UNIVERSITATEA DE ŞTIINŢELE VIEŢII
„REGELE MIHAI I" DIN TIMIŞOARA

FACULTATEA DE MEDICINĂ VETERINARĂ

LUCRĂRI ŞTIINŢIFICE

MEDICINĂ VETERINARĂ
TIMIŞOARA
VOLUMUL LVI (2)

SCIENTIFICAL PAPERS
VETERINARY MEDICINE

To be cited: LUCRARI STIINTIFICE: MEDICINA VETERINARA TIMISOARA (SCIENTIFICAL PAPERS: VETERINARY MEDICINE TIMISOARA)**,** vol. LVI (2), 2023

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

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ZOONOTIC PULMONARY PARASITES: A REVIEW

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Summary

Nowadays most diseases are zoonotic, i.e., they can be transmitted from animals to humans. After a period when this was obvious, during the Covid-19 pandemic, I think we should put an emphasis on these zoonotic diseases, especially zoonotic parasites, which have not been as well studied as infectious ones. Although the number of parasites is continuously decreasing due to the improvement of hygienic, socio-economic conditions, it is increasing in immunocompromised populations, due to climate change - which produces small changes/adaptations in the life cycle of parasites, transmission patterns, but also to travel/transport from one part of the world to another ("a parasite exchange/import"). In this paper we will talk about zoonotic pulmonary parasites belonging to the classes Protozoa, Trematodes, Cestodes and Nematodes.

Keywords: zoonotic pulmonary parasites, Protozoa, Trematodes, Cestodes, Nematodes.

Parasitic zoonoses can be defined as those parasitic diseases that can be transmitted naturally from animals to humans and vice versa (from humans to animals). The "parasite-host-environment" interdependence must be known in the smallest details, to determine the "weak link" of the evolution of the infestation and to be able to determine "when-how-and with what to intervene", so that the action is medically correct, ecologically, but also economically justified (9).

The number of parasites is continuously decreasing due to the improvement of hygienic, socio-economic conditions, but it is increasing in immunocompromised populations, due to climate change - which produces small changes/adaptations in the life cycle of parasites, transmission patterns, but also to travel/transport from one part of the world to another ("a parasite exchange/import")(24), thus it is important to know these zoonotic parasites to be able to effectively diagnose the people with respiratory clinical manifestations. The diagnosis of zoonotic pulmonary parasites is relatively difficult because symptoms and radiologic findings are non-specific. Therefore, high index of suspicion, a good anamnesis (travel history, and a detailed interrogation of personal hygiene) is crucial for diagnosis of parasitic lung diseases. The helminths can affect respiratory system in different phases of their life cycle (18).

Further on we will discuss the following zoonotic pulmonary parasites:

- Protozoa: *Entamoeba histolytica, Leishmania Donovani, Plasmodium vivax, Plasmodium falciparum, Plasmodium malariae, Babesia microti* and *Toxoplasma gondii.*
- Trematodes: *Schistosoma haematobium* and *Schistosoma japonicum.*

- Cestodes: *Echinococcus granulosus* and *Echinococcus multilocularis.*
- Nematodes: *Ascaris lumbricoides, Toxocara canis* and *Toxocara cati* (visceral larva migrans), *Ancylostoma duodenale, Necator americanus, Strongyloides stercoralis, Mammomonogamus laryngeus* (syngamosis*), Dirofilaria immitis, Brugia malayi* and *Wuchereria bancrofti* (tropical pulmonary eosinophilia), *Trichinella spiralis, Metastrongylus elongatus and Metastrongylus salmi.*

Protozoa

Entamoeba histolytica is found all over the world and is transmitted by eating mature cysts. Trophozoites invade the intestinal mucosa and enter the bloodstream which results in systemic infection (18). The disease is more severe in human infected immunodeficiency virus-infected patients (16). Symptoms seen in patients with amebiasis are fever, cough, chest pain, right upper quadrant abdominal pain. Live trophozoites can be found in sputum, pleural fluid, and lung biopsy. When making the diagnosis we must pay attention to the *Entamoeba* species, there are 2 other species that are not pathogenic. The diagnosis is preferable to be made after a PCR analysis. The treatment consists in the administration of metronidazole.

The visceral leishmaniasis is caused by *Leishmania donovani* and transmitted by various species of sand fly in Asia, Africa, Central and South America. The symptoms seen in pulmonary leishmaniasis are pneumonitis, pleural effusion, and mediastinal lymphadenopathy (42). Diagnosis can be made based on biopsy of a mediastinal lymph node showing histiocytes containing amastigotes of *L. donovani,* or PCR analysis. The treatment consists in the administration of pentavalent antimonials, liposomal amphotericin B and miltefosine.

Malaria is transmitted by infected Anopheles mosquito with *Plasmodium spp.* in tropical and subtropical areas (47). The symptoms are fever, cough, acute respiratory distress syndrome (ARDS). The gold standard for the diagnosis of *Plasmodium* infection is microscopic stained thick and thin blood smears. The treatment consists in the administration of artemisinin derivatives in humans infected by *P. falciparum* (37).

Pulmonary babesiosis is caused by *Babesia microti* and *B. divergens* in North America (44). The disease is a tick (*Ixodes scapularis*) borne infection. The symptoms are fever, drenching sweats, loss of appetite, myalgia, headache, and ARDS. In acute babesiosis it was reported splenic infarction and spontaneous splenic rupture (12). The diagnosis is made by the examination of thin blood smear (Giemsa-stained) which shows tetrads in the red blood cells. The treatment consists of a combination of clindamycin and quinine or atovaquone and azithromycin (20).

Toxoplasma gondii is a parasite that could be transmitted by eating contaminated or undercooked food with cyst, all over the world. Primary hosts, definitive hosts are cats (11). Clinical manifestations are myalgia and general lymphadenopathy, interstitial pneumonia and diffuses alveolar damage. Diagnosis is based on the detection of the bradyzoites in body tissue (Fig. 1). Toxoplasmosis is treated with a combination of pyrimethamine and sulfadiazine for 3-4 weeks (25).

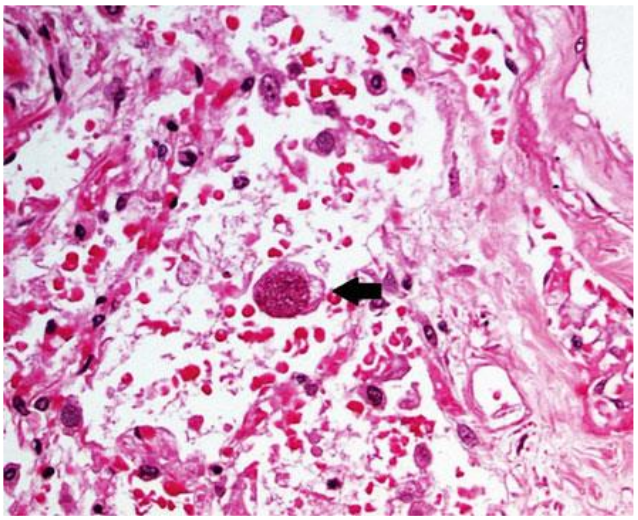

Fig. 1. Lung infected with Toxoplasmosis gondii (arrow) with diffuse alveolar damage (DAD) (H&E stain, ×100) (Courtesy of Danai Khemasuwan, MD, MBA, and Carol Farver, MD)

Trematodes

Schistosoma haematobium, S. mansoni, S. japonicum, S. intercalatum **and** ***S. mekongi*** are responsible for the disease schistomiasis. The disease is found in sub-Saharan, Africa, South America, and Far East (21). Schistosomiasis is the second most common cause of mortality among parasitic infections after malaria worldwide (24). The transmission is by penetration of the skin of schistosomal cercariae in fresh water. In acute schistosomiasis, patients present with dyspnoea, wheezing, dry cough, abdominal pain, hepatosplenomegaly, myalgia, and eosinophilia (3). Pulmonary involvement can occur because of the systemic migration of parasitic eggs from the portal system. The eggs trigger an inflammatory response that leads to pulmonary artery hypertension (40). The diagnosis could be performed through ELISA tests, or stool and urine examination, to detect the eggs. Acute schistosomiasis is treated with praziquantel. The treatment is repeated within several weeks since it has no anthelminthic effect on the juvenile stages of the parasites (43).

Paragonimus westermani is a parasite found in Southeast Asia, African, and South America, transmitted by ingestion of metacercaria from undercooked crustaceans. Undercooked meat of crab-eating mammals (wild boars and rat) can infect humans as indirect route of transmission (26) in acute paragonimiasis the clinical signs are fever, chest pain and chronic cough with haemoptysis (45). Pleural effusion had been described specifically with paragonimiasis and was reported in 40–70% of patients and associated with eosinophilia (32). The diagnosis is

confirmed by the presence of eggs or larvae in the sputum, stool, bronchoalveolar lavage fluid or pleural effusion (31). Serological tests with ELISA and a direct fluorescent antibody (DFA) are highly sensitive and specific for establishing the diagnosis (23). Praziquantel and triclabendazole are the treatments of choice with a high cure rate of 90 and 98.5 %, respectively (46).

Cestodes

Echinococcus granulosus and ***E. multilocularis*** are found in sheep-herding areas of the Mediterranean, Eastern Europe, the Middle East, South America, sub-Saharan Africa, and Australia. Humans become accidental hosts either by direct contact with the primary hosts (usually dogs) or by the ingestion of food contaminated with faeces containing parasite eggs (46). In most cases, lung hydatidosis is a single cyst (72–82 %). An echinococcal infection becomes symptomatic after 5–15 years, secondary to local compression or dysfunction of the affected organ. (30). In the case of an intact cyst clinical signs include cough, fever, dyspnoea, and chest pain. In the case of a cyst rupture into a bronchus - haemoptysis and/or expectoration of cystic fluid containing parasitic components (hydatoptysis) which is considered a pathognomonic finding (39). The patients may present with hydropneumothorax or empyema. Occasionally, a ruptured cyst can cause an anaphylactic-like reaction and pneumonia (21). The treatment is surgical - resection of the intact hydatid cysts and treat associated parenchymal and bronchial disease. Children especially those with small cysts, young cysts or cysts with daughter cysts may respond well to albendazole therapy without surgery (17).

Nematodes

Ascaris lumbricoides is transmitted through ingestion of eggs with L2, and is found in Asia, Africa, and South America. The symptoms are caused by larval ascariasis (Löffler's syndrome) - eosinophilic pneumonia, cough, wheezing, dyspnoea (13). Usually, the parasites eggs are found in faeces, but because pulmonary ascariasis is caused by migrating larval, adult parasites capable of reproduction are not yet found in the small intestine (5). Occasionally, the diagnosis can be confirmed by identifying larvae in the sputum. Solitary pulmonary nodules can also develop if the larva dies and evokes a granulomatous reaction (34). In the case of airway obstruction caused by adult ascaris, the mechanical removal through bronchoscopy is recommended (22). The most effective products against ascariasis are mebendazole and albendazole.

Toxocariasis is caused by migrating larvae of *Toxocara* canis and *T.cati*. Transmission is by ingestion of food contaminated with parasite eggs. The clinical signs are fever, cough, wheezing, seizures, and anaemia. The diagnosis is performed by an ELISA for the larval antigens (41). The treatment against larva migrans consist of diethylcarbamazine.

Ancylostomiasis is a disease caused by *Ancylostoma duodenale* and *Necator americanus* found in tropical and subtropical areas. *Necator americanus*

could be found in southern USA. The parasites larvae penetrate the skin of animals and humans, for *Ancylostoma duodenale* is possible also the orally transmission (15). Ancylostomiasis also like ascariasis is hookworm infestation that involves larval migration through the lungs via the bloodstream. Patients usually present with transient eosinophilic pneumonia (Löffler's syndrome) (15). Patients may ingest many *A. duodenale* larvae and develop a condition known as Wakana disease (nausea, vomiting, dyspnoea, cough, throat irritation, hoarseness, and eosinophilia) (15). Larval migration may also cause alveolar haemorrhage (38). The treatment consists of mebendazole and albendazole.

Strongyloides stercoralis is a parasite found worldwide, and endemic in tropical areas. The mode of transmission is by skin penetration by larvae, that migrate through the soft tissue via bloodstream to the lungs. The life cycle of *Strongyloides* can be completed entirely within one host. The term "hyperinfection syndrome" describes the presentation of sepsis from enteric flora, mostly in immunocompromised patients (33). The diagnosis is usually based on gastrointestinal and pulmonary symptoms, and the examination of the stool or feces to detect the larvae. A massive migration of larvae through the intestinal wall can result in sepsis from gram-negative bacteria (27, 42.) Strongyloidiasis has a fatal potential if untreated. The sensitivity of a stool exam for ova and larvae is 92 % when performed on three consecutive samples (8). The treatment against *Strongyloides* is oral ivermectin.

Syngamosis is caused by *Mammomonogamus laryngeus* a parasite that affects the respiratory tract of domestic mammals. Most cases of human syngamosis are reported from tropical areas, including South America, the Caribbean, and Southeast Asia (19). The life cycle of the parasite in human's hosts is not completely known. The diagnosis is made by bronchoscopy, or when the parasites are expelled by severe coughing. The mechanical removal of parasites through bronchoscopy is recommended like treatment.

Dogs are the definitive hosts for ***Dirofilaria immitis,*** humans are accidental hosts (no adult parasite found in humans, only immature forms). *D. immitis* is endemic in Southern Europe, Asia, Australia, and America. The parasite is transmitted by intermediate hosts, mosquitoes infected with third stage larvae. some patients may develop cough, haemoptysis, chest pain, fever, dyspnoea, and mild eosinophilia *5 %) (10). The nodule may show increased fluoro-deoxy-glucose avidity on a positron emission tomography scan (29, 35) and is often confused with malignancy. Calcification occurs within only 10 % of these nodules. The diagnosis is established by biopsy, identifying the parasite in the excised lung tissue. Dirofilariasis in humans is self-limiting and does not require any specific treatment (10).

Tropical pulmonary eosinophilia (TPE) is a mosquito-borne infestation, caused by microfilaria of *Brugia malayi* and *Wuchereria bancrofti*. in tropical and subtropical regions of South and Southeast Asia, Central and Latin America, Africa (west, coastal east and southern parts), India, Indonesia, Papua New Guinea and the western Pacific (7). The larvae reside in the lymphatics and develop into mature

adult worms. The clinical signs are eosinophilic pneumonia, cough, wheezing, dyspnea, restrictive pattern on spirometry, decreased diffusion lung capacity (18). Occasionally microfilaria can be identified on brushings or biopsies (36). The chronic phase of TPE may lead to progressive and irreversible pulmonary fibrosis (2). The treatment consists of diethylcarbamazine for 3 weeks.

Trichinella spiralis is a food borne disease from undercooked pork contaminated with trichinella larvae. Fertilized female parasites release first-stage larvae into the bloodstream and the lymphatics (4). Clinical signs are cough, pulmonary infiltrates, and dyspnea due to respiratory muscles involvement. The diagnosis is confirmed by muscle biopsy, which may demonstrate *T. spiralis* larvae. An ELISA using anti-Trichinella IgG antibodies can confirm the diagnosis in humans (14). A 2-week course of mebendazole with analgesics and corticosteroids is the recommended treatment (4).

Metastrongylus salmi and ***elongatus*** usually they are not transmitted to humans, but some cases were reported through the years. *Metastrongylus* are nematodes of the respiratory tract in wild and domestic pigs, transmission is by ingestion of the earthworms infected with third-stage larvae, or third stage larvae. Four cases were reported in Europe in 1845, 1855, 1888 and 1956 with *M. elongatus* (1, 28). One case was reported in 63 -year-old male, from Amazon region. The symptoms were productive cough with a bloody thick sputum, fatigue, chest pain, and progressive dyspnea. Sputum samples were examined. Macroscopically, dozens of worms were seen (6). The treatment was ivermectin, for two consecutive days.

Conclusions

For a good diagnosis, to confirm the pulmonary parasites, we need to recognize the epidemiology, life cycles, clinical signs, laboratory diagnosis, and treatments to make the proper management in patients. A detailed anamnesis is very important for the diagnosis in zoonotic pulmonary parasites.

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ANTIBIOTIC RESIDUES AND ANTIBIOTIC RESISTANCE OF *ESCHERICHIA COLI* IN RAW MEAT

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Summary

Antibiotic residue in meat is a serious public health concern due to its harmful effects on consumer health. This study aimed at estimating the presence of commonly used antibiotics in meat samples using a qualitative rapid test (*MeRA*, Liofilchem®, Italy), and evaluating the antibiotic susceptibility of *E. coli* bacteria isolated from the meat, using the automated compact system Vitek 2 (BioMérieux, Marcy l'Etoile, France). A total of 60 samples (pork, beef, goat and chicken) of raw meat from sales points were analysed. Overall, *MeRA* test analysis showed that 14 (23%; 95% CI 14.4-35.4) of the samples tested positive for the presence of antibiotic residues. Furthermore, six strains (60%; 95% CI 31.2-83.1) were resistant to ampicillin, norfloxacin and fosfomycin, and two strains (20%; 95% CI 5.6-50) of *E. coli* were resistant to the trimethoprim/ sulfamethoxazole combination. Consequently, the *E. coli* bacteria under investigation exhibited resistance to four antibiotics, belonging to three classes, namely penicillins, fluroquinolones and sulphonamides. The high percentage of positive meat samples to antibiotic residue highlights public health concerns. Moreover, the presence of antibiotic residues in raw meat is associated with the occurrence of multidrug-resistant strains of *E. coli*.

Keywords: meat, antibiotic residues, *E. coli* antibiotic susceptibility.

Antibiotics are widely used in food animal production to prevent and treat bacterial infections. However, the abuse of antibiotics has raised concerns about the presence of antibiotic residues in meat and other animal products, which can have negative consequences for human health (4).

Antibiotic residues in meat and meat products can occur when animals are given antibiotics before slaughter, or when antibiotics are used in the feed or water of animals that are used for food production. When animals are given antibiotics, an amount of the drug is excreted in the urine and feces, and part of it accumulates in animal tissues and products (9).

The presence of antibiotic residues in meat is a concern because it can lead to the occurrence of antibiotic resistance in human pathogens. When people consume food containing antimicrobial residues, they are exposed to low levels of drugs, which may induce the development of antibiotic resistance in microorganisms (10).

The use of antibiotics in food animal production is regulated by international agencies, such as the European Food Safety Authority (EFSA) and the U.S. Food and Drug Administration (FDA), which set limits on the antibiotic residues that are

allowed in meat and other animal products. These limits are designed to ensure that the levels of antibiotic residues in food do not pose a risk to human health (24,25).

However, there are concerns that these limits may not be adequate to prevent the development of antibiotic resistance. Several published studies found that even low levels of antibiotic residues in meat can promote the development of antibiotic resistance in human pathogens (1, 2, 4, 13, 18, 22).

In Romania, as in many other countries, there has been a trend towards the increased use of antibiotics in animal agriculture, in recent years. This has led to concerns about the presence of antibiotic residues in meat and other animal products, which can have negative consequences for human health. Several studies conducted in Romania reveal the presence of residues and the increase of antibiotic resistant bacteria in food produced in the country (3, 5, 6, 7, 12, 14, 15,16, 19).

In addition to the risk of antibiotic resistance, the presence of antibiotic residues in meat and food can also cause allergic reactions and other adverse health effects in some people (8).

Materials and methods

The study was carried out on raw meat of pork, beef, goat and chicken origin. The meat samples were purchased from various agri-food markets, in an area located in the north-west of Romania, which sell meat from veterinary registered farms. The 60 samples collected were classified by origin and anatomical region, as follows: 30 samples of pork (leg, neck, breast, ribs), 20 samples of beef (rump, shank), 6 samples of chicken meat (breast, thigh) and 4 sample of goat meat (rump, shank). After the meat samples were collected, they were individually packed in sterile nylon bags, labelled with a code and stored under refrigerated conditions (0-4°C). The samples were transported in a cooler box to the laboratory of Food Hygiene and Microbiological Risk Assessment, Faculty of Veterinary Medicine, Timișoara

Detection of the antibiotic residues using *MeRa* test. 2 grams of each grounded meat sample were introduced into a test tube with 6 ml of distilled water, the meat/ water ratio being 1/3. The pH of the mixture was then measured for each sample to assess if the sample had an optimal pH of 5.6. In samples where the pH value was not appropriate, a correction was made either by adding a 0.1 N NaOH solution to alkalinise the medium or a 0.1 N HCl solution to acidify the aqueous meat extract. The manufacturer advises the pH correction, because it is required to prevent false-positive or false-negative results, since a pH that is too acidic causes a yellow turn, which inevitably results in a false-negative result (invalidation of the test). The test tube contents were homogenized for 15 seconds in a homogenizer (13000 rpm), after which the samples were centrifuged (4000 rpm), for 15 minutes (27). The resulting supernatant was transferred into test tubes with a 10 ml volume, using sterile pipettes. A disc impregnated with *Geobacillus stearothermophilus* spores was inserted into each sample tube, then homogenized by gently shaking, for 10 seconds. In the following stage, the prepared samples were incubated at 64°

C, for 20 minutes. The samples were taken out of the thermostat and maintained at room temperature. From each sample one ml of the supernatant was put into a test tube filled with culture media and covered with a lid. The samples thus prepared were left at room temperature (20° C), for 20 minutes. The tubes were then put back into the thermostat for a second incubation, for 3.5 h, at 64°C (27).

The results have been interpreted based on the colour obtained after the incubation period. Samples with blue colour were considered positive - therefore with antibiotic residues, while the samples having yellow colour were interpreted as negative (27).

Within the limits outlined by EU legislation, the *MeRa* test enables the qualitative detection of residues of 29 antimicrobial agents that belong to 9 classes, including: aminoglycosides, betalactams, tetracyclines, macrolides, lincosamides, sulphamides, sulphanilamide, benzyl pyrimidine, and quinolones (27).

Microbiologic analysis. *Escherichia coli* was isolated using the procedures outlined in ISO 16649-2/2007 (28). From each sample 10 g of meat were homogenized in a Stomacher (BioMérieux, Marcy l'Etoile, France) with 90 ml of peptone buffered solution.

Next step involved serial dilutions of the samples in sterile peptone water 0.5% up to the dilution 10^{-3}. Subsequently, 1 ml from each dilution was transferred in a sterile Petri dish (in duplicate), and tryptone bile agar with X-glucoronide agar (TBX agar) was poured over the sample and then homogenised. The blue–green-coloured *E. coli* colonies that grew from the 10^{-3} dilution were examined, using Gram staining and enumerated, according to ISO 16649-2/2007 (28).

Antibiotic susceptibility detection for *Escherichia coli* strains was made using the automated Vitek 2 system (BioMérieux, Marcy l'Etoile, France). From the total number of *E. coli* colonies that were obtained a number of 10 colonies was analyzed for the antibiotic susceptibility.

The analyzed colonies were obtained after passages on brain-heart agar. The media thus prepared were incubated for 24 hours, at a temperature of 37° C. Next, bacterial suspensions with an optical density (OD) of 0.5-0.63 McFarland were prepared from the cultures developed on the agar surface. A volume of 128 µl was taken from each suspension, which was transferred to antibiotic susceptibility test cassettes and incubated in the incubation compartment of the Vitek2 system.

Antibiotic susceptibility was caried out using the AST-N204 card for Gram-negative bacteria. The used cards included a total of 16 antimicrobial substances, from 8 classes, as follows: ampicillin (**AMP**, MIC 16 µg/ml – penicillin class), amoxicillin/clavulanic acid (**AMC**, MIC 4 µg/ml), piperacillin-tazobactam (**TZP**, MIC ≤4 µg/ml) cefotaxime (**CTX**, MIC ≤1 µg/ml), ceftazidime (**CAZ**, MIC ≤1 µg/ml), cefepime (**CPM**, MIC ≤1 µg/ml), ertapenem (**ERT**, MIC ≤0.5 µg/ml), imipenem (**IMP**, MIC ≤0.25 µg/ml), meropenem (**MER,** MIC ≤0.25 µg/ml), amikacin (**AMK,** MIC ≤2 µg/ml), gentamicin (**GEN**, MIC ≤1 µg/ml), ciprofloxacin (**CIP,** MIC ≤0.25 µg/ml), norfloxacin (**NOR,** MIC ≤0.5 µg/ml), fosfomycin (**FOF**, MIC ≥256 µg/ml), nitrofurantoin (**NIT**, MIC ≤16 µg/ml), trimethoprim / sulfamethoxazole (**STX**, MIC ≤ 20 µg/ml).

Results and discussions

MeRA test result indicate that 14 (23.3%; 95% CI 13.7.4-35.4) of the samples tested positive for the presence of antibiotic residues. The distribution of positive samples depending on the species is as follows: pork - 8 (26.6%; 95% CI 14.1-44.4) samples, beef - 6 (30%, 95% CI 14.5-51.9) samples. The poultry and goat meat samples were negative for the presence of antibiotic residues. Table 1 shows the results obtained for the detection of antibiotic residues in the analyzed meat samples.

Table 1

Results of MeRa test for the presence of antibiotic residues in meat samples

Crt. No.	Meat sample origin	No of samples tested (species)/ no of positive samples	Percentage of positive samples (%)
1.	Pork	30/8	26.6
2.	Beef	20/6	30.0
3.	Goat	6/0	-
4.	Chicken	4/0	-
Total		**60/14**	**23.3**

Results from the antimicrobial resistance profile monitoring of the isolated *E. coli* strains (n= 10) are summarized in Table 2.

Table 2

Antimicrobial susceptibility profile of the *E. coli* strains isolated from meat samples

Antimicrobial		Susceptibility Test Resul	
Class	**Agent/ MIC Range (µl/ml)**	***S***	***R***
β-lactams	**AMP**/ ≤2	6	4
	AMC/ ≤2	10	-
	TZP/ ≤4	10	-
Aminoglycosides	**AMK**/ ≤2	10	-
	GEN /≤1	10	-
	ERT /≤0.5	10	-
Carbapenems	**MEM**/ ≤0.25	10	-
	IPM /≤0.25	10	-

Cephalosporins	**CTX**/ ≤1	8	2
	CAZ/ ≤1	8	2
	CPM/ ≤1	8	2
Fluoroquinolones	**CIP**/ ≤0.25	10	-
	NOR/ ≤0.5	2	8
Nitrofuran derivative	**NIT**/ 64	10	-
Phosphonic acid derivative	**FOF**/≤16	-	10
Sulfonamides	**STX**/ ≤20	8	2

Note: AMP- ampicillin, **AMC** - amoxicillin/clavulanic, **TZP** - acid piperacillin-tazobactam, **CTX** - cefotaxime, **CAZ** - ceftazidime, **CPM** - cefepime, **ERT** - ertapenem, **IMP** - imipenem, **MER** - meropenem, **AMK -** amikacin, **GEN** - gentamicin, **CIP** - ciprofloxacin, **NOR** - norfloxacin, **FOF** - fosfomycin, **NIT -** nitrofurantoin, **STX** - trimethoprim / sulfamethoxazole

The isolates (n=10) manifested the following resistance: FOF (100%), NOR (80%), AMP (40%), CTX (20%), CAZ (20%), CPM (20%), STX (20%). None of the isolates manifested resistance to AMC, TZP, AMK, GEN, ERT, MEM, IPM, CIP, NIT.

Out of the ten isolates, eight (80%, 95%. CI 44.2 – 96.4) manifested resistance to the association NOR-FOF, however a more serious concern represented the eight strains that were classified as multidrug resistant (MDR). Out of these six (20%; 95% CI 27.3 – 86.3) strains manifested a resistance to three antimicrobials from three different classes, and four (40%; 95% CI 13.6 – 72.6) manifested resistances to five antimicrobials form three different classes (Table 3).

Table 3

Multi-drug resistance phenotype combination of the tested *E. coli* isolates

Origin of meat	No. of isolates	No. of classes with resistance	Resistance to antimicrobial profile	Classes with resistance
Pork	6	3	AMP, NOR, FOF	β-lactams, fluoroquinolones, phosphonic acid derivative
	2	3	AMP, CTX, CAZ, CMP, FOF	β-lactams, cephalosporins, phosphonic acid derivative
	8	2	NOR, FOF	fluoroquinolones, phosphonic acid derivative

In agreement with the results of several previous investigations (12, 16, 24) our study pointed out that the isolation of antibiotic resistant *E. coli* strains from food samples is a common finding in different regions from Romania.

According to EFSA report of 2021 in the European Union the percentage of non-compliant samples was 0.17% showing a decrease compared to the previous 5 years where the percentages were between 0.19-0.37% (26).

Antimicrobial multi-drug resistance (MDR) is one of nowadays' major public health issues. Introduction of MDR bacteria into a human population is associated with increased rates of illness and even death (21).

To reduce the risk of antibiotic residues in meat, several strategies can be employed. These include:

(i) reducing the use of antibiotics in animal food production: by reducing the use of antibiotics in animal agriculture, the risk of antibiotic residues in meat and food can be minimized. This can be achieved by using alternative methods for disease prevention and treatment, such as vaccines and probiotics (11, 17);

(ii) improving monitoring and enforcement. International, European, and government agencies should improve monitoring and enforcement of regulations regarding antibiotic use in animal agriculture to ensure that the levels of antibiotic residues in meat and food are within safe limits;

(iii) improving consumer awareness. Consumers can also play a role in reducing the risk of antibiotic residues in meat and food by choosing products that are labeled as antibiotic-free or organic, and by cooking meat to appropriate temperatures to ensure that the antibiotic residues are destroyed during the heat treatment, although there are no guarantees of the breakdown of these components (20, 23).

Conclusions

Antibiotic residues in meat are a major concern as they can promote the development of antibiotic resistance and cause adverse effects on human health.

To reduce the risk of antibiotic residues in meat it is important to increase the awareness of farmers on the responsible use of antibiotics in meat-producing animals and to enhance surveillance and monitoring of antimicrobial use by the competent authorities.

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IDENTIFYING ANIMAL AND VEGETAL SPECIES AND INCORRECT LABELLING IN PET FOODS

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Summary

Mislabelling, falsifying and substituting food products is a growing problem in highly processed foods, including pet foods. Although there is a regulation for pet food, there needs to be more information on the prevalence of improper labelling of pet food. The study aimed to conduct market surveys of pet foods sold by specialised pet stores to identify the present animal and vegetal species and any instances of incorrect labelling. Twenty commercial dry products were collected from specialised pet stores. DNA was extracted from each product in duplicates, and the component species were identified using polymerase chain reactions (PCR). Species-specific analyses were used to verify the existence of meat species (bovine, pigeon, pig, chicken, turkey, pork and horse). Plant species were present in all the products analysed, a large share, in this case, corn and soybeans. Poultry meat products are the most used in the composition of the analysed products. They are detected in 80% of the samples and are correctly labelled.

Fish meat products are also widely used, especially in cat food. The percentage of 75% is also reached by using fish oils in the composition of these products. Products from ruminants are present in a high percentage, both as the main component and as animal protein or fat sources. Pig and horse products were detected in a reasonably low percentage and level. They are not mentioned on the labels and can be classified as animal protein and fat used in the composition of the analysed pet food. Although these results indicate that pet food labels are misleading, more studies are needed to determine the extent of misleading labels and identify the points in the production chain where misleading labels occur.

Keywords: pet food, mislabelling, species composition, DNA-based screening.

Products intended for feeding animals include various types of dry or wet feed, nutritional supplements, vitamins, minerals and other food additives.

These products are formulated to provide the nutrients necessary for the growth, development and maintenance of animal health, depending on the species, age, weight and physiological stage.

They can be made from ingredients of plant, animal or synthetic origin, and some products are specially formulated to meet the specific nutritional needs of certain animals, as the composition of food is a crucial aspect in maintaining a healthy life for small animals such as dogs and cats. Food influences the health, longevity, energy and behaviour of these pets. It is important to pay attention to the composition

of the feed to ensure that the animals receive all the necessary nutrients to maintain their health and enjoy an active and energetic life (17). Consumer trends are moving towards healthier, more natural food options (6, 21). Official agencies must, therefore, again enforce guaranteed compliance with food labelling, nutritional quality and food origin, as well as the perception of health and diet claims to avoid false claims by food manufacturers.

Legislation regarding adulteration of pet food varies by country and region. Regulatory authorities such as the FDA (United States) or ANSVSA (Romania) can set and regulate minimum standards for the quality of pet food.

Pet food quality standards vary but generally relate to food products' nutritional composition, safety and labelling. These standards may include requirements on the proportions of protein, fat and carbohydrates, including the specific types of ingredients used, such as meat, grains and vegetables. Standards may also set maximum level requirements for toxic or potentially harmful substances such as contaminants and food additives (20). They can also include labelling requirements, such as a full list of ingredients and detailed nutritional information, so pet owners can make informed choices about the food they buy.

Economically motivated adulteration was defined in 2009 by the Food and Drug Administration (FDA - USA) as "the intentional fraudulent substitution or addition of a product to increase the apparent value of the product or reduce the cost of its production"(3). It can often cover the effects of public safety by the unknown addition of allergens, toxins and hygiene risks (11). Food authenticity testing is important to rule out unfair competition to confirm labelling and product value. It is also essential to ensure consumer protection, both against fraud and to confirm the quality of the product. The pet food market is constantly developing, but the most popular are maintenance foods, which are over-the-counter diets. Chicken-based ingredients are commonly used in pet food. They are highly palatable, relatively inexpensive, and provide an excellent source of protein. However, individual products vary widely in nutrient composition and processing conditions that can affect protein quality and digestibility (15). For most dogs, chicken or ruminant meat is easy to digest, but its widespread use in dog food has probably led to more frequent allergies to this ingredient (20). Currently, protein detection in foodstuffs and feeds is based on several methods. For example, commercial animal feed contains herbicides (glyphosates) residues detected in each tested product using enzyme-linked immunosorbent tests (ELISAs). However, it was shown that the ELISA-based method was less sensitive and precise than the commercial canine food PCR method and that it was impossible to fully identify the presence of animal by-products (11).

DNA analysis techniques can detect feed adulteration by identifying the presence or absence of specific animal species such as pork, chicken or beef (16).

Materials and methods

The reference material in this project used, as a positive reaction control, suspensions of DNA isolated from various matrices from the collection of the Laboratory of Molecular Genetics within the Interdisciplinary Research Platform and from the collection of Laboratory A2 within the Complex of Research Laboratories – Horia Cernescu of USV, Timișoara. The plant reference material was represented by DNA solutions isolated from corn (Zea mays L.) and soybean (*Glicine max* L.) flours. The reference material of animal origin was represented by DNA solutions isolated from dried and ground muscle tissue from the species for which the detection experiments were performed: chickens, fish, ruminants, pigs and horses according to the methods described by Boldura et al., 2011.

The biological material is represented by 20 samples of dry food intended for dogs and cats, purchased from speciality stores on the domestic market.

Table 1

The analyzed products and the description of their labels

Sample No.	Label description
1	poultry, corn, other plant species, animal fats, animal proteins, yeast, fish oil, soybean oil.
2	poultry, corn, other plant species, animal fats, animal protein, fish oil, soybean oil.
3	poultry, corn, other plant species, animal fats, animal protein, fish oil, soybean oil.
4	poultry, corn eggs, other vegetable species, animal fats, animal proteins, fish oil, soybean oil.
5	poultry, corn, other plant species, animal fats, animal proteins, yeasts, soybean oil.
6	poultry, corn, other vegetable species, animal fats, animal proteins, fish oil,yeasts, soybean oil.
7	fish, poultry, corn, other vegetable species, animal fats, eggs, fish oil, soybean oil.
8	poultry, other plant species, animal fats, animal proteins, yeasts.
9	poultry, corn, other vegetable species, animal fats, animal proteins, fish oil,yeasts.
10	fish, other vegetable species, animal fats, animal proteins, fish oil,yeasts, soybean oil.
11	poultry, fish, whole grains, other vegetable species, animal fats, animal proteins, fish oil, soybean oil.
12	poultry, corn, other plant species, animal fats, animal protein, soybean oil.
13	poultry, corn, other vegetable species, animal fats, animal proteins, fish oil,yeasts, soybean oil.

14	poultry, corn, other vegetable species, animal fats, animal proteins, fish oil,yeasts, soybean oil.
15	corn, other vegetable species, animal fats, animal proteins, fish oil, soybean oil.
16	poultry, corn, other vegetable species, animal fats, animal proteins, fish oil, yeasts, soybean oil.
17	poultry, corn, other vegetable species, animal fats, animal proteins, fish oil, yeasts, soybean oil, algae.
18	lamb, bird, other vegetable species, bird fat, fish oil,yeasts, crustaceans.
19	lamb, bird, other vegetable species, bird fat, fish oil, yeasts, crustaceans.
20	lamb, bird, other vegetable species, bird fat, fish oil, yeasts, crustaceans.

Primer pairs used for species identification in this screening study and their bibliographic references are described in Table 2. They were selected from the collection of the A2 laboratory within the Complex of Research Laboratories – Horia Cernescu of ULS, Timișoara and were synthesised by Novogene, Amsterdam, Netherlands.

Table 2

PCR primers used in this project

Code	Species	Sequence 5'…3'	Reference
CW/CX	gena Rubisco - material vegetal	CGTAGCTTCCGGTGGTATCCACGT GGGGCAGGTAAGAAAGGGTTTCGTA	(22)
HA-nos	Lectina - soia	GCATGACGTTATTTATGAGATGGG GACACCGCGCGCGATAATTTATCC	(23)
zein3-zein4	Zeina - porumb	AGTGCGACCCATATTCCA GACATTGTGGCATCATCATT	(23)
Poultry/12S rRNA	pasăre	TGAGAACTACGAGCACAAAC GGGCTATTGAGCTCACTGTT	(9)
Fish/12S rRNA	pește	TAAGAGGGCCGGTAAAACTC GTGGGGTATCTAATCCCAG	(9)
Cattle/16S rRNA	rumegătoare	TAAGAGGGCCGGTAAAACTC GTGGGGTATCTAATCCCAG	(9)
Swine/12S rRNAtRNA	porc	CTACATAAGAATATCCACCACA ACATTGTGGGATCTTCTAGGT	(9)
Horse/mtDNA	cal	CCATCCCTACGCTCCATTCCC TGTTTTCGATGGTGCTTGCG	(4)

Materials and methods

DNA extraction and evaluation

Isolation and purification of DNA from the samples taken in the study was carried out with the help of the kit "NucleoSpin DNA Forensic" (Macherey-Nagel,

Düren, Germany), following the manufacturer's instructions. The DNA solution was qualitatively and quantitatively evaluated by the spectrophotometric method using the NanoDrop 8000 equipment (ThermoScientific, Waltham, Massachusetts, US), after quantification the samples were brought to the same concentration – 100 ng/ µl, by dilution.

End-point PCR analyses

PCR reactions were performed using the Surecycler Thermocycler, Agilent Technologies, Santa Clara, CA, US. The reagents that made up the amplification mixture were as follows: PCR kit: GoTaq Green Master Mix (Promega, Oregon, US)- 12.5 µl, 20 pmol of each primer, template DNA – 1 µl, adjusted with distilled water up to 25 µl. Amplification conditions were according to literature data and consisted of an initial denaturation step at 93°C for 3 min.; 35 cycles with 93°C – 30 sec., 58 – 60 °C – 30 sec. and 72°C – 45 sec.; a final extension step of 72°C for 3 min.

Agarose gel electrophoresis

PCR products were migrated in agarose gel in the presence of ethidium bromide and visualised under UV light. The gel image was captured and analysed using Vision Works software (UVP, Analytik Jena, Germany).

Results and discussions

In context, protein-based methods for species identification can fail after excessive proteolysis or heat-induced denaturation of indicator proteins. It is suggested that genomic DNA from milk somatic cells persists in the processed material and can be amplified and analyzed for species identification. Also, many of these methods are based on the properties of the proteins found in the meat of the species from which the raw material was taken. The identified methods are electrophoretic, chromatographic, immunological and molecular (1, 12, 23). These are just some of the methods used to detect the composition of pet food. Other methods can be used, depending on the needs and resources of each animal food production company, and these techniques based on DNA analysis appear to be the most accurate, rapid and reproducible (4, 13).

Food safety is an extremely important issue related to animal health. Both raw materials and food ingredients must be constantly monitored because some of the substances present in food are pollutants of anthropogenic origin, while others originate from food processing or storage. Unfortunately, food counterfeiting is a common phenomenon often encountered in the economic environment, where it causes unfair competition (7, 9). It also reduces food quality and can even pose a health risk. Both consumers and industry are calling on the research community to implement tests to help analyze food authenticity. The above-mentioned issues compel food safety and quality scientists to constantly seek new approaches and tools to address the main issues related to food quality, safety, and authenticity (7, 8). Recently, many techniques have been developed to trace food through DNA barcoding techniques, even for complex and highly processed mixtures such as pet

food. These techniques can identify adulteration in pet food, a growing problem among pet owners (5, 10). Properly selected methods can also provide a way to confirm that the pet food is truly consistent with what is described by manufacturers on labels or packaging. Given the importance of accurate pet food labelling, especially for animals with mild or life-threatening allergies, accurate detection of mislabeled or undeclared animal species is important for pet safety (18, 19).

Furthermore, wide variations among commercially available diets marketed to support the treatment of various medical conditions, such as skin and coat health or allergies, can confuse caregivers during diet selection (14). One of the problems with pet food mislabeling is the ingredient list itself. Generally or intentionally vague ingredients are often given (2, 3). Commission Regulation no. 767/09 allows listing ingredients by category in pet food (19). Despite existing mandatory traceability requirements for pet food, it has previously been reported that labels do not provide sufficient information about the sources of different nutrients in the product.

The combination of materials of vegetable and animal origin in the composition of pet food refers to the use of plant-based and animal-based ingredients in the formulation of pet food products. Considering their dietary requirements, this approach aims to provide pets with a balanced and nutritious diet. Plant-based ingredients commonly used in pet food include grains (such as rice, corn, and wheat), vegetables (like carrots, peas, and sweet potatoes), fruits, and various plant proteins (such as soybean meal or pea protein). These ingredients can provide a source of carbohydrates, fiber, vitamins, and minerals. Animal-based ingredients used in pet food typically include meat, poultry, fish, and their by-products. These ingredients are rich in high-quality proteins, essential fatty acids, and other vital nutrients for pets' overall health and well-being. Combining vegetable and animal ingredients allows pet food manufacturers to create a well-rounded and nutritionally complete diet for pets. By incorporating various ingredients from different sources, they can balance essential nutrients, including proteins, carbohydrates, fats, vitamins, and minerals, which are vital for pet optimal health and development. In this context, the first stage of the presented experiment was represented by a screening that allowed the identification of the material of plant origin by using its specific markers, namely for the ribulose-1,5-bisphosphate-carboxylase (RuBisCo) gene (Fig. 1, panel A). By identifying this gene, it was possible to certify the presence of material of plant origin and the amplifiable quality of the obtained DNA. The most frequently encountered plant species in the composition of pet food are soy and corn, thus the first screening analyses had in mind the highlighting of the presence of plant material originating from soy (Glycine max L., (Fig. 1, panel B) and of the plant material of corn (*Zea mays* L.), the presence of the latter being noted on most of the analyzed product labels both as a source of carbohydrates (corn starch) and as a source of protein (hydrolyzate) and is frequently used as an ingredient in pet food (Fig. 1, panel C) Being a qualitative analysis the results are interpreted only by the ***present/absent*** score.

From the analysis of Fig.1, panel B, it follows that the soybean-specific genetic material was at a level detectable by this analysis for 15 of the 20 analyzed samples. The presence of an amplification product with a molecular size of 118 bp certifies the presence of this species in the analyzed product. In the composition of five products, soy was impossible to detect by this method, namely samples 8, 9, 18, 19 and 20. Also from Fig. 1, panel C, it is found that the genetic material specific to maise was at a level detectable by this type of qualitative analysis for 16 of the 20 analyzed samples. The presence of an amplification band with a molecular size of 227 bp certifies the presence of this species in the analyzed product. In the composition of four products, maize was impossible to detect using this method, namely samples 10, 18, 19 and 20. The accuracy of the label in declaring the presence of these plant species in the composition of the products is shown in Table 3.

The following analyses were carried out to identify the presence of animal species in the composition of the analyzed pet food. The labels of all analyzed products mentioned the presence of material of animal nature, more or less processed, such as dried meat, animal fat, hydrolyzed animal protein or fish oils. The most frequently used species in the analyzed products were poultry, which can be used as meat by-products or eggs under different processing degrees. Notably, pork, poultry and beef are the animal species most commonly slaughtered in Europe for human consumption [28]. Thus, they provide the largest amount of animal by-products to pet food manufacturers. Accordingly, the most common animal species not declared on pet food labels appear to be pork, chicken and turkey.

The bird species included in this screening are hen (*Gallus gallus* L.) and turkey (*Meleagris meleagridis* L.). In the case of gene screening specific to bird species, PCR amplification, analysis of PCR products and interpretation of the results are carried out in the same way as in the case of plant species screenings (Fig. 2, panel A). In one of the largest studies reported in the literature (number of foods tested = 52), chicken was the most common meat species found in almost all (98%) pet foods tested (3).

The presence of biological material from fish required screening for the following species: sardines, *Sardinops melanostictus*, *Sardinella hualiensis* and *Pagrus major*, mackerel, *Tracurus japonicas* and salmon, *Salmo salar*. In the case of screening genes specific to fish species, the PCR amplification, the analysis of the PCR products and the interpretation of the results are carried out in the same way as in the case of the screening of the bird species. Fish-specific primers were designed from the 12S rRNA mitochondrial DNA region, generating amplification sequences of 228 base pairs (Fig. 1, panel B). It is found that the genetic material specific to the fish was at a detectable level through this type of qualitative analysis for 15 of the 20 analyzed samples. The presence of an amplification band with a molecular size of 228 bp certifies the presence of fish species in the analyzed products. In the composition of five of the products, genetic material from fish was not detected using this method, namely in samples 5, 8, 12, 19 and 20.

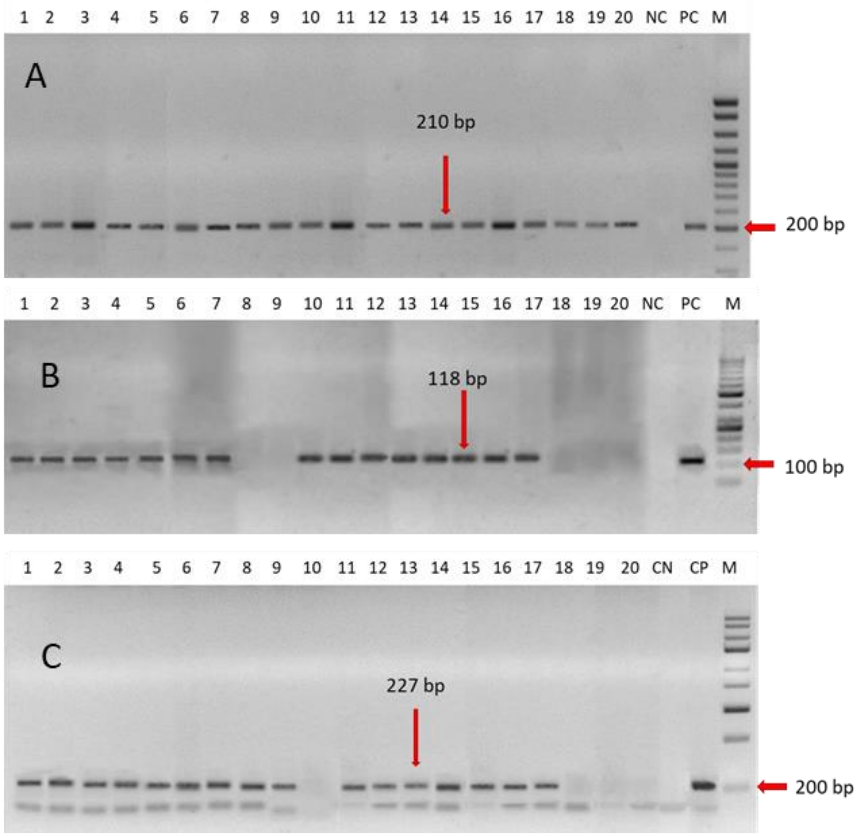

Fig. 1. The results of the PCR analysis using gene-specific primers from the plant material

Panel A - RuBisCo gene; Panel B – lectin gene; Panel C – zein gene. Lanes 1 – 20 Amplification products for the biological samples analyzed in this experiment; NC – negative reaction control (DNA isolated from material of animal origin); PC – positive reaction control – DNA solution isolated from material of vegetable origin (soy flour or corn) M – molecular weight marker for nucleic acids – PCR marker (Promega)

The following analyses were performed to detect some species not mentioned on the labels of the products analyzed in this paper. For this type of screening, three animal species were chosen that are frequently found in human food and whose by-products can easily end up being used in pet food as well. They may also be the source of those animal fats or proteins listed but not explicitly stated on the product label.

From this category, the first screening performed was to identify the presence in the composition of the products of genetic material from ruminants, namely: cattle - *Bos taurus*, goats - *Capra hircus*, and sheep - *Ovis aries*. Ruminant-specific primers were designed from their mitochondrial 16S rRNA DNA region, and generate amplification sequences of 204 base pairs, and can be successfully used on DNA samples degraded due to their exposure to high temperatures, as indicated by European laws regarding thermal processing of ingredients of animal origin (Fig 2, panel C). Even ruminants are not generally a species used for preparing pet food; it can be found in combinations, especially in the case of dog food. Thus, in this experiment, three samples were analyzed that were labelled as a mixture of lamb and poultry, which were intended for feeding dogs.

The analysis reveals that in 14 of the 20 analyzed samples, the level of specific genetic material is detectable by qualitative analysis for ruminants. The presence of an amplified band with a molecular size of 204 bp proves the presence of ruminant species in the analyzed material. This method detected no genetic material specific to ruminants in the composition of six products (samples 9, 10, 14, 15, 16 and 17).

A wide range of pet food products are commercially available, each with its ingredients and nutritional attributes. One of these ingredients is pork by-products, which can be found in many pet food products. However, there are many questions about the quality and safety of these products, but it is accepted that pork by-products are no less safe than any other source of protein for pets. However, some products may contain lower-quality by-products, such as tissue containing hormones or antibiotics, which may harm the animal's health (17). Considering all this, the products that can be purchased on the market do not specify the presence of material from the pig. Thus, the following analysis aimed to highlight the presence of pork by-products in the biological samples taken. In the case of the screening for the detection of components originating from the pig (*Sus scrofa*), primers were used that target the molecular markers of the genes specific to the pig (Fig. 2, panel D). In five of the 20 samples analyzed (1, 3, 7, 9 and 12), pig-specific genetic material was present at levels detectable by this type of qualitative analysis.

The presence of an amplified band with a molecular size of 204 bp demonstrates the presence of pork by-products in the analyzed products. In the case of the samples where the result was present, the genetic material belonging to the analyzed species was found to be relatively low compared to the positive control, as indicated by the intensity of the bands detected in the gel electrophoresis images. This indicates that the addition of by-products from this species is very low, most likely only traces to increase the animal fat content of the products.

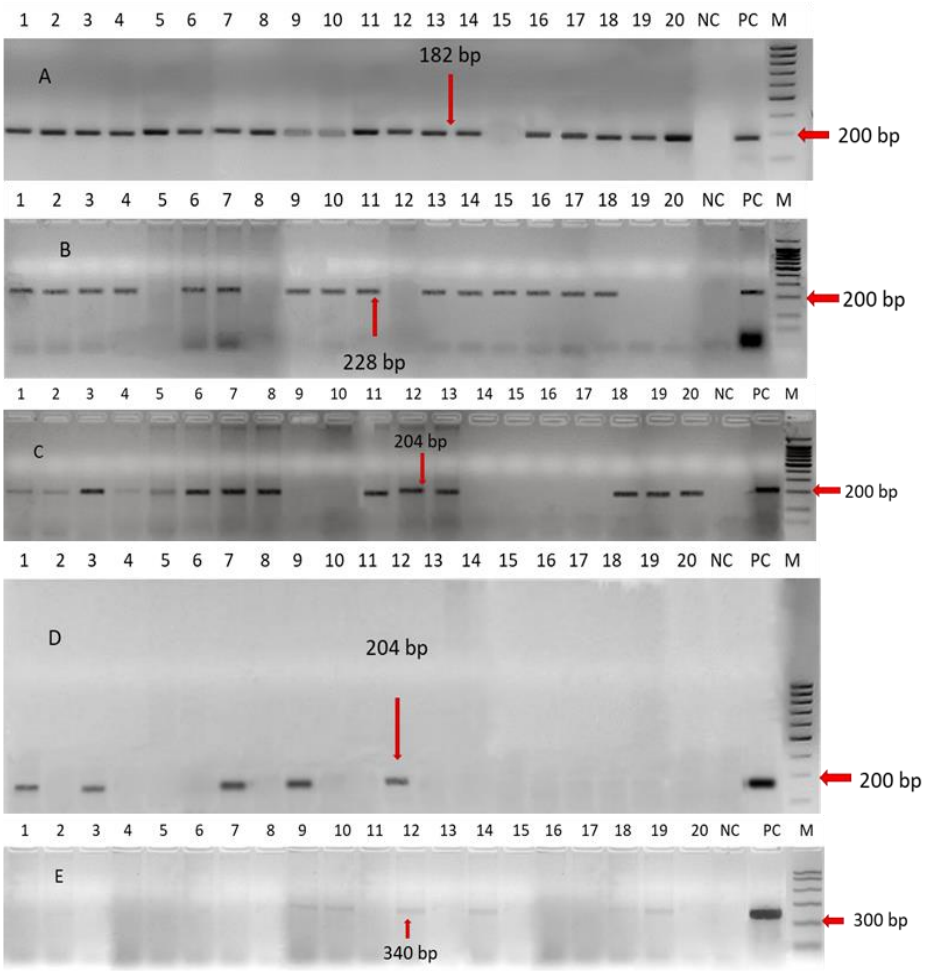

Fig. 2. The results of the PCR analysis using gene-specific primers from the animal material

Panel A - chicken; Panel B – fish; Panel C – ruminants; Panel D – pork; panel C - horses. Lanes 1 – 20 Amplification products for the biological samples analyzed in this experiment; NC – negative reaction control (DNA isolated from plant material); PC – positive reaction control – DNA solution isolated from material of animal origin (specific to each analyzed species – reference materials) M – molecular weight marker for nucleic acids – PCR marker (Promega)

One of these ingredients is pork by-products, which can be found in many pet food products. However, there are many questions about the quality and safety

of these products, but it is accepted that pork by-products are no less safe than any other source of protein for pets. However, some products may contain lower-quality by-products, such as tissue containing hormones or antibiotics, which may be harmful to the animal's health (12). Considering all this, in general, the products that can be purchased on the market, do not specify the presence of material from the pig. Thus, the following analysis aimed to highlight the presence of pork by-products in the biological samples taken. In the case of the screening for the detection of components originating from the pig (Sus scrofa), primers that target the molecular markers of the genes specific to the pig were used (Fig. 2, panel D). In five of the 20 samples analyzed (1, 3, 7, 9 and 12), pig-specific genetic material was present at levels detectable by this type of qualitative analysis. The presence of an amplified band with a molecular size of 204 bp demonstrates the presence of pork by-products in the analyzed products. In the case of the samples where the result was present, the genetic material belonging to the analyzed species was found to be relatively low compared to the positive control, as indicated by the intensity of the bands detected in the gel electrophoresis images. This indicates that the addition of by-products from this species is very low, most likely only traces to increase in the animal fat content of the products.

The last analysis had as a screening target the detection of the genetic material from the horse (*Equus ferus caballus*), a product not specified on the label but which, due to the slaughter of this species, may accidentally be found in the composition of products intended for feeding pets (Fig. 2, panel E). It is noted that small traces of genetic material of the target species were detected in six of the 20 analyzed samples (9, 10, 12, 14, 18 and 19). The presence of an amplified band with a molecular size of 340 bp demonstrates the presence of horse by-products in the analyzed products.

In the case of the samples where the result was present, the genetic material belonging to the analyzed species was very low compared to the positive control, as indicated by the intensity of the bands detected in the gel electrophoresis images. This indicates that adding by-products from this species is very low, most likely only traces to increase the products' protein or animal fat content.

The results of this screening were systematized and tabulated in Table 3.

Table 3

Results obtained from the screening study for species identification

Sample	Tagged species	Species identified
1	poultry, corn, other plant species, animal fats, animal proteins, yeast, fish oil, soybean oil.	Poultry, fish, ruminants, pigs, soybeans, corn
2	poultry, corn, other plant species, animal fats, animal protein, fish oil, soybean oil.	Bird, fish, ruminants, soybeans, corn.

3	poultry, corn, other plant species, animal fats, animal protein, fish oil, soybean oil.	Bird, fish, ruminants, pork, soybean, corn.
4	poultry, corn eggs, other vegetable species, animal fats, animal proteins, fish oil, soybean oil.	Bird, fish, ruminants, soybeans, corn.
5	poultry, corn, other plant species, animal fats, animal proteins, yeasts, soybean oil.	Bird, ruminants, soybeans, corn.
6	poultry, corn, other vegetable species, animal fats, animal proteins, fish oil, yeasts, soybean oil.	Bird, fish, ruminants, soybeans, corn.
7	fish, poultry, corn, other vegetable species, animal fats, eggs, fish oil, soybean oil.	Bird, fish, ruminants, pork, soybean, corn.
8	poultry, other plant species, animal fats, animal proteins, yeasts.	Bird, ruminants, corn.
9	poultry, corn, other vegetable species, animal fats, animal proteins, fish oil, yeasts.	Bird, fish, pig, horse, corn.
10	fish, other vegetable species, animal fats, animal proteins, fish oil, yeasts, soybean oil.	Bird, fish, horse, soybean.
11	poultry, fish, whole grains, other vegetable species, animal fats, animal proteins, fish oil, soybean oil.	Bird, fish, ruminants, soybeans, corn.
12	poultry, corn, other plant species, animal fats, animal protein, soybean oil.	Bird, ruminants, pig, horse, soybean, corn.
13	poultry, corn, other vegetable species, animal fats, animal proteins, fish oil,yeasts, soybean oil.	Bird, fish, ruminants, soybeans, corn.
14	poultry, corn, other vegetable species, animal fats, animal proteins, fish oil, yeasts, soybean oil.	Bird, fish, horse, soybean, corn.
15	corn, other vegetable species, animal fats, animal proteins, fish oil, soybean oil.	Fish, soybeans, corn.
16	poultry, corn, other vegetable species, animal fats, animal proteins, fish oil, yeasts, soybean oil.	Fish, soybeans, corn.
17	poultry, corn, other vegetable species, animal fats, animal proteins, fish oil, yeasts, soybean oil, algae.	Bird, fish, soybeans, corn.
18	lamb, bird, other vegetable species, bird fat, fish oil, yeasts, crustaceans.	Bird, fish, ruminants, horse.
19	lamb, bird, other vegetable species, bird fat, fish oil, yeasts, crustaceans.	Bird, ruminants, horse.
20	lamb, bird, other vegetable species, bird fat, fish oil, yeasts, crustaceans.	Bird, ruminants.

As for bird species, they are mentioned on 19 of the 20 analyzed product labels, and the detection was consistent with them, either as chicken or turkey.

Poultry genetic material is missing in sample number 15 but is not specified on the product label either. In all other 19 samples, bird species were detected, according to the product label, in a percentage of 95%.

Fish is also used a lot in pet food, both as a main ingredient (for cat food) and as an additive, an excellent source of vitamin D and fatty acids in the form of oil. Fish species are mentioned on 16 of the 20 product labels analyzed, and the detection was consistent with them, whether as sardines, mackerel or salmon. Fish genetic material is missing in samples numbered 5, 8, 12, 19, and 20, but in these cases, they are not specified on the product label either. In all the other 15 samples, fish species were detected, according to the product label, in a percentage of 75%.

Regarding ruminant species (cattle, goats, sheep), pigs and horses, no label mentions their specific presence, except for three products numbered 18, 19 and 20 that contain lamb meat. However, they can be present as by-products. Thus, the genetic material from the pig species was detected in 25% of the cases, the ruminants in 30% of the cases, specifying that half of them have a formula based on lamb meat, and the genetic material from the horse was detected at a very low level but still present in 30% of cases.

Conclusions

Molecular analyses based on the detection of nucleic acids can be successfully used to identify the species that enter the composition of pet food, allowing the correctness of the label to be accurately established. The standardisation of these methods, to be used in the authentication of commercial products, is necessary, as these methods are also applicable to highly processed products.

Plant species are present in all the analyzed products, a large share, such as corn and soy. Poultry meat products are the most used in the composition of the analyzed products; they are detected in 80% of the samples and are correctly labelled. Fish meat products are also widely used, especially in cat food. The percentage of 75% is also reached by the use of fish oils in the composition of these products.

Products from ruminants are present in a high percentage, both as the main component and as animal protein or fat sources. Pig and horse products were detected in a reasonably low percentage and level; they are not mentioned on the labels and can be classified as animal protein and fat used in the composition of the analyzed pet food.

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IDENTIFYING ANIMAL AND VEGETAL SPECIES AND INCORRECT LABELLING IN PET FOODS

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Summary

Mislabelling, falsifying and substituting food products is a growing problem in highly processed foods, including pet foods. Although there is a regulation for pet food, there needs to be more information on the prevalence of improper labelling of pet food. The study aimed to conduct market surveys of pet foods sold by specialised pet stores to identify the present animal and vegetal species and any instances of incorrect labelling. Twenty commercial dry products were collected from specialised pet stores. DNA was extracted from each product in duplicates, and the component species were identified using polymerase chain reactions (PCR). Species-specific analyses were used to verify the existence of meat species (bovine, pigeon, pig, chicken, turkey, pork and horse). Plant species were present in all the products analysed, a large share, in this case, corn and soybeans. Poultry meat products are the most used in the composition of the analysed products. They are detected in 80% of the samples and are correctly labelled.

Fish meat products are also widely used, especially in cat food. The percentage of 75% is also reached by using fish oils in the composition of these products. Products from ruminants are present in a high percentage, both as the main component and as animal protein or fat sources. Pig and horse products were detected in a reasonably low percentage and level. They are not mentioned on the labels and can be classified as animal protein and fat used in the composition of the analysed pet food. Although these results indicate that pet food labels are misleading, more studies are needed to determine the extent of misleading labels and identify the points in the production chain where misleading labels occur.

Keywords: pet food, mislabelling, species composition, DNA-based screening.

Products intended for feeding animals include various types of dry or wet feed, nutritional supplements, vitamins, minerals and other food additives.

These products are formulated to provide the nutrients necessary for the growth, development and maintenance of animal health, depending on the species, age, weight and physiological stage.

They can be made from ingredients of plant, animal or synthetic origin, and some products are specially formulated to meet the specific nutritional needs of certain animals, as the composition of food is a crucial aspect in maintaining a healthy life for small animals such as dogs and cats. Food influences the health, longevity, energy and behaviour of these pets. It is important to pay attention to the composition

of the feed to ensure that the animals receive all the necessary nutrients to maintain their health and enjoy an active and energetic life (17). Consumer trends are moving towards healthier, more natural food options (6, 21). Official agencies must, therefore, again enforce guaranteed compliance with food labelling, nutritional quality and food origin, as well as the perception of health and diet claims to avoid false claims by food manufacturers.

Legislation regarding adulteration of pet food varies by country and region. Regulatory authorities such as the FDA (United States) or ANSVSA (Romania) can set and regulate minimum standards for the quality of pet food.

Pet food quality standards vary but generally relate to food products' nutritional composition, safety and labelling. These standards may include requirements on the proportions of protein, fat and carbohydrates, including the specific types of ingredients used, such as meat, grains and vegetables. Standards may also set maximum level requirements for toxic or potentially harmful substances such as contaminants and food additives (20). They can also include labelling requirements, such as a full list of ingredients and detailed nutritional information, so pet owners can make informed choices about the food they buy.

Economically motivated adulteration was defined in 2009 by the Food and Drug Administration (FDA - USA) as "the intentional fraudulent substitution or addition of a product to increase the apparent value of the product or reduce the cost of its production"(3). It can often cover the effects of public safety by the unknown addition of allergens, toxins and hygiene risks (11). Food authenticity testing is important to rule out unfair competition to confirm labelling and product value. It is also essential to ensure consumer protection, both against fraud and to confirm the quality of the product. The pet food market is constantly developing, but the most popular are maintenance foods, which are over-the-counter diets. Chicken-based ingredients are commonly used in pet food. They are highly palatable, relatively inexpensive, and provide an excellent source of protein. However, individual products vary widely in nutrient composition and processing conditions that can affect protein quality and digestibility (15). For most dogs, chicken or ruminant meat is easy to digest, but its widespread use in dog food has probably led to more frequent allergies to this ingredient (20). Currently, protein detection in foodstuffs and feeds is based on several methods. For example, commercial animal feed contains herbicides (glyphosates) residues detected in each tested product using enzyme-linked immunosorbent tests (ELISAs). However, it was shown that the ELISA-based method was less sensitive and precise than the commercial canine food PCR method and that it was impossible to fully identify the presence of animal by-products (11).

DNA analysis techniques can detect feed adulteration by identifying the presence or absence of specific animal species such as pork, chicken or beef (16).

Materials and methods

The reference material in this project used, as a positive reaction control, suspensions of DNA isolated from various matrices from the collection of the Laboratory of Molecular Genetics within the Interdisciplinary Research Platform and from the collection of Laboratory A2 within the Complex of Research Laboratories – Horia Cernescu of USV, Timișoara. The plant reference material was represented by DNA solutions isolated from corn (Zea mays L.) and soybean (*Glicine max* L.) flours. The reference material of animal origin was represented by DNA solutions isolated from dried and ground muscle tissue from the species for which the detection experiments were performed: chickens, fish, ruminants, pigs and horses according to the methods described by Boldura et al., 2011.

The biological material is represented by 20 samples of dry food intended for dogs and cats, purchased from speciality stores on the domestic market.

Table 1

The analyzed products and the description of their labels

Sample No.	Label description
1	poultry, corn, other plant species, animal fats, animal proteins, yeast, fish oil, soybean oil.
2	poultry, corn, other plant species, animal fats, animal protein, fish oil, soybean oil.
3	poultry, corn, other plant species, animal fats, animal protein, fish oil, soybean oil.
4	poultry, corn eggs, other vegetable species, animal fats, animal proteins, fish oil, soybean oil.
5	poultry, corn, other plant species, animal fats, animal proteins, yeasts, soybean oil.
6	poultry, corn, other vegetable species, animal fats, animal proteins, fish oil,yeasts, soybean oil.
7	fish, poultry, corn, other vegetable species, animal fats, eggs, fish oil, soybean oil.
8	poultry, other plant species, animal fats, animal proteins, yeasts.
9	poultry, corn, other vegetable species, animal fats, animal proteins, fish oil,yeasts.
10	fish, other vegetable species, animal fats, animal proteins, fish oil,yeasts, soybean oil.
11	poultry, fish, whole grains, other vegetable species, animal fats, animal proteins, fish oil, soybean oil.
12	poultry, corn, other plant species, animal fats, animal protein, soybean oil.
13	poultry, corn, other vegetable species, animal fats, animal proteins, fish oil,yeasts, soybean oil.

14	poultry, corn, other vegetable species, animal fats, animal proteins, fish oil,yeasts, soybean oil.
15	corn, other vegetable species, animal fats, animal proteins, fish oil, soybean oil.
16	poultry, corn, other vegetable species, animal fats, animal proteins, fish oil, yeasts, soybean oil.
17	poultry, corn, other vegetable species, animal fats, animal proteins, fish oil, yeasts, soybean oil, algae.
18	lamb, bird, other vegetable species, bird fat, fish oil,yeasts, crustaceans.
19	lamb, bird, other vegetable species, bird fat, fish oil, yeasts, crustaceans.
20	lamb, bird, other vegetable species, bird fat, fish oil, yeasts, crustaceans.

Primer pairs used for species identification in this screening study and their bibliographic references are described in Table 2. They were selected from the collection of the A2 laboratory within the Complex of Research Laboratories – Horia Cernescu of ULS, Timișoara and were synthesised by Novogene, Amsterdam, Netherlands.

Table 2

PCR primers used in this project

Code	Species	Sequence 5'…3'	Reference
CW/CX	gena Rubisco - material vegetal	CGTAGCTTCCGGTGGTATCCACGT GGGGCAGGTAAGAAAGGGTTTCGTA	(22)
HA-nos	Lectina - soia	GCATGACGTTATTTATGAGATGGG GACACCGCGCGCGATAATTTATCC	(23)
zein3-zein4	Zeina - porumb	AGTGCGACCCATATTCCA GACATTGTGGCATCATCATT	(23)
Poultry/12S rRNA	pasăre	TGAGAACTACGAGCACAAAC GGGCTATTGAGCTCACTGTT	(9)
Fish/12S rRNA	pește	TAAGAGGGCCGGTAAAACTC GTGGGGTATCTAATCCCAG	(9)
Cattle/16S rRNA	rumegătoare	TAAGAGGGCCGGTAAAACTC GTGGGGTATCTAATCCCAG	(9)
Swine/12S rRNAtRNA	porc	CTACATAAGAATATCCACCACA ACATTGTGGGATCTTCTAGGT	(9)
Horse/mtDNA	cal	CCATCCCTACGCTCCATTCCC TGTTTTCGATGGTGCTTGCG	(4)

Materials and methods

DNA extraction and evaluation

Isolation and purification of DNA from the samples taken in the study was carried out with the help of the kit "NucleoSpin DNA Forensic" (Macherey-Nagel,

Düren, Germany), following the manufacturer's instructions. The DNA solution was qualitatively and quantitatively evaluated by the spectrophotometric method using the NanoDrop 8000 equipment (ThermoScientific, Waltham, Massachusetts, US), after quantification the samples were brought to the same concentration – 100 ng/ µl, by dilution.

End-point PCR analyses

PCR reactions were performed using the Surecycler Thermocycler, Agilent Technologies, Santa Clara, CA, US. The reagents that made up the amplification mixture were as follows: PCR kit: GoTaq Green Master Mix (Promega, Oregon, US)- 12.5 µl, 20 pmol of each primer, template DNA – 1 µl, adjusted with distilled water up to 25 µl. Amplification conditions were according to literature data and consisted of an initial denaturation step at 93°C for 3 min.; 35 cycles with 93°C – 30 sec., 58 – 60 °C – 30 sec. and 72°C – 45 sec.; a final extension step of 72°C for 3 min.

Agarose gel electrophoresis

PCR products were migrated in agarose gel in the presence of ethidium bromide and visualised under UV light. The gel image was captured and analysed using Vision Works software (UVP, Analytik Jena, Germany).

Results and discussions

In context, protein-based methods for species identification can fail after excessive proteolysis or heat-induced denaturation of indicator proteins. It is suggested that genomic DNA from milk somatic cells persists in the processed material and can be amplified and analyzed for species identification. Also, many of these methods are based on the properties of the proteins found in the meat of the species from which the raw material was taken. The identified methods are electrophoretic, chromatographic, immunological and molecular (1, 12, 23). These are just some of the methods used to detect the composition of pet food. Other methods can be used, depending on the needs and resources of each animal food production company, and these techniques based on DNA analysis appear to be the most accurate, rapid and reproducible (4, 13).

Food safety is an extremely important issue related to animal health. Both raw materials and food ingredients must be constantly monitored because some of the substances present in food are pollutants of anthropogenic origin, while others originate from food processing or storage. Unfortunately, food counterfeiting is a common phenomenon often encountered in the economic environment, where it causes unfair competition (7, 9). It also reduces food quality and can even pose a health risk. Both consumers and industry are calling on the research community to implement tests to help analyze food authenticity. The above-mentioned issues compel food safety and quality scientists to constantly seek new approaches and tools to address the main issues related to food quality, safety, and authenticity (7, 8). Recently, many techniques have been developed to trace food through DNA barcoding techniques, even for complex and highly processed mixtures such as pet

food. These techniques can identify adulteration in pet food, a growing problem among pet owners (5, 10). Properly selected methods can also provide a way to confirm that the pet food is truly consistent with what is described by manufacturers on labels or packaging. Given the importance of accurate pet food labelling, especially for animals with mild or life-threatening allergies, accurate detection of mislabeled or undeclared animal species is important for pet safety (18, 19).

Furthermore, wide variations among commercially available diets marketed to support the treatment of various medical conditions, such as skin and coat health or allergies, can confuse caregivers during diet selection (14). One of the problems with pet food mislabeling is the ingredient list itself. Generally or intentionally vague ingredients are often given (2, 3). Commission Regulation no. 767/09 allows listing ingredients by category in pet food (19). Despite existing mandatory traceability requirements for pet food, it has previously been reported that labels do not provide sufficient information about the sources of different nutrients in the product.

The combination of materials of vegetable and animal origin in the composition of pet food refers to the use of plant-based and animal-based ingredients in the formulation of pet food products. Considering their dietary requirements, this approach aims to provide pets with a balanced and nutritious diet. Plant-based ingredients commonly used in pet food include grains (such as rice, corn, and wheat), vegetables (like carrots, peas, and sweet potatoes), fruits, and various plant proteins (such as soybean meal or pea protein). These ingredients can provide a source of carbohydrates, fiber, vitamins, and minerals. Animal-based ingredients used in pet food typically include meat, poultry, fish, and their by-products. These ingredients are rich in high-quality proteins, essential fatty acids, and other vital nutrients for pets' overall health and well-being. Combining vegetable and animal ingredients allows pet food manufacturers to create a well-rounded and nutritionally complete diet for pets. By incorporating various ingredients from different sources, they can balance essential nutrients, including proteins, carbohydrates, fats, vitamins, and minerals, which are vital for pet optimal health and development. In this context, the first stage of the presented experiment was represented by a screening that allowed the identification of the material of plant origin by using its specific markers, namely for the ribulose-1,5-bisphosphate-carboxylase (RuBisCo) gene (Fig. 1, panel A). By identifying this gene, it was possible to certify the presence of material of plant origin and the amplifiable quality of the obtained DNA. The most frequently encountered plant species in the composition of pet food are soy and corn, thus the first screening analyses had in mind the highlighting of the presence of plant material originating from soy (Glycine max L., (Fig. 1, panel B) and of the plant material of corn (*Zea mays* L.), the presence of the latter being noted on most of the analyzed product labels both as a source of carbohydrates (corn starch) and as a source of protein (hydrolyzate) and is frequently used as an ingredient in pet food (Fig. 1, panel C) Being a qualitative analysis the results are interpreted only by the ***present/absent*** score.

From the analysis of Fig.1, panel B, it follows that the soybean-specific genetic material was at a level detectable by this analysis for 15 of the 20 analyzed samples. The presence of an amplification product with a molecular size of 118 bp certifies the presence of this species in the analyzed product. In the composition of five products, soy was impossible to detect by this method, namely samples 8, 9, 18, 19 and 20. Also from Fig. 1, panel C, it is found that the genetic material specific to maise was at a level detectable by this type of qualitative analysis for 16 of the 20 analyzed samples. The presence of an amplification band with a molecular size of 227 bp certifies the presence of this species in the analyzed product. In the composition of four products, maize was impossible to detect using this method, namely samples 10, 18, 19 and 20. The accuracy of the label in declaring the presence of these plant species in the composition of the products is shown in Table 3.

The following analyses were carried out to identify the presence of animal species in the composition of the analyzed pet food. The labels of all analyzed products mentioned the presence of material of animal nature, more or less processed, such as dried meat, animal fat, hydrolyzed animal protein or fish oils. The most frequently used species in the analyzed products were poultry, which can be used as meat by-products or eggs under different processing degrees. Notably, pork, poultry and beef are the animal species most commonly slaughtered in Europe for human consumption [28]. Thus, they provide the largest amount of animal by-products to pet food manufacturers. Accordingly, the most common animal species not declared on pet food labels appear to be pork, chicken and turkey.

The bird species included in this screening are hen (*Gallus gallus* L.) and turkey (*Meleagris meleagridis* L.). In the case of gene screening specific to bird species, PCR amplification, analysis of PCR products and interpretation of the results are carried out in the same way as in the case of plant species screenings (Fig. 2, panel A). In one of the largest studies reported in the literature (number of foods tested = 52), chicken was the most common meat species found in almost all (98%) pet foods tested (3).

The presence of biological material from fish required screening for the following species: sardines, *Sardinops melanostictus*, *Sardinella hualiensis* and *Pagrus major*, mackerel, *Tracurus japonicas* and salmon, *Salmo salar*. In the case of screening genes specific to fish species, the PCR amplification, the analysis of the PCR products and the interpretation of the results are carried out in the same way as in the case of the screening of the bird species. Fish-specific primers were designed from the 12S rRNA mitochondrial DNA region, generating amplification sequences of 228 base pairs (Fig. 1, panel B). It is found that the genetic material specific to the fish was at a detectable level through this type of qualitative analysis for 15 of the 20 analyzed samples. The presence of an amplification band with a molecular size of 228 bp certifies the presence of fish species in the analyzed products. In the composition of five of the products, genetic material from fish was not detected using this method, namely in samples 5, 8, 12, 19 and 20.

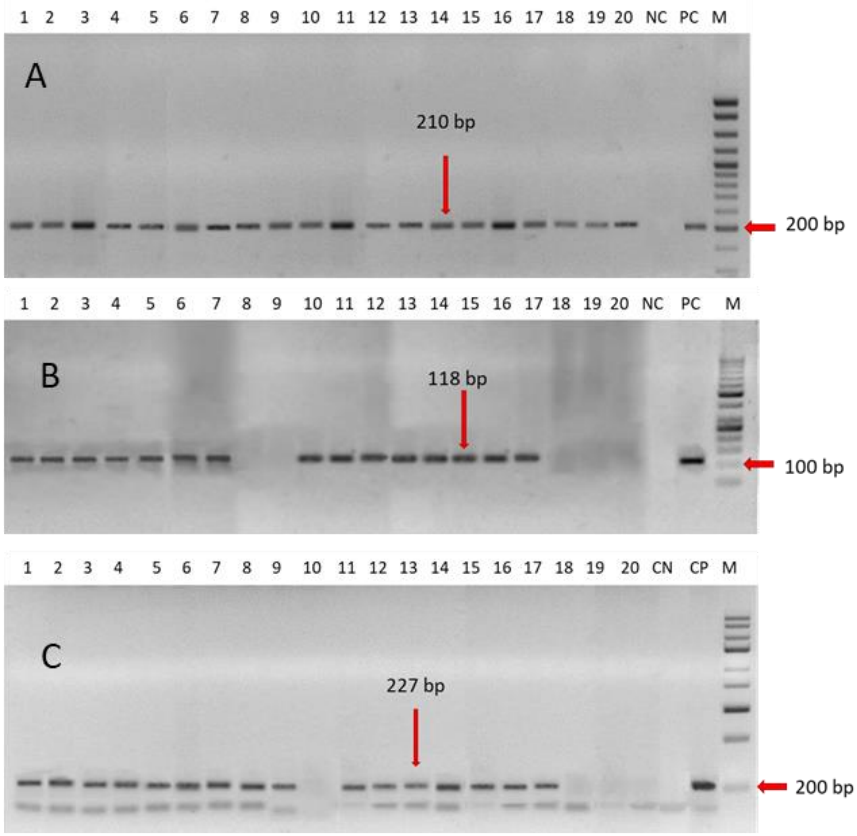

Fig. 1. The results of the PCR analysis using gene-specific primers from the plant material

Panel A - RuBisCo gene; Panel B – lectin gene; Panel C – zein gene. Lanes 1 – 20 Amplification products for the biological samples analyzed in this experiment; NC – negative reaction control (DNA isolated from material of animal origin); PC – positive reaction control – DNA solution isolated from material of vegetable origin (soy flour or corn) M – molecular weight marker for nucleic acids – PCR marker (Promega)

The following analyses were performed to detect some species not mentioned on the labels of the products analyzed in this paper. For this type of screening, three animal species were chosen that are frequently found in human food and whose by-products can easily end up being used in pet food as well. They may also be the source of those animal fats or proteins listed but not explicitly stated on the product label.

From this category, the first screening performed was to identify the presence in the composition of the products of genetic material from ruminants, namely: cattle - *Bos taurus*, goats - *Capra hircus*, and sheep - *Ovis aries*. Ruminant-specific primers were designed from their mitochondrial 16S rRNA DNA region, and generate amplification sequences of 204 base pairs, and can be successfully used on DNA samples degraded due to their exposure to high temperatures, as indicated by European laws regarding thermal processing of ingredients of animal origin (Fig 2, panel C). Even ruminants are not generally a species used for preparing pet food; it can be found in combinations, especially in the case of dog food. Thus, in this experiment, three samples were analyzed that were labelled as a mixture of lamb and poultry, which were intended for feeding dogs.

The analysis reveals that in 14 of the 20 analyzed samples, the level of specific genetic material is detectable by qualitative analysis for ruminants. The presence of an amplified band with a molecular size of 204 bp proves the presence of ruminant species in the analyzed material. This method detected no genetic material specific to ruminants in the composition of six products (samples 9, 10, 14, 15, 16 and 17).

A wide range of pet food products are commercially available, each with its ingredients and nutritional attributes. One of these ingredients is pork by-products, which can be found in many pet food products. However, there are many questions about the quality and safety of these products, but it is accepted that pork by-products are no less safe than any other source of protein for pets. However, some products may contain lower-quality by-products, such as tissue containing hormones or antibiotics, which may harm the animal's health (17). Considering all this, the products that can be purchased on the market do not specify the presence of material from the pig. Thus, the following analysis aimed to highlight the presence of pork by-products in the biological samples taken. In the case of the screening for the detection of components originating from the pig (*Sus scrofa*), primers were used that target the molecular markers of the genes specific to the pig (Fig. 2, panel D). In five of the 20 samples analyzed (1, 3, 7, 9 and 12), pig-specific genetic material was present at levels detectable by this type of qualitative analysis.

The presence of an amplified band with a molecular size of 204 bp demonstrates the presence of pork by-products in the analyzed products. In the case of the samples where the result was present, the genetic material belonging to the analyzed species was found to be relatively low compared to the positive control, as indicated by the intensity of the bands detected in the gel electrophoresis images. This indicates that the addition of by-products from this species is very low, most likely only traces to increase the animal fat content of the products.

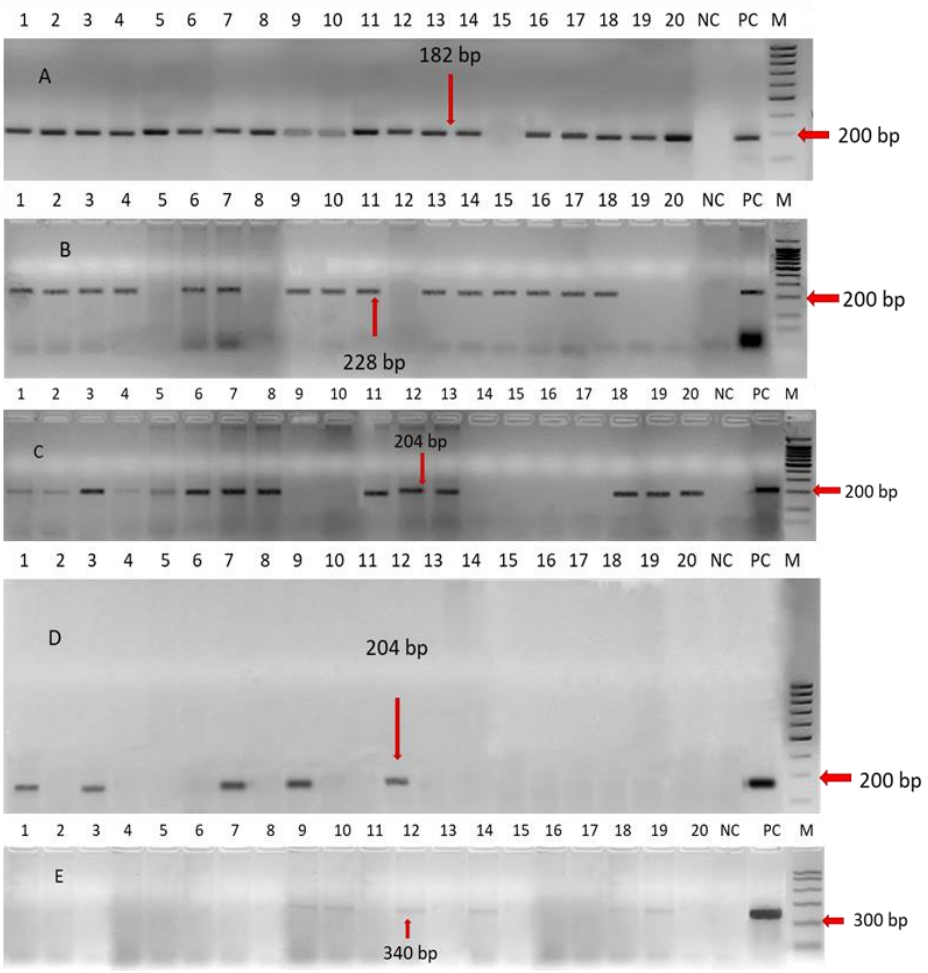

Fig. 2. The results of the PCR analysis using gene-specific primers from the animal material

Panel A - chicken; Panel B – fish; Panel C – ruminants; Panel D – pork; panel C - horses. Lanes 1 – 20 Amplification products for the biological samples analyzed in this experiment; NC – negative reaction control (DNA isolated from plant material); PC – positive reaction control – DNA solution isolated from material of animal origin (specific to each analyzed species – reference materials) M – molecular weight marker for nucleic acids – PCR marker (Promega)

One of these ingredients is pork by-products, which can be found in many pet food products. However, there are many questions about the quality and safety

of these products, but it is accepted that pork by-products are no less safe than any other source of protein for pets. However, some products may contain lower-quality by-products, such as tissue containing hormones or antibiotics, which may be harmful to the animal's health (12). Considering all this, in general, the products that can be purchased on the market, do not specify the presence of material from the pig. Thus, the following analysis aimed to highlight the presence of pork by-products in the biological samples taken. In the case of the screening for the detection of components originating from the pig (Sus scrofa), primers that target the molecular markers of the genes specific to the pig were used (Fig. 2, panel D). In five of the 20 samples analyzed (1, 3, 7, 9 and 12), pig-specific genetic material was present at levels detectable by this type of qualitative analysis. The presence of an amplified band with a molecular size of 204 bp demonstrates the presence of pork by-products in the analyzed products. In the case of the samples where the result was present, the genetic material belonging to the analyzed species was found to be relatively low compared to the positive control, as indicated by the intensity of the bands detected in the gel electrophoresis images. This indicates that the addition of by-products from this species is very low, most likely only traces to increase in the animal fat content of the products.

The last analysis had as a screening target the detection of the genetic material from the horse (*Equus ferus caballus*), a product not specified on the label but which, due to the slaughter of this species, may accidentally be found in the composition of products intended for feeding pets (Fig. 2, panel E). It is noted that small traces of genetic material of the target species were detected in six of the 20 analyzed samples (9, 10, 12, 14, 18 and 19). The presence of an amplified band with a molecular size of 340 bp demonstrates the presence of horse by-products in the analyzed products.

In the case of the samples where the result was present, the genetic material belonging to the analyzed species was very low compared to the positive control, as indicated by the intensity of the bands detected in the gel electrophoresis images. This indicates that adding by-products from this species is very low, most likely only traces to increase the products' protein or animal fat content.

The results of this screening were systematized and tabulated in Table 3.

Table 3

Results obtained from the screening study for species identification

Sample	Tagged species	Species identified
1	poultry, corn, other plant species, animal fats, animal proteins, yeast, fish oil, soybean oil.	Poultry, fish, ruminants, pigs, soybeans, corn
2	poultry, corn, other plant species, animal fats, animal protein, fish oil, soybean oil.	Bird, fish, ruminants, soybeans, corn.

3	poultry, corn, other plant species, animal fats, animal protein, fish oil, soybean oil.	Bird, fish, ruminants, pork, soybean, corn.
4	poultry, corn eggs, other vegetable species, animal fats, animal proteins, fish oil, soybean oil.	Bird, fish, ruminants, soybeans, corn.
5	poultry, corn, other plant species, animal fats, animal proteins, yeasts, soybean oil.	Bird, ruminants, soybeans, corn.
6	poultry, corn, other vegetable species, animal fats, animal proteins, fish oil, yeasts, soybean oil.	Bird, fish, ruminants, soybeans, corn.
7	fish, poultry, corn, other vegetable species, animal fats, eggs, fish oil, soybean oil.	Bird, fish, ruminants, pork, soybean, corn.
8	poultry, other plant species, animal fats, animal proteins, yeasts.	Bird, ruminants, corn.
9	poultry, corn, other vegetable species, animal fats, animal proteins, fish oil, yeasts.	Bird, fish, pig, horse, corn.
10	fish, other vegetable species, animal fats, animal proteins, fish oil, yeasts, soybean oil.	Bird, fish, horse, soybean.
11	poultry, fish, whole grains, other vegetable species, animal fats, animal proteins, fish oil, soybean oil.	Bird, fish, ruminants, soybeans, corn.
12	poultry, corn, other plant species, animal fats, animal protein, soybean oil.	Bird, ruminants, pig, horse, soybean, corn.
13	poultry, corn, other vegetable species, animal fats, animal proteins, fish oil,yeasts, soybean oil.	Bird, fish, ruminants, soybeans, corn.
14	poultry, corn, other vegetable species, animal fats, animal proteins, fish oil, yeasts, soybean oil.	Bird, fish, horse, soybean, corn.
15	corn, other vegetable species, animal fats, animal proteins, fish oil, soybean oil.	Fish, soybeans, corn.
16	poultry, corn, other vegetable species, animal fats, animal proteins, fish oil, yeasts, soybean oil.	Fish, soybeans, corn.
17	poultry, corn, other vegetable species, animal fats, animal proteins, fish oil, yeasts, soybean oil, algae.	Bird, fish, soybeans, corn.
18	lamb, bird, other vegetable species, bird fat, fish oil, yeasts, crustaceans.	Bird, fish, ruminants, horse.
19	lamb, bird, other vegetable species, bird fat, fish oil, yeasts, crustaceans.	Bird, ruminants, horse.
20	lamb, bird, other vegetable species, bird fat, fish oil, yeasts, crustaceans.	Bird, ruminants.

As for bird species, they are mentioned on 19 of the 20 analyzed product labels, and the detection was consistent with them, either as chicken or turkey.

Poultry genetic material is missing in sample number 15 but is not specified on the product label either. In all other 19 samples, bird species were detected, according to the product label, in a percentage of 95%.

Fish is also used a lot in pet food, both as a main ingredient (for cat food) and as an additive, an excellent source of vitamin D and fatty acids in the form of oil. Fish species are mentioned on 16 of the 20 product labels analyzed, and the detection was consistent with them, whether as sardines, mackerel or salmon. Fish genetic material is missing in samples numbered 5, 8, 12, 19, and 20, but in these cases, they are not specified on the product label either. In all the other 15 samples, fish species were detected, according to the product label, in a percentage of 75%.

Regarding ruminant species (cattle, goats, sheep), pigs and horses, no label mentions their specific presence, except for three products numbered 18, 19 and 20 that contain lamb meat. However, they can be present as by-products. Thus, the genetic material from the pig species was detected in 25% of the cases, the ruminants in 30% of the cases, specifying that half of them have a formula based on lamb meat, and the genetic material from the horse was detected at a very low level but still present in 30% of cases.

Conclusions

Molecular analyses based on the detection of nucleic acids can be successfully used to identify the species that enter the composition of pet food, allowing the correctness of the label to be accurately established. The standardisation of these methods, to be used in the authentication of commercial products, is necessary, as these methods are also applicable to highly processed products.

Plant species are present in all the analyzed products, a large share, such as corn and soy. Poultry meat products are the most used in the composition of the analyzed products; they are detected in 80% of the samples and are correctly labelled. Fish meat products are also widely used, especially in cat food. The percentage of 75% is also reached by the use of fish oils in the composition of these products.

Products from ruminants are present in a high percentage, both as the main component and as animal protein or fat sources. Pig and horse products were detected in a reasonably low percentage and level; they are not mentioned on the labels and can be classified as animal protein and fat used in the composition of the analyzed pet food.

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COMPARISON OF THE PROTEIN PROFILE AND MILK SUGARS OF DONKEY'S MILK WITH THAT OF HUMAN MILK

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Summary

Donkey milk is considered a potential substitute for human milk for infants affected by cow's milk protein allergy. In order to expand our knowledge about this valuable food, we explored the protein profile of donkey milk using the ELFO technique by comparing it with that of human milk, and the milk sugars were detected by the Brix method. Donkey milk showed a protein and sugar content similar to human milk and essentially different from cow's milk. The results of the performed analysis indicated that the protein profile of donkey's milk is more similar to human milk than that of cow's milk-based formulas, with higher content in sugars and lower in proteins. The total protein content is approximately at the same level in donkey and human milk, 1.14 g/dl and 1.1 g/dl, respectively, while cow's milk contains 2.8 g/dl. Also, β-caseins and κ-caseins are absent in donkey and human milk. Lysozyme content is slightly higher in donkey milk (0.12 g/dl compared to 0.1 g/dl), and lactoferrin is present in a slightly lower amount in donkey milk (0.2 g/dl) compared to human milk (0.24 g/dl). The two protein fractions are missing from cow's milk.

Keywords: donkey milk, protein profile, sugar content, ELFO technique.

The donkeys belong to the order *Perissodactyla* and are ungulates of the *Equidae* family, which consist of a single genus *Equus*, in turn, divided into five subgenres. There are more than 40 million donkeys worldwide, mostly in developing countries, where they are mainly used as animals for transportation. But in the last decade, there has been an increase in the number of donkeys, and they are bred as dairy animals (12,13). The advantages of using donkey milk have attracted considerable interest in its use for human consumption.

Donkey milk is white in colour with a slightly sweet taste with a very low number of somatic cells and germs (NTG) (19). The protein composition of donkey milk is significantly different from cow's milk: the total content is lower (1.5-1.8 g / 100g), and quite similar to that of human milk and mare milk (8). The higher proportion of whey protein in donkey milk by 35–50%, compared to only 20% of bovine milk, makes it more easily absorbed by the human body (9). The significant components of whey proteins present in donkey milk include β-lactoglobulin, α-lactalbumin, lactoferrin, serum albumin, lysozyme and immunoglobulins, and other

proteins of low abundances, such as enzymes and proteins that bind metals (8). As one of the most important milk allergens, β-lactoglobulin accounts for less than 30% of all whey protein, which has been suggested to be lower than that of bovine milk (>50%) but higher than that of human milk (10). The second major whey protein, α-lactalbumin, is a small-molecule protein that binds to calcium ions, with a very close amount of donkey milk to human milk and works with anti-inflammatory, antiviral and anti-tumor properties (15, 21). Furthermore, a high content of lysozyme is present in donkey milk (0.67-4,00 g/kg), which is much higher than that of human milk (0.04 to 0.5 g/kg) and bovine milk (17). Along with immunoglobulins, lactoferrin and lactoperoxidase, lysozyme works as one of the most important antimicrobials to reduce harmful bacteria in donkey's milk and the incidence of gastrointestinal infections in infants. Donkey milk has a high content of lactose (5.8-7.4), which is higher than in cow's milk and much more similar to human milk (1). The high lactose content also stimulates intestinal absorption of calcium and probiotic activity (17) because it is an ideal substrate for developing intestinal lactobacilli. Lactose gives a good taste to donkey milk and is also a precious source of galactose, which is essential for developing the nervous system (12). Donkey milk is lower in fat compared to human milk (12, 17). The pH of donkey milk and human milk is neutral or slightly alkaline, possibly due to the low content of casein and phosphates, compared to cow's milk. The pH value is around 7.2, slightly higher than in cow's milk (6.7), but closer to human milk (7.3) and mare's milk (7.18). This value varies depending on the period of lactation (13). Milk is the primary source of minerals. Donkey milk is also a rich source of minerals such as Ca, P, Na, K, Mg, Fe, Zn, Cu (). In donkey milk, except vitamin B3), thiamine (vitamin B1), riboflavin (vitamin B2), cobalamin (B12) and vitamin C are in more significant quantities than in human milk (4). Given its functional properties and nutritional values, donkey milk has become useful for health and the cosmetic industry. The effects of donkey milk consumption on health are related to low allergenicity, antimicrobial activity, iron homeostasis regulation, anti-inflammatory activity and modulation of the immune system, antihypertensive, antidiabetic, antitumor, stimulates development, anti-stress and anti-oxidant activity, and anti-osteoporosis (6, 7). Several studies show that donkey milk is a suitable alternative for children suffering from allergies to cow's milk proteins due to its low composition in casein, which is the main allergenic component of milk. Sarti et al., (15) showed that donkey milk does not negatively influence infants and children.

Materials and methods

Biological material

The milk samples from *E. asinus* and *Bos taurus* species were collected from authorised farms in Arad County, and the human milk sample was collected under a donation agreement by the subject in a volume of approximately 2 ml each. The milk samples were taken in sterile and sealed containers, frozen immediately

after collection, transported and stored under conditions that ensured the integrity of the proteins.

Sample preparation for analysis

The milk samples were thawed and homogenised on the analysis day by strong vortexing for 10 minutes. Initially, the milk samples were centrifuged at 3000 revolutions per minute for 15 minutes to remove fat from the sample according to the protocol described by Tudor et al., 2021.

ELFO type analysis

On-chip protein electrophoresis was performed on Bioanalyzer 2100 (Agilent Technologies, Santa Clara, US) Agilent patented and standardised technology following the laboratory standard developed under the guidance of the manufacturer of the analysis kit (16), using the Protein 80 kit, which uses electrophoresis with microfluidic capillary gel with laser-induced fluorescence (LIF) detection. The method involves obtaining the protein fingerprints on virtual support by recording the fluorescence, establishing the electropherograms and converting them according to the protein standards into numerical, quantifiable values (5).

Quantification of the content of sugars and dry matter by the Brix method.

The sugar content of milk was quantified using the refractometric Brix method, which is designed to measure the percentages in a liquid solution and express them in "degrees Brix". One degree of Brix is defined as 1 gram of sugars in 100 grams of solution. The analysis was performed with the BRIX refractometer - model DR201-95, KRUSS *Optronic, Germany.* Brix readings should always be taken using raw, unhomogenised whole milk or colostrum before pasteurisation. However, it was stated that the Brix technique practically evaluates the dry substance of whole milk, and for a relative quantification of it to the value obtained by refractometry, Brix is added with the value of two. In other words, a Brix refractometer reading of 10 would indicate a total solid estimate of 12 per cent, the 10 being the relative lactose content. Brix readings were taken using raw milk, following the following protocol indicated by the manufacturer.

Results and discussions

Even though a large amount of information was related to the characterisation of the proteins of donkey milk, the proteomics of donkey milk in the main producing areas of the world has yet to be well documented. The composition of protein, including caseins and whey protein in donkey milk, may be expressed differently between products from different zones or countries or between samples from different zones, which makes it difficult to standardise working methods (14).

The biochemical composition of mammalian milk is significantly different depending on the species. In the milk of ruminants, there are six types of significant proteins encoded by six non-polar genes, which are explicitly expressed in the epithelial cells of the mammary gland in lactation: αS1- casein (αS1-CN), -casein (β-

CN), αS2-casein (αS2-CN), κ-casein (κ-CN), β- lactoglobulin (β-LG) and α-lactalbumin (α-LA) (10).

Regarding the use of the microfluidic electrophoresis method on the chip (*patented Agilent technology*) for the detection and quantification of the protein fractions in the solution, from what is known until now, it has not been used until now as a biological matrix, the donkey milk. Thus, for each of the samples considered in this project, a rigorous analysis of all the protein fractions identified by electrophoresis was carried out, and by calibrating them according to the external control, the data for each of the protein fractions considered in consideration were recorded (Fig. 1).

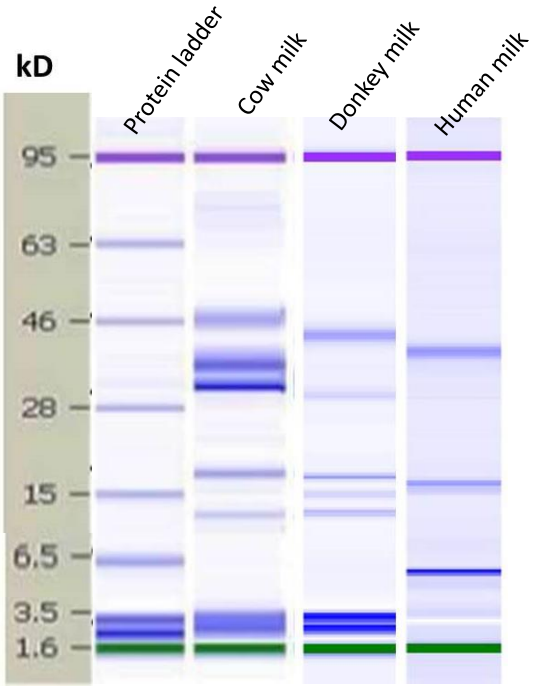

Fig. 1. Protein fingerprinting in the three types of milk. L – Molecular weight marker (Protein Ladder, Protein Kit 80; 1 - Cow's milk; 2 - Donkey milk; 3 - Human milk

The total protein content of donkey milk varies between 1 – 2.3 g/dl, and the casein fraction is about 35–45%, much lower than ruminant milk (>70%), but more similar to human milk (<30%). The available knowledge of donkey milk caseins is limited compared to conventional dairy species, and their heterogeneity has complicated a complete characterisation, partly due to post-translational processes,

genetic polymorphism, and non-allelic deletic forms (19). However, a combination of electrophoretic, chromatographic and proteomic methods allowed the identification of the four casein fractions (αs1-, αs2-, β- and k-casein). It is known that α-casein is the main protein in cow's milk, while donkey milk contains lactoferrin - approximate relative molecular weight (Mr) of 75 kDa, serum albumin (Mr about 60 kDa), casein (Mr: 21 to 35 kDa), β-lactoglobulin (Mr about 18 kDa), lysozyme (Mr about 15 kDa), α- lactoalbumin (Mr about 14 kDa). Of these, the three major proteins in whey from donkey milk are α-lactalbumin, β-lactoglobulin and lysozyme (2, 18).

The nutritional properties of donkey milk can be attributed to this fraction, which is mainly composed of β-lactoglobulins (β-Lg), α-lactoalbumin (α La) and lysozyme (Lyz). The other three minor immunoglobulin proteins (Igs) (not quantified in this project), serum albumin (SA) and lactoferrin (Lf) are also present. Even though the entire fraction of whey protein is responsible for the low number of bacteria in donkey milk [], antimicrobial activity is mainly attributed to Lyz and, to a lesser extent, Lf (3). It is believed that these minor proteins, along with Ig, work in synergy to inhibit microbial growth and reduce the incidence of gastrointestinal infections (7).

One of the main protein allergens in children is β-lactoglobulin, which is the main protein in whey in cow's milk and is absent in human milk. The content of β-lactoglobulin in donkey milk is about 40% of whey protein, being lower than that in cow's milk (4, 20, 22).

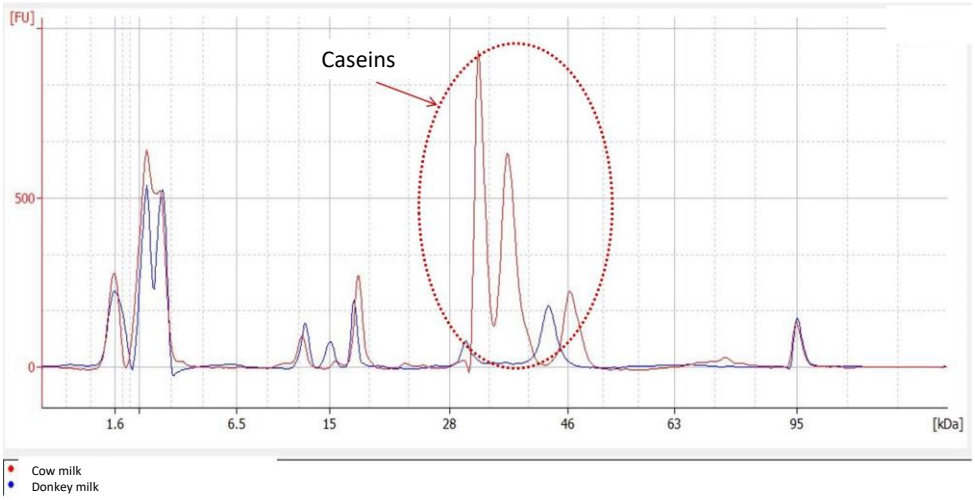

Fig. 2. Overlapped electropherograms of cow's milk (red) and donkey milk (blue)

An overlap of the electrophoregrams obtained in the case of cow's and donkey milk made it possible to highlight the differences between the two types of milk in terms of casein content (Fig. 2, Table 1). Thus, depending on the molecular weights

characteristic for the studied protein fractions, it can be seen that the β – casein and κ – casein are absent from donkey milk, and α - caseins are present in considerably reduced quantities.

From the images presented above and Table 3.1 we can see the major differences that can be found between the protein fingerprints of the three types of milk both in terms of the total protein content and of the protein fractions present. Thus, in addition to the differences in the content in caseins, the analyses made it possible to detect the absence of lysozyme and lactoferrin in cow's milk and their presence in donkey and human milk.

Table 1

Results of the analysis by comparison of protein concentrations in cow's milk, donkey milk and human milk

Protein fractions	Cow's milk (g/dl)	Donkey milk (g/dl)	Human milk (g/dl)
Total protein	**3.4**	**1,74**	**1.16**
α-lactalbumin	**0.15**	**0.16**	**0.12**
beta-lactoglobulin	**0.22**	**0.21**	**0.25**
beta-casein	**0.7**	**-**	**-**
α- casein	**0.73**	**0.3**	**0.23**
K-casein	**0.9**	**-**	**-**
lysozyme	**-**	**0.12**	**0.1**
lactoferrin	**-**	**0.21**	**0.24**
serum albumin	**0,1**	**0.14**	**0.16**

The analysis of the data presented in Table 1 and Fig. 1 and 2 shows that the variations in the data recorded for the protein fingerprints of the three milk types are significant, and a clear distinction can be made between cow's and donkey's or human milk, the latter having similarities in terms of the identified protein fractions.

In order to analyse the sugars and dry matter content, the three biological samples were removed from the freezer, left at rest for 30 minutes and then measured with the Brix refractometer, following the protocol recommended by the manufacturer. The raw data have been recorded and are presented in Table 2.

Table 3

Refractometric analysis data

Biological sample	Dry matter (g/dl)	Sugar content (g/dl)
Cow milk	**6.7**	**4.7**
Donkey milk	**8.8**	**6.8**
Human milk	**9.1**	**7.1**

Donkey milk contains a large amount of lactose (5.8-7.4 g%), which is higher than cow's milk (4.6 – 4.8 g/%) and much similar to human milk (approx. 7 g%). The high content is responsible for the good palatability of milk and facilitates the intestinal absorption of calcium, which is essential for the bone mineralization of the infant (7, 21).

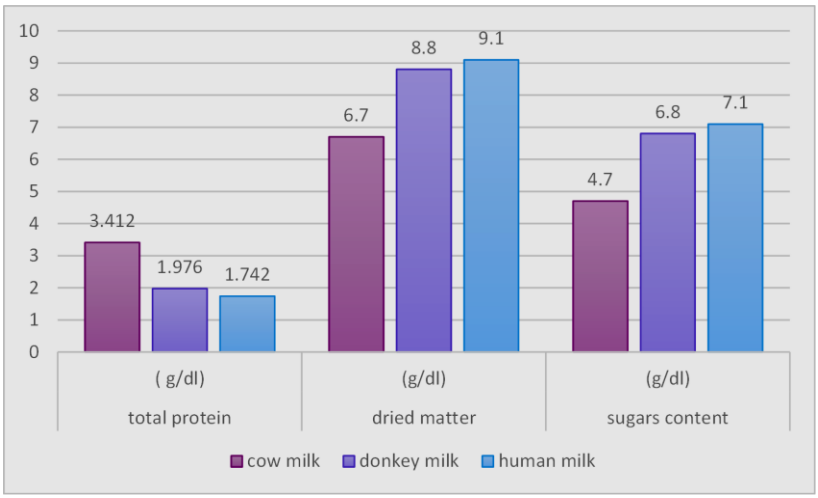

Fig. 3. Graphical representation of biochemical composition data of each sample collected in this experiment

The graphical representation of the data obtained from this experiment regarding the total content of proteins, sugars and dry matter highlights that the biochemical composition of donkey milk is much more similar to human milk than that of a cow (Fig. 3).

Thus, by comparison, the protein content of cow's milk is higher, but that in dry matter and sugars is lower than in the case of the other two studied. However, it should be taken into account that for this study, the variations that may exist were not considered for the three parameters depending on the breed peculiarities of the animals and the periods passed after the parturition.

Conclusions

In this study, two methods were used that allowed the identification and quantification of three biochemical parameters that ensure the nutritional quality of milk, but the amount of which can also be the source of some nutritional imbalances for the people who consume them.

The method of microfluidic electrophoresis on the chip allows the identification and quantification of milk proteins and has been successfully used to compare the protein fingerprints of the three types of biological samples. Thus, it was found that κ-casein (the major protein of cow's milk) is missing in the case of donkey milk and human milk. At the same time, cow's milk lacks lysozyme and lactoferrin, which are present in the milk of the other two species analyzed.

The Brix refractometry method was used to quantify the sugar content and dry matter in milk and made it possible to identify noticeable differences in these parameters as well. Thus, the content in the two biochemical parameters is higher in donkey and human milk than in cow's milk.

The data shows that donkey milk can be a quality substitute for human milk compared to cow's milk.

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RESEARCH ON THE EFFECT OF A PLANT EXTRACT ON SPECIFIC IMMUNITY IN BROILER CHICKS VACCINED AGAINST INFECTIOUS BURSAL DISEASE

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Summary

During four experiments, the effect on the specific immunity of two live attenuated vaccines against avian infectious bursal disease, whose composition includes the BIA virus, with an immunosuppressive but also immunogenic effect, namely Biavac and Biaromvac-PA, was tested. Of the four batches, two batches (E2 and E4) were given an aqueous extract of oregano (*Origanum vulgare L.*) in the drinking water. The research was carried out on batches consisting of 12 broiler chickens, aged 14 days, coming from a broiler farm, raised in alternative system (on litter, without outdoor acces) and in enriched cage system. In each experiment, the administration of the two vaccines was done only once, regardless of the batch, while the immunomodulatory preparation was administered in the drinking water. To evaluate the effect on the specific immune response, blood samples were collected from each chicken, in each experimental stage and the antibody titer was determined by the immunoenzymatic method (ELISA). Analyzing the obtained results, during the experimental period, differences were found from one chicken to another, as well as from one vaccine to another. The most conclusive results were found in the batches to which the aqueous extract of oregano was also administered, the differences between the batches being significant.

Keywords: avian infectious bursal disease, aqueous extract of oregano, specific immune response.

Avian infectious bursal disease (Gumboro disease) is an acute, highly contagious disease, considered one of the most important infectious diseases affecting the immune system of young birds, especially in the first days after hatching. In disease-free flocks, avian infectious bursal disease virus (IBDV) causes almost 100% morbidity and up to 90% mortality (16).

The disease evolves with morphological and functional alterations of the bursa of Fabricius, having as a direct consequence, the significant reduction of the immune response mechanisms mediated by antibodies (1, 3, 5, 7). IBD virus infection results in depletion of lymphoid tissue and eventual destruction of the bursa, which is the predominant feature of its pathogenicity (11).

So far, two serotypes of the IBD virus have been described, both capable of infecting gallinaceae and other species of domestic or wild birds, but only serotype

1 is pathogenic, causing the disease, strictly in gallinaceae. Immunization against serotype 2 does not protect against infection with serotype 1. The differentiation of the two serotypes can only be achieved by the viral seroneutralization test, with the help of polyclonal antibodies, while ELISA and the immunofluorescence test do not allow this (1, 16).

Due to the particular diffusibility of the avian infectious bursitis virus and its high resistance, the general prophylaxis is not sufficient, which requires a specific prophylaxis through vaccination. In this sense, different types of vaccines are used: inactivated, consisting of live germs with various degrees of attenuation, monovalent vaccines or polyvalent vaccines, each of which is indicated in certain circumstances, specified by the manufacturer.

Also, for the stimulation of specific immunity, recently, special attention is paid to various plant extracts and dietary spices, which have the ability to modulate both specific and non-specific immunity (2, 14, 15, 20, 21).

Materials and methods

The immunogen - was represented by two BIA vaccines, respectively Biavac and Biaromvac PA 100 DZ, with the following characteristics:

BIAVAC - live attenuated vaccine obtained from the BIA Cj virus strain. min. $10^{3.0}$ DIE_{50} (embryonic infectious doses 50), max$10^{3.5}$ DIE_{50} with PDZ Stabilizer (ST3) as excipients (casein hydrolyzate, monosodium glutamate, lactose, dipotassium phosphate, monopotassium phosphate). The vaccine was administered in the drinking water in a dilution of 100 doses in a liter of cold, clean water, in which 5 g of powdered milk were previously dissolved for vaccine protection.

BIAROMVAC PA 100 DZ - live, freeze-dried vaccine against avian infectious bursitis, which contains the avian infectious bursitis virus, PA strain, with a minimum titer of $10^{3.5}$ DIE_{50} and a maximum titer of $10^{5.5}$ DIE_{50}/dose, and peptone and lactose as excipients. The vaccine was administered in drinking water in a dilution of 100 doses in two liters of cold, clean water without disinfectants, in which 0.5% skim milk is added beforehand. The reconstituted vaccine was used within a maximum of two hours after preparation.

The immunomodulatory substance was represented by an Oregano extract, obtained according to the method modified by Hernandez et al. (6, 9, 10, 13). For this purpose, the Oregano leaves, dehydrated, were finely ground in the electric blender, the obtained powder being re-suspended in distilled water, in the ratio of 1 gram of powder to 2 ml of distilled water. The obtained solution was kept into the thermostat, for one hour, at 60°C and then left to extract with distilled water, for 24 hours, on a magnetic stirrer, respectively Hot Plate Stirrer (IDL LMS-1003, IDL GMBH&CO, England) at room's temperature. Afterwards the resulting mixture was filtered through filter paper and the resulting extract was again filtered using a Whatman Uniflo 25mm 0.2 µm syringe filter (Thermo Fisher Scientific Inc., France) and kept in a refrigerator until use.

The research was carried out on 48 broiler chickens, grouped into four experimental groups, as follows:

• group E1 (experimental 1), consisted of 12 chickens, which were administered live attenuated vaccine, Biavac, in the drinking water at a dilution of 100 doses per liter of water;

• batch E2 (experimental 2), made up of 12 chickens, which received live attenuated vaccine, Biavac, in the drinking water in a dilution of 100 doses per liter of water, together with the aqueous extract of Oregano, respectively 7 ml extract/l water (10);

• batch E3 (experimental 3), made up of 12 chickens, which received live attenuated vaccine, Biaromvac PA, in the drinking water in a dilution of 100 doses per two liters of water;

• group E4 (experimental 4), consisted of 12 chickens, which received live attenuated vaccine, Biaromvac PA, in the drinking water in a dilution of 100 doses per two liters of water, together with the aqueous extract of Oregano, respectively 7 ml extract/ l water.

To determine the antibody titer, blood samples were taken at the following time intervals: at the age of 14 days, on the day of vaccination, and later at the age of 24, 34, 44 and 54 days.

Anti-IBD antibody titer was determined by immunoenzymatic technique (ELISA) using a kit produced by IDEXX Laboratories, USA named "Infectious Bursal Disease Virus Antibody Test Kit".

Results and discussions

Analyzing the results obtained regarding the titer of individual antibodies, during the experimental period, in the chickens vaccinated with Biavac, differences can be found both from one chicken to another and from one stage to another (Table 1).

Thus, if at the beginning of the experiment, the antibody titer recorded an average value of 150.25±34.64 O.D. units, ten days after vaccination it showed a significant increase ($p<0.0001$) reaching the minimum value of 1016 units and the maximum value of 1298 units with an average of 1174.6 units and a rather low coefficient of variation (8.03%).

After 20 days post-vaccination, an increase in the antibody titer was recorded to an average value of 1725.67±87.27 O.D. units, so that after another 20 days an average antibody titer value of 3657.08±225.86 units was recorded, which is 24.38 times higher than that recorded at the time of vaccination (Table 1, Fig. 1).

In the case of experimental batch 2, chickens also vaccinated with Biavac, but to which aqueous extract of oregano was administered in the drinking water, it is observed that the antibody titer, although it registers different values from one chicken to another, shows the same tendency of growth, also found in the chickens from the first batch (Table 2, Fig. 1). Thus, the maximum average value was recorded

on day 54, respectively 4142.67 O.D. units, with a minimum of 3822 units and a maximum of 4314 units, the average value being 485 units higher than that recorded in chickens from the first batch, respectively the vaccinated chickens but which did not receive aqueous extract of oregano (Table 2, Fig. 1). It can also be mentioned that in this batch as well, the coefficient of variability was quite low, between 14.44% on the day of vaccination and 4.31% 40 days after vaccination.

Table 1

Anti-bursitis antibody titer in chickens administered the BIAVAC vaccine

No.	Antibody Titer				
	Day 14	**Day 24**	**Day 34**	**Day 44**	**Day 54**
1	184	1033	1722	2436	3322
2	113	1184	1668	2442	3419
3	214	1298	1742	2509	3545
4	166	1100	1815	2389	3408
5	194	1207	1651	2622	3661
6	138	1016	1612	2734	3817
7	172	1232	1593	2934	3618
8	115	1244	1799	2652	3553
9	138	1297	1899	2852	3901
10	125	1104	1725	2919	4094
11	132	1119	1731	2675	3741
12	112	1262	1751	2431	3806
X±SD	**150.25± 34.64**	**1174.6± 98.14**	**1725.67± 87.27**	**2632.92± 196.29**	**3657.08± 225.86**
C.V.	**23.06%**	**8.35%**	**5.06%**	**7.46%**	**6.18%**

Legend: X = mean value; SD = standard deviation; CV = coefficient of variation

Results similar to those found in the first two batches were also recorded in experimental batches 3 (chickens vaccinated with Biaromvac PA) and 4 (chickens vaccinated with Biaromvac and aqueous extract of oregano added to drinking water) (Tables 3, 4 and 5). Thus, compared to chickens from the first batch, vaccinated with Biovac, in chickens from experimental batch 3, vaccinated with Biaromvac PA, there was a slight increase in the antibody titer, especially after the first 20 days after vaccination, but also at the end the experiment.

Similar results were obtained by Scutaru et al. (12), following some experiments in which the dynamics of antibodies induced post vaccination with the Biaromvac PA vaccine were studied. Thus, the researchers mentioned above established that the antibody titer, in chickens vaccinated with Biaromvac PA, shows an increase starting on the fifteenth day after vaccination, the maximum values being recorded 25-30 days after the chickens' immunization (12). Also, Tîrziu et al. (17, 18), in an experiment where they used four different vaccines, including Biaromvac PA, achieved titers of over 3700 O.D. units, more than 28 days after vaccination.

Table 2

Anti-bursitis antibody titer in chickens administered BIAVAC together with oregano extract

No.	Antibody Titer				
	Day 14	**Day 24**	**Day 34**	**Day 44**	**Day 54**
1	224	1023	1922	2896	3822
2	293	1134	1818	2856	3998
3	314	1318	1912	2929	4160
4	231	1223	1815	2930	4308
5	296	1104	1651	2823	3981
6	238	1152	1722	3219	4227
7	199	1040	1971	3184	3988
8	285	1269	2191	3051	4253
9	297	1298	1803	2815	4381
10	241	1194	1922	3009	4314
11	227	1189	1796	2815	3992
12	282	1237	2151	3231	4288
X±SD	**260.58± 37.62**	**1181.7± 94.94**	**1889.50± 159.65**	**2979.83± 157.90**	**4142.67± 178.56**
C.V.	**14.44%**	**8.03%**	**8.45%**	**5.30%**	**4.31%**

Legend: X = mean value; SD = standard deviation; CV = coefficient of variation

Table 3

Anti-bursitis antibody titer in chickens administered BIAROMVAC PA

No.	Antibody Titer				
	Day 14	**Day 24**	**Day 34**	**Day 44**	**Day 54**
1	171	1311	1682	2514	3512
2	223	1121	1606	2961	4291
3	217	1076	1711	2808	4278
4	185	941	1561	2492	3882
5	211	1103	1683	2974	4320
6	218	1013	2232	3140	4202
7	231	1286	1986	2625	3621
8	229	1414	2101	2832	3628
9	221	1252	1841	2534	3394
10	242	1212	1824	2910	3704
11	212	1184	1715	2913	3923
12	219	1229	1922	2928	4185
X±SD	**214.92 ± 19.47**	**1178.50 ± 133.82**	**1822.00 ± 204.64**	**2802.58 ± 211.51**	**3911.67 ± 335.83**
C.V.	**9.06%**	**11.36%**	**11.23%**	**7.55%**	**8.59%**

Legend: X = mean value; SD = standard deviation; CV = coefficient of variation

The aqueous extract of oregano also had a positive effect on chickens vaccinated with Biaromvac PA (Tables 4 and 5, Fig. 1). Thus, after vaccination, the average titer of antibodies was higher, compared to the group of chickens that did not benefit from the administration of the oregano extract, at each sampling moment.

Table 4

Anti-bursitis antibody titer in chickens administered BIAROMVAC PA together with oregano extract

No.	Antibody Titer				
	Day 14	**Day 24**	**Day 34**	**Day 44**	**Day 54**
1	262	1222	1990	2814	3922
2	273	1120	2201	3762	4521
3	312	1186	2201	3108	4372
4	242	1242	1962	2902	3880
5	298	1108	1884	3278	4421
6	288	1210	1968	3442	4192
7	299	1209	2016	2824	3980
8	284	1322	2380	2931	4224
9	278	1431	2342	3232	4694
10	242	1312	1772	2810	3864
11	268	1380	2002	3013	4423
12	296	1418	2322	3128	4545
X±SD	**278.50 ± 22.21**	**1263.33 ± 108.74**	**2086.67 ± 196.33**	**3103.67 ± 289.58**	**4253.17 ± 286.35**
C.V.	**7.98%**	**8.61%**	**9.41%**	**9.33%**	**6.73%**

Legend: X = mean value; SD = standard deviation; CV = coefficient of variation

Table 5

Mean antibody titer values in the four experienced batches

Experimental batches		Antibody Titer				
		Day 14	Day 24	Day 34	Day 44	Day 54
E1 - Biavac	**Media**	150.25	1174.6	1725.67	2632.92	3657.08
	C.V.	23.06%	8.35%	5.06%	7.46%	6.18%
E2 - Biavac + extract	**Media**	260.58	1181.7	1889.50	2979.83	4142.67
	C.V.	14.44%	8.03%	8.45%	5.30%	4.31%
E3 - Biaromvac	**Media**	214.92	1178.50	1822.00	2802.58	3911.67
	C.V.	9.06%	11.36%	11.23%	7.55%	8.59%
E4 - Biaromvac + extract	**Media**	278.50	1263.33	2086.67	3103.67	4253.17
	C.V.	7.98%	8.61%	9.41%	9.33%	6.73%

For example, 20 days after vaccination, in the chickens that were administered the oregano extract (experimental batch 4), the average antibody titers were 2086.67 O.D. units, and in the chickens that were not administered the oregano extract (batch experimental 3) of only 1822.00 units O.D. At the end of the experiment in the chickens from group 3 the average value of the antibody titer was 3911.67 O.D. units, while in the chickens from group 4 an average value of 4253.17 O.D. units was recorded, which allows us to say that the aqueous extract of oregano

influences significantly the synthesis of specific immune effectors (Tables 4 and 5, Fig. 1).

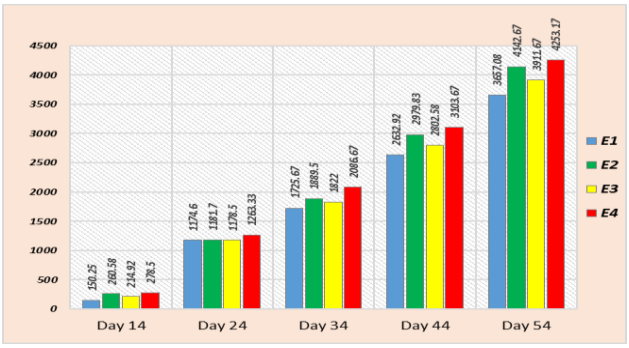

Fig. 1. Antibody titer dynamics in the four experimental batches

Our results have been confirmed by other researchers. For example, Vattem et al. (19) mention that oregano extract also influences the non-specific immune response, being able to have a positive effect in the case of some microorganisms that act through immunosuppression, its action being comparable to that of cyclophosphamide. The same authors state that these plants can also be used in cancer, diabetes and Alzheimer's disease (19).

In another study, Franciosini et al (4) found that oregano extract also influences the synthesis of the main classes of immunoglobulins. In the study carried out in broilers, the above mentioned researchers found that the administration of oregano influences the synthesis of total IgG, the values observed at the end of the study being significantly higher, compared to those reported in the specialized literature (4).

Conclusions

Insignificant differences were found between the antibody titers induced by the Biavac and Biaromvac vaccines.

The aqueous extract of oregano, administered to chickens in drinking water, positively influenced the synthesis of antibodies, with higher titers recorded in both groups to which the oregano extract was administered.

The highest antibody titer values were found 30 days after the administration of the vaccines and the immunomodulatory preparation.

It can be recommended to use biological preparations from Origanum vulgare L., in order to stimulate the immune response in broiler chickens.

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USUTU VIRUS IN ROMANIA CURRENT KNOWLEDGE AND FUTURE PERSPECTIVES

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Summary

Usutu virus (USUV) is a mosquito-borne flavivirus that has been emerging across southern and Central Europe since 2001. USUV is maintained by cycling through birds and mosquitoes and is accidentally transmitted to mammals. It shares many features with West Nile virus including a close phylogenetic relatedness and a co-circulation frequently observed in nature. The virus caused large outbreaks of severe neurological disease in birds, especially in blackbirds and, symptomatic neurological human cases. Continuous USUV activity confirmed by the detection or serologic evidence of USUV in birds, humans, horses, mosquitoes, and bats since 2001 indicates the endemization in many Southern and Central European countries. However, due to a limited number of human USUV infections, the zoonotic potential and clinical relevance of USUV need to be further investigated. It is considered that, since the virus activity in birds has increased, also human infection might become more frequent. *Eastern Europe and Romania have reported little data concerning* USUV. The present paper is aiming to discuss/describe the current state and future perspectives of USUV research in our country.

Key words: mosquito-borne flavivirus, Usutu virus, opinion, Romania.

Usutu virus

In addition to mosquito-borne West Nile, Japanese encephalitis virus and other human and animal pathogens, the African closely related Usutu virus (USUV), has been reported to cause encephalitis in Europe. USUV is a mosquito-borne flavivirus that has been emerging across southern and Central Europe since 2001. Originally non-pathogenic (6), USUV was first recognized and isolated in 1959 from *Culex neavei* mosquitoes collected near the Usutu River, in Swaziland, South Africa. In the subsequent years, USUV dispersed on the African continent and in recent decades, was found to circulate in European countries by mosquitoes displacement or infected migratory birds (4).

Usutu shares genetic and antigenic similarities with WNV, alongside belonging to the family *Flaviviridae* and the Japanese encephalitis antigenic complex. Therefore, USUV is antigenically related to pathogens such as Murray Valley encephalitis virus (MVEV) and St. Louis encephalitis virus (SLEV) (SLEV) (15). In addition, USUV and WNV share the same vector species and avian reservoirs involved in the transmission cycle. Moreover, these viruses can be

sympatric and co-circulate in the same environment (5).

Hosts, vectors and transmission cycle

USUV's natural life cycle is similar to WNV, involving ornithophilic mosquitoes (*Culex* and to a lesser extent *Aedes*) vectors (1) and birds (epornitic virus) as amplifying hosts (3).

Culex pipiens mosquitoes contribute most to USUV transmission in Europe. The plasticity in the behavior of this species, feeding on a variety of hosts, contributes to the amplification cycle among avian hosts, but also to the occasional spill-over of viruses into human and horse populations (18). In addition, vector competence studies have demonstrated that *Culex neavei* and *Culex quinquefasciatus* are competent vectors for USUV transmission (10).

As regards the vector species, unlike the closely related WNV where tick-borne virus transmission has been reported (16), for USUV, the role of ticks as carriers and spreaders is insignificant (17)

Amplifier Hosts

Unlike Africa, where bird mortalities were not reported, in Europe USUV was shown to be highly pathogenic, resulting in a massive die-off of several bird species, like blackbirds (*Turdus merula*) and great gray owls (*Strix nebulosa*) (26). Avian species, such as the kestrel (*Falco tinnunculus*) and the babbler (*Sylvia curruca*), are assumed to be responsible for the viral introduction into Europe, while blackbirds, magpies, or sparrows are believed to have disseminated the virus throughout the continent (9).

Incidental hosts

Humans and other mammals are considered incidental hosts to this virus (3), corresponding to a "dead end" for the epidemiological propagation of the virus, as a result of a short-lasting and low viral titer not high enough to ensure transmission via mosquito bites.

The transmission cycle seems mostly determined by different biotic and abiotic factors (24) influencing the developmental rate of the mosquito vectors, the extrinsic incubation period of the virus in its mosquito hosts (8) and its geographical dispersal (24).

Introduction in Europe and genetic diversity of Usutu virus

Following its first identification in South Africa in 1959, the first demonstration of USUV circulation was reported in Austria in 2001 (4), with a silent viral circulation retrospectively recognized in 1996 – 2001. In 2018, the virus spread rapidly in Western Europe, associated with a large epidemic of WNV, suggesting both the continued expansion of the virus and the colonization of new ecological niches. In the subsequent years, USUV gained the attention of the scientific community due to its extensive spread in Europe (10), expanding to neighboring countries, such as

Italy, Germany, Spain, Hungary, Switzerland, Poland, England, Czech Republic, Greece, and Belgium. After its emergence in Europe, USUV was serologically documented in a wide range of animals (birds, horses, squirrels, wild boars, chimpanzees, deer and lizards) (26), humans, whilst viral isolation was performed from mosquitoes, birds, and bats.

Although the first report of USUV in Europe occurred in 2001, studies performed to assess the drivers of viral migration and explore the origin of the outbreaks, suggested that at least three USUV introductions have been recorded in Europe, with endemization in some countries along the migratory routes from Africa (14). The three introductions of USUV in Europe, before 2001, are hypothesized to date for more than 50 years. It is believed that the virus was first introduced by migratory birds in Spain, between 1950-1960. The second introduction is believed to have occurred in Italy and Spain, between 1970 - 1980, while the third took place also in Spain (1984-2006) (15).

USUV introductions into Europe follow the main migratory hubs: the first introduction from Africa to Spain overlaps with the East Atlantic migratory network, and that from Africa to Central Europe overlaps with the Black Sea/Mediterranean migratory flow (9).

The genetic diversity of the USUV strains discovered in Europe emphasizes the numerous entries from Africa, as well as the plasticity of the strains circulating there (3). Furthermore, it is considered that USUV genetic diversity in Central Europe is shaped primarily by *in situ* evolution rather than extensive migration (14).

In Europe, long-term USUV circulation diversity and variability have been explored by phylogenies over full-length sequences and NS5 and envelope genes (21).

Based on the origin of the isolates, USUV can be divided into eight genetic lineages (29), clustered into two groups: African and European. Studies on the phylogenetic analysis of the NS5 gene demonstrated that strains from Africa belong to three lineages (Africa 1 to 3) and the European group comprises five distinct lineages (Europe 1 to 5) (15).

Zoonotic potential of USUV

Far less common than its closely related WNV, USUV's clinical relevance and potential was reported in 2009, when the first human cases of severe encephalitis due to USUV infection were reported from Italy in two immunocompromised persons (26), demonstrating its neurotropism (8). Also, the number of recent reports exceeding 100 acute human infections described in Europe is a reason for concern and increasing evidence of a role in human disease (5).

Clinical manifestations in USUV infections are rather similar to WNV (27), leading to misdiagnosis and suboptimal case management (18). Infection in humans is usually asymptomatic or associated with a clinical outcome ranging from moderate to severe symptoms (meningoencephalitis, polyneuritis, encephalitis, or facial

paralysis) (29). With both viruses being circulated by the same vector species for over two decades, their co-circulation represents a concern for public health.

The zoonotic potential of the virus has been highlighted by seroprevalence studies. These studies have suggested that USUV can be, in most cases, asymptomatic or associated with mild forms. The symptomatic phase of the infection is usually characterized by moderate fever, sometimes associated with skin rashes and febrile jaundice. In rare cases, in patients with other comorbidities, the infection has been described with neurological manifestations, meningitis and meningoencephalitis (9).

It is considered that significant circulation of USUV in both reservoirs and vectors, at a given time and place, increases the relevance and probability of human cases.

USUTU and WNV Co-circulation and Co-Infection Dynamics

The expansion of the geographical range of transmission of arboviral infections has consequences for the arising of concerns on public health issues, on the increasing of mosquito borne viruses (MBV) co-circulation and the emergence in new areas.

The simultaneous circulation of these viruses alters the epidemiology, as seen in the replacement of the Saint Louis encephalitis virus as a consequence of WNV introduction in California (20). Transmission kinetics of MBVs are influenced by biological and environmental factors (21). USUV and WNV co-circulation has implications in terms of overlapping transmission cycles, geographically and within hosts, vector populations and immunological cross-reactions (18) Studies showed that viral replication of USUV is compromised by the increased replication rate of WNV. Pre-exposure to a blood mass loaded with USUV particles reduced the susceptibility of *C. pipiens* upon subsequent oral infection with WNV compared to mosquitoes not previously exposed to USUV. The observed reduction in infection and transmission rates is due to a competitive relationship between two viruses in the midgut (28). Such competition has epidemiological consequences, so in regions where both viruses are circulating, the vector competence for USUV of mosquitoes previously infected with WNV could be reduced. In addition, USUV circulation in WNV-free countries could prevent transmission and dissemination. Further, experimental studies conducted on *C. pipiens* species have shown that it is a more effective vector for USUV than for WNV in high temperature conditions (28°C) (10).

Although there are many ecological similarities between the two viruses, unlike WNV, USUV has been found to be more harmful and lethal for some avian species, while rarely affecting humans (5).

Co-circulation of the two MBVs in Europe was reported in mosquitoes, birds, horses and humans (21). Positive serology in humans has been reported in different countries, such as France (11) Italy (22), Austria (2, 7) or Croatia (25).

Research in Romania

While WNV is endemic in certain areas of Romania, few data regarding USUV infections have been documented.

In mosquitoes collected within a longitudinal arbovirus surveillance program between 2014 and 2016 in a rural/urban environment (Letea, Sulina) in the DDBR a blood meal analysis showed that one mosquito (*Anopheles hyrcanus)* that fed on a dog in Sulina, Danube Delta was positive for USUV specific antibodies. The study published in 2019 is the first indication of USUV in Romania in vector species (23).

In animals, USUV antibodies were documented for the first time in 2020, in a domestic dog sampled in 2019. In humans a study on healthy blood donors from north-western Romania (12) sampled from November 2019 and February 2020 showed a 0% seroconversion for USUV but 3.17% for WNV. In avian hosts a retrospective study on migratory and resident birds from the South-Eastern region of Romania sampled in 2018-2019, showed a 2.71% seroprevalence for USUV and 8.72% for WNV (13). Even so, seroprevalence data are still very scarce for assessing the incidence of USUV and there are no efficient serological diagnostic tools for large-scale screening. The technical disadvantages of serology testing due to lack of specificity linked to the antigenic cross-reactions with closely related flaviviruses (9).

Recently, a retrospective molecular study in mosquito vectors collected in South-Eastern Romania in 2012-2013 and 2019 detected USUV by real-time RT-PCR in *C. pipiens s.l.* female pool collected from Bucharest in 2019. The partial genomic sequences obtained from the positive sample showed that the virus belongs to the Europe 2 lineage (EU2) (19).

Current surveillance program for mosquito-borne viruses in Romania

Entomological surveillance is an essential component of vector control strategies, providing information on vector species, their spatio-temporal distribution, bionomics and density.

Despite the impact on human and animal health, in Romania, the available measures to control arboviruses are limited and rely solely on research activities. Although currently there is no specific treatment or vaccine for WNV or USUV available for humans (11), there are no surveillance procedures in the field, nor the blood transfusion centers.

Recommendations and conclusions

Involved in repeated epizootics since 1996 in the European bird population, USUV is now recognised as responsible for potentially severe neurological affections in humans.

Considering that USUV spreading is likely to continue, multidisciplinary

interventions in the frame of the “One Health” concept, should be conducted to increase USUV awareness and implement appropriate monitoring and prevention methods for this emerging arboviral infection. An integrated veterinary and human surveillance system, including virus detection in mosquitoes, avian reservoirs, mammals, and humans, is needed to estimate USUV risk and spatial dynamics

The true prevalence, regional range, and seasonality of USUV infection still needs to be determined through the above-mentioned integrated surveillance and control measures. In Romania, no national mosquito vector-monitoring program is implemented making the detection of emerging moboviruses challenging in terms of specific laboratory facilities and public awareness.

The recent findings in Romania confirm the virus presence and raise a concern. We consider that similar to other European areas, in our country, due to its co-circulation, antigenic relatedness and similar clinical outcome with WNV the detection of USUV is suboptimal. The first signals in Romania were registered starting with 2016 but more data showed a higher percent of positivity in animals and vectors in 2019. This can be considered as a starting point for the virus introduction in the country.

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THE IDENTIFICATION OF *SARCOPTES* SPP. MITE IN RED FOX (*VULPES VULPES*) SKIN LESIONS - CASE REPORT

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Summary

Sarcoptic mange, caused by the mite Sarcoptes spp., is a highly contagious parasitic disease affecting various animal species, including wildlife. This case report describes a red fox (Vulpes vulpes) from the hunting grounds Giarmata from Timis County (Romania) which was received from the hunters. A thorough physical examination revealed specific lesions associated with sarcoptic mange, including alopecia, erythema, and crusts, primarily in the head, neck, and tail regions. This emaciated fox female body was examined at the Parasitology Department of the Faculty of Veterinary Medicine. To establish the diagnosis we performed skin scrapings that were collected from the affected areas and microscopic analysis confirmed the presence of *Sarcoptes* spp. mites. The mites were identified based on their morphology and typical pathological signs.

Keywords: *Sarcoptes* spp., red fox, Timis County.

The fox (*Vulpes vulpes*) is a species of wild carnivore from the *Canidae* family and is widespread throughout Europe, including Romania. In Europe, foxes are present in various habitats such as forests and plains, even mountainous areas. Romania offers a varied habitat for foxes, having remarkable geographical and climatic diversity. Foxes can also adapt to urban and rural areas, becoming present in towns and villages. This ecological adaptability and variability give foxes an advantage in survival and population growth. Red foxes play an important role in ecosystem dynamics (2, 3, 7).

Red foxes are a natural reservoir for both endoparasites and ectoparasites. Foxes are known to carry many species of ectoparasites such as fleas, mites, and ticks, the majority of which are potentially transmissible to humans and other animals (4, 9, 13).

Mange is one of the most important skin diseases affecting both domestic and wild carnivores. In foxes, sarcoptic mange is caused by the mite *Sarcoptes* spp. Infestation can occur through direct contact between animals during interactions or through the environment in which the fox has lived or moved (2, 3).

In this context, the aim of the study was to identify the etiological agents involved in skin lesions from a red fox.

Materials and methods

Case presentation

A corpse of a female fox, about 2 years old, was received from the hunters, coming from the hunting fund 59 Giarmata in Timiș county. The study was carried out at the Parasitology and Parasitic Diseases clinic within the Faculty of Veterinary Medicine, University of Life Sciences "King Mihai I" from Timisoara (Fig. 1. a, b).

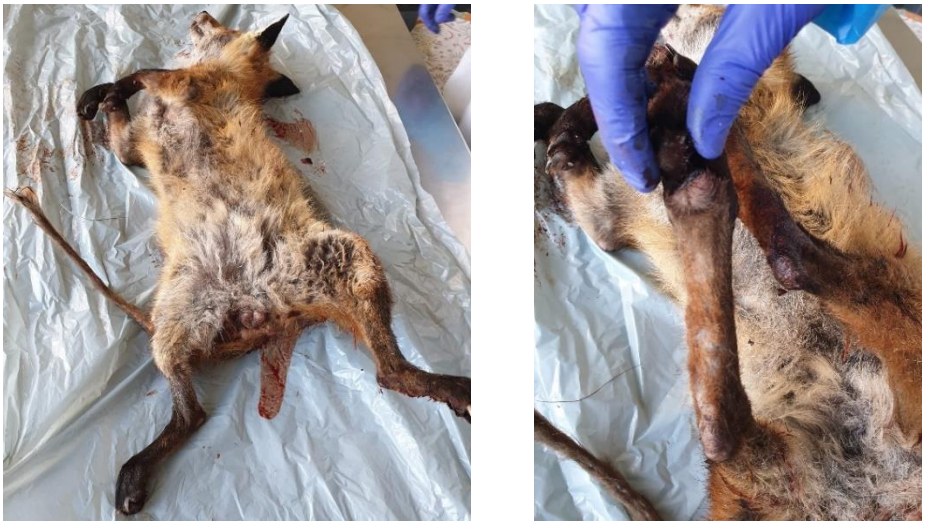
The steps of the diagnosis were:

- The general inspection
- Identification of skin lesions
- Scraping of the skin

Skin scraping is commonly used to identify mites such as *Demodex*, *Sarcoptes*, and *Cheyletiella*. The skin scraping technique used can vary depending on the specific ectoparasite suspected, as described here (6, 10).

We use a laboratory spatula or a No. 10 scalpel blade dulled by repeatedly scraping the blade's edge on a hard surface and we apply paraffin oil to the dull blade and the microscope slide, as well as to the lesion to be scraped. After scraping, we mix the accumulated debris on the blade into the paraffin oil on the microscope slide, apply a coverslip, and examine the slide under 4X to 10X magnification.

- Microscopic examination of the slide (6).



a. b.

Fig. 1. General examination

Results and discussions

At general inspection, the corpse was emaciated, had skin lesions, and, associated with them, flea parasitism was evident.

The skin lesions resulted in alopecia, erythema, crusts, and hyperkeratosis, their location being on the neck, limbs, tail, and in abdominal and groin regions.

The skin scraping confirmed the presence of the *Sarcoptes* spp mite, from a morphological point of view is characteristic: round body, and short limbs that do not exceed the rostrum anteriorly nor the body outline posteriorly.

Based on these results, we established the diagnosis of sarcoptic mange (Fig. 2).

Fig. 2. *Sarcoptes* spp.

This study provides an additional understanding of sarcoptic mange as a disease, potentially allowing the management of wildlife infected with sarcoptic mange. This disease has the ability to infect wildlife, domestic animals, and humans. Infected wildlife can approach human settlements in search of various resources such as shelter and food, potentially spreading mange to domestic dogs and humans. Identifying modes of morbidity in wildlife, such as altered microbiome, can lead to the management of mange dynamics across host systems (10, 12, 15, 16).

Red foxes are one of the most widespread canid species globally and are highly susceptible to sarcoptic mange. The majority of research on mange in red foxes has occurred in Europe, where the disease has affected this species since the late 1600s (2, 11, 17).

The results obtained in the present study can be compared with the reports of researchers from Spain, Hungary, Italy, and Switzerland, which reveal the involvement of the mite *Sarcoptes* spp. in skin lesions from wild carnivores (1, 5, 8, 14).

Conclusions

This case report emphasizes the importance of early detection, prompt diagnosis, and effective management of sarcoptic mange in wildlife populations.

The finding raises concerns for both animal health since the parasite can infect both species, with hunters having the highest risk of contact.

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ASSESSMENT OF WELFARE IN THE DAIRY COW THROUGH THE WELFARE QUALITY SYSTEM

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Summary

In this paper, we performed a welfare assessment in dairy farms. Welfare assessment in farms allows the farmer (owner) to integrate into the EU requirements, and to have better economic value when it comes to production services. We used evaluation criteria established for dairy cows by the Welfare Quality System (WQS). Welfare Quality defines four principles for assessing animal welfare: proper feeding, proper housing, good health, and the possibility to express specific behaviour, each of them subdivided into specific criteria. We collected data from five farms, and observations were made with reference to the following criteria: body condition score, lifting behaviour, collision with the equipment in the shelter, lameness, body hygiene, the presence or absence of nasal, ocular and vulvar discharge, and the approach test. The results were analyzed using the software program provided by WQS®. According to the grading system, two out of the five farms were classified as being “GOOD” while the other three were “ACCEPTABLE”.

Keywords: cows, welfare, evaluation, health, farm.

Since the beginning of time, animal welfare has piqued curiosity, and various definitions have been offered before a comprehensive and intricate one was finally obtained (1, 14). Aspects that convey the physical, mental, and natural status of the animal were commonly used to define animal welfare (5). An animal's level of *well-being* can be expressed by evaluating its physical and mental health as well as its capacity to respond to its surroundings as naturally as possible. The expression of typical and expected behaviors for the species, sex, age group, and physiological state are indicators of complete welfare (15). Knowing the behavioral characteristics of the relevant species, raised in freedom or at least in extensive systems, is required to develop this state in animals exploited in intense systems. Observations of behavior during eating, drinking, resting, sleeping, standing up, and lying down are more pertinent. Animals' distinctive physiological, physical, and behavioral factors are used to determine their welfare, which calls for the investigation of a wide range of parameters (2, 5, 7). The productive indicators - which measure an animal's capacity to function in a productive manner - can be added to the existing physiological, pathological, and ethological welfare indicators. The welfare evaluation system's goal is to inform dairy cow operators and owners about the requirements and standards they must be aware of (8, 10). This approach is designed for all individuals who are in charge of determining the welfare of dairy cows, pigs, and birds. Every person in charge of an animal, including its owner, is

required by this act to uphold the animal's welfare (11, 17, 19). The analysis of variations in various parameters is a multidisciplinary process that is necessary for the welfare of dairy cows. In order to assess an animal's welfare, Welfare Quality identifies four criteria: appropriate feeding, appropriate housing, health, and specific behaviour (1, 5). The assessment of the welfare of dairy cows gives us a mental picture of how to include the dairy farmer in EU regulations. The farmer can learn about the importance of shelter, food, how to maintain health, and last but not least, how an animal can express specific behaviours, through this activity.

Materials and methods

The research was carried out in five dairy farms (A, B, C, D, E) in the western part of Romania. In each farm, observations were made with reference to the welfare assessment criteria, as follows:

1. physical condition;
2. lifting behaviour;
3. collision with shelter equipment;
4. lameness; lifting behaviour;
5. the state of bodily hygiene at the level of the tarsus, the hindquarters and the udder;
6. nasal, ocular and vulvar secretions;
7. animal approach test (5, 17, 20).

At **Farm A**, 38 cows were kept in tied stalls, in a covered structure with natural lighting. **Farm B**'s herd of 150 cows was housed in a closed-off structure with natural lighting. In **Farm C**, 100 cows were kept in a free stall with natural lighting inside a wooden structure. **Farm D** has been registered as having 100 cows where they were housed in free stalls under natural lighting. **Farm E** had a herd of 70 cows that were kept in free stalls in a shelter with natural lighting that was open in the summer and closed in the winter. **The body regions** are examined to determine the condition of the body, and the cows are then categorized according to the examination of these indicators:

0 - good physical condition;
1 - very weak, the score is given to cows, which obtained the very weak indicator in at least three body regions;
2 - very fat, given to cows that obtained the very fat indicator in at least three body regions.

The lifting habit of these animals starts with head motions that travel forward and backward before lifting the rear and anterior train. On a scale from 1 to 4, lifting behaviour was measured and scored as follows:

1 - normal movement, with a pause of up to 5 seconds on the knees;
2 - normal movements, with a pause longer than 5 s in the knees;
3 - long break on the knees with difficulty in lifting (repeated forward and backward movements of the head);

4 - abnormal lifting with total deviation from the normal sequences of lifting behaviour (sitting dog position).

Body hygiene for dairy cows involves giving them scores for their udders, acropodium, and hindquarters-three major anatomical locations (5, 16, 20). Using a scale that reads as follows, these regions are rated:

0 - very good condition

0.5 - some portions are slightly dirty;

1 - some dirty portions, which in total cover more than half of the body area;

1.5 - dirty portions, which in total cover more than half of the body area;

2 - entire body area covered with dirt.

For dairy cows, the level of cleanliness is crucial since it might affect mammary gland infection.

Health condition

Lameness is a condition that can affect one or more limbs or the spine. Lameness can be seen while someone is in an orthostatic position, getting up from the floor, moving, or assuming a recumbent position (4, 14, 15). Direct observation of cows in motion is used to assess lameness, and the results are as follows:

0 - no lameness;

1 - lame with conditions in the initial stage of development;

2 - severe lameness with serious foot ailments.

Nasal discharge is described as a distinct, observable discharge that happens in the nostrils. Stereotypies, vaginal secretions, dyspneic breathing, and ocular conditions were solely assessed based on their presence or absence at the time of the consultation. The secretions' colors range from translucent to yellowish-greenish, and it is noted:

0 - absent secretions;

1 - present secretions.

Following the animals' standing up, **the animal approach test**, for fear assessment was carried out at a distance of 2 m in front of the animals that would be put to the test. We approach the animal at a speed of one step per second with the arm extended at a roughly 45° angle after ensuring that it is alert or has become aware of our presence (6, 9, 11). On a scale from 1 to 5, the behavioural response was rated in accordance with the appraiser's proximity to the cow:

1 - the cow stays in place and allows touching,

2 - the cow stays in place, but does not allow touching,

3 - the cow remains in place, but takes a step back when the evaluator extends his hand,

4 - the cow moves back, before the evaluator stops,

5 - the cow avoids the evaluator completely.

Results and discussions

According to the criteria of the Welfare Quality system, the farms were classified as follows: **AT FARM A**: Body Condition: good; Lifting Behaviour and Interaction with Equipment in the Shelter: well; Body Hygiene: acceptable; Health: acceptable; Human-Animal Relationship: acceptable. The final scoring made, and given for farm A being **ACCEPTABLE**.

The need for methods to assess the overall level of animal welfare on farms has been stressed frequently (3, 4, 18). An overall level of farm animal welfare can facilitate product labelling, encourage producers to improve animal welfare, and, in the future, might become part of export legislation (5, 11). Various measures are used to assess animal welfare; for example, animal behaviour, heart rate, or cortisol levels in blood. Measures need to be combined, however, to determine an overall level of animal welfare on farms. Although it has been argued that science should not attempt to perform overall welfare assessment because value judgments are inherently involved (5, 13), others state that overall welfare assessment is not arbitrary and a high level of accuracy can be achieved (12, 19). In spite of different viewpoints, various models have been developed to assess overall level of animal welfare. By using an objective evaluation, we can compare the management system between the farms and see which protocol of management is the best as emulating the needs of the animals, and thus ensuring the welfare.

AT FARM B: Body Condition: well; Lifting Behaviour: acceptable; Body Hygiene: good; Health: good; Human-Animal Relationship: acceptable. The final scoring made, and given for farm B being **ACCEPTABLE**.

The objective of the present study was to compare housing and management factors of dairy cattle farms showing larger variations within selected animal welfare indicators he percentages of the most examined animal welfare indicators (except lameness) and scores of the welfare criterion “social behaviour” were in accordance with the findings of other studies using the WQP.

Comparing the two farms shown in Table 1, we can see a uniformity throughout the analysis, even though the number of individuals placed under observation is significantly different. As shown in Table 2, in farms C, D and E, it can be seen and taken into account the uniformity of the results, given that the management practises are similarly between these three farms. It can be seen a correlation between the practice and the state of being of the animals, more so the Welfare criteria being met in every assessment made over the course of the study. The most important assessment, and the indicator of welfare that is important to be assessed weekly is the health, being that the state of health of one animal can change the behaviour and thus affecting all the other indicators that should be assessed.

Table 1

Values of welfare assessment in Farm A and B

Criteria	Description	Farm A		Farm B	
		Number	%	Number	%
B.C	Normal	32	84	120	80
	Slim	1	2.6	7	4.6
	Overweight	5	13.4	23	15.4
Lifting Beh.	<5 Sec	15	39.47	119	79.33
	> 5 Sec	20	52.63	20	13.33
	Difficult	2	5.26	10	6.66
Hitting	Abnormal	1	2.64	1	0.68
	0	20	52.63	139	92.66
	1	1	2.64	1	0.68
	Can Not Be Seen	17	44.73	10	6.66
Hygine Tarsus	Clean	28	73.68	112	74.66
	Areas Of Dirt (10 Cm Diameter)	10	26.32	38	25.34
Hygine Posterior Area	Clean	29	76.32	75	50
	Areas Of Dirt (10 Cm Diameter)	9	23.68	75	50
Udder Hygine	Clean	30	78.95	75	50
	Areas Of Dirt (10 Cm Diameter)	8	21.05	75	50
Lameness	No Lameness	35	92.1	145	96.66
	Moderate	2	5.2	3	2
	Severe	1	2.7	2	1.34
Nasal Discharge	Absent	37	97.3	137	91.33
	Present	1	2.7	13	8.67
Ocular Discharge	Absent	38	100	149	99.33
	Present	0	0	1	0.67
Vulvar Discharge	Absent	28	73.7	127	84.66
	Present	10	26.3	23	15.34
Human-Animal Rel	No Movement+Touching Is Allowed	10	26.3	10	6.66
	No Movement+ No Touching Allowed	10	26.3	40	26.66
	Movemet Is Made 1	5	13.2	50	33.36
	Movement Is Made	10	26.3	40	26.66
	Flight	3	7.9	10	6.66

Table 2

Values of welfare assessment in Farm C, D and E

Criteria	Description	Farm C		Farm D		Farm E	
		Nr.	%	Nr.	%	Nr.	%
B.c	Normal	83	83	91	91	55	78.6
	Slim	8	8	1	8	8	11.4
	Overweight	9	9	8	1	7	10
Lifting beh.	<5 sec	80	80	97	97	58	82.87
	> 5 sec	10	10	1	1	4	5.71
	Difficult	7	7	1	1	5	7.14
	Abnormal	3	3	1	1	3	4.28
Hitting	0	99	99	99	99	61	87.15
	1	1	1	1	1	4	5.71
	Can not be seen	0	0	0	0	5	7.14
Hygine tarsus	Clean	78	78	80	80	58	84.28
	Areas of dirt (10 cm diameter)	22	22	20	20	11	15.72
Hygine posterior area	Clean	82	82	87	87	49	70
	Areas of dirt (10 cm diameter)	18	18	13	13	21	30
Udder hygine	Clean	87	87	97	97	58	82.86
	Areas of dirt (10 cm diameter)	13	13	3	3	12	17.14
Lameness	No lameness	96	96	99	99	66	94.28
	moderate	3	3	1	1	2	2.86
	severe	1	1	0	0	2	2.86
Nasal discharge	Absent	99	99	99	99	69	98.57
	Present	1	1	1	1	1	1.43
Ocular discharge	Absent	100	100	100	100	70	100
	Present	0	0	0	0	0	0
Vulvar discharge	Absent	87	87	91	91	59	84.29
	Present	13	13	9	9	11	15.71
Human-animal relationship	No Movement+Touching Is Allowed	40	40	42	42	42	60
	No Movement+ No Touching Allowed	40	40	42	42	12	17.14
	Movemet Is Made 1	10	10	11	11	10	14.28
	Movement Is Made	5	5	3	3	4	5.71
	Flight	5	5	2	2	2	2.87

At Farm C: Body Condition: good; Lifting Behaviour and Interaction with The Equipment in the Shelter: well; Body Hygiene: good; Health: good; Human-Animal Relationship: good. The final scoring made, and given for farm C being **GOOD**.

At Farm D: Body Condition: good; Lifting Behaviour and Interaction with Equipment in the Shelter: well; Body Hygiene: good; Health: good; Human-Animal Relationship: good. The final scoring made, and given for farm D being **GOOD**.

At the Farm E: Body Condition: acceptable; Lifting Behaviour and Interaction with Equipment in the Shelter: acceptable; Body Hygiene: acceptable; Health: acceptable; Human-Animal Relationship: good, the final scoring being **ACCEPTABLE**.

Conclusions

The farms were classified in two of the four possible welfare categories based on the scores obtained for the four welfare principles: acceptable (nr. 3) and good (nr. 2). This study demonstrated that the welfare of dairy cows is significantly influenced by the housing system, and that the loose system has advantages when it comes to the feeding, housing and behaviour of the dairy cow.

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SCREENING OF *CLOSTRIDIUM DIFFICILE* INFECTIONS IN PETS - PILOT STUDY

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Summary

Clostridioides (Clostridium) diificile is an opportunistic toxigenic bacterium involved in mild or severe digestive infections in animals and humans. This condition is often associated with prolonged antibiotic treatment or with immunocompromised patients. In pets, the main reservoir for this microorganism is largely represented by dogs and cats with clinically disease, asymptomatic carriers or contaminated external environment. In this pilot study we tested the presence of toxigenic *Clostridium difficile* in 35 feces samples collected from dogs (n=25) and cats (n=10) with enteritis. A Combo immunochromatography test was used to detect glutamate dehydrogenase (GDH), an antigenic marker of the bacteria proliferation, and, most important, the toxins A and/or B. The results revealed the presence of *Clostridium difficile* in feces samples from 5 dogs and one cat, but in canine samples only 2 revealed to be toxigenic, toxin A being identified in both cases.

Keywords: *Clostridium difficile*, pets, Combo immunochromatography, GDH, A/B toxins.

Clostridioides (Clostridium) difficile (*C. difficile)* (27) represents a complex of genetically diverse bacteria strains, some of them toxigenic and others non-toxigenic, that colonize the gastrointestinal tract in humans and animals (25, 51, 58). *C. difficile* is associated with mild to severe diseases in humans, but also in veterinary medicine, in various animal species.

C. difficile is a spore-forming, gram-positive bacteria, which, in anaerobic conditions, produces the most important virulence factors, the toxins responsible for digestive symptoms (60, 65). Because in the presence of oxygen can sporulate, this pathogen can survive in environmental conditions, as well as under the action of gastric acids (40).

Among animals, the infection or the carrier state has been reported in primates, horses (56), farm animals (pigs, cows, sheep, goats), dogs, cats, poultry (58) laboratory animals (rabbits, rats, hamsters), ostriches, white-tailed deer, wild birds, zebras, Asian elephants and ocelots (2, 25, 57). *C. difficile* appears to be a normal component of the colonic microbiota in mammals, particularly in young individuals (62).

Although the sources of infection are diverse, direct contact with the feces of animals with clinical signs (52) or ingestion of food (64) or water contaminated with spores (39) causes infections in susceptible animals. However, it seems that

asymptomatic animals can be a much greater source of infectious spores than symptomatic animals that eliminate the vegetative form of the bacteria. The survival of the vegetative form in the intestine for extended periods can cause sporulation due to the deprivation of nutritional elements (34).

In humans this enteropathogen is considered responsible for the majority of nosocomial antibiotic - and health care - associated infectious diarrhea cases (28, 54), but, in the same time, it is a common cause of enteritis in various animas species (16). Although initially the enteritis produced by this germ was considered a complication of extensive antibiotic treatments, currently it can become a real challenge for immunocompromised patients (13) and community - acquired infections became more common than hospital - acquired infections (19). Thus, toxigenic strains can produce symptoms ranging from low-severity diarrhea, pseudomembranous colitis, toxic megacolon, colon perforation and death (51).

The evolution of the process following contamination can be diverse. After spore ingestion and their germination in the intestine**,** *Clostridium difficile* produces three toxins: enterotoxin A (TcdA), responsible for severe inflammation of the epithelial intestinal cells, damage of the intestinal mucosa (25), cytotoxin B (TcdB), responsible for apoptosis of epithelial cells (45, 51, 55) and the binary toxin (CDT) with the role of increasing adherence and colonization (44). Usually, CDT is associated with increased severity of the disease (3, 9) and recurrent infections, being identified as an additional toxin in 4-12% of toxigenic strains (20, 50). The A/B toxins encoding genes are placed on a chromosomial region named PaLoc (pathogenity locus) (7). CDT instead is encoded by cdtA and cdtB genes, located on a distinct region from the PaLoc, named the Cdt locus or CdtLoc (10). Strains that lack genes encoding for TcdA, TcdB, and CDT are considered nontoxigenic and clinically irrelevant (58). In the same time, it become clear that those strains are able to acquire the PaLoc from toxigenic strains through horizontal gene transfer, becoming toxin producing strains (8).

The purpose of this pilot study was to evaluate the presence of the toxigenic strains of *C. difficile* in pets (dogs and cats) with clinically expressed enteritis, some of them recurrent.

Materials and methods

For this study 35 pets (25 dogs and 10 cats) with enteritis were evaluated. The pathological material was represented by fresh feces collected from animals with diarrhea. Most of them expressed recurrent digestive problems and previously received antibiotic treatment. The microscopic examination of the Gram-stained smears with the Leica optical microscope (100x) revealed the presence of a relatively large number of germs with *Clostridium difficile* - like morphology. Based on the anamnesis and clinical symptoms it became necessary to confirm or deny the presence of the *C. difficile* and the toxins in the feces sample.

The diagnosis was based on specific toxins A/B identification using immunological tests, much more efficient and fast compared to the bacteriological exam. For this purpose, a lateral flow immunochromatographic test was used, following the manufacturer instructions. Rapid Combo test Clostridium difficile GDH + Toxin A + Toxin B Dr. Smart allowed the qualitative detection of *C. difficile* GDH antigens (Glutamate-Dehydrogenase), together with toxins A and B.

Results and discussions

The study concerning *C. difficile* infections screening was performed on fecal samples collected from adult dogs (n=22), adult cats (n=9) with obvious signs of enteritis, and 4 samples collected from young pets (3 puppies and 1 kitten). However, it should be taken into account that infectious enteritis can be caused by a multitude of bacteria (*Campylobacter, Salmonella, E. coli, C. perfringens,* etc.), viruses, parasites (*Giardia*), but also non-infectious causes (allergies or various inflammatory processes) (46). Frequent symptoms observed in the animal patients were diarrhea, loss of appetite, fever, soft stools with mucus, semi-liquid feces, persistent for 1-2 weeks, streaks of blood in the feces, dehydration with varying degrees, vomiting. A 9-year-old half-breed female from a private shelter developed enteritis after 4 days of enrofloxacin administration.

Microscopic examination aimed to triage samples, immunochromatography being used only if microorganisms with characteristic morphology for clostridia were observed. Because there are other bacteria with similar morphology, specific identification is required. Usually, bacteria identification is based on the isolation, followed by cultural, morphological, biochemical, pathogenicity and serological characters analysis or molecular methods. In this particular case, because the isolation is difficult and because animals can be carriers of non-toxigenic strains of *C. difficile*, the diagnostic was based on the identification of the bacterial antigens and the toxins produced and released in feces.

Fecal samples were collected from dogs (n=25) aged between 2 weeks and 12 years, (n=11 females and n=14 males). The patients came from shelters (n=3), the rest of them being animals with owners (n=22). No breed predisposition was observed in individuals with digestive disorders, 16 being mixed breeds and 9 animals from the Golden Retriever, German Shepherd, Yorkshire Terrier, Maltese Bichon, Husky breeds.

The microscopic examination revealed Gram positive bacilli with clostridium-like morphology in 17 of the 25 smears. The lateral flow immunochromatography test was positive for 5/25 cases. In 3/5 (12%) half-breeds (a 12-year-old male, a 10-year-old female and a 6-week-old female), only the GDH antigen was identified. *Clostridium difficile* GDH is an enzyme produced in large amounts by toxigenic and non-toxigenic strains, being considered a marker for these bacteria. In the absence of a positive result for a toxin as well, this microorganism can be considered non-toxigenic or the amount of toxin is too small, titer considered undetectable. In the

other 2/5 cases (2 males aged 6 and 8 years), toxin A was identified. In both cases the animals presented gastrointestinal clinical signs.

The results obtained in this study showed a prevalence of 20% *C. difficile* in dogs, with toxigenic and non-toxigenic strains. Referring strictly to the toxigenic strains, only 8% of dogs were infected.

For this study, feces samples were harvested also from felines aged between 3 weeks and 12 years, with an equal distribution between sexes. Regarding the origin, the samples came from public and private shelters (n=5), the rest being animals with owners (n=5). From the breed point of view, 7 were from the European breed, and the rest from the Siamese, Persian or British shorthair breeds. The same protocol was followed, and due to the presence of Gram positive bacilli with morphology similar to *C. difficile* in 8/10 examined smears, immunochromatography tests identified the pathogen and the toxin A only in 1/10 case (a 4-year-old female, European breed, with profuse diarrhea, streaks of blood, fever).

Concerning cats, out study revealed a prevalence of 10%. Other studies showed the presence of *C. difficile* or toxins A and B in different proportions. In 2013, in Brazil, Silva et al. identified toxins A/B in 36.8% cases, 76.2% being from diarrheic dogs (45). In 2002, Marks et. al (32) identified toxin A in 12.5% diarrheic dogs and 8.7% (15).

In cats, a study conducted by Madewell et. al (31) in 1999 revealed 9,4% presence of *C. difficile* and toxin A and B sequences in 8/23 isolates. A study in Australia showed 39.5% fecal carriage of both toxigenic and nontoxigenic isolates (38). In 2020, Silva et al. (48) revealed the presence of the bacteria at 5% of the cats, 1.6% being the toxigenic type. In Eastern China, in 2019, Wei et al. (60) showed that shedding is not common in healthy pets, with 7% in cat faecal sample and 0.7 in dogs. According to others healthy dogs and cats can shed *C. difficile* at rates of 3.4%–5.5% and 2.5%–3.4%, respectively (36, 43).

Another study in Germany, in 2018 revealed 3% isolation in pets and 2.9% in owners. (36). According to Bjöerdorff et al. (6) in 2021, various studies on dogs without gastrointestinal clinical signs revealed the lowest carriage rates in Europe (3,7-4.4%) and higher rates in Malawi (12.0%), USA (16.8%), Brazil (28.6%) and Japan (29.2%).

Toxin A is a potent enterotoxin with pro-inflammatory activity (25) and the release of pro-inflammatory cytokines causes inflammation and damage to the intestinal mucosa (42). Some authors consider that in domestic animals clostridial infections are mediated by toxin A, since toxin B probably lacks the ability to bind in neonatal pigs and to induce lesions in porcine intestinal explants (26, 49).

The results obtained in our study, but also in similar studies have multiple valances. In animals, the symptoms that draw attention are acute or chronic diarrhea and colitis (5, 15). The identification of the etiological agent and most important, the toxins, establishes a diagnostic, but the isolation offers the possibility to determine a sensibility profile and antibiotics useful for the treatment. It is possible, like stipulated in other cases, that this pathogen doesn’t have a primary role in the appearance of

the clinical signs, but to have a role in worsening them. In dogs, the involvement of this bacteria in the production of primary or secondary infections is still controversial (33).

Some studies have shown that it is possible that an increased prevalence of the enterotoxin-encoding cpe gene (*Cl. difficile*) in faecal samples positive for *Clostridium perfringens* to influence the occurrence of infection with the latter. In other cases, it was observed that dogs with diarrhea and hematochezia, following treatment with metronidazole, had an obvious improvement in clinical signs, although A/B toxins were still present in these individuals, suggesting that they may contribute to worsening symptoms as secondary agents (46).

In dogs, vancomycin is rarely used, but metronidazole is widely administered for control of acute diarrhea and chronic enteropathy (24). Regarding the susceptibility of *C. difficile* strains isolated from animals, the results are quite controversial. Although in humans most strains are resistant, those isolated from dogs are still sensitive to metronidazole and vancomycin (61), although metronidazole resistant strains were identified in horses (23). With the same importance, vancomycin, cefoxitin, clindamycin, ampicillin, erythromycin resistant strains were isolated (18, 60). This situation can change dramatically, if drugs are used without discerning, producing a really scary movie, but a real fact, when no active antibiotic are available.

Although the main transmission route is fecal – oral one, according to some studies, due to the fact that animals use the nose as a detection tool, the prolonged contact of their nose with the soil showed a portage of 19% of the spores in the proximal and distal areas of the nose, from where they can reach the environment by secretions or, through toileting, in the digestive tract (40) In cats, a direct contamination is observed as a result of using the tongue to groom the fur.

On the other hand, ill animals are a source of infection for other susceptible animals or even humans. Farm animals can be used as a source of animal origin food for humans or other animals (5).

From this point of view genotyping *C. difficile* strains allows the evaluation of the similarity degree between the strains isolated from humans and animals, which may suggest a zoonotic transmission (51). What predisposes to the appearance of these toxic genotypes is the high degree of plasticity of the genome with the possibility of recombination (3).

C. difficile is recognized as an emerging pathogen in both humans and animals, being considered an emerging community-acquired pathogen associated with a zoonotic and/or foodborne transmission (41). In time, many authors stipulated that pets could be implicated in the transmission of *C. difficile* to humans because similar genotypes were revealed in both (2). Among the strains isolated from dogs, there are several PCR-ribotypes of international interest, such as RT078, RT014/020 and RT045 (14).

In humans, the severity of infections is associated with certain ribotypes 078, 027 014 and 020 (40). Worldwide, ribotype 014/020 is considered the most

frequently present in dog samples (6, 22, 61), but in Brazil, for example, ribotype 106 is the one implicated in human and animal infections, being identified in dogs and cats (47, 48, 58). Ribotype 078 is considered the etiological agent of infectious enteritis in pigs and cattle (25), being in the same time isolated from human patients in Europe, raising concerns about zoonotic or food origin (58). 027 is a hypervirulent strain linked to humans' infections in Europe and Northern America, being isolated from cattle and horses but rarely in companion animals (21, 36). A study of Albuquerque in 2021, identified ribotypes 039, 010, 020, 012 (1).

Another aspect that should not be neglected is the fact that asymptomatic animals can function as a reservoir of toxigenic ribotypes common to humans and animals, constituting a source of infection for susceptible individuals. Although there is no clear evidence of direct transmission between pets and owners, this potential should not be neglected (25). Loo in 2016 (29) revealed that 22% cats and 40% dogs belonging to owners with *C. difficile* infections carried these bacteria with identical profiles as their owners *(*37*).* The real relevance of *C. difficile* as a cause of feline diarrhea remains unclear relatively. There are not available a lot of information regarding the frequency of *C. difficile* fecal shedding in cats and the molecular characteristics of these strains (48, 59).

Clostridial infections evolves as a general infection with a specific localization in the digestive tract and with varied clinical manifestations. The immunopathogenetic mechanism of the disease is approximately common for all susceptible species, being influenced by a number of factors such as: physiological state, age, bacteria strain and the infectious dose, maintenance and feeding conditions, the ability of the host microbiota by composition and competition for nutrients to oppose the colonization of the colonic epithelium (62). Prolonged antibiotic treatments create a profound imbalance in the colon microbiota, mainly in extremely young animals (with an incompletely formed microbiome), thus allowing colonization and subsequent multiplication, followed by the release and action of toxins (30).

Considering the sporulated character of this bacteria, the spores, under the action of primary bile acids from the small intestine, germinate and transform in metabolic active vegetative cells able to multiply rapidly, to adhere and to colonize the intestinal epithelial cells following antibiotic - induced dysbiosis (11, 53). *C. difficile* shows a special affinity for the large intestine, the organ where the most obvious lesions are usually found (35). It should be noted that the presence of spores is not always followed by colonization, some hosts serving as transient carriers of bacteria or spores (51). Approximately 1-3% of spore-bearing human individuals are asymptomatic (4).

In order to exert their effect, toxins attach to specific receptors represented by cell surface proteins, one possible protein receptor for TcdA, sucrase-isomaltase, being found on cells in the ileum of rabbits, but not on cells targeted within the human colon (35). Other receptors are expressed on the brush border from the small intestin. Following endocytosis, they will induce pore formation and cell lysis. TcdA

and TcdB act on the epithelial cells, inducing the rupture of the tight junctions and cell death. Under toxin action, colonic cells synthesize proinflammatory cytokines and inflammatory cells release chemo-attractants (interleukin-8/IL-8, monocyte chemoattractant protein-1), mediators of the inflammatory response. Because of it, the permeability will increase, the natural protective local barrier will be affected and toxins and microorganisms can enter in the intestinal cells, stimulating macrophages and dendritic cells to release inflammatory mediators (tumor necrosis alpha, IL-1, IL-6, IL-8). The process can be mediated also by the neurons and mast cells scattered in intestinal mucosa, releasing histamine and other cytokines. A prolonged inflammatory process will attract more cells that can finally damage and destroy the intestinal wall (11).

Some studies revealed the fact that TcdB is able to induce apoptosis when is produced in low amounts or necrosis mediated by increased production of reactive oxygen species when is released in high amounts (12, 17, 63).

Although the natural mechanisms of the innate immunity try to maintain or to regain the balance at intestinal level, sometimes is really complicated once the toxin interact with their receptors. For this kind of situations, researchers tested various methods for protecting the organism in a specific manner.

Conclusions

It is very easy to observe that *Clostridiodes (Clostridium) difficile* is a very intriguing bacteria, with a lot of resources to survive and to adapt to new hosts, finding breach and making individuals susceptible. Animals are potential infectious reservoirs, being able to create carriers or to infect humans and other animals. Healthy animals can retain spores on various body external or internal surfaces and spread it to owners, or the reverse. It is important to evaluate the presence of the toxigenic and non-toxigenic *C. difficile* strains in ill or healthy animals. Our pilot study focused on identification of the toxigenic strains in samples harvested from dogs and cats with digestive symptoms. Next steps will target their isolation and genotyping.

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THE INFLUENCE OF THE BREEDING TECHNOLOGY ON THE HEALTH OF THE CALVES FROM TWO FARMS IN TIMIŞ COUNTY

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Summary

The purpose of this work was to assess the efficacy of two different breeding technologies in dairy farming, based on the weight of the calves at weaning and on the frequency of veterinary interventions during this period. Three lots of calves were selected from Farm A, an intensive breeding farm, where the calves are separated from the cows shortly after birth and fed using artificial feeding until they are 2 months old (weaning age). A group of calves were selected from Farm B which is using a traditional breeding technology, the cow and the calf being separated after de 15th-day post-birth. The separated calves are kept in groups and fed exclusively with collected milk for a 30-day period. The results revealed that the lot of calves from Farm B didn't need any type of medical intervention, while the groups from Farm A displayed respiratory and digestive pathologies during this time interval. The calves from Farm B had a higher average weaning weight than those from Farm B.

Keywords: technology, housing, treatment, calves.

Increasing efficiency in livestock production is needed to ensure that enough food can be produced to meet the demands of a growing population, help reduce the environmental impact, and ensure that food is available and affordable (15, 22). Like other automatic monitoring technologies, feeding calves through a robotized system can decrease human-animal interactions, which may not be favorable (10, 21). Good colostrum management is still recognized as the single most important factor to preventing calf morbidity and mortality; however, it is now known that immunoglobulins are only one of many components of colostrum that are vital for the calf's development (2, 3, 14). A biologically normal (intensive) milk-feeding programme is subsequently necessary for optimal body growth, organ development and resistance to infectious diseases. Ad-libitum or close to ad-libitum feeding in the first three to four weeks of life also leaves calves less hungry thus improving calf welfare. Only calves fed intensively with colostrum and milk are able to reach their full potential (1, 4, 15) for performance throughout their life. Bovine colostrum contains bioactive proteins in higher abundance then mature milk. When the proteomes of colostrum and milk are categorized according to their biological function, the largest difference is observed in the category of proteins responsible for metabolic processes. Colostrum quality is defined by Ig concentration and remains one crucial factor for successful passive transfer (5, 8, 12). Since direct laboratory methods for the determination of Ig in colostrum are elaborate and

expensive, the use of cow-side testing of colostrum quality has been intensively investigated (9, 10).

On most of the last century research in the area of calf nutrition and management focused on reducing the amounts of whole milk fed to dairy calves prior to weaning by all means possible. In two seminal studies Kahn et al. (6, 7) presented a method which allows feeding high volumes of milk in the first three to four weeks of live without the drawback of stagnating growth during weaning due to poor starter intake. Calves in the step-down group did not only gain weight and grow much faster than conventionally fed calves, they also showed higher starter and forage intake after the reduction of feeding volumes. Thus, profound changes in current calf management practices are needed to improve dairy calf health and survival, enhance long-time performance of dairy heifers and satisfy consumer interests in farm animal welfare.

Materials and methods

For monitoring animals, pedometers are used, which record the number of steps, standing time, rest time and the amount of milk given. The forage front consists of a place for the feeding bucket, the bucket of water and a container for the fodder to be given in granulated form. The removal of manure from under the berths is done by washing with a large volume of water at regular intervals. Water from the mains is collected in basins that drain under the berths when they reach a certain level.

Ventilation is commonly associated with respiratory health in animals; the quality of the air the animals breathe directly influences the health and diseases of the animals. But ventilation, directly and indirectly, has an impact on many other aspects of animal health:

The calculation necessary to determine the state of thermal comfort, implicitly the effective temperature can be made, for all species, according to a formula designed and experienced by Thom (11, 17), taking into account the fact that of the pregnant females of the farm animals, prolonged stress, regardless of the triggers (cold, heat, etc.), has negative consequences on the adaptability of newborns. This is mainly due to the transition of maternal corticosteroids into the placental circulation, with the effect of inhibiting the activity of the adrenal cortical foetus (5, 12, 16).

Technological flow

At 225 days of gestation, the heifers receive the first vaccine, Kolibin©, against neonatal diarrhea of calves, a vaccine that includes antigens against gastroenteric diseases caused by *Rotaviruses*, *Coronaviruses* and enteropathogenic *E. coli*, with a booster every two weeks. At 249 days gestation is confirmed and the heifers are moved to the calving preparation cages, to the primiparous boxes with common permanent litter. At the time of calving, the heifer is moved to the calving box. After calving, a marking tape of freshly calved is placed on it to be more easily

observed on the parlor, in view of collecting colostrum (at the first and two milkings) (19). The colostrum is tested with the lactodensiometer, it is placed in three-litre bags, and then the bag is put in plastic cassettes and frozen. On the colostrum bag, the number of cows, the date of collection and the executioner are noted. The cow stays for 15 days in the group of freshly calving cows, where the ration is rich in energy and protein, and after 15 days it is moved to the primiparous group. 50-60 days after farrowing, the first artificial sowing takes place. After calving, the calf receives an elastic band on the anterior right leg with the identification number of the mother. He will be left with his mother to be licked, but he will be supervised not to suck or be trampled by the mother. Within 20 minutes after calving, a calf regardless of gender receives three liters of colostrum with the help of an oesophagal probe and will be moved to individual berths, depending on gender. In addition to milk, they receive a mixture of musli and clean water at discretion. After 58 days, a week they receive three liters in the morning, and the next week only granules. On the second day of life, calves are vaccinated with intranasal vaccine with modified virus strains (Rispoval RS) and the level of serum postcolostral proteins is tested. On day 35, vaccination against *Trichophyton* takes place, and the booster is made on day 49th. On day 65, calves are dewormed orally with Toltrazuril©.

Results and discussions

The first lot consisted of ten females born from 01.01.2020 to 05.01.2020, these having the crotarations 3460, 3461, 3462, 3463, 3464, 3465, 3466, 3466, 3467, 3468 and 3469 (Table 1). The average weight at calving was 37.5 kg, with a minimum value of 28 kg and a maximum of 43.5 kg. At weaning, on the 65th day of life, the average weight was 84.5 kg. In this batch, 6 out of 10 calves had respiratory diseases, two of which also had digestive disorders. Domestic animals, especially hybrids obtained in the last 10 years, are dependent on human care, especially for housing and fodder, because being unilaterally selected, they need special living conditions (13, 20, 17). Of the physical environmental factors, temperature exerts the strongest influence on the metabolism and implicitly on the systems that ensure the state of health. The state of health implies an optimal development of the biological functions of the organism, which are conditioned by the complex of relations between the organism and the environment. It is a feature of the way of existence of living matter, differentiated and organized structurally and functionally in the body. That is, a healthy organism is characterized by a balanced unfolding of all biological processes, which ensures its adaptation to the various demands of the environment (10, 15). Thus, the first bovine (3460) received treatment for respiratory diseases on 30.04, the treatment consisting of taking the antibiotic Forcyl© for five days, Melovem©, which has an anti-inflammatory role and the Intravit© product containing a vitamin complex. At the end of May on the 29th, the same therapeutic protocol was administered because the respiratory symptoms returned. The second vine (3661) received a single administration of antibiotic Draxxin© on 11.02.2020,

and on 29.05 followed the therapeutic protocol consisting of the administration of Melovem©, Forcy©l and Intravit to treat respiratory diseases. The third calf (3462) showed digestive disorders on the 16th day after calving and received the same treatment (Synulox, Melovem© and Introvit©). On 28.01. Florkem©, Melovem© and Intravit© were administered to treat respiratory conditions, and on 19.02 he received a single dose of Draxxin. Calves four, five, six and nine (3463, 3464, 3465, 3468) did not show any ailments until 07.06.2020 when the monitoring was completed. The seventh-day calf (3466) had respiratory diseases and was treated on 23.01 with the antibiotic Florkem©, Melovem© and Introvit©. The eighth calf (3467) received treatment on 21.01.2020 for respiratory diseases, the treatment consisting of the administration for 5 days of Baytril©, Melovem© and 5 days of administration of vitamin C.

Table 1

Median values of experimental lots

Crt. No.	Reg. Number	Weight at birth	Weight at farrowing	Clinical signs	No. of treaments	
1	3462	32	82	D+R	3	
2	3465	43,5	89	-	0	
3	3467	36	84	D+R	5(+)	
4	3678	42	85	R	1	
5	3673	30	80	-	0	
6	3682	44	83	R	2	
7	3460	30	78	R	1	
8	3649	32	78	-	0	
9	3466	36	82	-	0	
10	3464	35	80	D+R	2	
Median Values		**36.05**	**82.1**	**Total calves with problems**	**6**	**1.4**

D- digestive problems, R- respiratory problems, (+)- deceased

On 28.01.2020, the second lot consisting of ten females, which were born from 02.03.2020 to 11.03.2020, had at calving, an average weight of 35 kg, the minimum value being 30 kg and the maximum value of 42 kg, and at weaning, the average weight was 83 kg. In this batch, the prevalence of respiratory diseases was 8 out of 10. The third lot was formed of calves calving in May, starting with 01.05.2020 until 11.05.2020. They had, at calving, an average weight of 33.5 kg, the minimum weight was 29 kg, and the maximum was 38.5 kg. In this group 4 out of 10 had respiratory diseases, two calves also showed digestive disorders, and one of them showed only digestive symptoms. The eighth calf showed respiratory disorders two weeks after calving, the treatment. Because the symptoms did not go away, on 28.05 he was given Oxytetracycline, Melovem® Vitamin C and Glucose 10%. After two days the vine died from complications.

In contrast (lot 4) 15 calves were monitored from a dairy (B) cow farm, where the calf was checked next to the cow for 15 days, after which they received collecting milk. Of the 15 calves, only two had a slight diarrhoea on day 16, which did not require therapeutic intervention. The average weight at calving was 28 kg, and at weaning, 85 kg. It can also be seen that the calves in group 4, although they had a lower fattening weight than the calves in the other three groups, had higher growth increases, reaching weaning at higher body masses. In the fourth lot there were neither mortality nor specialized medical intervention was required, as the transient diarrhoea of the 16th day appeared against the background of the transition from breast milk to collecting milk.

Conclusions

Technological drilling requires a higher, sometimes unjustified (18), contribution of sanitary actions, which most often are to the detriment of the animal (calf), but also of man, both from an economic and health point of view, so in the first three lots, mortality was also recorded as a result of respiratory and/or digestive complications, and in lot 4 there were no significant problems. It can thus conclude that depending on the chosen breeding method, pathologies can be observed in the breeding systems, which are directly proportional to the change in the technological flow in terms of the animal's nutrition.

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DISORDERS OF SEX DEVELOPMENT IN CATS – TWO CASE STUDIES

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Summary

Disorders of sex development (DSD) include any congenital or developmental abnormality of any part of the female or male reproductive tract, definition that is used also in veterinary medicine and science, not only in humans. In feline's DSD, abnormalities of chromosomal sex, gonadal sex or phenotypic sex are reported. In this article we want to shortly review the embryogenesis of the reproductive tract, to describe the possible causes that can generate such abnormalities of sexual development and to provide two examples – one case with bilateral ovarian agenesia and another one with unilateral ovarian agenesia and complete unilateral aplasia of the uterine horn, abormalities identified during routine elective ovariohisterectomy. The aim of this paper is to contribute to a better understanding of DSD feline's case studies seen in practice.

Keywords: sexual development, reproductive abnormalies, Mullerian aplasia, disorder of sex development, ovarian agenesia.

Developmental abnormalities of the ovaries or/and utero-vaginal segments in animals can be accompanied by clinical signs or, more often, can be encountered during elective ovariohysterectomy. Some examples of patologies included here with different clinical aspects from severe to less severe we named: ovarian agenesis, ovarian hypoplasia, cystic epoophoron, uterus unicornis, unilateral or bilateral segmental aplasia of the uterine horns, segmental aplasia of the uterine body or the cervix, uterus didelphus, segmental aplasia of the vagina/vestibulo-vaginal constriction, imperforate hymen etc (9). For a better understanding of the etiopathogenesis of these developmental disorders, we describe the main embryonic stages through which the genital tract is formed in mammals. Knowledge of embryogenesis of the genital tract is very important, because if during this period there are inherited even *de novo* gene mutations, or external factors that interfere with the stages of development, clinically, sooner or later in the development of the individual, changes in the reproductive function will be identified.

Embryogenesis of the reproductive system

Normal embryonic sexual development takes place in three stages: chromosomal, gonadal and phenotypic or somatic stage. Different mechanisms such as differentiation, migration, fusion and canalization manage to complete this dynamic process (6).

The chromosomal stage occurs at the moment of fertilization, when the zygote obtains the normal number of chromosomes. Each blastomere maintains its normal number of chromosomes during embryonic mitotic divisions. At this stage, the embryos are sexually indifferent. Sexual differentiation begins when the genital ridge forms, the place where the gonads are formed.

The gonadal stage is the beginning of embryonic sexual differentiation and takes place under the influence of the expression of specific genes such as SRY, located on the short arm of the Y chromosome, as well as autosomal genes, such as SOX9, located on chromosome 17 which has an important role in testicular development. For the initiation of ovarian development, there is no single gene signal, this inevitably takes place in the absence of the SRY master gene.

The phenotypic stage is the last stage in the development of sex and takes place mainly under the influence of steroid hormones. The internal and external segments of the reproductive system, as well as the urinary system, have their embryonic origin in the intermediate mesoderm. The formation process of these segments is a staged one, during which some structures regress. Thus, the pronephros, mesonephros, metanephros, Muller's ducts and Wolff's ducts coexist parallel at the level of the genital ridge. Their formation takes place at different postfertilization days, depending on the species.

Among these structures, the pronephros degenerates early, the mesonephros also regresses almost entirely, and the main urinary organ, the kidneys will form from the metanephros. From Muller's ducts (the paramesonephric ducts) in the female fetus, in the absence of the Mullerian inhibitory hormone produced by Sertoli cells, the oviducts, uterine horns, uterine body, cervix and cranial vagina will form, and from Wolff's ducts (mesonephirc ducts), in male embryos, under the influence of androgenic hormones produced by the testes, will form the epididymis, the vas deferens and the seminal vesicles (2, 7, 10, 32).

At the beginning, the Mullerian canals are paired throughout their entire length, and as they approach the urogenital sinus, they will merge. In carnivores, fusion progresses cranially only a short way beyond the cervix. Caudally, the fusion ends where the ducts make contact with the urogenital sinus (9). Focal defects during the development of the paramesonephric canals cause segmental aplasia or stenosis of the uterine horn.

The rest of the internal genital segments, as well as the external genital segments, will be formed from a bipotential structure - cloaca. The cloaca is divided by the urorectal fold in the rectum - the dorsal part and in the urogenital sinus - the ventral part. From the urogenital sinus in females the caudal vagina, vaginal vestibule and hymen will form, and in males the urethra, prostate, bulbo-urethral glands will form. Other important embryonic segments are the genital tubercle - from which the clitoris or the penis will be formed, as well as the labioscrotal folds from which the vulvar labia or the scrotum will be formed (10, 32).

The complete classification of the genital tract abnormalities is based on the histological analysis. Thus hypoplastic uterine horns are defined as being

underdeveloped, but having the histological layers and the normal functional lumen. The unicornuate uterus has only one uterine horn, the other being missing. In this situation, the tissue in place of the uterine horn that connects the uterine body to the uterine tube lacks normal layering and a lumen and is often reduced to a fibrous cordiform remnant. Segmental agenesis implies the presence of an underdeveloped portion of the uterine horn (6, 14).

Congenital anomalies of the uterine development have been reported in most species of professional interest, such as cow, mare, deer, sheep, sow, ferrets, rats etc. (4, 16, 23, 26, 31). In Table 1 are presented the main ovarian and uterine-vaginal abnormalities in animals that were reported in literature.

Table 1

Classification of ovarian and uterine-vaginal abnormalities in animals

Type of ovarian and uterine-vaginal abnormality	Cause	References
Ovarian agenesis/Ovarian aplasia	Failure of migration and synchronous mitotic division of primordial germ cells	(10,20,21,22)
Ovarian hypoplasia		
Agenesis of the uterine horns	Failure of Mullerian ducts to develop	(6)
Uterus unicornis/ Unilateral aplasia of the uterine horn		(6)
		(6,23)
Unilateral or bilateral segmental aplasia of the uterus		(6)
Segmental aplasia of the uterine body	Failure of the caudal parts of the two Mullerian ducts to fuse appropriately and to form a single lumen	(6,18)
Segmental aplasia of the cervix		(6,18
Logitudinal septation of the uterine body/ *Uterus didelphus*		(6)
The cranial vaginal septation		(6)
Segmental stenosis of the vagina/ Aplasia of the vagina/ Vestibulo-vaginal constriction	Failure of the caudal parts of the Mullerian ducts to fuse with invaginated urogenital sinus to establish anatomical continuity	(6,18)
Longitudinal septum in the cranial vagina		(6,18)
Imperforate hymen		(6,18)

The frequency of these disorders in female cats was 0.09% (49/53,258) and 0.05% (15/32,660) in female dogs, data reported by McIntyre et al. (14). The uterine anomalies identified were unicornuate uterus, segmental horn agenesis and hypoplasia of the uterine horn. Ipilateral renal agenesis was present in 29.4% (10/34) of cats and 50.0% (6/12) of dogs with uterine abnormalities in which the kidneys were also evaluated, and the ovaries and oviducts were present in most cases with uterine anomalies (14).

The causes of these congenital abnormalities can be multifactorial, such as genetic, endocrine or environmental factors and hence is difficult to identify (6).

A case of congenital vaginal obstruction in a Bull-Mastiff dog was reported by Gee (9). Clinically the dog had an enlarged vulva with a whitish mucopurulent content, enlarged uterus observed during the ultrasound examination and at the exploratory laparotomy a congenital obstruction of the vagina of approximately 9 cm was identified. The cranial vagina, cervix, uterus and oviducts developed normally. The surgical treatment to remove the obstructed vaginal region was carried out without affecting the reproductive function of the female. The offspring obtained at the second heat cycle after the surgical intervention did not present such problems. The probable cause of this vaginal obstruction was the lack of fusion and canalization of the Mullerian duct system with the urogenital sinus (9).

Similar case with congenital segmental agenesis of the uterine body, cervix and vagina was described in a five-year-old Pomeranian female who presented anorexia, abdominal distension, and absence of vaginal discharge during estrus. The cause in this case was the non-fusion of caudal parts of Mullerian ducts in order to develop a single lumen (18).

In cattle, segmental aplasia of Muller's ducts is known as *"White Heifer Disease"*, being associated with the gene for white coat color in Shorthorn and Belgian Blue cows, but similar cases have been reported in other breeds such as Holstein, Jerseys, Senepol and Zebu, with a prevalence of 0.15% to 0.2%. The trait of inheritance is sex-linked recessive. The frequency of the pathological form is higher on the right paramesonephric duct, which probably indicates an asynchronous development of the two channels (16). Segmental aplasia or stenosis of the uterine horn must be considered as a differential diagnosis in cattle with uterine enlargement that is nonresponsive to prostaglandin treatment. Multiple ultrasound examinations are necessary to have a correct diagnosis in this situation (11, 16).

In goats, disoders of sexual development (e.g. XX sex reversal, polled intersex syndrome - PIS), may be related to the polled gene. The cause identified in intersex Chinese goats was ~0.48 Mb duplicated fragment (including *ERG* and *KCNJ15*) downstream of the ~20 Mb PIS region that was reversely inserted into the PIS locus (8). Other abnormalities of the female genital tract seen in small ruminants is freemartinism, with 5% incidence from 80 ewes genital tract collected from a slaughterhouse (27).

The purpose of this work is to briefly review the embryogenesis of the reproductive tract, to describe the possible causes that can generate abnormalities of sexual development, to provide two examples that are included in these categories of anomalies and to give other cases as examples for these abnormalities.

Materials and methods

The two clinical cases describe in this article and identified during routine ovariohisterectomy are presented in Fig.1.

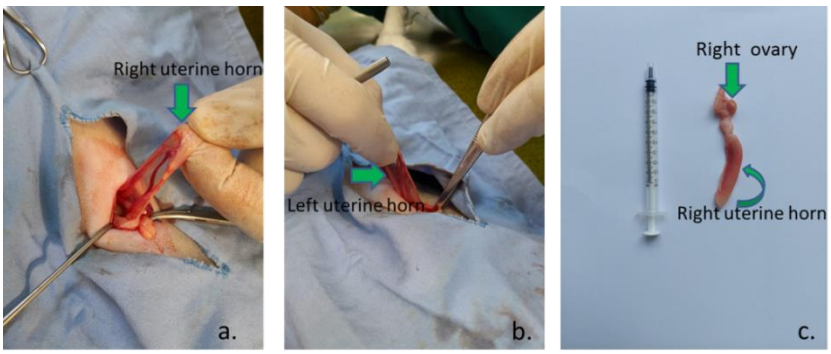

Fig. 1. Case no1. Female domestic cat during elective ovariohisterectomy with bilateral ovarian agenesia, both uterine horns are presented (a-b); Case no2. Right uterine horn with right ovary identified during elective ovariohisterectomy (c)

The first case, was a 2 years old female domestic cat that was presented to the Small Animal Reproduction Clinic from Faculty of Veterinary Medicine, Timisoara in December 2022 for routine ovariohisterectomy. The cat didn't had cycled regularly and no other significant reproductive history. On presentation the patient was in good condition. After the anesthesia protocol was carried out with Xylazine 1-1.5 mg/kg body weight IM, Ketamine 10-15 mg/kg body weight IM and Propofol 1-2 mg/kg body weight IV the surgical procedure was done. During surgical intervantion bilateral ovarian agenesia was identified with the present of the uterine horns.

The second case, was an average 2 years old female domestic cat that was presented to a veterinary clinic (SC.SANROVET-DUO.SRL) in Sânmihaiu Român in March 2023 for routine ovariohisteroctomy. No reproductive history was known for this case due to unknown origin of the cat. During surgical intervention unilateral ovarian agenesia and complete unilateral aplasia of the left uterine horn was observed.

Results and discussions

The two cases with abnormalities in the female genital tract were identified during elective ovariohyterectomies. In both cases there were no clinical signs. In the first case presented, bilateral ovarian agenesis was found, with the presence of Mullerian segments (Fig. 1.a – Fig. 1.b), and in the second case, it was observed unilateral ovarian agenesia and complete unilateral aplasia of the left uterine horn (Fig. 1.c).

Congenital anomalies of the reproductive tract in cats are diverse, ovarian agenesis or ovarian aplasia (present of an undifferentiated ovarian tissue) has a low incidence and causes permanent anestrus, ovarian hypoplasia (differentiated ovarian tissue), supernumerary ovaries and atypical development (hermaphtodites) are other clinical forms that can occur (21). These pathologies can be associated with the absence of the ipsilateral kidney because they have a common embryonic origin, an aspect that cannot be confirmed in the two cases presented. Chromosomal changes such as 37, XO; 37, X/39, XXX, 37, XO/38, XX karyotypes are associated with ovarian aplasia or hypoplasia in the cat (21, 28).

Ovarian agenesis has been reported in dogs and cats, either unilaterally or bilaterally. The histopathological examination is the sure way to diagnose aplasia (lack of development of the gonads) or their hypoplasia (21).

In human medicine, unilateral absent ovarian has a prevalence of 1 in 11241, but this number is possible underestimate since the pathology is often asymptomatic. This disorder is often associated with the partial or complete absence of the ipsilateral oviduct and occasionally there are other associated anomalies. Among the possible causes are those of embryonic origin (disturbance in the formation of this structure), vascular (ischemia following a vascular accident) or torsion (torsion of the ovary maybe preceded by the formation of an ovarian cyst that leads to adnexal autoamputation). The last two causes can occur in utero or during early postpartum development (30). Knock-out mice for *LHX1, LHX9, EMX2, NR5A1* and *Wt1* genes lack gonads completely, but agenesis affects also the kidneys due to their common origin. Other genetic factors, such as *OXL2*, *WNT4*, *TCF21* (*POD1)*, *SIX1/4, INSR, IGF1R AND INSRR* has been shown to affect gonadal development. Such deletions usually results in gonadal hypoplasia, dysgenesis. The phenotype in such situations is greatly influenced by the time and the tissue where the mutant gene should be expressed (5).

Ovarian hypoplasia can be seen in animals as a consequence of failure of migration and synchronous mitotic division of primordial germ cells (PGCs). Mechanisms of PGC migration vary by organism, though the process is highly conserved (3). In mouse, for example the process last for approximately four hours and can be dividid into: transepithelial migration across the midgut, reorientation to the dorsal side of the midgut and finally bilateral migration into the mesoderm toward genital ridge (20). In cattle, gonadal hypoplasia is common in the Scandinavian Northern Finncattle and Swedish Mountain cattle breeds, being inherited in an autosomal recessive form with incomplete penetrance. In these cases the body development is normal, the genital segments being present. The cause identified in the Finncattle breed through a genomic analysis was a translocation between chromosomes 6 and 29 involving a 500 kb duplication in the *KIT* oncogene is the underlying genetic mechanism responsible for gonadal hypoplasia, normal karyotype XX (29). On the other hand, if gonadal hypoplasia or agenesis is associated with the small stature of the animals, a possible cause may be monosomy X, being found in different species, such as buffaloes, cats, dogs, horses, llamas,

alpacas, sheep, pigs, foxes (22). In dogs, gonadal dysgenesis syndrome appears also in mosaicism cases, e.g. 77, XO – 78, XX (17) and phenotipically can be associated with small stature, but also excessive skin in the ventrum of the neck, supernumerary digits etc (26), or with gonadoblastomas, rare mixed germ cell neoplasms (19). An unusual case of ovarian dysplasia associated with granulosa cell hyperplasia and a benign vaginal fibroleiomyoma was identified in a young intact Golden Retriver, where the most likely cause was the excessive production of estrogen from the hyperplastic granulosa cells (15). It is known that sex steroid hormones have a role in the initiation, promotion and progression of the carcinogenesis cascade and the presence of estrogen-α and progesterone receptors in canine leiomyomas indicates that these hormones can lead to genital tumors (13). In alpacas, the minute chromosome syndrome (MCS) has been associated with ovarian hypoplasia, but the molecular mechanisms are yet unknown (1).

An interesting DSD case was described by (25) in an intact 8-year-old domestic cat, where they identified during routine ovariohysterectomy segmental aplasia of the uterus, cervix and vagina. The failed canalization of the cervix had as consequence a blind dilation of the cranial vagina in which normal secretions collected, forming an atypical cystic lesion. Also Salar et al. (24) presents a case encountered in a 2-year-old cat with left ovarian agenesis with the left uterine horn without any abnormality and on the right side both the ovary and uterine horn were identified, without an adverse effect on sexual activity of the queen (24).

In cases of unilateral segmental aplasia of the uterine horn with partial or complete occlusion the major consequence is the accumulation of fluid in the affected uterine horn or in the entire uterus, depending on the degree and position of the occlusion. In most cases, these developmental disorders of the genital system are identified on routine ovariohysterectomy, because the reproductive function is supported by the unaffected uterine horn (21). Another case of segmental aplasia of the uterine body was reported in a 5-year-old mixed breed dog by Ki-Seok Oh et al. (12). In this case, a dilatation of the uterine horns with a hydrometer was identified radiographically and sonographically, without highlighting a uterine body, this being represented by a piece of cord-like tissue was identified as an aplastic/dysplastic remnant which made the connection with the cervix and the right uterine horn (12).

The discussions carried out on account of the similar cases reported in the literature underline the varied clinical symptoms as well as the importance of knowing the embryogenesis of these anatomical structures in order to be able to explain the occurrence of the cases.

Conclusions

Sexual development is a complicated, staged process with multiple interactions between different genes, hormones, hormone receptors etc. DSD deviations can occur at any stage of the sexual development process. Affected animals generally show infertility and/or ambiguous sex, but also other complications such as pyometra, hydrometra, cysts. Definitive diagnosis requires genomic, cytogenetic and histopathologic investigations.

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STUDY ON THE EXPERIMENTAL INFESTATION OF RATS WITH LARVAE OF *TRICHINELLA* SPP.

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Summary

Nematodes of the *Trichinella* genus are among the most widespread zoonotic parasites located in the muscle tissue of a wide variety of terrestrial vertebrate species native to all continents. *Trichinella spiralis* is the species most adapted to domestic and wild pigs and rats, having a cosmopolitan distribution. This species is also the most important etiological agent of human disease. Rats play a role in the transmission of *Trichinella* spp. from domestic to sylvatic animals and vice versa. In this context, the aim of the study was to evaluate the viability of *Trichinella* spp. larvae and the parasite load of the muscles with which the rats were experimentally infested. Experimental infestation in rats proved to be possible by administering samples from pigs (70 g) with a parasite load of 500 larvae, respectively with samples from foxes (5 g) with a parasite load of 2300 larvae. The obtained results emphasize the fact that the rat remains the most important vector in the transmission of trichinellosis and its maintenance in animals.

Keywords: trichinellosis, experimental infestation, rat.

Nematodes of the genus *Trichinella* are among the most widespread zoonotic parasites, located in the muscle tissue of a wide variety of terrestrial vertebrates originating from all continents (3, 4, 6, 7).

Trichinella spiralis is the species most adapted to domestic and wild pigs and rats, having a cosmopolitan distribution. This species is also the most important etiological agent of human disease (15, 17, 16, 19).

Infestation is achieved by ingesting meat infested with first-stage larvae (L1), which decapsulate in the stomach and invade the lining of the small intestine, where they molt four times in the first 24-30 hours. After reaching sexual maturity, adult females start releasing larvae as early as day 5 after infection. The newborn larvae migrate throughout the host's body and localize in the striated muscle cells (4, 5, 6).

Rats play a role in the transmission of *Trichinella* spp. from domestic to sylvatic animals and vice versa (20).

In this context, the aim of the study was to evaluate the viability of *Trichinella* spp. larvae and the parasite load of the muscles with which the rats were experimentally infested.

Materials and methods

The research was carried out in the Parasitology Clinic of the Faculty of Veterinary Medicine, Timișoara. The experiments were performed on 2 batches of 10 rats each (20 rats), 10 males and 10 females. The experiments were carried out in collaboration with the "Victor Babeș" University of Medicine and Pharmacy in Timisoara.

- ✓ Experiment 1 – an experimental infestation of rats with muscle from pigs containing cysts of *Trichinella* spp.

The batch used for this experiment consisted of 10 rats, 5 males, and 5 females. The rats were housed separately, one per cage, and were observed daily throughout the experiment. To be able to identify them, the letter "M" followed by a number from 1 to 5 (M1, M2, M3, M4, M5) was written on the cage of each male rat, and the letter "F" on the cage of the female rats (F1, F2, F3, F4, F5) (Fig. 1).

Fig. 1. Housing rats in separate cages

Striated muscles with *Trichinella* spp. cysts from pigs were used to infest rats.

The trichinelloscopic examination allowed us to confirm the presence of *Trichinella* spp. larvae in the muscle samples that served as materials for infestation. The samples were kept in the refrigerator until the time of rat infestation. They were starved for 1-2 days before being infested to ensure the consumption of muscle samples.

The 10 rats were given 70 g of striated muscle from the pig (Fig. 2. a, b).

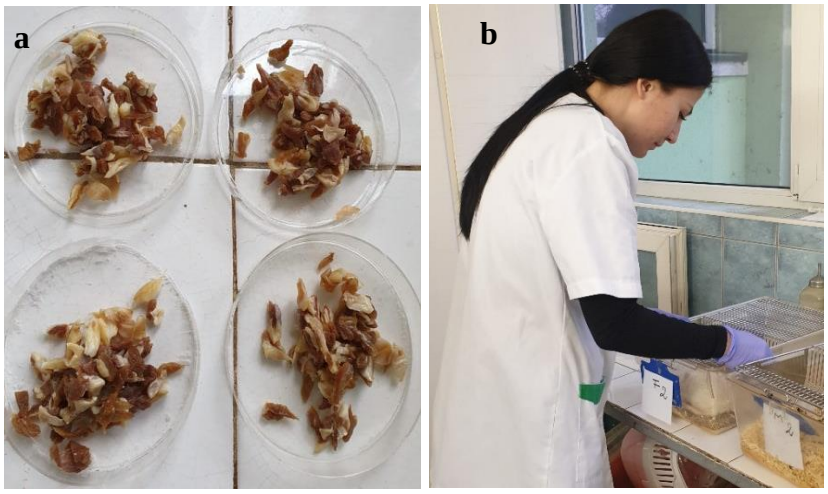

Fig. 2. a, b. Rats infestation – an experiment I

Rats consumed all *Trichinella* spp. larvae-infested muscle provided.

After a period of 30 days post-infestation, the rats were sacrificed and each individual subject to the study was examined by direct trichinellscopy.

- ✓ Experiment 2 – an experimental infestation of rats with muscle from a fox containing cysts of *Trichinella* spp.

The batch for this experiment also consisted of 10 rats, 5 males, and 5 females. They were assigned one rat per cage and scored as in the first experiment.

For the infestation of the rats in this batch, striated muscles with cysts of *Trichinella* spp. from the fox were used.

The trichinelloscopic examination allowed us to confirm the presence of *Trichinella* spp. larvae in the meat samples that served as materials for infestation. The samples were kept in the refrigerator until the time of rat infestation. They were starved for 1-2 days before being infested to ensure the consumption of muscle samples.

The 10 rats were given 5 g of fox striated muscle (Fig. 3. a, b).

After a period of 30 days post-infestation, each rat was sacrificed, striated muscle samples were collected from each individual, and we performed the examination by direct trichinelloscopy.

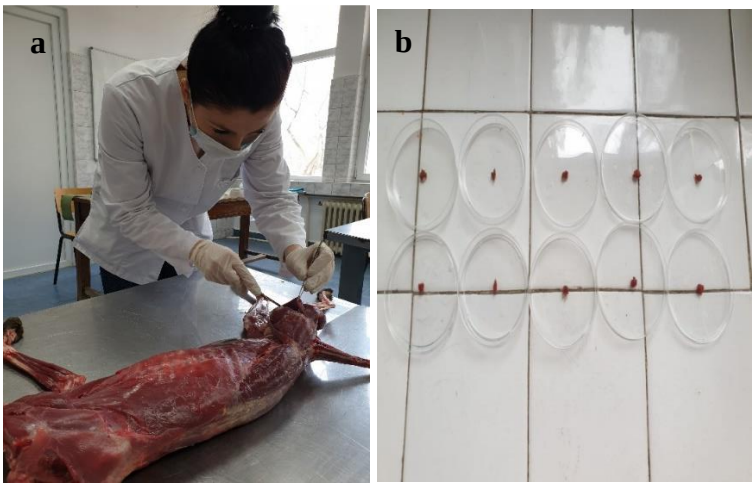

Fig. 3. a, b. Rats infestation – experiment II

Results and discussions

✓ Experiment I:

The number of larvae contained in the 70 g of muscularity of the pig with which the rats were infested (500 larvae) proved to be sufficient to achieve the experimental infestation in all individuals in the batch (Fig. 4. a, b).

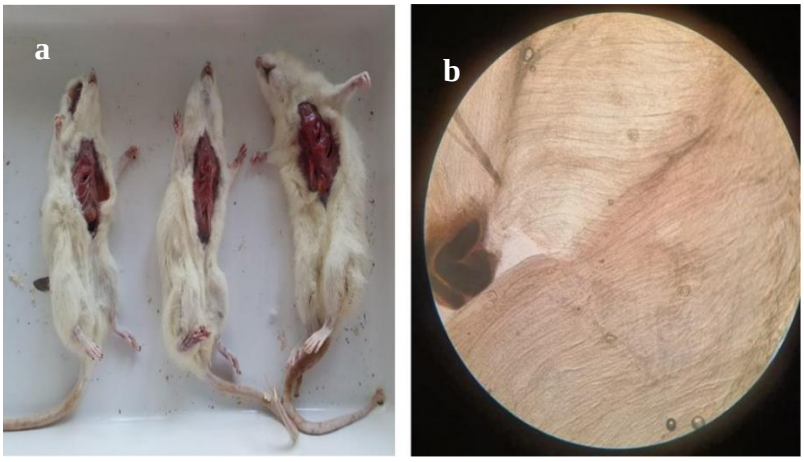

Fig. 4. a, b. Cysts of *Trichinella* spp. – rat muscle

✓ Experiment II:

The experimental infestation with 5 g of fox meat, with a concentration of 2300 larvae, proved to be effective in achieving the experimental infestation in all individuals in the batch (Fig 5. a, b)

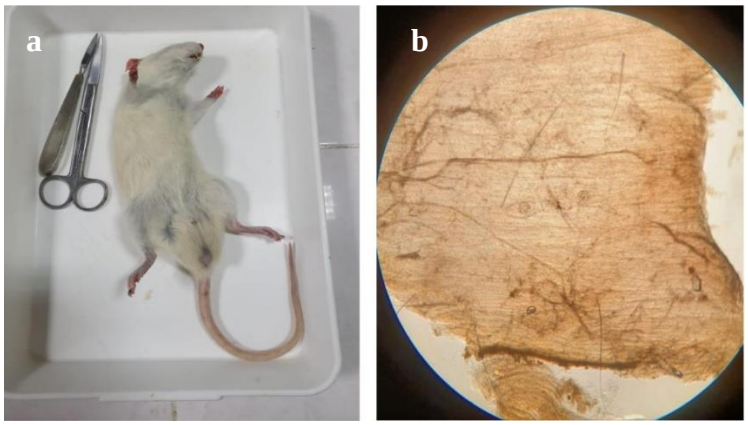

Fig. 5. a, b. Cysts of *Trichinella* spp. – rat muscle

In Romania, numerous studies have reported the identification of *Trichinella* spp. in wild species (2, 8, 9, 11, 12). Of these, wild boar is a significant source of human contamination (13, 14).

For most specialized authors, the rat remains the most important vector in the transmission of trichinellosis and its maintenance in livestock, a fact demonstrated by the high prevalence of the disease in pigs from farms where infested rats live (1, 18).

Marian I. et al. (10) conducted a study to assess the effect of *T. spiralis* on the effort capacity of experimentally infected mice. The study demonstrates the reduction of muscular capacity in mice experimentally infected with *Trichinella spiralis*, in correlation with the infective dose, providing new insights into this parasite's transmission strategy (10).

Wang N. et al. (21) have carried out a study to investigate the infectivity and antibody response of four *Trichinella* species (*Trichinella spiralis*, *Trichinella britovi*, *Trichinella pseudospiralis*, and *Trichinella murrelli*) in experimentally infected pigs. The results showed that the larvae first be detectable for *T. spiralis, T. britovi*, and *T. pseudospiralis* at 16 dpi, 17 dpi, and 16 dpi, respectively. These results provide important information in the primary characterization of pigs infected with *Trichinella* (21).

Conclusions

Experimental infestation in rats from batch 1 proved to be possible by administering striated muscle samples from pigs, in the amount of 70 g with a parasite load of 500 larvae.

Experimental infestation of rats in batch 2 was shown to be possible with samples from foxes in the amount of 5 g with a parasite load of 2300 larvae.

Acknowledgments

This scientific paper was carried out in the Laboratory of Parasitology and Parasitic Diseases at the Faculty of Veterinary Medicine Timisoara, Laboratory within the Animal Hygiene and Pathology Research Center*/* ULS Timisoara.

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USSING CHAMBER: THE STUDY OF TRANSEPITHELIAL TRANSPORT IN THE SMALL INTESTINE

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Summary

The Danish zoologist and physiologist Hans Henriksen Ussing invented the device named after him in the 1950's as a means to measure the short-circuit current with interest to the passage of ions through frog skin. The device suffered modifications over time as today there are many models in use. The Ussing chamber system provides a physiological method for studying the transport of ions, nutrients and farmaceutical compounds across epithelial tissues *ex vivo* in health and disease. Studies can be done either on native preparations of epithelial tissues or on cell monolayer cultures. The technique is suitable for quantifying transport and barrier functions of tissues. The focus of this study is directed towards the conformation, principle and method applied in the study of intestinal absorption and secretion with interest to the Ussing chamber and also to outline the advantages and limitations of the technique. The importance of the technique resides in the fact that it is versatile regarding the substances that can be used in the experiments, yields well controlled experimental conditions and offers possibilities of better understanding the dynamic of ions, nutrients and drug compounds transport across the intestinal tissue and can provide precious data to the medical researchers and developers of pharmaceutical preparations.

Keywords: rat, intestine, epithelial transport, Ussing chamber.

The danish zoologist Hans Ussing invented the device (Fig.1) in the 1950's. He used the Ussing chamber to determine the value of the current that passes through short circuited frog skin and with the help of Na^{24} isotope marker he quantified the influx and eflux of sodium, therefore establishing the permeability of the tissue (22, 40). The frog skin (S) is placed between the two celluloid tubes (C) as a separation membrane for the Ringer solution that was used to fill the two C tubes. Two agar bridges (A and A') are placed in the close proximity of the two sides of the epithelial tissue: mucosal and serosal side. The KCl-calomel electrodes come in contact with the exterior ends of the agar bridges. The potentiometer, noted with P, is placed for measuring the potential difference. The electrodes immersed in a KCl saturated solution (saturated in AgCl) are placed as far as possible from the tissue, at the ends of the C tubes. In the saturated KCl solution lay immersed the ends of a thick silver spiraled wire through which voltage can be sent to the tissue. A battery is needed as a voltage source (noted with D), and also a potential splitter (W), meant to maintain the value read on the potentiometer at 0 mV. For reading the value of current that passes through the epithelia, a microammeter is needed (M) (40).

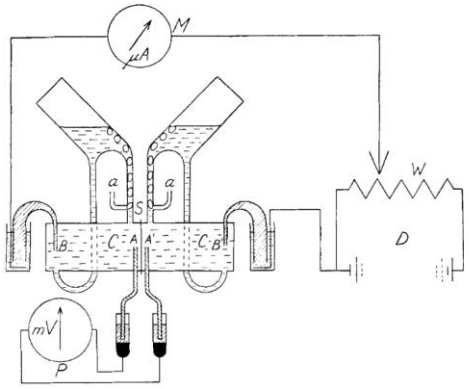

Fig. 1. The classical Ussing chamber device (37)

Materials and methods

In time, the device has undergone changes, so at the present time there are many models in use. The main components (Fig. 2) of a Ussing chamber are:

- The chambers
- A perfusion system
- A water circulating system with heating jackets
- An amplifier
- A data aquisition system.

Besides those components, there's also need for inserts (specific models for each type of tissue) and two electrodes placed in each half of the chamber: one Ag/AgCl pellet electrode for voltage sensing and one Ag wire electrode for passing of current). The electrodes are connected to the chamber via agar salt bridges (34, 38, 43, 44).

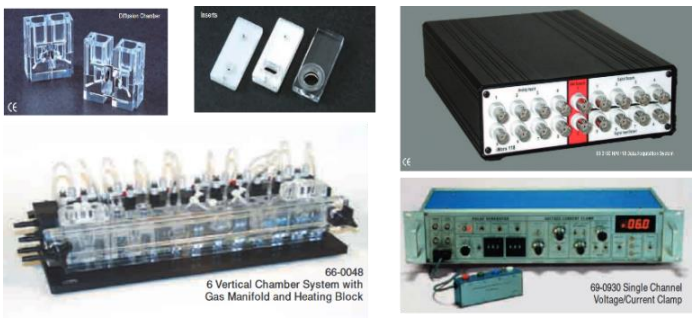

Fig. 2. Components of a Ussing chamber (44)

The chambers are made of a solid transparent compound (either an acryl derivate or tetrafluoroethylene). There are many types of chambers, with round and slotted openings for the placement of tissues. Chambers are available in vertical (Multi-channel chamber systems like Navicyte Vertical and EasyMount models) and horizontal configuration (Classical Ussing chamber systems) (43, 44).

The chambers are divided in halves. They are separated by the mounted tissue in the inserts, so each side of the epithelia mounted (the mucosal or apical side and the serosal or basolateral side) is oriented towards a half of the chamber. The system also supports the use of cell monolayers (26, 29, 43, 44). The volume of liquid that can be introduced in each half of the chamber can vary with the model. Each half of the chamber has specific entries for the perfusion system, the carbogen gas mixture, the placement of electrodes and the water circulating system (9, 43, 44). The perfusion solutions that are used can differ slightly, based on the aim of the experiment. The composition of the perfusion liquid can alter the transepithelial transport. Physiologically relevant buffers have to be used in order to satisfy the needs of the tissue. Some examples of buffers used in the experiments regarding mammalian intestinal tissue are: Krebs-Ringer buffer, HEPES-buffered Krebs-Ringer solution, Krebs-Ringer phosphate buffer, or other modified variants of Krebs-Ringer solution. The Krebs-Ringer buffer used by Hempstock et al. (21) for the assessment of native intestinal tissue paracellular permeability for ions, attributed by the measurements of transepithelial conductance and dilution potentials, has in composition: 115 mM NaCl, 25 mM $NaHCO_3$, 2.4 mM K_2HPO_4, 1.2 $CaCl_2$, 1.2 mM $MgCl_2$, 0.4 mM KH_2PO_4, at pH 7.4, at a temperature of 37°C and gazed with 95% O_2 and 5% CO_2. Since the study comprises whole samples harvested from the entire length of the intestine, they elaborated a slightly different recipe of KBR for the small intestine (adding 1mM glutamine) and the large intestine (no glutamine added, but 10mM of glucose) (21). Clarke (2009) mentions the addition of 10mM glucose to the serosal bath as an energetic substrate and 10mM mannitol to the mucosal bath to maintain the osmotic balance (9, 10, 21).

In most studies regarding cell expression systems, because it is difficult to sustain CO_2 tension in cell culture preparations, the HEPES buffer is used instead, which does not interfere in the cells' buffering capacity. The HEPES buffer, used for the dilution potential experiment by Hemptock et al. (21) contains: 10 mM HEPES, 10 mM glucose, 1 mM glutamine, 150 mM NaCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 µM indomethacin, at pH 7.4 (using 1 M Tris buffer), at a temperature of 37°C (21).

The two circulating reservoirs (one in each half-chamber) are made of borosilicate glass and have two outputs at their base side for the perfusion system. The gas mixture, driven under low pressure, comes in contact with the buffer, creating bubbles that facilitate the circulation and provide the oxygenation of the solution (43, 44). During the experiments, the temperature of the chambers is kept at 37°C (34).

The amplifier is connected to each chambers' electrodes and is responsible for the amplification and transmission of electrical signals in the form of current and

voltage. The data acquisition system centralizes and transposes the information gathered to the software, where it can be translated into numeric values over time and interpreted (43, 44).

The principle of the method relays on mounting a native or stripped portion of the intestine inside the chamber, each half of the chamber facing either the mucosal side or the serosal side, therefore separating the fluid that bathes each side of the intestinal tissue independently. The mucosa can be separated from the sero-muscular layer under a stereomicroscope, either by blunt dissection or by pulling a taut thread longitudinally between the two layers. The mounted tissue has to follow an equilibration period of 20-30 minutes before the beginning of the experiment, so it can adjunst to the chambers' conditions (9).

The study types that can be done with the Ussing chamber are electrophysiology-based, diffusion-based or both. The electrophysiology-based systems are focused on recording the electrical paramethers: transepithelial resistance (R), short-circuit current (I_{sc}) and transmembrane voltage (V_t) and the response of the tissue to various changes that can be induced experimentally. This type of system lends itself to assess the functioning of electrogenic transport pathways, therefore it is useful in studies that imply ion movement across biological membranes. The electrophysiology-based system requires an amplifier and a data acquisition system related to the software, in comparison to the diffusion-based system (43, 44).

Diffusion-based systems are designed for measuring the water or solute transepithelial transport but without acquiring specific information about the underlying mechanism. This type of system is meant to be used for the measurements of electroneutral transport across leaky epithelia (e.g. intestine, renal tubules, gallbladder) (22, 43, 44).

Transport across the intestinal epithelia refers to absorption and secretion. While absorption is defined as the movement of nutrients, electrolytes and water from the intestinal lumen to the blood stream, the secretion represents the movement of water and solutes from the intracellular space to the luminal space (27).

The transport of ions and nutrients across the intestinal epithelia can take:

- the transcellular pathway, which implies the movement of molecules from the luminal space, through the brush border of the enterocytes, into the cell and then to the basolateral membrane towards the blood stream (absorption) or from the enterocyte intracellular space towards the lumen (secretion); an example of trancellular transported molecule would be fructose which is absorbed by the GLUT5 transporter located at the apical side of the enterocytes (18);
- the paracellular pathway, which refers to the transport of molecules from the luminal space, through the tight junctions, towards the basolateral space and the blood stream; this represents the main route of water absorption in the intestine (10, 12); the inflammatory process of the intestine, associated with inflammatory cytokines act on the tight junctions, resulting in altered intestinal permeability *in vivo* (e.g. Chron's disease) (24, 28, 36);

- both the transcellular and the paracellular pathway; an example of this type of transport would be the transport of water that passes paracelullarly through the tight junctions via a passive mechanism and trancellularly via the Na^+/glucose cotransporter (which delivers in the intracellular space 2 ions of sodium, 1 molecule of glucose and 260 molecules of water) (30);
- the cell mediated transport route, which involves the reaction of the solute with the carrier protein, leading to the formation of a solute-carrier complex, this complex then diffuses through the cell membrane and releases the solute in the intracelullar space (15).

The transcellular and paracellular passage can be altered via administration of different compounds, for instance fitotherapeutical preparations. Jabri et al. (25) investigated how *Matricaria recutita L.* decoctin influences the intestinal transport of glucose in *ex vivo* mouse intestine (25).

Channel blockers can be used to highlight the transfer of certain ions. Ma et al. (31) identified thiazolidinone as a CFTR inhibitor (39). The use of stimulants can underline certain effects on the epithelial tissue transfer of molecules. Cermak et al. (1998) investigated flavonol quercitin effects on rat colon (8).

The passage of molecular species can also be altered by pathological entities. Ysnes and Robertson (39) researched the effects of *Giardia duodenalis* on transcellular and paracellular transport in enterocytes.

Investigating how molecules are transported across the intestinal epithelia and figuring out specifically which route they take and what changes they bring, can give insight to the processes taking place under physiological conditions. Yvonne et al. (2019) investigated prediction of passive drug permeability under fasted and fed states across different regions of the small intestine of pigs (4), followed by a 2020 study which highlighted a possible use of the Ussing chamber system for identification of new molecular entities that are substrates for membrane transporters with interest to drug development (3). Studying the passage of molecules through epithelia can also be done under pathological circumstances. El Mecherfi et al. (14) studied whether the peptic hydrolysis of bovine beta-lactoglobulin (BLG) under microwave treatment reduces its allergency in *ex vivo* murine alergy model, considering the reaction of mice jejunum previously exposed to BLG after stimulation of the gut-assosciated immune system.

Results and discussions

The Ussing chamber system records:

- Potential difference (PD), measured in mV/cm^2;
- Transepithelial resistance (TEER), measured in Ωcm^2;
- Short-circuit current (I_{sc}), measured in $\mu A/cm^2$ (32);

The intestinal tissue is only viable for a limited amount of time so the recordings must be done within the first 3 hours after harvesting the intestine (9).

An important step after mounting the preparate and before beginning the experiment recordings is to assess the viability of the tissue, which is done by evaluating the baseline measurements and then adding glucose or forskolin and checking whether the I_{sc} value has rissen adequately (resulting in values between 100-300 µA/cm^2 in mice intestinal tissue) (21). The baseline measurements of the electrical parameters from domestic and commonly used experimental animals intestinal segments can vary (Table 1) (1, 4, 5, 7, 11, 16, 32, 37).

Table 1

Electrical parameters of the small intestine in different animals

No.	Species	Parameter	Intestinal segment		
			Duodenum	Jejunum	Ileum
1	Chicken	PD (mV/cm^2)	-	1.54±0.50 (1)	~5 (1)
		Isc (µA/cm^2)	2.61±0.95 (1)	130±9.4 (1)	205±16.5 (1)
		TEER (Ωcm^2)	196±30 (1)	471±34 (1)	21-42 (1)
2	Horse	Isc (µA/cm^2)	0.78±0.20 (7)	−0.59±0.18 (7)	-
		G[b] (Ωcm^2)	34.6±3.7 (7)	19.3±1.3 (7)	-
3	Rat	PD (mV/cm^2)	−9.2±0.4 (37)	0.8±0.2 (5)	-2.2±1 (16)
		Isc (µA/cm^2)	-	269±42 (20)	49.1±4.0 (16)
		TEER (Ωcm^2)	97.2±5.1 (32)	66.4±7.1 (32)	60-80 (16)
4	Pig	TEER (Ωcm^2)	57.33±20.66 (4)	41.77±13.79 (4)	40.85±15.08 (4)
5	Rabbit[a]	PD (mV/cm^2)	1.89±0.99 (11)	2.21±0.17 (11)	1.40±0.10 (11)
		Isc (µA/cm^2)	0.85±0.11 (11)	1.10±0.11 (11)	1.20±0.10 (11)
		G[b] (Ωcm^2)	12.00±1.36 (11)	14.10±1.70 (11)	18.50±3.4 (11)

[a]Stripped tissue; [b]Conductance.

One major quality of epithelia relays on the electrogenic activity of ion pumps and it revolves around the ability to maintain the potential difference of the cell membrane (34).

PD is determined via the Ag/AgCl electrodes (38).The barrier function and integrity of the tissue can be assessed based on the values of TEER and the PD, while I_{sc} value points to the active transport of molecules. The values of PD or I_{sc} can and will change drastically following an erratic pattern, if a bubble appears at the tip of the salt bridge (38).

TEER value is determined by the electrical resistance of the paracellular route. It can indicate the overall integrity of the tissue. Low values of TEER can relate to either low permeability, the presence of an inflammatory process or if the low value is accompanied by a rise in I_{sc} and PD, this could indicate the concurrent usage of paracellular and transcellular pathways (38, 42).

According to studies regarding electrical parameters of whole intestinal preparates from pigs, sheep, horse and chicken the highest values of the TEER are

recorded in the duodenum, compared to the other segments of the small intestine (1, 3, 4, 7).

I_{sc} represents the current that is necessary to nullify tissue PD. It can be calculated using the PD and TEER value and it represents the sum of all ionic currents across the epithelium. The rise in cytosolic concentration of Na^+ at the brush border level of enterocytes contributes greatly to the depolarization of the membrane, which stimulates the Na^+/K^+-ATP pump. This determines the increase of Na^+ ions passage from the mucosal side to the serosal side, which translates to the alteration of electrical parameters, like PD and I_{sc} (38).

A rise in the value of I_{sc} is usually indicative of a high rate of ion passage through the epithelia, while the use of inhibitors such as phlorizin (inhibitor of the SGLT2 transporter) will result in a decrease in the I_{sc} value (21, 38, 42, 45).

If the values of I_{sc} decrease over time, it could mean that the tissue viability is declining. The highest values of I_{sc} were recorded in the duodenum of pigs and adult sheep (7).

Making use of the Ussing chamber technique, the permeability of the tissues can be determined. A way to assess the permeability and the integrity of the epithelia with interest to the transcelullar transport, is the use of permeation fluorescent markers (e.g. fluorecently labeled particles of bacteria, horseradish peroxidase) while simultaneously evaluating the elecrophysiological parameters. As for paracellular permeability markers, there can be mentioned: fluorescein isothiocyanate-dextran, polyethylene glycols polymers, carbon-mannitol and carbon-inulin, lucifer yellow and biotin-labelled probes (2, 34).

Following the contents of this paper, some advantages and limitation of the Ussing chamber technique can be mentioned.

Advantages of the method:

- Allows the study of regional transport (duodenum, jejunum, ileum and colon), with the possibility to record the parameters individually, but at the same time;
- High level of control of the experimental conditions of each individual sample;
- Useful for assesing intestinal tissue integrity in animal and human models;
- Allows the study of absorption and secretion across the intestinal epithelia in physiological and pathological conditions (healthy tissues and tissues affected by various diseases);
- Experimental data can be reproduced accurately considering the same experimental conditions;
- Enables the estimation of absolute absorption across epithelia that takes place both via the transcellular and paracellular route, whether the compounds used are found in suspension or solution form;
- Useful application in pharmacokinetics with interest in the effects of drug-drug interaction on absoption (17, 19, 33, 38).

The most important limitations of the method are:

- Limited clinical use;

- Requires rigurous training of staff for the preparation of samples;
- Limited viability of the intestinal tissue;
- The method should be used in combination with *in vivo* experiments to prove the real effects of the studied pharmacological compounds (10, 19, 22, 34, 35, 41).

Conclusions

There is a vast applicability for the technique, providing valuable understanding of the physiological transport proccesses that take place in the intestine. It presents interest mainly in domains like biology, medicine, pharmacology and their related branches.

Although there are some limitations of the technique, the advantages can surpass them taking in consideration the accuracy of the information that can be gathered and the high understanding regarding physiological proceses as a result of applying the Ussing chamber technique.

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MORPHOLOGICAL ASPECTS OF THE CELIAC ARTERY IN SHEEP CORRELATED WITH THE STAGES OF DEVELOPMENT OF THE GASTRIC COMPARTMENTS

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Summary

The study of the development of the digestive system in ruminants, especially the gastric compartments, has been and still is a subject of interest to many researchers. The main characteristic of adult ruminants is their ability to consume large amounts of forage, which they break down into simpler, easily absorbed components. Thus, fermentative digestion is initially carried out, followed by gastric and intestinal digestion. The different development of the gastric compartments, depending on the age of the animal and the type of feeding, leads to variations in the morphology and topography of the arteries supplying these components of the digestive tract. The aim of this research is to identify possible variations in the celiac artery in terms of its origin and its collaterals and to observe whether these variations are the result of changes in the proportions between gastric compartments. Thus, in animals fed exclusively on maternal milk, the abomasum is the only functional compartment, which is reflected by the strong development of the gastric and gastroepiploic arteries. As the animal is weaned and the diet changes, the volume and especially the functional importance of the rumen increases, as the compartment plays a key role in carbohydrate degradation. Similar aspects regarding the vascularization have been observed in individuals belonging to different age groups, but also some differences, such as: origin of the celiac artery (from a common trunk with the cranial mesenteric artery, but also independent from the aorta artery), numerical and topographical variations of the splenic artery.

Keywords: celiac artery, newborn sheep, gastric compartments.

The understanding of the arteries branching from the abdominal aorta (their origin, the presence of atypical variants of common artery origin or the presence of additional arteries) plays an important role in the diagnosis and treatment of some digestive pathologies, as well as in surgical interventions concerning the abdominal cavity (10). The possibility of variations in the trajectory and origin of celiac artery collaterals has a significant impact on the difficulty of surgery and the risks that may occur during surgery. There are studies analyzing variations of the celiac artery in ruminants, such as those by Mohamed et al. (16) where the vascularization of gastric compartments in a breed of sheep (Barbados Black Belly Sheep) is described, but also more detailed research comparing variations of the celiac artery in different species belonging to the family *Bovidae* (14, 15, 16).

However, details on the particularities of the celiac artery in small ruminants according to the age category of the animal are missing, and morphological

correlations between the caliber and distribution of the branches of the celiac artery and the size of the gastric compartments at different stages in their developmental dynamics as a result of changes in diet, are very briefly described.

Materials and methods

The study was conducted on 5 lamb carcasses aged between 2 and 3 days and one adult small ruminant carcass. The animals used in the study came from breeding farms and were destined for dissection and research in the Comparative Anatomy Laboratory of the Faculty of Veterinary Medicine in Bucharest.

The most common method used was dissection. Shortly after the animals were slaughtered, the abdominal cavity was opened through a longitudinal incision in the median plane between the xiphoid appendix and the pubis. The aortic artery was revealed and the site of origin of the celiac artery was observed. A low-viscosity epoxy resin (mixed with a red pigment to highlight the arterial vascularization) was injected centrifugally at this level. Thus, the contrast substance reached the branches of the celiac artery through the celiomesenteric trunk. Complete cure time of the bicomponent epoxy resin was achieved within 24 hours at a constant temperature of 25°C. The solidification of the substance used in the arteries helped to highlight vascular formations and facilitated dissection and following their trajectory.

To make conclusive measurements of the stomach in the ruminant, a moderate amount of polyurethane foam was introduced inside the gastric compartments to restore their shape and volume, while preventing the gastric wall from overstretching.

Results and discussions

The special development of the stomach in ruminants consists in its division into rumen, reticulum, omasum and abomasum. A very important aspect of compartmentalization is the change in the proportions of the resulting components in close correlation with the age of the animal and the type of feed (11, 13). In the pictures below, the differences between gastric compartments in newborn and adult are illustrated (Fig. 2, 3, 4, 5). In the young animals examined in the research, maternal milk was the only source of food. In these animals, the strong development of the abomasum, which is the only compartment involved in milk digestion, is remarkable (Fig. 2, 3). This also implies a topographical change of the digestive organs in the abdominal cavity, with the rumen, reticulum and abomasum projecting on the left side of the cavity (17, 19).

Significant anatomical development of the rumen, both morphometrically (its volume) and structurally, occurs concomitantly with the increased intake of solid food (12, 20, 22). Dietary modification and the transition from a preruminal to a ruminal functional status represents one of the most spectacular adaptations in mammals,

both anatomically and physiologically. In adult ruminants, the solid components of the diet are broken down in the ruminal portion by fermentative digestion unlike in newborn ruminants where digestion is glandular (4, 7, 21).

Measurements of the gastric compartments in moderate state of fullness have been made in both young and adult animals. The results obtained were then represented in the graph below (Fig. 1).

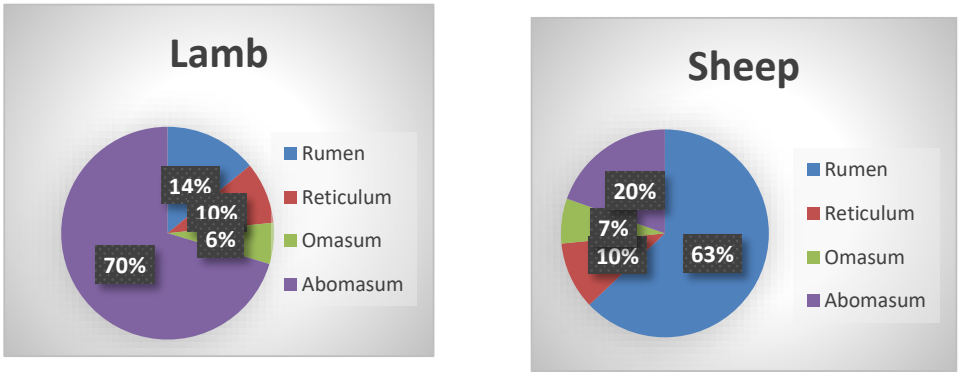

Fig. 1. The change in proportions between gastric compartments in the young and adult ruminant

The values were similar to those in the literature, Samir and Ghadbane, 2021 and Baldwin and Connor, 2017 describing the rumen as representing 50-60% of the digestive tract volume in the adult ruminant (3, 18).

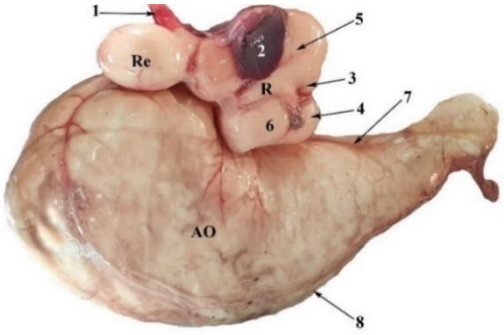

Fig. 2. Gastric compartments in newborn lamb - left side (original)
R- rumen; Re- reticulum; AO- abomasum; 1- esophagus; 2- spleen; 3- caudodorsal blind sac; 4- caudoventral blind sac; 5- dorsal ruminal sac; 6- ventral ruminal sac; 7- lesser curvature of abomasum; 8- greater curvature of abomasum

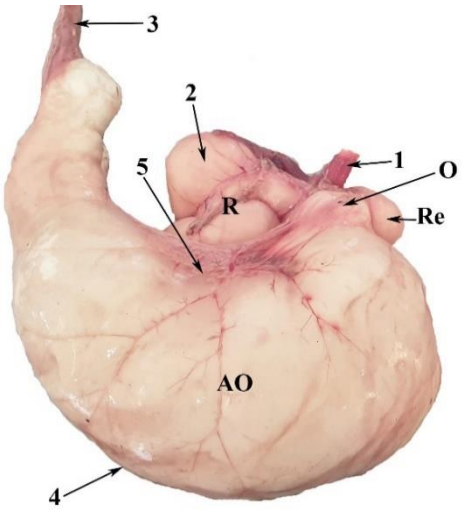

Fig. 3. Gastric compartments in newborn lamb - right side (original)
R- rumen; Re- reticulum; O- omasum; AO- abomasum; 1- esophagus; 2- dorsal ruminal sac; 3- duodenum; 4- greater curvature of abomasum; 5- lesser curvature of abomasum

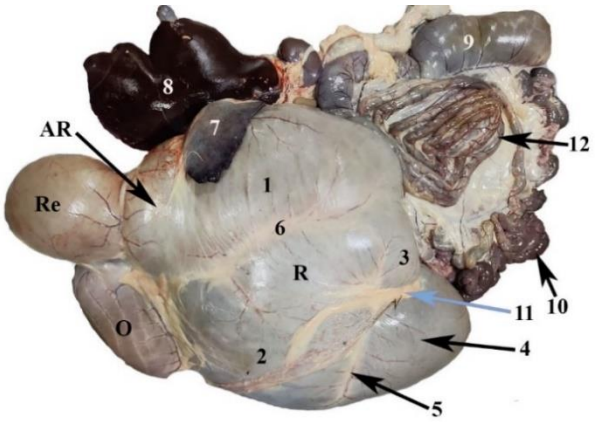

Fig. 4. Gastric compartments of the adult small ruminant - left side (original)
R-rumen; AR- ruminal atrium; Re- reticulum; O- omasum; 1- dorsal ruminal sac; 2- ventral ruminal sac; 3- caudodorsal blind sac; 4- caudoventral blind sac; 5- ventral coronary groove; 6- left accessory groove; 7- spleen; 8- liver; 9- caecum; 10- jejunum; 11- caudal groove; 12- ascending colon.

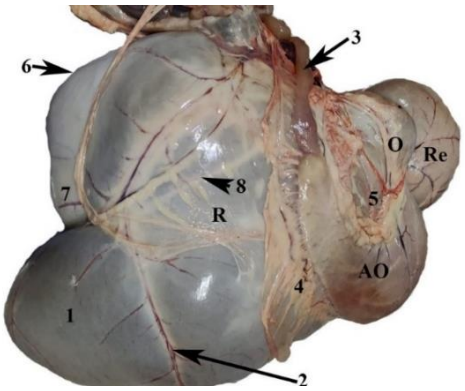

Fig. 5. Gastric compartments of the adult small ruminant - right side (original)
R- rumen; Re- reticulum; O- omasum; AO- abomasum; 1- caudoventral blind sac; 2- ventral coronary groove; 3- duodenum; 4- the greater omentum; 5- the lesser omentum; 6- dorsal curvature of the rumen; 7- caudodorsal blind sac; 8- right longitudinal groove

The following are the variations of the celiac artery that were observed after dissection in young specimens between 2 and 3 days of age.

Between the bodies of the first two lumbar vertebrae (L_1-L_2), ventral to them, the common celiomesenteric trunk branches off from the abdominal aorta and after a 0.5 cm course divides into the celiac artery and the cranial mesenteric artery (Fig. 6). In one specimen, the celiomesenteric trunk was absent, the celiac artery branching independently from the abdominal aorta, before the origin of the cranial mesenteric artery, at a distance of about 0.2 mm from it.

According to the literature, the existence of the common celiomesenteric trunk is common in small ruminants, compared to cattle, where the separate detachment of the two arteries has been generally reported (2, 9).

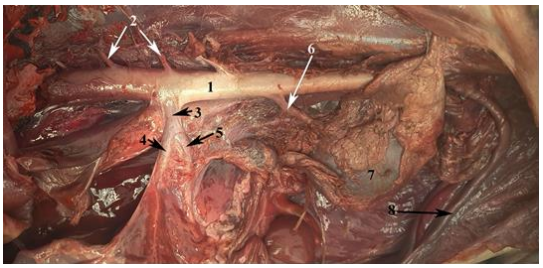

Fig. 6. Origin of the celiomesenteric trunk (original)
1- abdominal aorta; 2- lumbar branches; 3- celiomesenteric trunk; 4- celiac artery; 5- cranial mesenteric artery; 6- renal artery; 7- left kidney; 8- umbilical artery

Therefore, **the celiac artery** (*A. celiaca*) branches above the dorsal ruminal sac and emits 3 branches: hepatic artery, left gastric artery and splenic artery.

The hepatic artery (*A. hepatica*) splits off from the celiac artery near the origin of the left gastric artery, a fact also noted in the study by Berlea et al. 2019 (5). The hepatic artery orients towards the visceral side of the liver, entering at the level of the hepatic hilum and divides into 2 branches: right and left (Fig. 7). The left branch continues its course along the visceral face, entering the hepatic parenchyma at the limit between the quadrate lobe and the left lobe. In all specimens examined, the right gastric artery was seen to detach from the left branch of the hepatic artery approximately 1 cm from the origin of this branch. The right branch is smaller in size and caliber compared to the right and gives a distinct branch to the caudate lobe of the liver, then emits the cystic artery destined for the gallbladder.

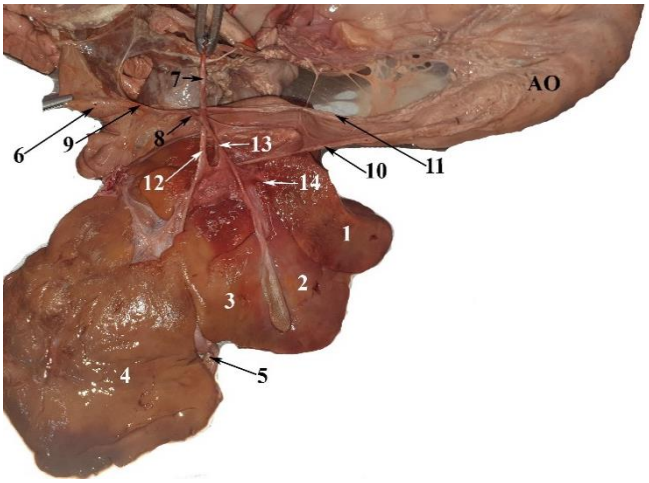

Fig. 7. Terminals of the hepatic artery in newborn sheep (original)
AO-abomasum; 1- caudate lobe; 2- right hepatic lobe; 3- quadrate lobe; 4- left hepatic lobe; 5- umbilical artery; 6- duodenum; 7- hepatic artery; 8- gastro-duodenal artery; 9- cranial pancreatico-duodenal artery; 10- right gastric artery; 11- right gastro-epiploic artery; 12- left branch; 13- right branch; 14- branch for caudate lobe

Before the division of the hepatic artery into two branches, the gastro-duodenal artery (*A. gastroduodenalis*), which has a short path (0.5 cm), separates. This, in turn, divides into the right gastro-epiploic artery (*A. gastroepiploica dextra*) and the cranial pancreato-duodenal artery (*A. pancreaticoduodenalis cranialis*).

The right gastroepiploic artery runs along the great curvature of the duodenum and sends 6-7 branches to this portion of the small intestine and 2 pyloric branches, then continues its course along the greater curvature of the abomasum.

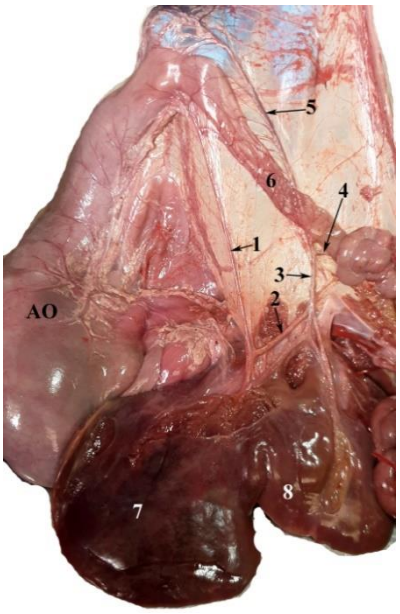

Fig. 8. The origin of the right gastric artery (original)
AO- abomasum; 1- right gastric artery; 2- left branch of hepatic artery; 3- gastro-duodenal artery;4- cranial pancreatico-duodenal artery; 5- right gastro-epiploic artery; 6- duodenum; 7- left hepatic lobe; 8- caudate lobe

In all young specimens examined, **the right gastric artery** (*A. gastrica dextra*) originates from the left branch of the hepatic artery (Fig. 8). It runs ventro-caudally towards the pylorus, in the thickness of the lesser omentum, then follows the lesser curvature of the abomasum where it anastomoses with the left homologue artery. Reaching the pyloric orifice, the artery sends a branch to the lesser curvature of the duodenum over a 4-5 cm portion. In the study by Berlea et al. (5), the right gastric artery is described as a collateral of the hepatic artery, while Mohamed et al. (16) supports its origin from the left branch of the hepatic artery.

The left gastric artery after a 2 cm path from its origin, gives the left gastroepiploic artery which follows the reticulo-omasal junction on the right side, sends 2 reduced branches on the right side of the omasum, then reaches the lesser curvature of the abomasum, an aspect also observed in studies conducted in goats (6, 14). The left gastric artery after following a third of the length of the lesser curvature, deduplicates for about 4 cm, then anastomoses with the right gastric artery. The accessory reticular artery, reduced in development, branches off from the gastric artery, irrigating the right side of the reticulum.

The splenic artery (*A. lienalis*) runs to the left to reach the splenic hilum, where it enters the parenchyma of the organ. After the separation of the two

collaterals: the right ruminal artery and the left ruminal artery, in one specimen the splenic artery is duplicated for 2.5 cm (Fig. 9). The two ruminal arteries have a reduced calibre, making it difficult to follow their course due to the poor development of the rumen in newborn lambs. The reticular artery is reduced in caliber and separates from the left ruminal artery.

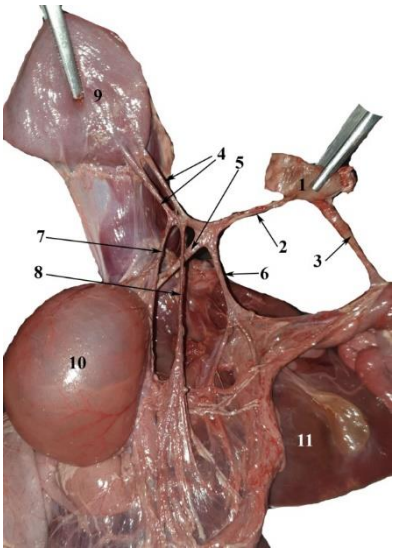

Fig. 9. Origin of the main trunks of the celiac artery (original)
1- abdominal aorta; 2- celiac artery; 3- caudal mesenteric artery; 4- splenic artery (double); 5- left gastric artery; 6- hepatic artery; 7- left ruminal artery; 8- right ruminal artery; 9- spleen; 10- rumen; 11- liver

In the adult ruminant, the splenic artery and the left ruminal artery have been observed to split into a common trunk, thus the celiac artery emits four main collaterals: splenic artery, hepatic artery, left gastric artery and left ruminal artery (Fig. 10, 11). In contrast to young specimens where the left ruminal artery represented a collateral of the splenic artery. Research in goats (1, 8, 14) notes that the left ruminal artery is more frequently a branch of the splenic artery than of the left gastric artery. In the study by Mohamed (15), the left ruminal artery originated either from the splenic artery or from the celiac artery individually or as a common trunk with the left gastric artery. In newborn animals, both ruminal arteries originated from the splenic artery. Also, the left gastric artery and hepatic artery appear to form a common trunk for about 0.5 cm.

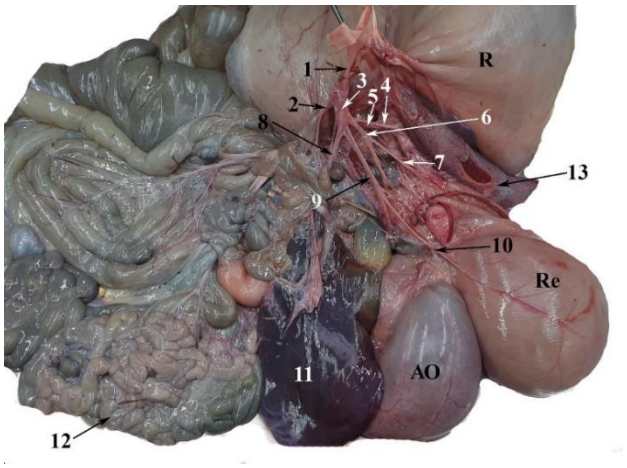

Fig. 10. The main collaterals of the celiac artery in the adult ruminant
R-rumen; Re-reticulum; AO- abomasum
1- the common celiomesenteric trunk; 2- cranial mesenteric artery; 3- celiac artery; 4- splenic artery; 5- right ruminal artery; 6- left ruminal artery; 7- reticular artery; 8- hepatic artery; 9- left gastric artery; 10- accessory reticular artery; 11- liver; 12- jejunum; 13- spleen

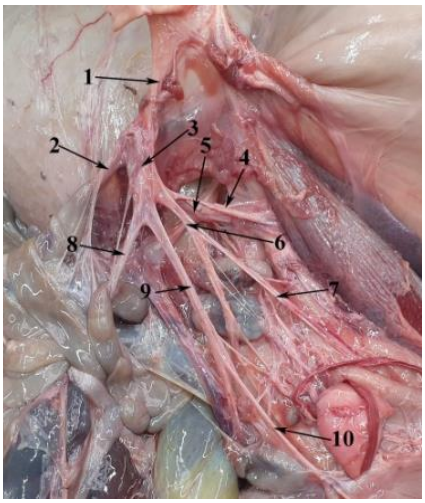

Fig. 11. Detail image of the main collaterals of the celiac artery in the adult ruminant
1- the common celiomesenteric trunk; 2- the cranial mesenteric artery; 3- the celiac artery; 4- the splenic artery; 5- the right ruminal artery; 6- the left ruminal artery; 7- the reticular artery; 8- the hepatic artery; 9- the left gastric artery; 10- the accessory reticular artery

Conclusions

In 17% of the cases examined the celiac artery detaches independently from the aortic artery, in the remaining specimens a common celiomesenteric trunk is formed.

The most significant variations were noted in the splenic artery, with the possibility of a common trunk separation with the left ruminal artery. Also, in newborn lambs the splenic artery emits two main collaterals: the left ruminal artery and the right ruminal artery, poorly developed in this age group, consistent with the fact that the rumen is not yet functional. In 10% of newborns, it was observed that the splenic artery doubled over a 2.5 cm course before entering the splenic hilum.

Detailed studies on the celiac artery, but also the possibilities of variations of its branches are needed, both on different age groups, but also taking into account the different sheep breeds and their particularities.

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THE ENDOPARASITISM IDENTIFICATION IN JACKALS FROM TIMIS COUNTY HUNTING GROUNDS

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Summary

The involvement of jackals in regulating small mammal populations is vital for preserving ecological balance and biodiversity. However, the focus on parasitic infestations in wildlife, including jackals, has intensified due to their detrimental effects on animal well-being and potential consequences for human populations. Understanding the prevalence or the co-infestations of cestodes with other parasites is crucial for assessing the health risks associated with jackal populations and the potential transmission to humans. The purpose of this study was to evaluate the endoparasites infection of the jackals from the Timis County hunting grounds. Over a one-year period, necropsies were performed on 41 jackals. The necropsies were conducted at the Faculty of Veterinary Medicine, Discipline of Parasitology, where the gastrointestinal tracts of the jackals have been examined. Among the 41 necropsied jackals, 29% (12) were infested with cestodes. Furthermore, 16% (2) of the infested jackals were positive for both cestodes and roundworms, while 75% (9) were infested with cestodes and other nematodes, and 8% (1) were exclusively infested with cestodes. These findings highlight the high prevalence of cestode infestations in jackals from the hunting grounds of Timis County, as well as the co-occurrence of cestodes with other nematodes. These parasites can have a significant impact on the health and survival of wild animals, as well as the health of domestic animals that may come into contact with infected wildlife.
Keywords: jackals, Timis County, parasitic infestations.

The golden jackal (*Canis aureus*) is one of the most widespread canid species with a range covering areas of central, eastern, and southern Europe, northern Africa, and parts of Asia. The epidemiological importance attributed to this species is relevant when the jackal plays the role of host for some parasites, with a zoonotic character (1, 11, 20).

Jackals, are wild carnivores that can be infested with both endoparasites and ectoparasites (13).

Cestode infections in golden jackals have been recorded across all their distribution range, with relatively high species diversity. The most commonly reported tapeworms in golden jackals are *Dipylidium caninum, Mesocestoides* spp., *Echinococcus granulosus*, and *Taenia* spp. (8, 10, 14, 16).

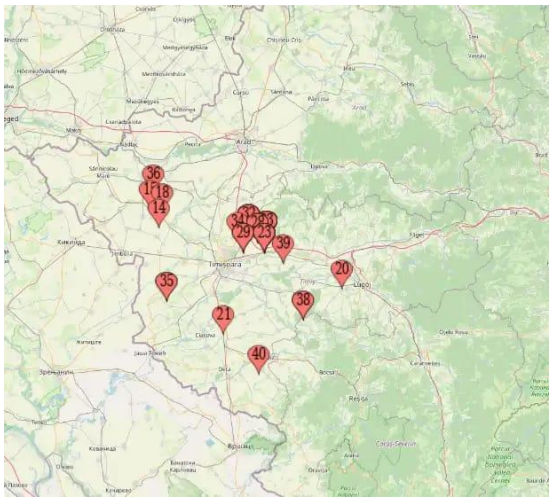
The cosmopolitan character of all these cestodes is attributed to the abundance and diversity of intermediate hosts and the lack of specificity for the

definitive hosts. Hence, the jackal and other carnivores represent an important source of environmental contamination. Several of these species are known to be zoonotic (9).

In this context, the purpose of the study was to identify parasitic fauna in jackals from hunting funds in Timiș County, using classical coproparasitological methods, macroscopic and microscopic examination of the intestinal mass from hunted animals.

Materials and methods

The study was carried out over a period of one year, on 41 jackals, males, and females, from 10 hunting grounds in Timis County (Fig. 1). The samples collected from these studies were represented by feces and intestinal tracts of the hunted animals.



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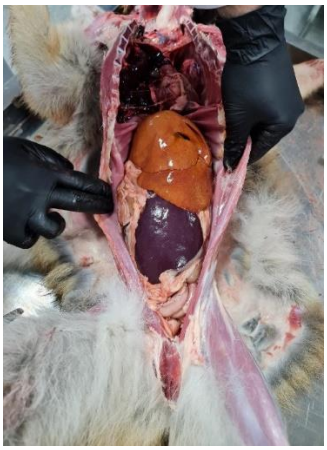

a.

b.

Fig. 2. a, b Necropsy examination

Results and discussions

The results of the coprological examination performed by the flotation method revealed the presence of the following parasitic elements (Fig. 3., Fig. 4):

- negative jackals - 6/41 (14.63 %)
- the presence of morulated eggs - 29/35 (82.85 %)
- the presence of morulated eggs and roundworm eggs - 1/35 (2.85%)
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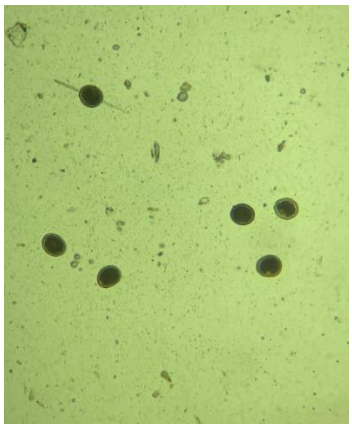

Fig. 3. Roundworm eggs

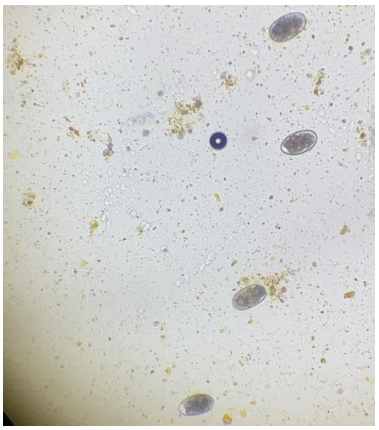

Fig. 4. *Ancylostoma*/ *Uncinaria* eggs

Pulmonary nematode larvae were not evident in the Baermann examinations.

The results of the necropsy examination of the intestinal mass revealed (Fig. 5., fig. 6).

- negative jackals – 11/41 (26.82%)
- the presence of tapeworms – 1/30 (3.33%)
- the presence of *Ancylostoma*/ *Uncinaria* – 18/30 (60%)
- the presence of tapeworms and roundworms – 2/30 (6.66 %)
- the presence of tapeworms and *Ancylostoma*/ *Uncinaria* - 7/30 (23.33 %)
- the presence of tapeworms, roundworms, and *Trichocephalus* spp. – 2/30 (6.66 %)

Fig. 5. Cestod

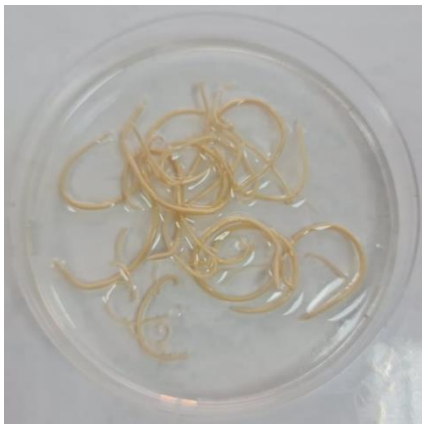

Fig. 6. Nematods

It is important to investigate and monitor the parasite load of wildlife in order to develop strategies to control and prevent important zoonoses, as well as emerging infections in wildlife, domestic animals, and humans (7, 18).

A study carried out between 2005 and 2010, on a number of 447 jackals from six localities in Serbia, revealed polyparasitism, among the highlighted species being *Mesocestoides lineatus* și *Mesocestoides litteratus* (3).

Twenty species of endoparasites have been identified in Greece, in feces collected from foxes, wolves, jackals, and wild cats. From 314 samples examined, *Mesocestoides* spp. It showed a prevalence of 73.2%, followed by *Uncinaria* spp and *Toxacara* spp. The results are discussed in light of the feeding characteristics of wild carnivores in rural areas of Greece (15).

To investigate the presence of *Echinococcus* spp. in wild mammals in Kenya, 832 fecal samples were collected from wild carnivores. Cestode eggs were

identified in 120 samples (14.4%) and the species *Echinococcus felidis* and *Echinococcus granulosus* (12).

The prevalence of adult cestode infestation in wild carnivores is reported to be present on all continents, reaching values ranging from 4.37% to 91% (2, 4, 17, 19).

Conclusions

The co-occurrence of cestodes with roundworms and other nematodes emphasizes the complexity of parasitic infestations in these animals.

Implementing effective control measures, including regular monitoring, is necessary to reduce the prevalence and impact of these parasites in both wildlife and domestic animals.

Continued monitoring and research are required to better understand the health impacts on jackals and the potential transmission risks to other animals and humans in the region.

Acknowledgments

This scientific paper was carried out in the Laboratory of Parasitology and Parasitic Diseases at the Faculty of Veterinary Medicine Timisoara, Laboratory within the Animal Hygiene and Pathology Research Center*/* ULS Timisoara.

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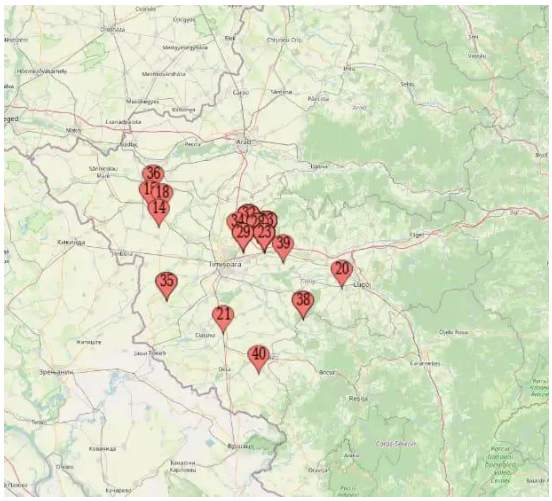

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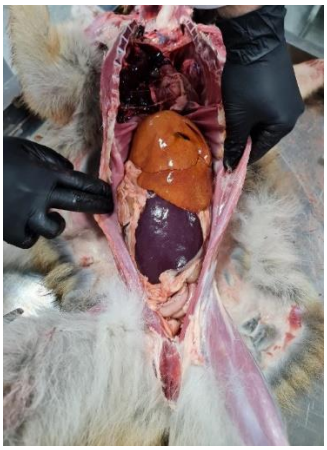

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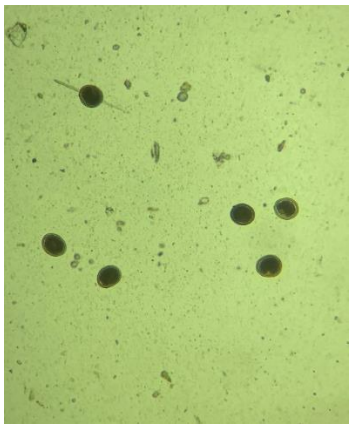

Fig. 3. Roundworm eggs

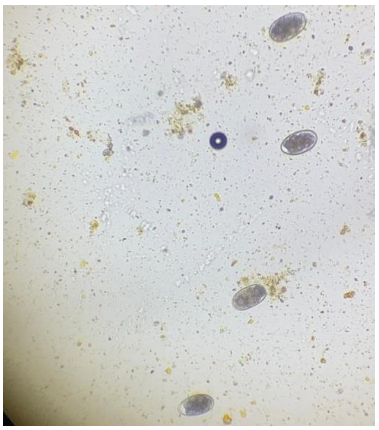

Fig. 4. *Ancylostoma*/ *Uncinaria* eggs

Pulmonary nematode larvae were not evident in the Baermann examinations.

The results of the necropsy examination of the intestinal mass revealed (Fig. 5., fig. 6).

- negative jackals – 11/41 (26.82%)
- the presence of tapeworms – 1/30 (3.33%)
- the presence of *Ancylostoma*/ *Uncinaria* – 18/30 (60%)
- the presence of tapeworms and roundworms – 2/30 (6.66 %)
- the presence of tapeworms and *Ancylostoma*/ *Uncinaria* - 7/30 (23.33 %)
- the presence of tapeworms, roundworms, and *Trichocephalus* spp. – 2/30 (6.66 %)

Fig. 5. Cestod

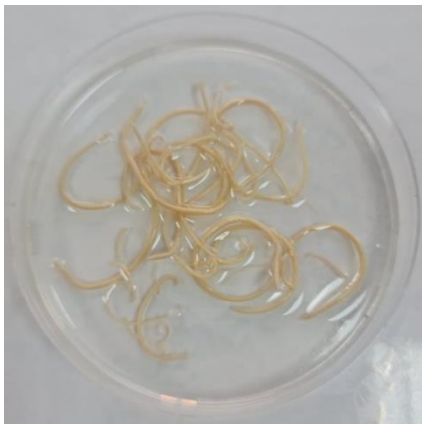

Fig. 6. Nematods

It is important to investigate and monitor the parasite load of wildlife in order to develop strategies to control and prevent important zoonoses, as well as emerging infections in wildlife, domestic animals, and humans (7, 18).

A study carried out between 2005 and 2010, on a number of 447 jackals from six localities in Serbia, revealed polyparasitism, among the highlighted species being *Mesocestoides lineatus* și *Mesocestoides litteratus* (3).

Twenty species of endoparasites have been identified in Greece, in feces collected from foxes, wolves, jackals, and wild cats. From 314 samples examined, *Mesocestoides* spp. It showed a prevalence of 73.2%, followed by *Uncinaria* spp and *Toxacara* spp. The results are discussed in light of the feeding characteristics of wild carnivores in rural areas of Greece (15).

To investigate the presence of *Echinococcus* spp. in wild mammals in Kenya, 832 fecal samples were collected from wild carnivores. Cestode eggs were

identified in 120 samples (14.4%) and the species *Echinococcus felidis* and *Echinococcus granulosus* (12).

The prevalence of adult cestode infestation in wild carnivores is reported to be present on all continents, reaching values ranging from 4.37% to 91% (2, 4, 17, 19).

Conclusions

The co-occurrence of cestodes with roundworms and other nematodes emphasizes the complexity of parasitic infestations in these animals.

Implementing effective control measures, including regular monitoring, is necessary to reduce the prevalence and impact of these parasites in both wildlife and domestic animals.

Continued monitoring and research are required to better understand the health impacts on jackals and the potential transmission risks to other animals and humans in the region.

Acknowledgments

This scientific paper was carried out in the Laboratory of Parasitology and Parasitic Diseases at the Faculty of Veterinary Medicine Timisoara, Laboratory within the Animal Hygiene and Pathology Research Center*/* ULS Timisoara.

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STUDY REGARDING THE INFESTATION WITH ENDOPARASITES IN RED FOXES FROM TIMIS COUNTY HUNTING GROUNDS

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Summary

Parasitic infestations in wildlife, including foxes, have been the subject of numerous studies due to their impact on animal health and potential implications for human populations. Infestation with cestodes and nematodes in wild animals is common and highlights the importance of monitoring and understanding the impact of these parasites on wildlife populations. The aim of this study was to identify the endoparasitism infestations in foxes from hunting grounds in Timis County. Over a one-year period, a total of 47 foxes have been necropsied at the Faculty of Veterinary Medicine, Discipline of Parasitology. Among the examined foxes, 25 (53%) were found to be infested with cestodes. Out of these 25 positive cases, 4 (16%) foxes were exclusively infested with cestodes, 5 (20%) foxes were infested with both cestodes and roundworms and 18 (72%) foxes were infested with cestodes along with other nematodes. The results of this study indicate a significant prevalence of cestode infestations in the fox population from the hunting grounds in Timis County. Furthermore, the co-occurrence of cestodes with nematodes highlights the complex nature of parasitic infestations in these animals. Further research is necessary to explore the impact of these infestations on the health of foxes and the potential transmission risks to other animals and humans in the region.

Keywords: red fox, Timis County, endoparasitism.

The red fox (*Vulpes vulpes*) is widespread in the world, in Central Europe, it is the most representative wild species in the family *Canidae*. The epidemiological importance attributed to this species is relevant when the fox plays the role of a definitive host for some intestinal parasites, including zoonotic helminths. Zoonotic infections can be transmitted directly from the environment when infective stages of parasites contaminate water or food (1, 8).

Nematode infestation in carnivores attracts a zoonotic risk, being incriminated species *Toxocara spp., Ancylostoma spp*., and *Trichocephalus spp. T. canis* is a causative agent of larval toxocarosis in humans. The role of the red fox in the dissemination of eggs of *T. canis* into the environment is significant. A large number of epidemiological surveys were undertaken of this ascarids nematode (13, 14).

Tapeworm infections are among the most relevant parasitic diseases in both human and animal health. Several tapeworms rely on wild animals to complete their life cycle, among them, taeniids from the Genus *Echinococcus* are particularly important as they are the causative agents of cystic and alveolar echinococcosis (6).

In this context, the purpose of the study was to identify parasitic fauna in native red foxes from hunting funds in Timiș County, using classical coproparasitological methods, macroscopic and microscopic examination of the intestinal mass from foxes.

Materials and methods

The study was carried out over a period of one year, on 47 red foxes, males, and females, from 13 hunting grounds in Timis County (Fig. 1). The samples collected from these studies were represented by feces and intestinal tracts of the hunted animals.

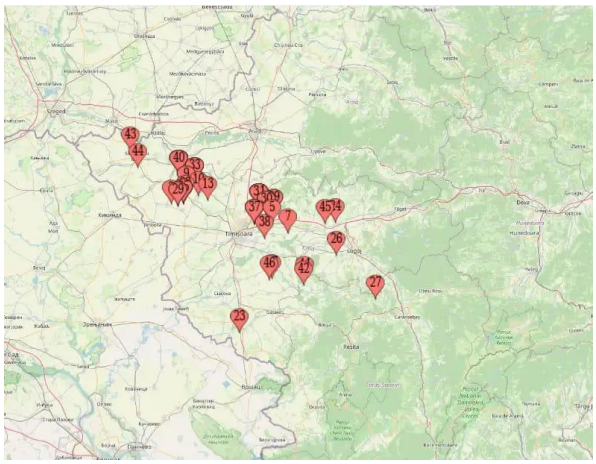

Fig. 1. Map of Timis County

The samples have been processed in the Parasitology and Parasitic Diseases Clinic of the Faculty of Veterinary Medicine Timisoara by the following methods:

- Qualitative method - identification of the parasitic load of the whole digestive tract with light eggs of nematodes, cestodes, protozoan oocysts
- Larvoscopic method - highlighting parasitism with pulmonary nematodes
- Necropsy examination - according to the technical instructions of necropsy (Fig. 2. a, b) (7).

a.

b.

Fig. 2. a, b. Necropsy examination

Results and discussions

The results of the coprological examination performed by the flotation method revealed the presence of the following parasitic elements (Fig. 3, Fig. 4):

- negative foxes - 9/47 (19.14 %)
- the presence of morulated eggs - 8/38 (21.05 %)
- the presence of roundworm eggs – 8/38 (21.05 %)
- the presence of Trichocephalus eggs – 1/38 (2.63 %)
- the presence of morulate eggs + roundworm eggs - 8/38 (21.05 %)
- the presence of morulated and *Trichocephalus* eggs – 6/38 (15.78 %)
- the presence of morulated eggs and oocysts of protozoa – 1/38 (2.63 %)
- the presence of roundworm eggs and oocysts of protozoa – 2/38 (5.26 %)
- the presence of morulated eggs, roundworm eggs, and *Trichocephalus* eggs – 3/38 (7.89 %)
- the presence of morulated eggs, *Trichocephalus* eggs, and oocysts of protozoa - 1/38 (2.63 %)

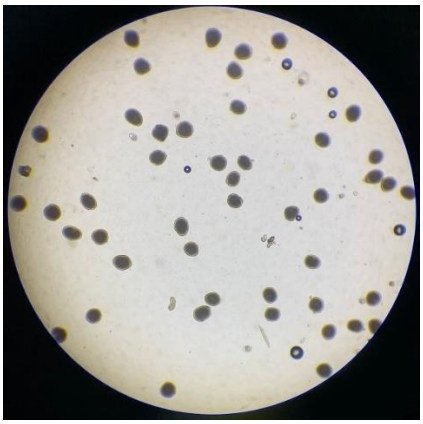

Fig. 3. Roundworm eggs

Fig. 4. *Ancylostoma*/ *Uncinaria* eggs

Pulmonary nematode larvae were not evident in the Baermann examinations.

The results of the necropsy examination of the intestinal mass revealed (Fig. 5, a, b):

- negative foxes – 12/47 (25.53 %)
- the presence of tapeworms – 4/35 (11.42 %)
- the presence of roundworms – 4/35 (11.42 %)
- the presence of *Ancylostoma*/ *Uncinaria* – 3/35 (8.57 %)
- the presence of tapeworms and roundworms – 5/35 (14.28 %)
- the presence of roundworms and *Trichocephalus* spp. 4/35 (11.42 %)
- the presence of tapeworms and *Ancylostoma*/ *Uncinaria* - 5/35 (14.28 %)
- the presence of tapeworms, roundworms and *Ancylostoma*/ *Uncinaria* – 10/35 (28.57 %)

a. b.

Fig. 5. a, b. Helminths isolated from the small intestine

The results obtained in the present study can be compared with the reports of researchers from Europe, and Asia (2, 3, 4, 15, 16, 17, 20).

The red fox (*Vulpes vulpes*) is widely distributed in the world, in central Europe, it is the most representative wild species of the Canidae family. The epidemiological importance attributed to this species is relevant when the fox plays the role of a definitive host for many intestinal parasites, including zoonotic helminths. Thanks to its geographical position, Poland is an interesting area for parasitological investigations of this species. Thus, in red foxes different genera and species of intestinal parasites were highlighted, some of them presenting a real danger for human infestation: *Alaria alata* (78.7%), *Mesocestoides spp*. (78.2%), *Taenia spp.* (53.2%), *Toxocara/Toxascaris* (43.1%) and *Echinococcus multilocularis* (18.5%) (13, 14).

From the center of Europe, we turn our attention to the Mediterranean area, where Alvarez et al. (1) studied the intestinal fauna of 201 foxes captured in Galicia (northwest Spain). The following were identified: *Toxocara canis* (23%), *Toxocara cati* (0.5%), *Toxascaris leonina* (1%), *Uncinaria stenocephala* (28%), *Mesocestoides litteratus* (2.5%) and *Dipylidium caninum* (0.5%). The study reveals the possible relationship between the prevalence of fox infestations with zoonotic species and the risk of contamination of the human population, an aspect highlighted by Sanchis-Monsonis et al. (19) in a more recent study (2020), but also in Italy by Fiocchi et al. (10).

In Slovenia, Rataj et al. carried out an extensive study, on a batch of 428 foxes, in which they identified *Uncinaria stenocephala* (58.9%), *Toxocara canis* (38.3%) and *Mesocestoides spp.* (27.6%) (18).

In 2021, in northern Italy, the highest prevalence of endoparasitism in the red fox belongs to the species *E. multilocularis*, followed by the species *T. crassiceps* and *T. polyacantha* (5).

The bibliographical references regarding the isolation of *Taenia polyacantha* from the intestine of red foxes are significant, but without accepting the zoonotic nature of this cestode (5, 10, 18, 19).

Fecal samples were collected from 528 foxes and 277 stray dogs. *E. multilocularis* was the most prevalent species with a prevalence of 4% in foxes and 15.2% in stray dogs. *E. shiquicus* had a prevalence of 1.5% in foxes and 0.7% in stray dogs, while *E. granulosus* was identified only in stray dogs with a prevalence of 1.8% (11).

According to the study by Jesudoss Chelladurai et al. in 2021, the prevalence of *Mesocestoides* spp. infestation in foxes was 35.79% (12).

It is important to investigate and monitor the parasite load of wildlife in order to develop strategies to control and prevent important zoonoses, as well as emerging infections in wildlife, domestic animals, and humans (9, 20).

Conclusions

Necropsical examination allowed us to identify adult roundworms and adult cestodes harvested from the gut of foxes.

This study brings new information about the parasitic intestinal fauna of red foxes from Timis County and warns of the risk of human contamination with zoonotic species.

We recommend the implementation of permanent coproscopic controls in order to assess the carrier status of various parasitic agents and to identify possible risks of human contamination.

Acknowledgments

This scientific paper was carried out in the Laboratory of Parasitology and Parasitic Diseases at the Faculty of Veterinary Medicine Timisoara, Laboratory within the Animal Hygiene and Pathology Research Center*/* ULS Timisoara.

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PREVALENCE OF BOVINE MASTITIS AMONG DIFFERENT INDEGINOUS COW BREEDS FROM SELECTED HERDS IN NIGERIA

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Summary

Bovine mastitis continues to be a burden in the dairy sector worldwide, despite the many mastitis control programs. Nigeria is not exempt from this problem, though there is still a high potential for exploitation in the dairy sector, as about 60% of the consumed dairy products are imported. Nigeria has the sixth-largest cattle population in Africa, fully understanding its cattle structure and dynamics can help harness its potential. This study aimed to determine the prevalence of bovine mastitis among common breeds of dairy cows in Nigeria. Lactating cows in selected peri-urban dairy farms and pastoral cattle herds were evaluated for mastitis by physical examination and using the California Mastitis Test. Milk samples were collected from cows with mastitis for cultural isolation and phenotypic characterization of *Staphylococcus* species. The prevalence of mastitis among the Friesian x Bunaji (FB), Bunaji (BJ), Gudali (GD), and Azawak cattle breeds was 72%, 56%, 41%, and 0%, respectively. Three percent of the udders of the BJ cows were found to have blind teats. Seventy-three percent of the milk samples had isolates suggestive of *Staphylococcus* species based on cultural isolation on Mannitol salt agar and their biochemical profile. Indigenous breeds of cows are known to be less susceptible to mastitis compared to exotic breeds; this could be explained by their udder structure and low milk production potential, which are part of the risk factors for mastitis. This study shows a high prevalence of mastitis in Bunaji of 56% in comparison to other indigenous cow breeds. This can serve as information to guide the breeding selection of indigenous cows for dairy potential with reduced mastitis risk.

Keywords: Bovine mastitis, dairy, prevalence, indigenous cows.

Mastitis, which is the inflammation of the parenchyma of the mammary glands, has caused and still causes devastating economic losses among dairy cattle worldwide. Losses reported in different countries vary greatly (18). Different mastitis control structures and programs have been tried for the control of the disease, but it still remains a raging problem in the dairy sector (2, 20, 21). One of the identified risk factors for mastitis is the cow's breed (1, 16). Recent trends in research focus on genetic selection for mastitis resistance in dairy cows using various methods, including indirect methods like lower somatic cell counts (8, 19). The impact of bovine mastitis in Nigeria is poorly estimated, though Nigeria has the sixth largest cattle population in Africa, next to Ethiopia, Chad, Sudan, Tanzania, and Kenya (22). Breeds of cattle indigenous to Nigeria vary from Bunaji "white Fulani" (the most

dominant with a wide national spread), Gudali (the second largest cattle population in Nigeria), Azawak, Red Bororo, Wadara, Muturu, Keteku, Ndama, and Kuri (7). The three most commonly reared breeds of cows in Nigeria for meat and milk are the Bunaji, Gudali, and Red Bororo (7). The Bunaji and Gudali cow breeds are being explored for dairy potential in Nigeria by crossing them with exotic dairy breeds.

This study seeks to show the breed-based prevalence of mastitis using the California Mastitis Test evaluation of the individual cow somatic cell count.

Materials and methods

Study location

This study was carried out in two states (Kaduna and Kano States) located in the north-western geopolitical region of Nigeria, also known as the north-western Nigeria. The North Western zone is one of the six geopolitical zones in Nigeria (Fig. 1). North-Western Nigeria comprises seven states, namely: Sokoto, Kebbi, Zamfara, Katsina, Kaduna, Kano, and Jigawa States (Fig. 1). The zone lies between longitude 12° 10′ North and latitude 6° 15′ East, occupies a land mass of about 214395 km^2 and has a human population of about 35786944 (2006 national census). Two seasons are experienced in the region: the wet season (April–October) and the dry season (November–March) (14). Agriculture is the main activity carried out in this region.

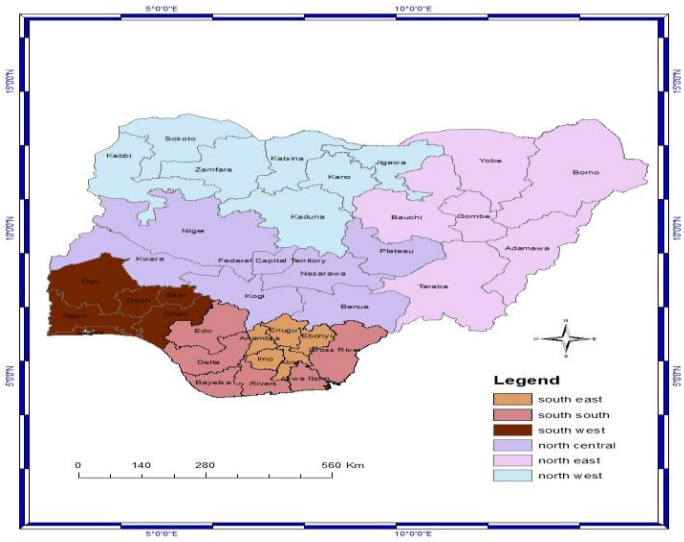

Fig. 1. Map of Nigeria indicating the geopolitical zones

Sampling design

A cross sectional study of pastoral and peri-urban dairy farmers was conducted in two states (Kaduna and Kano states) in the North-West zone of Nigeria. Two senatorial zones were randomly selected from each state, and then local government areas in each of the selected senatorial zones were randomly selected. Based on the available records of dairy farms and clusters, at least five dairy farms and clusters with at least ten lactating cows were sampled.

Sample collection and analysis

Milk samples were collected from cows for visual and somatic cell assessment using the strip cup and California mastitis test, respectively. This was done after subjecting the cows' udders to a visual examination for signs of inflammation. Test samples were scored as 0 (negative), indeterminate, +1, +2 and +3. Cows with test scores of +1, +2 and +3 in at least one teat were considered positive for mastitis and selected for milk sample collection for bacteriological analysis.

Milk samples were collected from cows with mastitis and transported to the Bacteriology laboratory from the department of Veterinary Public Health and Preventive Medicine, Ahmadu Bello University, Zaria, where they were cultured on mannitol salt agar and blood agar for bacterial isolation and hemolysis determination. A panel of biochemical tests (catalase, coagulase, DNase, Gram staining and sugar fermentation) were carried out for phenotypic identification of the bacterial pathogen.

Data analysis

Data collected were entered into Microsoft Excel 2008, organized and presented in tables. The association between mastitis and the different breeds of cows was tested using the JMP 13.0 statistical software.

Prevalence of mastitis was calculated as follows:

$$\text{Prevalence} = \frac{\text{Number of positive samples}}{\text{Total samples collected}} \times 100\ (\%)$$

Results and discussions

Three hundred and forty-eight cow udder and milk samples were visually assessed. No visible signs of inflammation were noticed in any of the udders examined. A visual examination of the udder teats revealed that twelve of the examined cows had between one and two blind teats (3%). Eleven out of the twelve cows with blind teats were of the Bunaji breed and multiparous (parities 5-7, Table 1). This finding is probably the result of an untreated, long-standing infection, either due to poor screening for subclinical mastitis or poor clinical mastitis management, which might have resulted in damage to the milk-producing cell. A similar finding was

reported by Sarba and Tola (15), who reported a 5.5% prevalence of blind teats among studied herds in Ethiopia.

One hundred and eighty-four (53%) of the examined cows were shown to have mastitis (clinical and subclinical) from the California Mastitis Test and physical examination results.

Table1

Blind teats among different cow breeds and parity in Kaduna and Kano states, Nigeria

S/N	Breed	Parity
1	FB	3
2	WF	7
3	WF	6
4	WF	7
5	WF	6
6	WF	5
7	WF	6
8	WF	5
90	WF	6
10	WF	7
11	WF	5
12	WF	5

Key. WF white Fulani, FB Friesian x Bunaji

There was a highly significant variation ($p<0.001$) in mastitis infection among the studied cow breeds (Table 2). Friesian x Bunaji had the highest number of episodes of mastitis, followed by the Bunaji breed of cow and the Gudali cows. Breed-specific prevalences of mastitis recorded were 72%, 56%, and 41% for the Friesian x Bunaji, Bunaji, and Gudali cows, respectively (Table 2). Studies on the comparative prevalence of bovine mastitis within indigenous breeds of cows are hard to find in Nigeria. Among the Bunaji breeds, Olufemi (12) reported a lower prevalence (12.3%) of mastitis, though his diagnosis was based on visible abnormal changes in the cow's udder or milk. This method can only identify clinical mastitis, which might explain the lower prevalence observed in his study. Thomas et al. (17) reported a higher prevalence of 70%. The diagnostic methods also used in his study were different from those in this study. The breed distribution and parity of the cows with mastitis are shown in Table 2. This finding indicates a high rate of mastitis occurrence among the indigenous cattle breeds, as opposed to the popular belief that mastitis is not a disease of indigenous cattle.

Table 2

Prevalence of mastitis among the different cow breeds in Kaduna and Kano states, Nigeria

Cow breeds	Number of cows tested	Number positive (%)	X^2	P value
Friesianx Bunaji	36	26 (72)	0.0286	0.0001**
Bunaji	253	142(56)		
Gudali	39	16 (41)		
Azawak	20	(0)		
Total	348	184 (53)		

Key: ** Highly significant

The prevalence of subclinical and clinical mastitis among the cows studied is shown in Table 3. There was a significant difference ($p<0.05$) between the clinical and subclinical mastitis prevalence among the studied cows. There was a higher likelihood of subclinical mastitis infection in the cows. This indicates that subclinical mastitis may be more prevalent than clinical mastitis among dairy cows in the study area. Subclinical mastitis has been shown to have a worse economic impact on dairy cows due to decreased milk quality and quantity (4, 13). It has also been linked to reduced reproductive performance in cows (10).

Table 3

Prevalence of sub-clinical and clinical mastitis among dairy cows in Kaduna and Kano states, Nigeria

Disease status	No of cows tested	No positive (Percentage)	95% CI
Sub clinical mastitis	348	169 (46%)	1.080-1.440
Clinical mastitis	348	15 (4%)	0.909-1226
Blind teats	348	12 (3%)	-

Fifteen of the studied cows tested had clinical mastitis (4%), with visibly abnormal milk ranging from clots or flakes in milk to blood in milk. The Friesian x Bunaji breeds were more affected 8 (53%) than other breeds 7 (47%) with clinical mastitis (Table 4). Studies by Sarba and Tola (12) in Ethiopia also reported a higher prevalence of mastitis in exotic breeds than local breeds. This may be explained by the findings by researchers that breed (3, 6, 9, 11) and high genetic potential for milk production are risk factors for bovine mastitis (1, 5).

One hundred and forty cultural isolates suggestive of *Staphylococcus* species from phenotypic and biochemical analysis were obtained.

Table 4

Clinical mastitis in different breeds and parity of cows in Kaduna and Kano states, Nigeria

S/N	Breed	Parity
	FB	1
2	FB	2
3	FB	2
4	FB	3
5	FB	6
6	FB	6
7	FB	4
8	FB	4
9	GD	4
10	WF	6
11	WF	5
12	WF	5
13	WF	5
14	WF	5
15	WF	5

Key. WF white Fulani, FB Friesian x Bunaji, GD Gudali

Conclusions

This study showed a prevalence of mastitis of 53% in both indigenous and crossbreeds dairy cows in Kaduna and Kano states. The highest breed-specific prevalence of mastitis was recorded in the crossbred cows, but the Bunaji cow had the highest prevalence of mastitis among the indigenous breeds. More blind teats were encountered in Bunaji than other cow breeds in the study. This registers the importance of mastitis among even the indigenous cow breed. Proper enlightenment campaigns on the importance of bovine mastitis and it associated economic cost implication has to be organized for dairy farmer, including pastoral, peri-urban, and commercial dairy cow owners, if there is a goal to control the disease nationally.

Acknowledgement

The Authors are grateful to the National Animal Production Research Institute, Zaria, Nigeria, for their support of this study. We are also grateful to the academic and technical staff of the department of Veterinary Public Health and Preventive Medicine, Ahmadu Bello University, Zaria, Nigeria, for their role in making this study possible. We appreciate the dairy farms' and herd owners' cooperation throughout the fieldwork.

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SCREENING OF BRUCELLOSIS IN DOGS USING ROSE BENGAL PRECIPITATION TEST (RBPT) AND CANINE BRUCELLOSIS ANTIBODY RAPID DETECTION TEST (GENOMIX)

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Summary

Serological screening of canine brucellosis was conducted using Rose Bengal Precipitation Test (RBPT) and Genomix Canine brucellosis Antibody Rapid Detection Test Kit. A total of 91 canine serum samples (31 from asymptomatic and 60 from dogs with clinical signs suggestive of brucellosis) were screened with RBPT and Canine brucellosis Antibody Rapid Detection Test (Genomix). The results showed an overall positivity of 4.4 and 1.1 per cents by RBPT and Canine brucellosis Antibody Rapid Test (Genomix) respectively. High percentages of positive cases were seen in n ≥5 year's age group, females and exotic breeds. Also, high positive cases were seen in dogs with infertilility and skeletal problems compared to apparently normal. The study arrived at the conclusion that the screening of brucellosis in dogs could be done with RBPT and Canine brucellosis Antibody Rapid Detection Test (Genomix) for *Brucella abortus* and *Brucella canis* respectively.

Keywords: Canine brucellosis, RBPT, Genomix brucellosis antibody detection kit, infertility, skeletal problem.

Brucellosis is of particular concern in India because nearly 80% of the Indian population resided in rural areas in close contact with livestock like cattle, sheep, goat etc (3). Hence, human population stands at a greater risk of acquiring zoonotic diseases including brucellosis (14).

Brucellosis is an infectious disease affecting domestic and wild animals which impose serious health implications to both human and animals. Brucellosis causes considerable economic losses at the capacity of livestock farmers and also has a reasonable health impact, particularly among occupational groups since it is anthropozoonotic in nature and associated with male and female infertility. The disease has been identified as a vital public health problem in various parts of the world. Almost all the species of livestock including cattle, goats, pigs, horses and dogs plays significant role in the transmission of brucellosis to man (3).

B. melitensis is the most pathogenic species of *Brucella* for humans, with the exposure to only 1–10 CFU (colony forming units) being sufficient for establishment of infection, whereas *B. suis* and *B. abortus* have intermediate zoonotic potential. *B. canis* has the lowest zoonotic potential among the classic *Brucella* spp., and there are no documented cases of human infection with *B. ovis* (18).

B. canis is the most common cause of canine brucellosis (16), associated with abortions and stillbirths in pregnant dogs, lymphadenitis, epididymitis, scrotal edema and orchitis (occasionally) in male dogs. Discospondylitis is the most common orthopaedic disorder attributed to *B. canis* infection in dogs. In these cases, back pain, lameness, and neurologic deficits may be present. In India, the first report of Brucella infection in dogs from Small Animal Clinic of the Madras Veterinary College, Chennai (13).

Diagnosis of *B. canis* infection is very challenging. Although the dog is the most common host of *B. canis*, canine infections with other *Brucella* spp. such as *B. suis* (10) and *B. abortus* (17) may occur. Importantly, *B. canis* is serologically distinguished from *B. melitensis, B. abortus,* and *B. suis,* which carry a smooth Lipopolysaccharides (LPS), and therefore their antigens do not react with anti-*B. canis* antibodies (4). However, none of the serological tests currently used for the diagnosis of canine brucellosis are completely satisfactory. Serologic diagnosis of *B. canis* infection is challenging, and a combination of different tests is highly recommended (11).

Since the *Brucella* spp. infects not only their preferred hosts but also other domestic and wild animal species, which in turn can act as reservoirs of the disease for other animal species and humans. The present study aim to screen brucellosis in dogs using RBPT to detect *B. abortus* antibodies which carry a smooth LPS and Canine Brucellosis Antibody Rapid Detection Test (Genomix) which is specific in detecting *B.canis* antibodies which carry a rough LPS.

Materials and methods

Experimental design

This study was designed to screen for brucellosis in dogs by RBPT and Canine Brucellosis Antibody Rapid Detection Test (Genomix) from small animal clinic, obstetrics and gynecology and orthopaedics units of Madras Veterinary College teaching hospital, MVC, Chennai. The study area and animals were sampled using simple random sampling to represent the target population. The samples were collected randomly from dogs with history of abortion, infertility, spondylitis and apparently healthy ones of different ages and sexes.

Sample collection

Sera samples were collected from randomly selected dogs from small animal clinic, obstetrics and gynecology and orthopedics units of Madras Veterinary

College teaching hospital, MVC, Chennai, India. An about 3 ml blood sample was collected from each of the 91 dogs by cephalic vein puncture, into sterile test tubes of 5 ml capacity. The tubes were left undisturbed until the sera cleared, and centrifuged at 2000 rpm for 15 minutes. All the sera samples were numbered and stored at – 20^{o}C until further use.

Serological tests

Rose Bengal plate test (RBPT)

The colored antigen required for RBPT was obtained from the Division of Biological products, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh, the test was performed as per the standard protocol of agglutination test (20). Briefly, a drop of serum (30 µl) was placed on clean grease-free glass slide and an equal quantity of antigen was added and mixed thoroughly using an inoculation loop. The mixture was observed for clumping / agglutination for one minute and the results were recorded as agglutination (+) or no agglutination (-).

Canine Brucellosis Antibody Rapid Detection Test Kit (Genomix) (Hyderabad)

The testing device from the foil pouch was removed by tearing at the “notch” and then placed the testing device on a level surface. 5µl of specimen was added without air bubbles into the sample well by holding the Sample dropper vertically followed by addition of 2 drops of Sample diluents marked with an arrow on the testing device. The appearance of distinct pink colored bands at the control and test line regions were taken as positive for *Brucella canis* antibody and the appearance of distinct pink colored band only at the control line region and no colour development at the test line region even after 20 minutes, the test were taken as negative for *Brucella canis* antibody (Fig. 1). Also, if there is no visible pink band at control region, the test was taken as invalid tests systems and the test was repeated with a new test device.

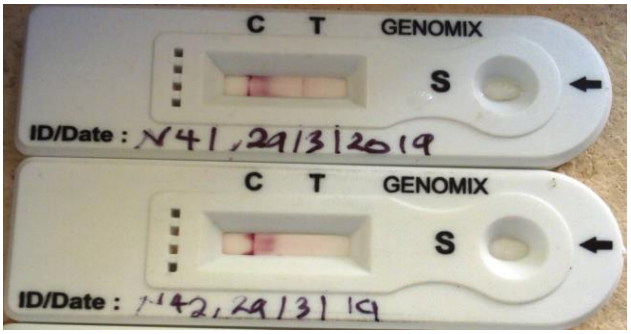

Fig.1. Canine brucellosis Ab Rapid Test (Genomix)

Results and discussions

In this study, overall seropositivities of 4.4% and 1.10 % were observed respectively by RBPT and Canine Brucellosis Ab Rapid Test (GENOMIX®) (Table 1). Among the 91 sera samples screened by RBPT and Canine Brucellosis Ab Rapid Test (GENOMIX®), the highest percentage of positivity was observed in the ≥5 years of age group, with 3.30% and1.10% respectively. Sex distribution of the cases had high positivity in females (3.3%) compared to males (2.2%). Pedigreed dogs showed high seroposivitity with 4.40% in RBPT compared to non-descsripts with 1.10% in Canine Brucellosis Ab Rapid Test kit (Table 1). Base on health status of an animal, the study recorded 2.2% in each of infertility and spondylitis cases and 1.10% apparently healthy ones (Table 1).

Among the serum collected from clinical cases with various clinical signs (mainly, infertility and spondylitis cases) and also from apparently healthy ones, the RBPT and Canine brucellosis Ab test kit showed a positivity of 4.4 and 1.1% respectively. The variation in the positive percentage may be due to the fact that, the RBPT was performed using *Brucella abortus* colored antigen which could detect only *B. abortus* species and the canine brucellosis Ab Rapid Test kit could detect only *Brucella canis* antibodies in the serum.

Table 1

Percentage positivity of canine serum samples for brucellosis based on Canine brucellosis Ab Rapid Test and RBPT

Tests	Status of dog (91)				
	Infertility	**Skeletal problems**	**General population**	**Total positives**	**Total % positives**
Canine brucellosis Ab Rapid Test (Genomix)	-	-	1.10% (1/91)	1	1.10(1/91)
RBPT	2.2% (2/91)	2.2% (2/91)	-	4	4.4(4/91)

Another study (5) reported a higher seropsitivity of 27.78 % than our findings using RBPT in epidemiological investigation for brucellosis in dogs of Thrissur, India. Also, Ayoola et al. (7) had reported a higher positivity of 12.72% by RBPT in a study on sero-epidemiological survey and risk factors associated with brucellosis in dogs. But, in the same study, sero positivity of 1.06 % in ELISA is very much similar to the 1.1% positivity of our study by employing canine brucellosis Ab Rapid Test. Similarly,

Aulakh et al. (6) reported a seropositivity of 9.8 % by using canine brucellosis antibody test kit in a study on canine brucellosis in Punjab state of India and their public health significance. In Bangladesh, Talukder et al. (15) recorded an overall seroprevalence of canine brucellosis in 30 stray dogs at 10.0% with ELISA.

Female dogs had a higher seropositives percentage (3.3%) than male dogs (2.2%). A major contributing factor to higher rates in females could be that a single male dog, if infected, is used in mating different females, it can transmit the infection through infected semen (9). However, Radostits et al. (19) have shown that erythritol, a polyhydric acid found in higher concentration in the placentas and foetal fluids of females than in seminal vesicles and testis of males, can be responsible for females being more susceptible than males. This result was in agreement with the findings of Cadmus et al. (9) who reported a prevalence of 6.17% in females and 4.9% in males and Momoh et al. (12) who reported prevalence of 29.3% in female dogs and 28.6% in male dogs. However, it disagrees with findings in a previous study where a slightly higher rate in males (29.6%) than in females (26.7%) (2).

Seropositivity was lower among the young animals screened as compared to the older ones (Fig. 2.). Usually young animals are protected by maternal immunity and thus they are less susceptible to infections. This shows that the infection increases with age. The high prevalence seen in older animals shows the chronic nature of brucellosis as it has been shown to increase with age, and most affected animals carry the infection throughout their lives (19).

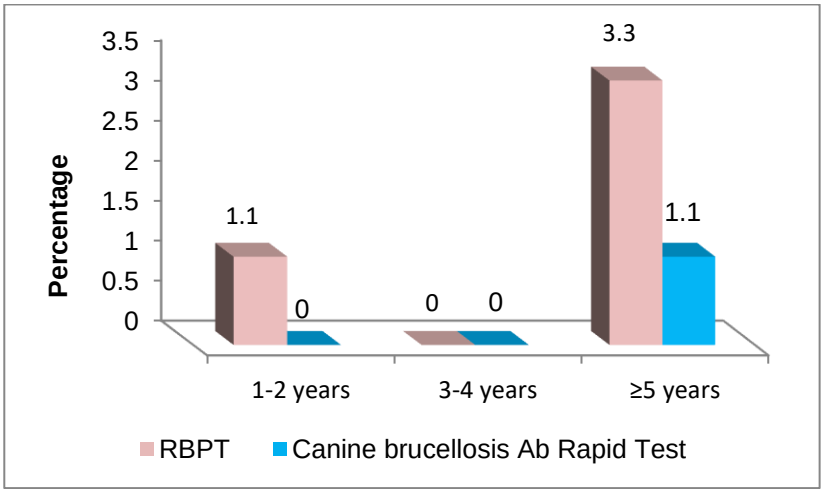

Fig. 2. Age wise distribution of brucellosis in dogs (%)

The reason for the increase in prevalence as the animal age increases may be due to the fact that the bacteria localizes mainly in the reproductive tracts, especially in gravid animals. There is also evidence that the mammary gland may be even a more probable area of localization than the reproductive tract (1). Age-wise prevalence studied by Abubakar et al. (1) and Momoh et al. (12) showed that the incidence is higher in sexually mature animals. Therefore, the increase in age, increases probability of exposure to infection in dogs. However, the results in this study do not agree with previous study by Cadmus et al. (9), as they reported more prevalence in dogs below one year old than in adult dogs.

Pedeegre dogs showed high seroposivitity with 4.40% (Fig. 3) and on examination of breed involvement, Great Dane showed a higher percent of overall positivity (2.2%) (Fig. 4). The detection of canine brucellosis in exotic dogs may indicates a new source of infection from abroad as these dogs may be imported from countries and regions where the disease is endemic (8). The higher prevalence among the exotic breeds is in agreement with the findings of Behzadi et al. (8); they recorded a prevalence of 19.35% in exotic breeds. It is also in agreement with the findings of Cadmus et al. (9) who got 50.55% in Alsatian breeds of dogs.

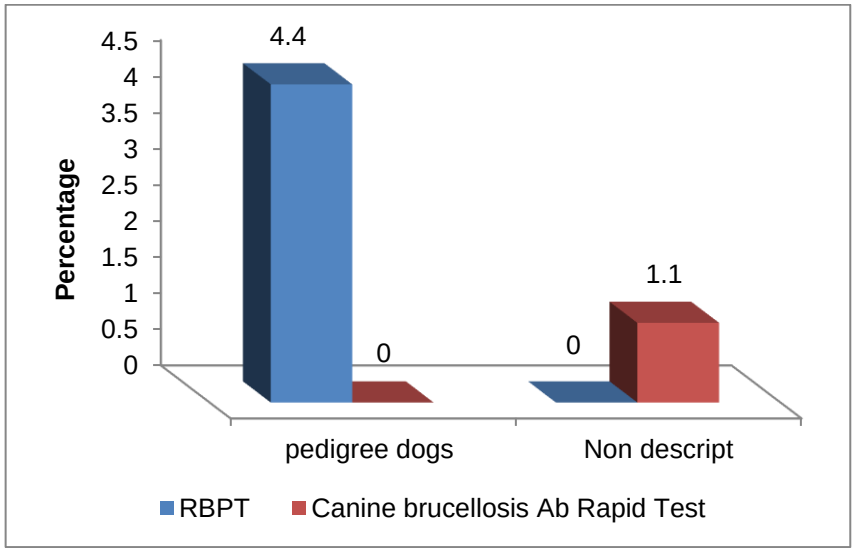

Fig. 3. Breed wise distribution of brucellosis in dogs (%)

Base on health status of an animal, the study recorded a high seropositive of 4% (2.2% in each of infertility and spondylitis cases) and 1.10% apparently healthy ones (Table 1). A study was conducted by Sharma et al. (21) on canines exhibiting clinical symptoms of brucellosis in Punjab state in which 112 serum samples of dogs

were analyzed and serologically positive samples were 9.8%. Out of which 32.6% positive samples were among those, which showed clinical symptoms.

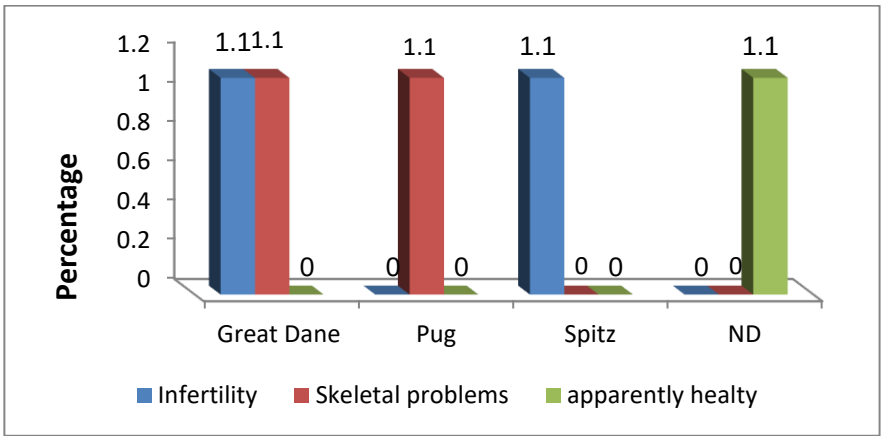

Fig. 4. Correlating Breed and clinical condition in dogs (%)

Conclusions

The overall positive case rates detected by RBPT and Canine Brucellosis Ab Rapid Test (GENOMIX®) were 4.40 and 1.10% respectively, showing that there is a conspicuous presence of Brucella antibodies in the dogs population in the study area, indicating the presence of Brucella infections in the population and justifying the need for continued screening and confirmatory programs in a wider region of the disease in the study area which in turn might be useful for strategic planning to establish appropriate control measures and prevent further spread of an infection. The study also supported the evidence of cross reaction of brucella species in different animal species, as *Brucella abortus* antibodies was detected from dogs sera in this study.

Acknowledgements

The authors are thankful to the Dean, Madras Veterinary College, for providing funds and facilities to conduct this experiment; and also thankful to Dr. P. Sridevi, Professor Department of Clinics (retd), Madras Veterinary College Chennai, India, for providing free of charge Canine Brucellosis Ab Rapid Test Kits (GENOMIX®) to carry out a part of this study.

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STUDY REGARDING THE USE OF MODERN MEANS OF VIDEO RECORDING AND GPS-TRACKING IN MONITORING THE DIPSIC BEHAVIOR IN DOMESTIC CATS

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Summary

Currently, in the specialty literature, the studies about the physical activity, as well as the dipsic and nutritional behavior monitoring in cats are insufficient, despite the current interest of researchers in this direction. An important part of the currently existing studies focuses on analyzing the spatial behavior of domestic cats with outdoors access, being especially directed on studying the territorial and predatory behavior manifestations. In the present study we performed the behavioral analysis of domestic cats living strictly indoors, without access to the outside environment, with an emphasis on the level of physical activity, as a physiological factor with impact on the dipsic behavior. The study was conducted on a number of 15 healthy individuals, adults - aged between 1 and 8 years old, whose physical activity level was monitored using the IKATI GPS collar correlated with the Tractive IKATI software, while the dipsic behavior was monitored using the MiHome video camera. The GPS tracking and the video monitoring was performed 24 hours a day, 5 days in a row for each individual. The aim of this research was to establish a direct correlation between the physical activity level and the dipsic behavior in adult cats, and also to investigate and perfect the modern GPS tracking and video recording means in monitoring different types of behaviors and performing complex ethograms in domestic cats.

Keywords: dipsic behavior, GPS tracking, domestic cats.

In cats, the water resulting from metabolic processes provides only 5% to 10% of the body's total water requirement, the rest being procured through direct water intake or through food (1, 4). Dipsic behaviour, like nutritional behaviour in this species, is profoundly influenced by the high level of selectivity that individuals exhibit vis-à-vis the food/water ingested (10, 14, 21).

Among the physiological factors, according to the specialty literature, those that present the most relevant impact on dipsic behaviour in cats are: age, weight, level of physical activity, type of food ingested and the physiological status - gestation/lactation (7, 11).

The level of physical activity is inversely proportional to the age of the individual, thus, according to specialty literature cubs and youth show the highest level of physical activity, ludic behaviour being best represented at ages younger than 1 year (3, 9).

At the same time, an exacerbation of the exploratory and social behaviour is observed in youth, compared to adult and geriatric cats (2, 3). In the case of geriatric cats, exploratory, social and especially ludic behaviours are much less represented

(8, 12, 15, 16), the level of physical activity being, in general, reduced, short periods of wakefulness, alternating with long periods of sleep/inactivity (5, 6, 13).

The more intense the level of physical activity is, the greater the loss of water through evaporation (7, 14), therefore the body replenishes the level of lost water by exacerbating the sensation of thirst and stimulating the manifestation of the dipsic behaviour (9, 17).

The most important tool for the behavioural research is considered to be, by the speciality literature, the ethogram (3, 20). Therefore, in our studies, we used all the data gathered by video-recordings and GPS tracking for performing various types of ethograms.

Materials and methods

In order to monitor the level of physical activity of the 15 individuals, we used the Tractive IKATI GPS collar, special designed for cats.

The GPS device comes with a special adjustable leather collar and also with a special clip-on device that makes it easy to attach to the normal collar of the cat (Fig. 1).

Fig. 1. The Tractive IKATI GPS device attached to the regular collar of the cat *(original photos)*

Also, the GPS device is connected to a mobile app that records permanently the location of the animal, but also the level of physical activity per 24 hours in the form of time spent active (with specific distribution per hours/day) and number of calories consumed.

For the dipsic behaviour monitoring we used the Xiaomi Smart Camera C300, the data being stored and processed using the Xiaomi Home mobile app, Android version (Fig. 2).

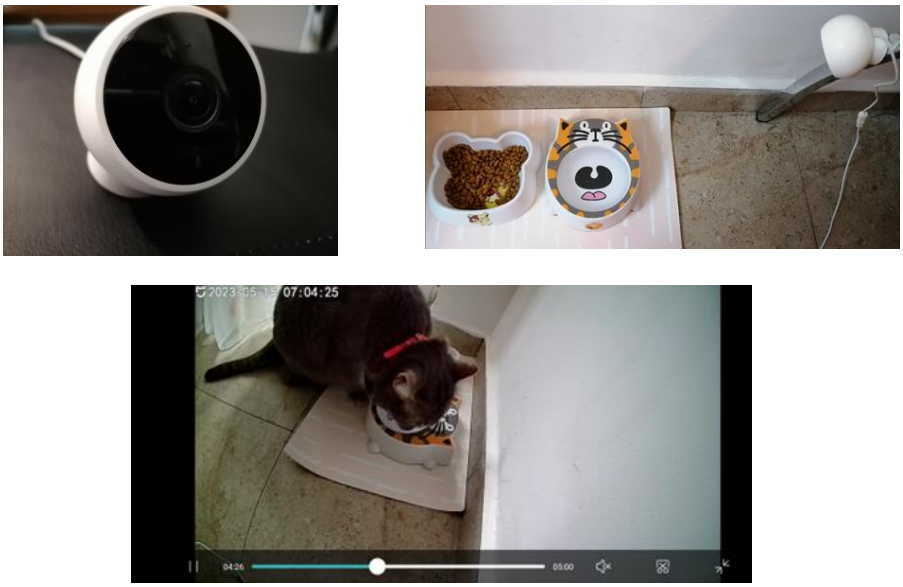

Fig. 2. The Xiaomi Smart Camera C300 – monitoring the drinking behaviour in cats
(original photos)

The 15 individuals were monitored for 5 days, the video recording being afterwards analysed in order to perform ethograms that comprised the frequency of the dipsic behaviour manifestation, as well as the time per drinking session.

Results and discussions

For the 5 days of monitoring we performed 5 synthetic ethograms, based on the video recordings. Each ethogram comprised the time when the manifestation of the dipsic behaviour occurred in each cat (allowing us to establish the frequency per 24 hours per individual and also the average per group) and also the time duration of each drinking session.

The ethogram for day 3 of monitoring is presented bellow in Table 1. Similar ethograms were performed also in the other 4 days.

The synthetic data for the physical activity level in all 15 patients are presented below in Table 2 – for the 3rd day of monitoring. We proceeded the same for all 5 days of monitoring.

The mean active time (minutes/24 hours) referes to time spent running, playing etc., not to the awake time and was calculated by the Tractive app. on each

day of monitoring based on the data gathered by the GPS tracker attached to the collar.

Table 1

Ethogram of the dipsic behaviour in the studied group of patients – 3rd day of monitoring –

Time interval	PATIENT NO.														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
0^{00}-1^{00}				21s			32s			24s					
1^{00}-2^{00}			38s					46s			33s		29s		
2^{00}-3^{00}					32s										42s
3^{00}-4^{00}						29s			39s						
4^{00}-5^{00}		44s		17s						31s		41s			
5^{00}-6^{00}							38s				21s				
6^{00}-7^{00}													34s		
7^{00}-8^{00}	32s		26s			18s		33s						41s	
8^{00}-9^{00}					43s										
9^{00}-10^{00}											19s				
10^{00}-11^{00}	29s			19s										20s	
11^{00}-12^{00}		27s				21s									
12^{00}-13^{00}												32s			
13^{00}-14^{00}				11s						30s					
14^{00}-15^{00}															
15^{00}-16^{00}							43s								
16^{00}-17^{00}													36s		
17^{00}-18^{00}						16s			45s						
18^{00}-19^{00}											25s				36s
19^{00}-20^{00}					33s										
20^{00}-21^{00}			28s												
21^{00}-22^{00}	40s			26s				48s			16s			23s	
22^{00}-23^{00}						19s				37s			18s		
23^{00}-0^{00}				12s								36s			
Drinking sessions/ 24 hours	**3**	**2**	**3**	**6**	**3**	**5**	**3**	**3**	**2**	**4**	**5**	**3**	**4**	**3**	**2**
Average number of drinking sessions/ 24 hours	**3.53 times**														
Mean time / drinking session	**37 sec**	**36 sec**	**31 sec**	**18 sec**	**36 sec**	**21 sec**	**38 sec**	**42 sec**	**42 sec**	**31 sec**	**23 sec**	**36 sec**	**29 sec**	**28 sec**	**39 sec**
Mean time / drinking session /group	**32.46 sec.**														

Dipsic behaviour
Nutritional behaviour

Table 2

The synthetic data regarding the physical activity level for the studied group of patients – 3rd day of monitoring

Patient no.	Mean active time minutes/24 hours	Mean number of calories consumed/24 hours	Time interval of the day when the cat was most active
1	118	254	4^{00} P.M. – 7^{00} P.M.
2	112	249	2^{00} P.M. – 5^{00} P.M.
3	182	312	1^{00} P.M. – 4^{00} P.M.
4	210	336	11^{00} A.M. – 2^{00} P.M.
5	192	321	6^{00} P.M. – 9^{00} P.M
6	226	342	10^{00} A.M. – 1^{00} P.M
7	178	296	7^{00} A.M. – 10^{00} A.M
8	154	281	10^{00} P.M. – 1^{00} A.M
9	108	188	11^{00} A.M. – 2^{00} P.M
10	214	203	1^{00} P.M. – 4^{00} P.M
11	232	216	9^{00} A.M. – 0^{00} P.M
12	148	196	4^{00} P.M. – 7^{00} P.M
13	164	203	7^{00} P.M. – 10^{00} P.M
14	156	198	11^{00} P.M. – 0^{00} A.M
15	110	242	3^{00} P.M. – 6^{00} P.M

The physical activity level reffered not only to the active time, but also to the number of calories consumed in 24 hours. The mean number of calories was calculated by the Tractive app for each individual, daily, based on the activity level, age, gender and weight. Although it was direct proportional to the mean active time, it did not only depended on that parameter, being influenced also by the individual profile of each cat.

As concerning the time interval of the day when the cats were most active, it was also estimated by the Tractive app, 33.4% of the individuals being active in the afternoon (after 3 P.M.), their activity level being increased around the time when the owners get home. This findings are consistent with the ones mentioned in the specialy literature (9).

For the 3rd day of monitoring the average frequency of the dipsic behaviour manifestation was 3.53 times per 24 hours, with a maximum frequency in patient #4 (6 times/24 hours) and patients #6, #10 and #11 (5 times/24 hours). The rest of the patients had 2-3 drinking sessions/24 hours.

Also, the mean time per drinking session for the group was 32.46 seconds. The patients that showed shorter time per drinking session were also the ones that showed highest drinking sessions frequency per 24 hours.

Therefore we observed, in the given examples (table 1 and 2) for the 3rd day of monitoring that in individuals with high levels of physical activity the frequency of the dipsic behavior manifestation was higher (individuals #4, #6, #10 and #11), this individuals being also the youngest patients in our study (1 year old, 2 years old, 1,5 years old and 2 years old). This cats also had the highest mean active time/24 hours

and the highest number of calories consumed. The results are consistent with the ones mentioned in the specialty literature (9, 21).

As concerning the aplicability of the GPS collar in monitoring behavioral manifestations, we did not find it suitable for actually tracking the individuals with only indoors acces, explaing why in the specialty literature (18) the GPS tracker was almost exclusively used for outdoor or stray cats.

However, we observed that the Tractive device and mobile app are extremely reliable in providing data regarding the time of the day when the individual was active, the total active time per day, the number of calories consumed and also different statistics per day and per period of monitoring overall.

Conclusions

The modern monitoring means, video recording and GPS-tracking, proved to be extremely suitable for performing ethograms and establishing direct correlations between the level of physical activity and the manifestation of the dipsic behaviour in cats.

Individuals with the most intense physical activity levels, translated into long periods of active time per 24 hours and a high number of consumed calories, also showed a more pronounced manifestation of the dipsic behaviour, translated into a higher frequency of the drinking sessions in 24 hours.

The level of physical activity can also be correlated with the age of the individuals, as the youngest cats showed the highest physical activity indicators and also manifested the dipsic behaviour more often than the other studied individuals.

We highly recommend the correlation between video monitoring and GPS collar tracking for the study of different types of behaviour and for identifying behavioural patterns in this species.

The modern apps – connected to the devices (video camera and GPS collar) facilitate the real time monitoring and the data storage, in our opinion, making the ethological studies accessible and accurate.

Acknowledgements

The presented materials, original pictures and results are part of the complex studies and research carried out during the doctoral studies of the PhD student Călin (Nicolae) Simona and will be presented and widely interpreted in the doctoral thesis: "*Research on the physiological and pathophysiological factors involved in the modification of dipsic and urinating behavior in cat*".

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LEGISLATION REGARDING WILDLIFE DISEASES SURVEILLANCE IN ROMANIA

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Summary

National wildlife diseases surveillance programmes are essential in monitoring, preventing, and controlling programs of human and animal diseases. Regulation (EU) 2016/429 of the European Parliament and the Council completed by Commission Delegated Regulation (EU) 2020/689 provides the rules concerning surveillance, eradication programmes, and disease-free status. At the national level, surveillance diseases in regulated by Order 35/ 2016 on the approval of the Methodological Rules for the implementation of the Program of actions for the surveillance, prevention, control and eradication of animal diseases, those transmissible from animals to humans, animal protection and environmental protection, for the identification and registration of bovine, porcine, ovine, caprine animals and Equidae and the Methodological Rules for the implementation of the Food Safety Surveillance and Control Program.
Keywords: wildlife diseases, legislation, surveillance.

Wildlife disease epidemiological surveillance is an essential part of both human and animal diseases.

Many of the pathogens on the OIE (World Organisation for Animal Health) List have the ability to infect and survive in wild animals for a long or a short period of time (11, 16). Moreover, not all infections may produce visible clinical signs associated with disease, knowing that some species represent some pathogens' natural reservoir. Even if clinical signs of infection appear, in most situations, wild animals do not have part of close observational vigilance and monitoring to recognise the illness, so there is a limit to the detection and reporting of diseases in wildlife (9). The risks of transmitting diseases to domestic animals and to humans are variable depending on wildlife species that live in different regions or areas and the types of livestock interfaces present (17). All these being exposed, it is understood that national wildlife disease surveillance programmes are essential tools for understanding local risks to animal health and potential zoonotic disease transmission (8, 11). These programmes provide data about wild animal morbidity and mortality, changes in patterns of disease occurrence over time and, last but not least, assist in the early detection of disease outbreaks, including those linked to emerging diseases (12).

Results and discussions

Epidemiological surveillance represents the systematic collection, analysis, and dissemination of data to plan, implement, and evaluate health programmes. It is the key to immediate and long-term strategies for combating infectious diseases. The actions authorities develop for each wildlife disease differ from country to country, depending on national risk (6, 10). Still, common points are implemented at the European or even global level. The rules concerning surveillance, eradication programs and disease-free status are provided in Regulation (EU) 2016/429 of the European Parliament and the Council, completed by Commission Delegated Regulation (EU) 2020/689 (19).

All diseases programs consist of 4 essential components, the specific one for wildlife being noted below:

1. Detection of pathogens and diseases. The detection of the disease is based on the collection of samples, the entire surveillance program being dependent on this stage. For the efficiency of this stage, it is important to train the stakeholders.
2. Identification of pathogens and diseases. Many of the pathogens of wild animals are easily identified using specific laboratory tests capable to identify the microorganism in domestic animals. And yet there are only wildlife-specific diseases or even newly identified pathogens, so investing in wildlife-specific laboratory equipment is justifiable.
3. Analysis and communication. Epidemiologists, wildlife biologists and ecologists must review the information obtained. Of course, the test results' validity and accuracy must be considered, especially if diagnostic tests used have not been validated in wildlife.
4. Information management. OIE member countries are encouraged to submit the obtained data to WAHIS-Wild, as part of the voluntary notification by the OIE wildlife diseases not on the OIE List (16).

Two types of category of wildlife category disease surveillance are described, both of them being designed with the four components. The first type- *Scanning wildlife disease surveillance*, also called “passive” surveillance, is designed to detect diseases and pathogens in wild animals (pathogens prevalence estimates) rather than obtain statistical data on one or a few pathogens. The second type- *Targeted wildlife disease surveillance*, also named “active” surveillance, refers to collecting data on one or more particular pathogens in one or more wild animal species to obtain statistical data regarding the prevalence, age and sex distribution of infection or geographic distribution of the pathogen (1, 20).

In Romania, epidemiological surveillance of wild animals is regulated by Order 35/ 2016 on the approval of the Methodological Rules for the implementation of the Program of actions for the surveillance, prevention, control and eradication of animal diseases, those transmissible from animals to humans, animal protection and environmental protection, for the identification and registration of bovine, porcine, ovine, caprine animals and Equidae and the Methodological Rules for the

implementation of the Food Safety Surveillance and Control Program. In the following the actions to be taken according to the order are listed (4, 9, 10).

- ***The wild boar surveillance program*** in Romania is applied for classical swine fever and African swine fever. Serological and virological surveillance for both infections is mandatory for all hunted wild boars, whether found dead or sick. Samples for serological examination shall be accompanied by samples taken from the same animal for virological examination (17, 20).

For classical swine fever, the serological tests used refer to ELISA, with the detection of antibodies on serum or thoracoabdominal fluid samples and the virus neutralisation test in case of positive ELISA samples. The virological examination involves performing RT- PCR and immunofluorescence tests; the virus's isolation on cell cultures is mandatory in all RT-PCR and IFD-positive cases (13, 19).

Election tests for African swine fever are PCR for detection of PCR tests for viral genome detection and an ELISA test for antibodies, according to Commission Decision 2003/422 / EC approving the diagnostic manual for PPA. A second test will follow a positive ELISA assay to confirm infection, respectively, indirect immunofluorescence or immunoperoxidase assay (1).

- ***The wild birds surveillance program*** includes the surveillance for influenza viruses program running for the entire calendar year with the half-yearly online transmission of data to the European Commission. Thus, the inspection is done periodically, with the prelevation of samples from sick or dead wild birds and testing by molecular biology tests. Positive samples for molecular biology tests are sent to the LNR within the IDSA and are subjected to virological investigations (virus isolation, typing, sequencing and phylogenetic analysis). The target species to be tested are listed in Annex II to Decision 2010/367 / EU (19).
- ***The wild ruminant surveillance program*** considers the surveillance for the chronic wasting disease of Cervidae (CWD) and transmissible spongiform encephalopathies (EST) in another wild ruminant, tuberculosis, and brucellosis (19).

The CWD and EST surveillance and/or monitoring of bovine, Cervidae and other wild ruminants from the forest or captivity is carried out based on Decision 2007/182 / EC. According to this decision, the brain is prelevated from all Cervidae over 18 months with or without clinical signs of disease, hunted, injured/ accidentally killed or dead. The samples are collected only by trained veterinarians, in compliance with Chapter I of the Veterinary Sanitary Norm approved by the Order of the President of ANSVSA no. 25/2008 (15, 18). The rapid tests registered and accredited in Romania are ELISA for EST with the double sandwich protocol, chemical polymers, and monoclonal antibodies protocol. These tests are performed by Morphopathology Laboratories within the D.S.V.S.A. designated for EST diagnosis and by LNR-EST within IDSA. LNR-EST exclusively confirms the disease by Western- Blot test for EST or immunohistochemistry, applying the legal provisions of Decision 2007/182 / EC and Regulation no. 999/2001 with subsequent amendments (2, 19).

Tuberculosis surveillance programs include passive surveillance of

susceptible species; thus, the hunted ruminants are subjected to morphophonological examination, direct microscopic examination and biological tests on lab rats. Additional tests include cultural examination, phenotypic and genotypic typing (3, 14).

Serological examination by the Rose Bengal test and RFC applied to the hunted susceptible species represents the test used for brucellosis passive surveillance. From the animals with lesions, samples are prelevated to confirm the diagnosis by isolation and identification of the etiological agent of the disease (direct microscopic examination, cultural examination, phenotypic and genotypic characterisation) (16).

- The program for the eradication, control and monitoring of ***rabies*** in Romania has been technically and financially approved by the European Commission since 2011, in accordance with European Union Veterinary Legislation and with the criteria set out in Commission Decision 2008/341/EC of 25 April 2008 on Community criteria for national programs for the eradication, control and monitoring of animal diseases and zoonoses (5, 18). Foxes from all over Romania are orally vaccinated in two annual campaigns according to Order no. 642/2002 for the approval of the Sanitary Veterinary Norm regarding the recognition of rabies vaccines. Samples of foxes shot are taken at least 45 days after the vaccination campaign (4 foxes/100 km2) and are subjected to ELISA test to evaluate the vaccination efficiency. Vaccination activity is evaluated by direct immunofluorescence (13, 14). All positive direct immunofluorescence samples are tested for virus-specific genome identification and characterisation (R-PCR). According to the legislation, if Romania is at risk for rabies, the ANSVSA deposition will be implemented clinical inspections and serological tests for this early diagnosis of this viral disease (7, 19).

Notification of infectious diseases is made based on the legal provisions of the Order of the President of ANSVSA no. 79/2008 with subsequent amendments.

The surveillance systems should be regularly reviewed based on explicit usefulness, cost, and quality criteria.

Conclusions

In Romania, the legislative basis for epidemic-surveillance of infectious diseases in wild animals is vast. The measures applied for active and passive surveillance are intended to diagnose early infections in these groups of animals in order to avoid the appearance of epidemic outbreaks. Surveillance programs are constantly being reviewed, and the emergence of infections sets new trajectories for monitoring diseases diagnosed to reduce incidence, mortality, and morbidity.

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DETERMINATION OF ANTIMICROBIAL DRUG RESIDUES AND THE ROLE OF BACTERIOPHAGES IN FALSE POSITIVE MICROBIAL RESIDUE DETECTION TEST IN MILK IN KADUNA STATE, NIGERIA

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Summary

Antimicrobial residue in milk causes financial losses to dairy farmers due to milk rejection or low pricing. Detection of antimicrobial residues can be a bit challenging due to the presence of bacteriophages in milk, which could cause false-positive results. The aim of this study was to determine the occurrence of antimicrobial residues and lytic bacteriophage in fresh bulk cow milk from peri-urban dairy centers and the influence of phages on antimicrobial residue detection in milk. Antimicrobial residues in fresh bulk cow milk were determined using the standard microbiological methods, the disc diffusion assay and the Delvotest Sp-Nt. The plaque assay method was used for the isolation of lytic bacteriophages. Out of the overall milk sample of 204, antimicrobial residues were detected in 43.6% and 42.2% of the samples using the Delvotest and the disc diffusion tests, respectively. The inter-rater reliability (Kappa) for rating the performance between the Delvotest SP-Nt and Disc diffusion assay test was found to be 0.890 and statistically significant (P-value = 0.00). Lytic bacteriophages were isolated from 30% of all milk samples collected (204 fresh bulk cow milk samples). Ten percent of the antimicrobial positive milk samples were confirmed to be false positives after conducting the two tests in parallel. The false-positive samples were confirmed for the presence of lytic bacteriophage, and 55.5% were found to be contaminated with lytic bacteriophage. Thus, the overall prevalence of antimicrobial residues recorded in this study using the disc diffusion test exceeded the maximum residue levels. Raw milk harbors natural inhibitory agents such as bacteriophages, which often cause the misinterpretation of antibiotic contamination in milk during antimicrobial detection since tests are not specific on the types of antimicrobial residues. It is recommended that more advanced detection kits be used for antimicrobial detection to limit the levels of false positives.

Keywords: residue, milk, bacteriophages.

Milk and milk products are important sources of animal protein which contribute greatly to human growth and development worldwide (43). Milk contains all the essential food constituents for all age groups and meets the nutritional needs of the body better than any single food (29). Fermented dairy products, such as cheese, kefir, cultured buttermilk, cultured cream, koumiss, Kindirmo, Nono, Warankasi and yoghurt, are products of high value known as functional food and widely consumed due to their positive health impact. Fermented milk are known to represents a distinct food culture in every community in the world, symbolizing the heritage and socio-cultural aspects of the people (39).

Starter cultures are those microorganisms (bacteria, yeasts, and molds or their combinations) that initiate and carry out the desired fermentation essential in manufacturing cheese and other fermented dairy products (40). Lactic acid bacteria (LAB) starters can be affected by many factors, such as temperature, pH, strain capability, growth medium, antibiotics and bacteriophage (2).The growth and activity of this starter cultures in milk are unfavorably influenced because of the presence of residual antibiotics and sanitizers in milk. Additionally, the production of antibiotic-like substances (bacteriocins) by certain wild strains of *Lactococcus lactis* subsp*. lactis* and other lactic cultures in raw milk also contributes (12).

In dairy animals, mastitis is the most reason for antibiotics use (28, 32). Antibiotic residues in milk exceeding tolerance levels not only present potential public health risks when consumed, but also interfere with fermentation processes during milk processing (4). Antibiotic residues in animal derived foods in many African countries have mostly been found to exceed the WHO maximum residue levels (11). Though, common antimicrobial residues detection methods are qualitative, thus, having a drawback. The limitation of these methods is inability to specify types of antimicrobial residues that can inhibit starter culture i.e., bacteriocins and bacteriophage, this can result in false positive microbial residues detection.

Bacteriophages are viruses that infect and kill bacteria without any negative effect on both human and animal host, and they are ubiquitous in nature (14). Bacteriophage infection of dairy starter cultures remains one of the causes of fermentation failures in the dairy industry and can lead to substantial economic losses due to manufacturing delays, waste of ingredients, lower quality product, or even total production loss (34). It is, therefore, crucial to identify the sources or reservoirs of bacteriophages within a factory in order to introduce corrective action to limit their propagation (38). The presence of bacteriophages in milk can also makes detection of antimicrobial residues a bit challenging leading to a false-positive results.

Accordingly, the present study was to determine the occurrence of antimicrobial residues and lytic bacteriophage in fresh bulk cow milk and their influence on false positive results in microbial residues detection tests in milk, using Delvotest® Sp-Nt kit and disc diffusion assay and to compare their performance of the two methods.

Materials and methods

Study Area: The study was conducted in the peri-urban dairy centers in Kaduna State.it is located in the northern Guinea Savannah Zone of Nigeria and lies between Latitude 09^0 02'N and $11^0$32'N and between Longitude 06^0 15'E and 08^0 38'E. It is made up of twenty three local Government areas and is characterized by rain-fed agricultural activity which spans from May to October and irrigation farming in the dry season which occurs between November and April. It has a tropical climate

with mean and annual rainfall of 1092.8mm and a monthly mean temperature which ranges between 13.8^{o}C to 36.7^{o}C (1).

Sample Collection: A total of 204 fresh bulk cow milk samples were collected. About 100mls of fresh bulk cow milk sample from each milk churn were collected from the peri-urban dairy centers. The fresh bulk cow milk were sampled once weekly between November to January from two Peri-urban dairy centers in Kaduna State, Nigeria. The samples were collected using sterile universal bottles and transported on ice to the Bacterial Zoonoses Laboratory of the Department of Veterinary Public Health and Preventive Medicine, Ahmadu Bello University, Zaria, for laboratory analysis.

Disc Diffussion Assay Method: This was done using the method described by Kabir (16). Sterile Mueller Hinton agar plates were inoculated with 1ml of broth culture of *Bacillus subtilis* ATCC6633 and air dry for about 5-10 minutes. The milk were tested by dipping a sterile 1.2cm diameter Whatman filter paper disc into the test milk sample, until the disc was saturated ,then shaking off the excess milk. A sterile thumb forceps was used to impregnate disc directly on the surface of the inoculated Mueller Hinton agar plate and labeled accordingly. It was incubated at 37^{o}C for 18-20 hours. The presence of antimicrobial residues in the milk sample was indicated by the presence of inhibition zone of the growth of *Bacillus subtilis* around the disc while absence of antimicrobial residues was indicated by the absence of inhibition zone growth around the disc.

Delvotest®SP- NT Method: The Delvotest SP-NT kit was used as described by the manufacturer (DSM, Netherlands). For each milk sample, 0.1ml of milk sample was pipetted using a fresh disposable pipette and transferred to the ampoule containing solid agar embedded with *Bacillus stearothermophilus* var. *calidolactis* and Bromocresol purple indicator. The ampoule was placed in a preheated dry incubator specially designed for Delvotest® at 64^{o}C after which the ampoules were incubated for 3hours. The ampoule that change from purple to yellow colouration indicate negative results while the positive result retained its purple colouration after 3 hours.

Bacteriophage Isolation: Bacteriophages isolation from raw milk **sa**mples were done as described by Clokie and Kropinski*.* (10). Briefly, 5ml of each raw milk sample was centrifuged at 2,000rpm for 5minutes. Each supernatant was filtered through a 0.22µm sterile syringe membrane filter in to a sterile test tube. Then, 2.5mls of each filtrate were inoculated in 5ml of Luria Bertani broth and 0.lml of an overnight culture of *Bacillus substilis.* The tube was incubated at 37^{o}C for 24hr. After 24hr of incubation, the content of the tubes was centrifuged at 2,000rpm for 5minutes. Then, each supernatant was pipetted into a new sterile test tube and 0.5ml of chloroform was added, then, filtered into a sterile test tube. Each filtrate was considered to be a clean source of phage and stored at 4^{o}C for further analysis.

Plaque Assay: 10µl of bacteriophage filtrate sample was transferred into the test tube using micropipette and allowed to sit undisturbed for 5-10 minutes. Two Petri dishes were labeled for each sample; one and the other as control. Eight

milliliters of Luria Bertani broth was pipetted and dispensed into the phage test tube and 90µl of $CaCl_2$ and 4.5ml agar solution were added using micropipette and pipette respectively. This was mixed and poured into the Petri dish labeled as test and allowed to solidify. Five milliliters agar solution was dispensed into the bottom of the Petri dish labeled as control. Both petri dishes were swirled in both directions for even coating of the agar and incubated at 37^{o}C for 24 hours.

Plaque Picking Method: each plaque was picked using a Pasteur pipette into a 100µl of bacteriophage buffer (1mM$CaCl_2$) in a labeled test tube. The solution in test tube was mixed well by vortexing for thirty seconds. The resulting solution containing bacteriophage sample was re-plated and incubated at 37^{o}C for 24 hours.

Statistical Analysis: Statistical Package for Social Science (SPSS) version 20.0 was used for data analysis. The correlation between Disc diffusion assay and Delvotest® was determined using kappa statistic. Prevalence was calculated using the formula:

$$\text{Prevalence}(\%) = \frac{\text{Number of positive samples}}{\text{Total samples collected}} \text{ X } 100$$

Results and discussions

The present study was undertaken to determine the presence of antimicrobial residues in fresh bulk cow milk samples from the peri-urban dairy centers in Kaduna State, Nigeria. Out of the 204 fresh bulk cow milk samples tested (Table 1) for antimicrobial residues, 43.6% had antimicrobial residues using Delvotest Sp –Nt kit, this was higher than (14.7%) report by Muhammad et al., (24) in Zaria, Kaduna State. Antimicrobial residues found in this study was also above the 33.8% reported by Salman et al., (33) in Sudan, 21 % in Kenya (36), 14% in Iran, (22), 13% in Uganda (35), and 3.1% in Ghana (3).

Table1

Distribution of Antimicrobial Residues in Fresh Bulk Cow Milk from the Peri-Urban Dairy centers in Kaduna State using Delvotest SP-Nt Kit

Sampling location	Total number of Samples examined	Number of positive Samples (%)
PUC1	42	13 (31.0)
PUC2	162	76 (46.9)
TOTAL	204	89 (43.6)

PCU1 Peri-urban center 1, PCU2 Peri-urban center 2

Using disc diffusion assay (Fig. 1), 42.2% (86) of fresh bulk cow milk samples out of 204 samples had antimicrobial residues (Table 2), which is higher than the 40.8% reported by Olatoye et al., (26) in Oyo State and 76% in Delta state

(37) who recorded high occurrence rate of antimicrobial residues in fresh and fermented (Nono) cow milk in Nigeria. Nevertheless, lower occurrence rates were reported in various studies from Kenya, Kosovo, and Iraq with a total occurrence range from 10% to 18.4% (6, 7, 18, 25, 27).

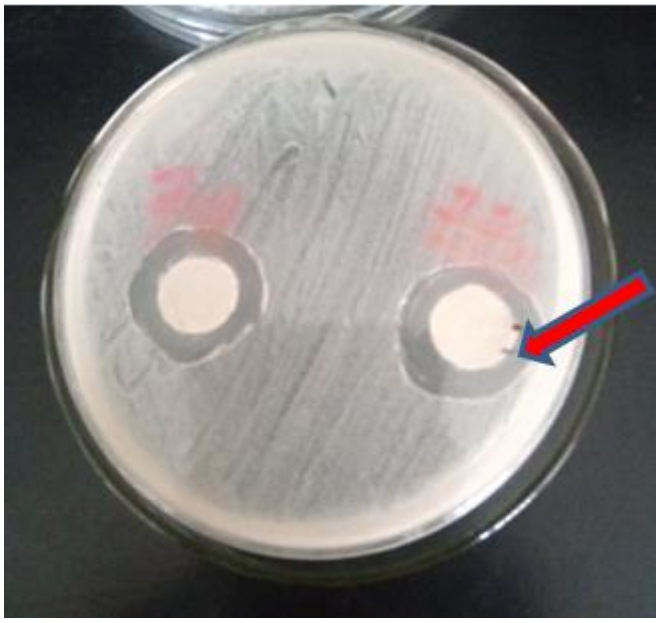

Fig.1. Inhibition zone of a positive disc-diffusion assay test

Table 2

Distribution of Antimicrobial Residues in Fresh Bulk Cow Milk from the Peri-Urban Dairy centers in Kaduna State using Disc Diffusion Assay

Sampling location	Total number of Samples examined	Number of positive Samples (%)
PUC1	42	12 (28.6)
PUC2	162	74 (45.7)
TOTAL	204	86 (42.2)

PCU1 Peri-urban center 1, PCU2 Peri-urban center 2

The comparison between the Performance of Delvotest and Disc Diffusion Assay Test using Cohen's Kappa statistic (K) was 0.89% (Table 3), which mean, there is almost perfect agreement between the two test methods. This finding is in

line with finding of Ahmed et al., (5) who found 1.0% agreement between the two test in assessment of microbial loads and antibiotic residues in milk supplied in Sudan. This is contrary to what is reported by Salman et al. (33) in Sudan, who had 0.47%, representing moderate agreement between the two tests method. The presence of antimicrobial residues in milk is considered as an infringement of food safety standards (42). This occurrence could be as a result of antibiotics used in dairy farming for various purposes such as treatment or prevention of numerous infectious diseases, increase milk production, increase feed efficiency, growth promotion, improving digestion, weight gain, and rise feed conversion ratio (9, 15, 41). The overall prevalence of 43.6% antimicrobial residues recorded in the present study which has exceeded the maximum residue levels by WHO, indicates that consumption of such milk products can be sources of potential risk to the health of the consumers, as well as interfering with fermentation processes, during milk processing (4). This problem could be due to the lack of awareness of dairy farmers on withdrawal periods of drugs among other factors.

Table 3

Comparison between the Performance of Delvotest® and Microbial Inhibition Test in Detection of Antimicrobial Residues in Cow Milk

Type of test	Total number of samples examined	Number of positive samples	Prevalence (%)	Kappa statistic (k)
Disc diffusion Assay	204	86	42.2	0.890
Delvotest SP-NT	204	89	43.6	

P-value = 0.00

Using plaque assay method (Fig. 2), 30% of lytic bacteriophages were isolated from 204 fresh bulk cow milk samples that were collected from the peri-urban dairy centers (Table 4). This finding is similar to that by McIntyre et al., (21); who isolate lytic bacteriophages from pooled bulk raw milk in New Zealand cheese plants and Madera et al., (20), who also reported 10% of lytic bacteriophage isolated from raw milk in Spain. This is contrary to the finding of Arbon, (8), who recorded absence of lytic bacteriophage in raw and pasteurized milk samples from Utah in the United States. The presence of lytic bacteriophages in fresh bulk cow milk in this study indicate that raw milk could be source of milk processing failure, as determined by some researchers (13, 23), that 10% of fermentation failures in the dairy sector are caused by lytic bacteriophage infections.

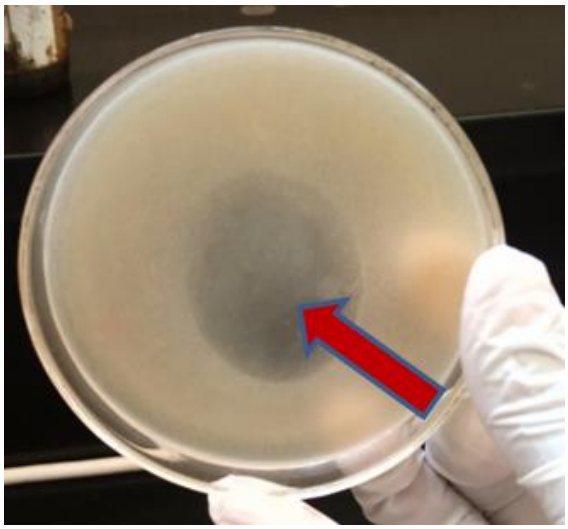

Fig. 2. Positive plaque assay test, arrow indicating plaque formed

Table 4

Isolation of Lytic Bacteriophages in Fresh Bulk Cow Milk Sampled in the Peri Urban Dairy Centers in Kaduna State

Sampling location	Total Samples examined	Samples positive (%)
PUC1	42	20 (47 .6)
PUC2	162	42 (25.9)
TOTAL	204	62 (30)

PCU1 Peri-urban center 1, PCU2 Peri-urban center 2

The false positive test result in the disc diffusion assay method (Table 5) was found to correlate with the presence of lytic bacteriophage. Around fifty six percent of the nine false positive test results from the milk samples had lytic bacteriophages present, similar findings were reported by kang et al., (17) and Ahmed et al., (5), who recorded 2 and 3 false positive test results from raw milk samples respectively. Kosikowski, and O'Leary, (19) demonstrated the characteristics of the natural inhibitory reaction in Irish manufacturing milks which give rise to false positive test results that are misinterpreted for pharmaceutical antibiotic reaction in disc assay testing. This study has confirmed that the presences of lytic bacteriophage in raw milk which could be one of the major reason why raw milk could act as natural inhibitory agent to the test organism during microbial inhibition test because of their bactericidal nature to bacteria, hence, inhibiting the bacteria growth. Though there

are other natural bacteriostatic substances in milk, such as protein, fat, somatic cells, other bacteria lactoferrin, lysozyme, and lacto-peroxidase system, which can permeate the detection medium (30, 31).

Table 5

The Contribution Effect of the Isolated Bacteriophage on the Occurrence of False Positive in Disc Diffusion Assay Test Result

S/N	Sample ID	Mit inhibition zone	Delvo-sp-nt test	Phage test
1	MZ3	+	–	+
2	ML9	+	–	+
3	MZ26	+	–	–
4	MZ29	+	–	–
5	MZ33	+	–	+
6	MG57	+	–	–
7	MG136	+	–	+
8	N201	+	–	–
9	NB204	+	–	+

Positive +, Negative –

Conclusions

The overall prevalence of antimicrobial residues recorded in this study using microbial inhibition test exceeded the maximum residue levels. There is almost perfect agreement between the performance of Delvotest Sp-Nt and disc-diffusion assay in detection of antimicrobial residues in the fresh bulk cow milks, in which Cohen's Kappa statistic (K) was 0.89%. The Delvotest showed more positive test results than the disc diffusion assay, therefore, it is considered to be more sensitive than the disc assay. Raw milk harbors natural inhibitory agents such as bacteriophages, which often cause the misinterpretation of antibiotic contamination in milk during antimicrobial detection since tests are not specific on the types of antimicrobial residues. It is recommended that more advanced detection kits be used for antimicrobial detection to limit the levels of false positives.

Acknowledgment

The authors would like to thank the staffs and technicians of the Department Of Veterinary Public Health and Prevent Medicine, ABU, Zaria, Peri-urban dairy centers in Kaduna State for their contributions toward the success of this work.

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STUDY REGARDING THE ACCURACY OF THE CHEMICAL COMPOSITION OF COMMERCIAL DIETS FOR DOGS

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Summary

The aim of this study was to compare the accuracy of the chemical composition of dry dog food with the nutritional values printed on their labels. To conduct this study, five diets were randomly selected from each type of commercial diets: economy, basic and premium class. The results showed an increased in the dry matter content, compared to the values written on the labels. For all three categories of commercial diets analysed, the crude protein content showed values below those registered on the labels by 1-4%. The fiber content was higher, especially for the economy class and the ether extract showed higher values in 3 premium class diets, varying by 1.18%.

Keywords: dog, commercial diets, nutritive value, chemical composition.

In recent years, there has been an increase in the production of pet food due to the growing number of pets (6, 19). There are many pet diets available on the market, from different producers and different quality categories (6, 7, 20). Most pet owners feed their dogs commercial food, the majority of them using this type of food exclusively (1-4).

Dry pet food is very popular among the owners because it is easier to store and it represents an economical and simple option for feeding dogs. Thus, animal owners and veterinarians are interested in using, respectively, recommending optimal commercial diets that are as close as possible to the nutritional requirements of dogs, in order to maintain their growth and development (18, 19).

The purpose of this study was to compare the nutrient content obtained by chemical analysis of dry dog food with the nutrient content declared by the manufacturer of the commercial dog diets and printed on the label of the commercial products. To conduct this study, five diets were randomly selected, from each type of class: economic, basic and premium.

Materials and methods

This study aimed to analyze commercial dog diets by determining their chemical composition. To this end, five commercial brands from each category: economic, basic and premium were selected. A total of 15 commercial dog diets were examined. The selection of these diets was made by considering the most marketed dog food category for adult dogs with moderate physical activity.

The commercial diets were analyzed for their proximate chemical composition in the physical and chemical feed analysis laboratory, of the Animal Nutrition and Agronomy discipline, Faculty of Veterinary Medicine. In order to preserve the anonymity of the producers, the diets were divided into 3 main groups: economic (E), basic (B) and premium (P) and numbered, within each category, from 1 to 5.

To determine the proximate chemical composition of the diets, the following parameters were analyzed: dry matter (DM%), moisture (U%), crude protein (CP%), ether extract (EE%), crude fiber (CF%), and crude ash. For the analysis of the chemical parameters, standard methods were used in accordance with the equipment manufacturer's notes.

The values of the chemical parameters were compared with the values written by the producer on the packages of the commercial diets, with the aim of observing the fidelity of the nutritional value declared by the manufacturer, to the crude chemical composition of the diets.

The obtained results were statistically analyzed using Excel Data Analysis. Statistical correlations and descriptive statistics were carried out, and the data were analyzed by calculating the t-Test and the positive or negative correlations between parameters, or of them with the nutritional value of the food presented on the product label.

Results and discussions

After the chemical analysis of the commercial diets, the obtained results are presented in table 1.

The results obtained from the analysis of the chemical composition of the commercial dry food were compared with the values registered on the labels by the producers. Thus, for the dry matter, values between 94.34% and 98.16% were obtained for all 3 categories of food, and on the labels the values of the dry matter were 90% for each of them, except for one diet from the premium class, which had a 92% value written on the label (Table 1).

Regarding the moisture content of the analyzed samples, lower levels were obtained during the analysis, between 1.84 and 5.66%, compared to the labels which, in 14 of the 15 analyzed diets, presented a moisture content of 10%, and for one brand, from the premium category, a moisture of 8% was described (Table 1).

When analyzing the crude protein, the values obtained using the Kjeldahl method showed different variations compared to the values of the same parameter written on the labels. For all 15 diets studied, the crude protein values were lower than the values written on the labels, as follows: in the economy class the values were lower by 10-12%, and in the premium class by 18-23% lower (Fig. 1).

Table 1

Analysis of the proximate chemical composition of the studied diets

Diets/ Analysis	*DM%*		*M%*		*CP%*		*EE%*		*CF%*		*ASH%*	
	A*	L**	A*	L**	A*	L**	A*	L**	A*	L**	A*	L**
E1	98.16	90	1.84	10	15.4	20	6.67	7	6.26	3	5.82	9
E2	95.79	90	4.21	10	17.81	18	4.2	8	4.62	3.5	8.65	7.5
E3	96.77	90	3.23	10	14.94	17	4.8	8	4.99	3	8.18	8
E4	96.69	90	3.31	10	18.78	21	7.27	13	4.37	1.8	8.69	7.5
E5	96.66	90	3.34	10	23.23	26	10.28	16	4.31	3	8.25	6.2
B1	96.8	90	3.2	10	22.13	26	11.5	16	7.4	2.5	6.08	7.2
B2	97.56	90	2.44	10	21.87	25	9.09	12	3.67	3	8.25	8
B3	97.19	90	2.81	10	16.59	20	7.54	10	3.8	3.5	8.77	8
B4	97.32	90	2.68	10	20.27	23	9.34	11	3.63	3.5	9.43	6.5
B5	94.73	90	5.27	10	17.7	22.5	8.49	16	6.79	2.5	8.49	7.5
P1	96.61	92	3.39	8	29.05	33	11.92	17	4.54	3.5	8.4	9
P2	97.41	90	2.59	10	24.87	38	15.74	20	6.48	3.2	8.92	8
P3	95.9	90	4.1	10	21.75	26	16.51	16	3.57	2.5	7.33	7.5
P4	94.34	90	5.66	10	19.86	25	15.74	14	4.22	1.5	6.42	5.8
P5	95.2	90	4.8	10	17.82	21.8	16.03	14.8	3.8	1.7	2.72	4.8

*A – analysed
**L - label

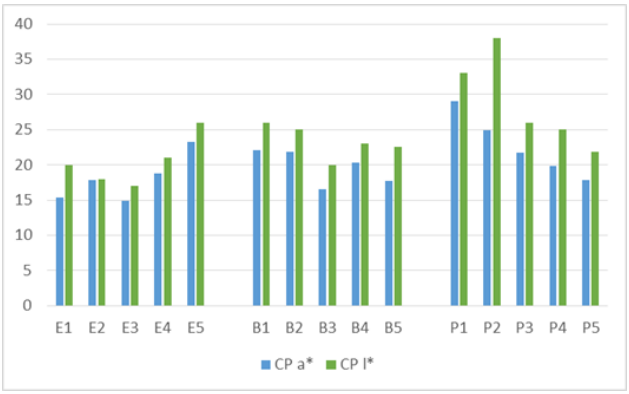

Fig. 1. Comparison of the values obtained during the analysis with those written on the labels, for *crude protein*
*A – analysed
**L - label

For the analysis of ether extract using the Soxhlet method, the following results were obtained: for the economic class, the values obtained (4.2% - 10.28%) were lower than those labeled (7% - 16%); for the basic diets, lower values were also obtained (7.54% - 11.5%, against 10% - 16%); likewise with premium diets, the values obtained by analysis were lower than those written on the label (11.92% - 16.51%, against 14% - 20%) (Fig. 2).

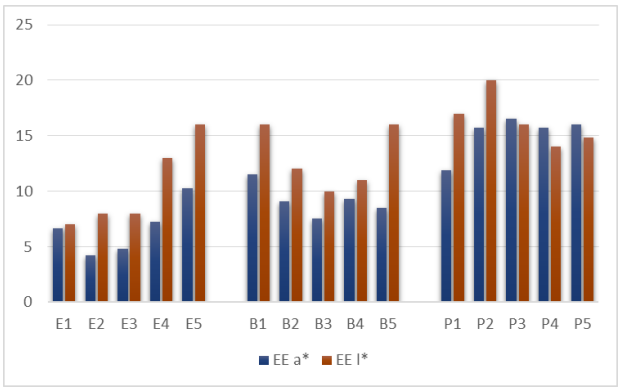

Fig. 2. Comparison of the values obtained during the analysis with those written on the labels, for *ether extract*

*a – analysed
**l - label

When analyzing the crude fiber (crude cellulose) content, the values identified showed different variations. The commercial food from the 2 categories, economy and basic, had a lower crude fiber content than the labeled values, while the premium class diets presented, on analysis, a higher fiber content compared to the one on the labels (Fig. 3).

The total mineral content (ash) showed lower values compared to those labeled, for all the studied diets, according to Fig. 4.

Fig. 3. Comparison of the values obtained during the analysis with those written on the labels, for *crude fiber*
*a – analysed
**l - label

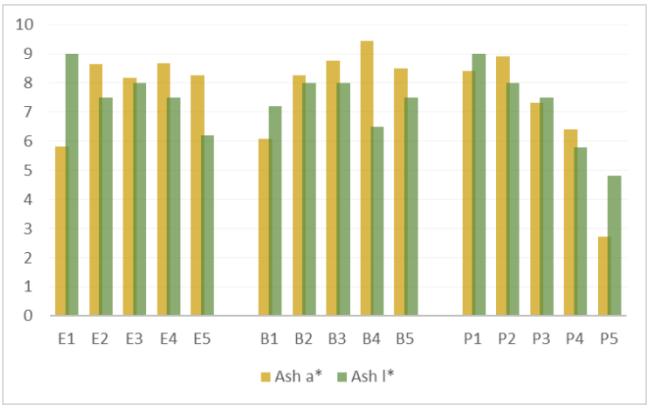

Fig. 4. Comparison of the values obtained during the analysis with those written on the labels, for *crude ash*
*a – analysed
**l – label

Fallowing the statistical analysis, the most significant differences ($p < 0.05$) were identified in the *economic class diets*. For dry matter and fiber, the significant differences were positive, and for moisture, crude protein and crude fat, the significant differences were negative.

Commercial diets from the basic category presented fewer significant differences ($p < 0.05$) compared to the diets belonging to the economic class.

Significant positive differences were observed only for dry matter. In contrast for moisture, crude protein and crude fat the significant differences were negative.

For commercial diets in the premium category, statistical analysis revealed transitional differences, compared to the other two categories of diets studied. Significant ($p < 0.05$) positive differences were observed for dry matter and crude cellulose, for which the values obtained by chemical analysis were higher than the labeled values.

For all the commercial diets studied, crude fiber showed higher values in the chemical analysis compared to the data written on the labels. This growth in the crude fiber content is due to a quantitative increase of plant base ingredients added to dogs diets (5, 8). This increase influences the nutritional value of the diets, theoretically influencing the digestibility of the product, knowing that cellulose is more difficult for the dog to digest (11-13). For all three categories of commercial diets analyzed, the crude protein content was lower in chemical analysis compared to the values written on the labels. Knowing that protein has a plastic role, forming new tissue in the body, a correct estimation of the quantity of protein administered to dogs would be necessary (1, 3, 10). Furthermore, the ether extract content of the diets presented lower values in the chemical analysis compared to the values listed on the labels, for all the diets analyzed. These differences can have an effect on the general condition of the animal because they mislead the veterinarian or the animal owner regarding the energy intake of the ration (10, 17).

To date, few studies have been conducted on this subject. In 2015, Rolinec et al. (16) carried out a similar study, in which they compared the chemical analysis of commercial diets with the values written on the packaging. They observed that only 6 out of 15 dry dog food samples were within ± 5% of the crude protein concentration declared by the producers; neither one of the samples was within ± 5% of the declared ether extract concentration; only 2 out of 15 dry dog food samples were within ± 5% of the declared crude fiber concentration. The highest discrepancy was detected by the authors in the fat (ether extract) concentration. The largest excess was detected at the fiber concentration. The authors concluded that the analysis of dry dog food samples had a very different nutritional values compared to the nutritional value written on the package (16). Similar results, with differences in nutrient concentration in dog food, were also published by Harlow in 1997 (9) or Lasek et al., in 2013 (11).

Another study published by Daumas et al. (7) carried out in France, evaluated eight commercial diets from the super-premium, basic and economy categories. The range values of nutritive parameters, for crude protein, ether extract, crude fiber and crude ash, were between 20.9-30.6%, 20,9–30,6%, 6,8–19,7%, 2,3–3,3% and 4,6–9,7% (7).

Analyzing commercial dog diets would be very important, considering that they have a different nutritional value compared to the declared nutritional value. Over time, the result of feeding such feed is that, some dogs will be overfed or underfed with certain nutrients, which can lead to the development of various

pathologies (15-17). The concern of animal breeders regarding the health status of their companions could support the development of a program for analyzing commercial pet diets in the veterinary medical field.

Conclusions

Regarding the chemical content, the commercial diets showed different values from those written by producers on the labels. The nutritional and energetic values of the commercial diets were different compared to those noted on the labels, thus changing the caloric intake of the diets consumed by dogs. The highest differences in the chemical composition were observed in the economical commercial feed, identifying important nutritional imbalances in the diets. Knowing the real chemical composition of commercial diets is important for the prevention of various nutritional and metabolic pathologies, frequently encountered in the medical-veterinary practice.

Acknowledgement

This research paper is supported by the project " Increasing the impact of excellence research on the capacity for innovation and technology transfer within USAMVB Timișoara" code 6PFE, submitted in the competition Program 1 - Development of the national system of research - development, Subprogram 1.2 - Institutional performance, Institutional development projects - Development projects of excellence in RDI.

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MICROSPORUM CANIS STRAINS SENSITIVITY TO ANTIFUNGAL DRUGS

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Summary

Researches presented in this study underlines results obtained in sensivity of *Microsporum canis* strains from dogs and cats on different antifungal drugs. It was found that of the five antifungal substances tested by the diffusimetric method, in terms of efficacy on isolated *M. canis* strains (n = 116), Clotrimazole showed special activity, with the inhibition zones diameter ranging from 21 mm to 40 mm, with an average of 31.28 ± 5.18 mm. With lower efficacy, causing areas of inhibition smaller than Clotrimazole, from intermediate to sensitive, Miconazole and Nystatin acted. There were strains sensitive to Miconazole, but intermediate sensitive to Nistatin, suggesting the need to test the sensitivity to the antifungal substance before starting a treatment.

Keywords: dermatophytosis, *Microsporum canis*, antifungal substances, sensitivity test.

Dermatomycoses are of all fungal infections the most common forms of infection in humans, affecting more than 20% - 25% of the world's population (7). It is estimated that in the human population, 30% - 70% of adults are asymptomatic carriers of these pathogens (17). From studies conducted so far, it is estimated that zoophilic species are responsible for about 30% of human dermatophytosis and usually cause acute inflammatory conditions. Anthropophilic species account for approximately 70% of infections in these hosts, causing a chronic infection with slow progression, suggesting that the fungus has adapted to the human host (19).

Compared with the importance given to fungal infections in humans, in animals fungal diseases are relatively neglected, even if they are a source of up to 80% of human skin problems in rural areas and 20% of infections in urban areas (21).

Another aspect that must be reported in fungal infections, both in humans and animals is the manifestation of resistance to certain antifungals used in treatment. The most important element that can induce the appearance of antifungal resistance seems to be the improper prescription of systemic antifungal agents and their indiscriminate use (9).

The incidence of fungal infections, including resistant infections, has increased during the last few years, and may be due to inadequate or irregular use of drugs or increased incidence of immunodeficiency states (23).

There is no clear evidence of the dosing strategy to be used during treatment and prophylaxis to avoid the best resistance, especially for animals (23).

There are medical studies that have suggested measures to prevent and suppress the occurrence of antifungal resistance, which specify the prudent use of

antifungals and their appropriate dosage, treatment with an antifungal appropriate to the identified etiological agent, after establishing its sensitivity to antifungal substances (5).

In veterinary medicine for the treatment of dermatophytosis in pets there are a limited number of drugs, specially conditioned for dogs and cats. For this are used medical antifungal ointments recommended for the treatment of human dermathosis. Also, for pets systemic treatments of this type of disease, commercially veterinary products are limited, and as result, drugs used for human dermatophytosis treatment are used, but these are difficult to dose.

In addition, it should be noted that due to the transmission of the disease, the owners of animals with dermatophytosis could be also infected. They ask for the help and advice of a veterinarian, for themselves or their family members. As a result it is found that the prescription of topical or systemic antifungal drugs is done without a antifungal susceptibility test, which can lead in time to the development of the resistance on antifungal drugs.

In view of these aspects, the aim of this study was to test the sensitivity of strains of *Microsporum canis*, isolated from dfifferent cases encountered in Timisoara veterinary practices, in order to assess the efficacy of some products that are frequently recommended for the treatment of this infection and to observe the antifungal resistance in some strains of this dermatophyte specie.

Materials and methods

A total of 116 samples composed of fur, nail and skin scraping specimens were collected from 40 (25%) dogs and 76 (62.5%) cats with clinical suspicion of dermatophytosis, in veterinary clinics from Timisoara. Samples were microscopically examined and cultivated in Mycology Research Laboratory of the Faculty of Veterinary Medicine from Timisoara.

The skin samples were collected by scraping with a sterile scalpel blade, and the hairs were taken from the center and from the edges of the lesions with a sterile forceps. The samples were deposited in sterile containers for laboratory processing. Their inoculation was performed after their fragmentation with a sterile scalpel blade and immersion in a 70 ° alcohol solution for 30 seconds. Sabouraud's dextrose agar (SDA - 1% peptone, 2% dextrose, and 2% agar) with the addition of chloramphenicol, previously poured into sterile Petri dishes, was used for cultivation. Incubation of the plates was performed at 27 - 28 ° C for 7-10 days. The identification of the species of *Microsporum canis* was made on the basis of cultural and microscopic characteristics (14).

Microsporum canis produces colonies that become visible 4-5 days after inoculation, but then grow rapidly. The colonies have a fluffy to woolly appearance, being white-yellow on the obverse and yellow or yellow-orange on the reverse (Fig. 1).

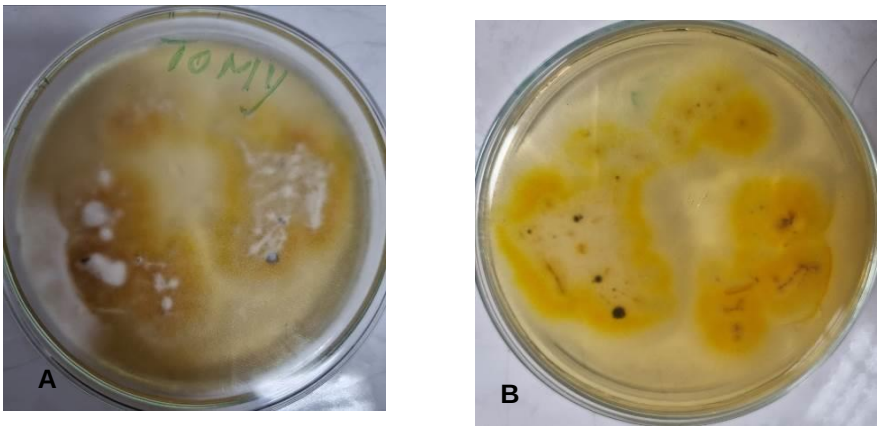

Fig. 1. Microsporum canis - macroscopic characters:
obverse (A) and reverse (B)

Direct microscopic examination (wet mount) was performed from pure culture, using lactophenol blue solution. Optika microscope with video camera was used for examination. Initially, the 10 x objective was used for the overall examination of the blade, and then for the magnification of the image and the study of the characteristic elements (macroconidia) the 40 x was used. In the microscopic preparations, the septate mycelial filaments and the typical, fusiform, long, sharp, pointed macroconidia were observed at both ends or with a slightly curved extremity (15). The wall is thick, smooth on some stems or strongly ornamented, even echinous on others. The interior of the macroconidia is puriseptate with 7-14 cells (Fig. 2).

Microsporum canis strains antifungal sensitivity testing was performed by diffusimetric method. For this, antifungal substances frequently used in this type of dermatophytosis therapy, both in animals and in humans, in topical applications, baths, but also in systemic treatment were chosen. The antifungal substances used for this study are: 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 synthesis pathway of ergosterol by blocking the activity of the enzyme 14 dimethylase, causing changes in the structure and permeability of the cytoplasmic membrane, and the other two (Amphoteric B). Nystatin), from the Poliene group, which acts by binding to the cell membrane, causing changes in the structure and permeability of the fungal membrane by selective binding to ergosterol (Amphotericin B, Nistatin) (11).

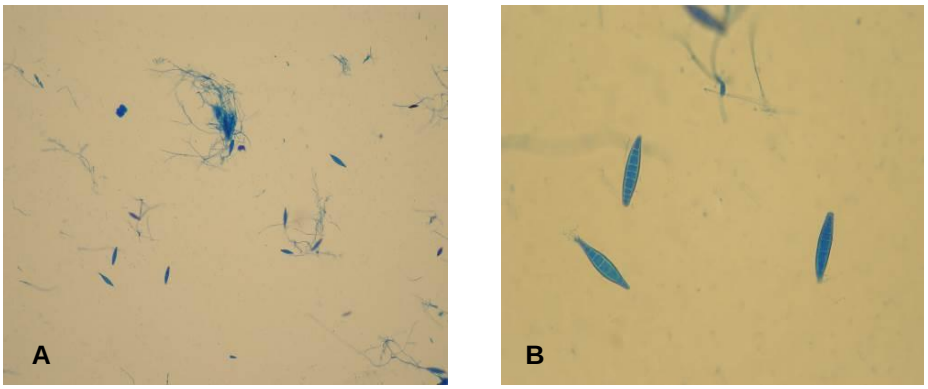

Fig. 2. *Microsporum canis* – microscopic aspect
A - Hyphae and macroconidia *M. canis* - 10x objective;
B - Macroconidia of *M. canis* - 40x objective

The diffusion method was performed on solid media Sabouraud Dextrose Agar and is based on the ability of the substances present in the disc to diffuse into the culture medium, from the place where they are deposited. The test is also called a fungigram (24).

In order to perform the test, fresh cultures were prepared from all 116 strains of *Microsporum canis* isolated and preserved in the discipline. Inoculations from each strain were made on the Sabouraud medium, with the addition of chloramphenicol, poured inclined (sloping) into test tubes, which were incubated for 7 days at 28°C. A spore suspension was obtained by adding the 10 ml of sterile nutrient broth over the fresh cultures and with the help of the microbiological loop the culture was scraped. The spore suspension thus obtained (at a concentration of 10^6) was used to inoculate on solid medium (Sabouraud with the addition of chloramphenicol). From each test tube, which contained the spore suspension, 1 ml was taken and inoculated by flooding on the surface of the solid medium in the plate. The uniform dispersion of the inoculum on the surface of the medium was then ensured by performing rotational movements with the plate slightly inclined. The plates were then left to stand for 15 minutes to ensure good adhesion of the spores to the surface of the medium, and then sterilely removed, with a pipette, the excess liquid from the surface of the medium. At the end, on the surface of the culture medium, from each plate, the discs with fungicide subtance were deposited, respecting the distance of 30 mm between them and 15 mm from the edge of the Petri dish.

After 10 - 15 minutes (pre-diffusion time), the Petri dishes were incubated at 28°C. A first reading of inhibition zone was made after 3-4 days, and the final interpretation after 7 days.

For the interpretation was taken into account the diameter of the inhibition zone, measured in mm with the ruler, in two - three directions, including the disk and the criteria specified by the discs manufacturer (HI Media Laboratories) that present the corresponding values in qualitative attributes: resistant strains, intermediate resistance or sensitive strains (Table 1).

Table 1

Criteria for assessing the action of tested antifungal substances (mm)

Interpretation	**Sensitive**	**Sensitive intermediary**	**Resistant**
Diameter of inhibition zone (mm)	≥ 20	10 -19,9	5-9

Results and discussions

The results regarding the testing of the sensitivity to certain antifungals of some strains of *Microsporum canis* isolated from dogs and cats colected from veterinary clinics from Timisoara are presented in Table 2.

Table 2

Efficacy of antifungal substances on some strains of *Microsporum canis*

No of samples	Diameter of inhibition zone (x± sdx - mm)				
	Miconazole	Ketoconazole	Clotrimazole	Amphotericin B	Nystatin
116	19.21±1.79	6.4±1.81	31.28±5.18	5.36±0.94	14.78±3.47

Overall, it was found that of all the five antifungal substances tested by the diffusimetric method, in terms of efficacy on isolated *M. canis* strains (n = 116), Clotrimazole showed outstanding activity. Compared to all strains tested (116 strains) this substance showed remarkable efficacy, with the diameter of the induced inhibition zones ranging from 21 mm to 40 mm, with an average of 31.28 ± 5.18 mm. A good antifungal action was found in both Miconazole and Nistatin.

Miconazole determined an inhibition area with an average of 19.21 ± 1.79 mm, with diameters ranging from 16 mm to 22 mm. Out of a total of 116 strains, 70 were sensitive to this substance (60.34%) and 46 strains were intermediate sensitive (39.65%).

Nystatin exibit an inhibition zone average of 14.78 ± 3.47 mm, but out of a total of 116 strains, only 30 (25,86) were sensitive to this substance. The other 86 (7413%) strains *of M. canis* proved to be intermediate sensitive.

However, analyzing in detail, the sensitivity of each strain was found that all 116 were sensitive to Clotrimazole, but the sensitivity to the other two antifungals (Miconazole and Nistatin) was different, in the sense that there were strains that were sensitive to Miconazole (70 strains), but intermediate sensitive to Nistatin (86 strains).

Compared to Ketoconazole and Amphotericin B all tested strains can be considered resistant. The diameter of the zones of inhibition induced by the two antifungals was non-existent (microcompet diameter = 5 mm) or extremely small, averaging 6.4±1.81 mm for Ketoconazole and 5.36±0.94for Amphotericin B.

From the literature, for all tested substances are known how to act and it is also known the group of fungi on which they act predominantly (5). Ergosterol is the predominant component of the fungal cell membrane and serves as a bioregulator of membrane fluidity and consequently of membrane integrity in fungal cells (17, 18).

Changes in the sterol or on the phospholipid composition of the fungal cell membrane may result into a decrease in azole uptake by the fungal cell. Similarly, reduced intracellular accumulation of the drug may occur due to increased active transport of the drug out of the cell. The antifungal action of azoles, polyenes, allylamine and thiocarbamates consist into inhibition of ergosterol synthesis or direct interaction with ergosterol (5, 6).

Miconazole is an imidazole that was introduced into therapy at about the same time as Clotrimazole. It is mainly used in local applications on dermatophyte-induced lesions in dogs and cats (lotion with Miconazole nitrate 1%) and in the treatment of otitis in carnivores (otic solutions with miconazole nitrate 23 mg / ml). Miconazole has fungistatic or fungicidal action at high concentrations and acts by inhibiting the biosynthesis of ergosterol, which is the essential component of the fungal cell membrane, thus causing a change in its permeability (11).

Ketoconazole is an imidazole compound recommended for the fungicidal and fungistatic effect on dermatophytes, yeasts and certain species of filamentous fungi. At oral administration of 400 mg of Ketoconazole, in dogs, absorption is very good due to the acidic environment in the stomach, but availability for absorbtion varies from 4 to 89%, and long-term treatments have been accompanied by clinical side effects due to toxicity (11). Ketoconazole has a relatively broad spectrum of action against dermatophytes, yeasts and some species of filamentous fungi, especially those of the genus Aspergillus (12). Ketoconazole is used in veterinary medicine in dogs, cats and other small species for local treatments or baths, but also in the therapy of primary mycoses. There are no specific veterinary products, but products for human use are recommended: Ketoconazole 200 mg tablets and Ketofungol - shampoo with 2% ketoconazole (11).

Clotrimazole is also a derivative of imidazole. It acts fungistatically and / or fungicidal against some species of dermatophytes, against yeasts and against dimorphic fungi (11). Clotrimazole acts by interacting with 14-α demethylase and blocking the conversion of lanosterol to ergosterol, the predominant component of

the fungal membrane. It is recommended in topical applications, Clotrimazole 1% cream, being commercially available only preparations for human use (11).

Amphotericin B is a polyenic macrolide antifungal, being very effective especially against yeasts of the genus Candida but also against some pathogenic fungi. It acts by irreversibly binding to ergosterol in the membrane of fungal cells forming membrane pores that allow the loss of ions (K^+, Ca^{2+} and PO43-). Due to its ability to bind to cholesterol present in the cell membranes of higher organisms, it has an important toxic potential (11).

Amphotericin B is usually indicated in humans in some severe systemic fungal infections, when treatment with other antifungal substances does not work, due to the installation of their resistance to the low spectrum of action. In veterinary medicine, Amphotericin B is used in dogs, but has also been used in other species such as cats, horses, llamas and birds. In animals with nephropathy it is contraindicated or used provided that renal function is monitored. There are no veterinary products based on Amphotericin B, but those for human use, injectable products with different concentrations, as well as products for topical applications can be used (11).

Nystatin is a polyene antifungal that has a mechanism of action similar to that of amphotericin B. In veterinary medicine, Nystatin is used in dogs, cats and birds for gastrointestinal fungal infections. As there is no conditioned products for veterinary use, for animal are prescribed products for human use, especially the product Nystatin-oral suspension with 100 000 units /ml (11).

It is known from the literature that, although *M. canis* infection has always been relatively simple to treat with antifungal agents, there are still, in human medicine, patients with recalcitrant infection, whose number is increasing (8, 10). Resistance to treatment with an antifungal substance is considered to be clinical expresed when in a clinical case there is a persistent infection or recurrences that reappear 4 weeks after stopping treatment, at a correct dosage of that antifungal drug (5).

There are various mechanisms by which microbial cells might develop resistance (5). Martinez-Rossi (13) described the antifungal resistance mechanisms in dermatophytes (13). He concludes that the main biochemical and molecular mechanisms that induce the antifungal resistance coud be the reduced uptake of the drug, an active transport out of the cell or modified drug metabolic degradation of the cell (13, 22).

Moreover, in the results published in the literature there is no uniformity in terms of testing the sensitivity to antifungal substances of the species *Microsporum canis*. In a recent study realised by Aneke et al. (3) for *M. canis*, high values of the minimum inhibitory concentration for Floconazole were found, using the method of micro-dilution in broth and a reduced inhibition of this antifungal in case of diffusion in agar method. Similar aspects, increasing the resistance to Floconazole of *Microsporum canis* strains have been previously reported by other authors (2, 4, 20).

High values of the minimum inhibitory concentration and low diameters of inhibition zones have been reported for Griseofulvin, using both the broth micro-dilution method and the agar diffusion method (20). Compared to this substance, values greater than 3 µg / mL (for the minimum inhibitory concentration) and a diameter of the inhibition area of less than 16 mm (25 pg / disc) were considered in *M. canis* strains from people indicate an installation of resistance. As these values are considered a limitation of the efficacy of therapy for *T. rubrum*, it is recommended that Griseofulvina not be the first choice for the treatment *of M. canis* infection in both humans and animals (3, 10).

Extensive research, conducted by Afshari et al. (1) on different strains of dermatophyte species, evaluated the antifungal sensitivity to commonly used antifungal drugs using standard fluconazole discs (6.0 mm Dia., MAST Diagnostics) (25 µg), itraconazole (10 µg), 30 mg terbinafine (30 µg), griseofulvin (25 µg), and ketoconazole (10 µg) on glucose (2%) and methylene blue supplemented Mueller Hinton agar 5 µg / ml). These studies showed that the sensitivity of isolated strains of the genus *Trichophyton,* to ketoconazole was 23 ± 0.35 mm, to itraconazole was 22 ± 0.25 mm, to griseofulvin was 16 ± 0.10 mm, and terbinafine was 14 ± 0.25 mm. In strains of the genus *Microsporum,* the mean sensitivity to ketoconazole was estimated at 22 ± 0.15 mm, for itraconazole it was 23 ± 0.15 mm, for griseofulvin it was 16 ± 0.20 mm, for terbinafine it was ± 0.12 mm, for the genus *Epidermophyton,* the mean sensitivity to ketoconazole was estimated to be 23 ± 0.35 mm, for itraconazole it was 24 ± 0.10 mm, for griseofulvin it was 16 ± 0.10 mm, and for terbinafine was 16 ± 0.23 mm. Comparative results of drug sensitivity of different dermatophyte species to the antifungal agents examined indicated that the highest sensitivity to ketoconazole was in *E. floccosum* and *T. mentagrophytes* species, the highest sensitivity to itraconazole was found in *T. rubrum* and *E floccosum*, the highest sensitivity to griseofulvin was found in *T. verrucosum*, and the highest sensitivity to terbinafine was revealed for *T. verrucosum* (1).

Overall, susceptibility to ketoconazole and itraconazole tested in this study was higher than in other types of antifungals, including griseofulvin and terbinafine. However, these results are different from those obtained in our study.

In vitro demonstration of resistance does not necessarily equate to in vivo resistance (6, 8). Other determinants in the selection of resistance include host-related factors, e.g. immunosuppression, the site and severity of infection and drug pharmacokinetics (12, 16).

Conclusions

Of the total of five substances tested for antifungal efficacy on some strains of Microsporum canis isolated from dogs, Clotrimazole showed remarkable activity, determining zones of inhibition well above the sensitivity limit given by the manufacturer for the microdiscs used

Miconazole and Nystatin acted with lower efficacy, given smaler inhibition areas smaller than Clotrimazole, from intermediate to sensitive.

Of the total strains tested, all presented sensitivity to Clotrimazole, but sensitivity to the other two antifungals, was different, being observed strains sensitive to Miconazole but intermediate to Nystatin.

In Ketoconazole and Amphotericin B, all tested strains were considered resistant, the area of inhibition being non-existent or very small.

Sensitivity tests for antifungal substances for dermatophytes should become common in veterinary and human practice both to ensure the effectiveness of treatment and to prevent the onset of resistance.

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EVALUATION OF ENDOPARASITISM IN FALLOW DEER (*DAMA DAMA L.*) FROM BIHOR COUNTY (ROMANIA) HUNTING GROUNDS

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Summary

The fallow deer (*Dama dama L*.) is a notable representative of the *Cervidae* family in the Romanian fauna, particularly of the genus *Dama,* being an allogeneic species reintroduced into the country's fauna during antiquity by the Romans. The species do not live alone, at the habitat or ecosystem level they form a union, a conglomerate, together with other species between which various relationships or interactions are established. This interaction between two or more species takes various forms: competition, commensalism, mutualism, predation, or parasitism. From this point of view, the fallow deer (*Dama dama L.)* is no exception. In this context, the purpose of the study was to identify the possible presence of endoparasites in fallow deer from Bihor County using classical coprological methods, macroscopic and microscopic examination of the gastrointestinal mass and organs and evaluation of the impact of the results on the host. We identified, morulated strongilid eggs (gastrointestinal nematodes), *Nematodirus* spp. eggs and *Dicrocelium lanceolatum* eggs. These results disclose the importance of the presence of parasitic endofauna with obvious repercussions on the health status of the parasitized host and, equally, with implications in its ecological relationships.

Keywords: fallow deer, endoparasites, Bihor County.

The relatively recent history of the European fallow deer from the Early Holocene period (starting 11700 years ago), after the end of the last great glaciation (Würm), is still not fully revealed and provokes numerous discussions and approaches in the scientific and research environment regarding spread and distribution of this species on the European continent. During the last glaciation, fallow deer disappeared from the European area due to severe climatic conditions, taking refuge in favorable areas in Asia Minor, the southern Balkan peninsula or southern Sicily from where anthropochore dissipation with the help of the Hittites and Phoenicians and later the Gauls, Romans (who called them "cervi palmati") and Normans repopulated southern and central Europe, including the British Isles (1, 4, 17).

Currently, the status of fallow deer as an autochthonous faunal element is not yet fully recognized, some specialists continue to include it in the category of

potentially invasive allochthonous species, although it is a certainty its existence on the territory of Romania since the Neolithic period in fossil deposits found at Liubcova-Ornița whose age is dated back to the fourth millennium BC (2).

Despite its troubled past, the fallow deer was not a highly studied species in Romania, and the purpose of our research is precisely the desire for in-depth knowledge of the biology and peculiarities of this species, especially in terms of parasitic fauna analysis.

Materials and methods

The study was carried out over a period of one year, on 28 fallow deer, males, and females, from three hunting grounds in Bihor County (Fig. 1). The samples collected from these studies were represented by feces, organs (lung, liver, and nervous system) of the hunted animals, and gastrointestinal tracts.

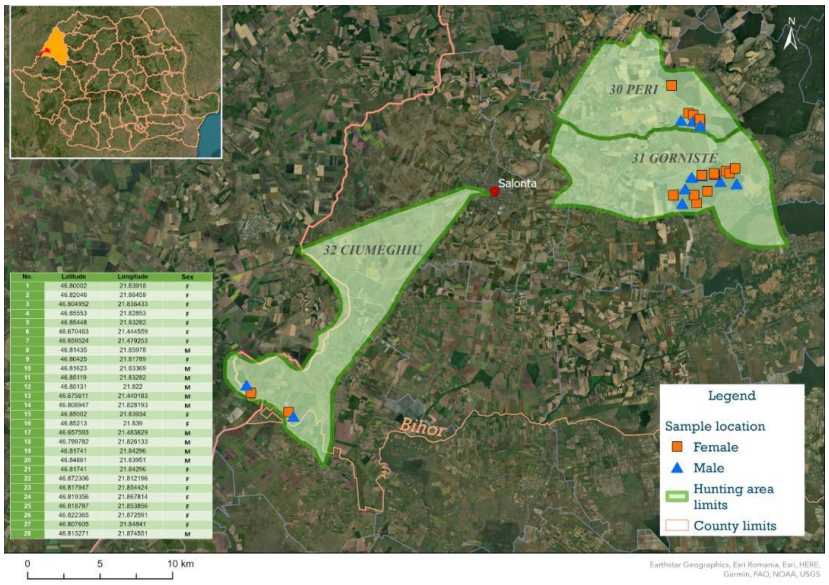
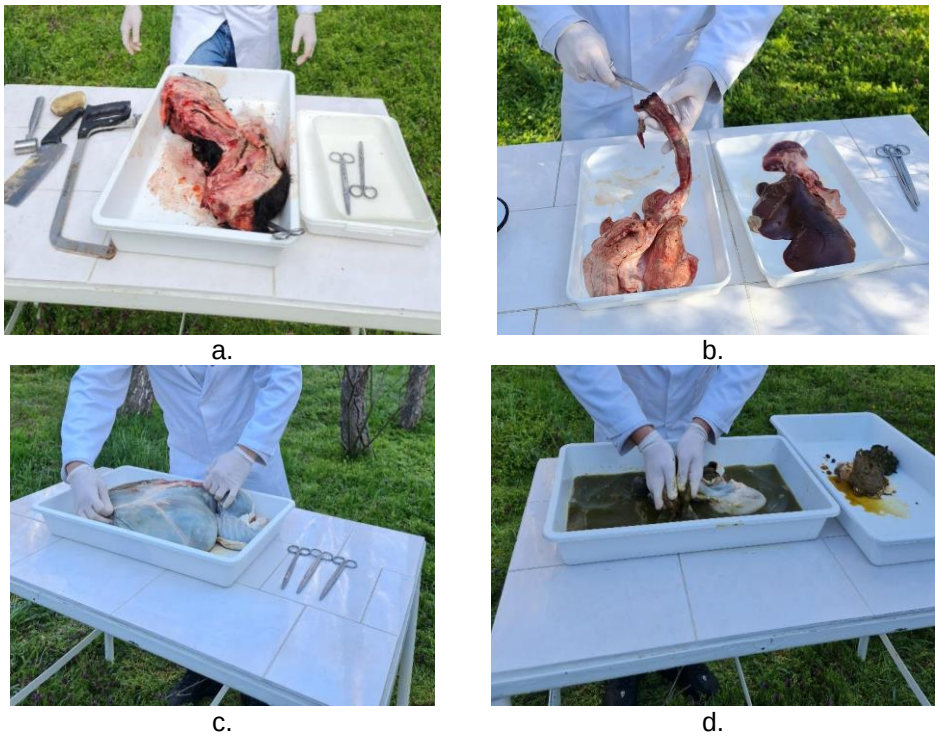


Fig. 1. Map of Bihor County

The samples have been processed in the Parasitology and Parasitic Diseases Clinic of the Faculty of Veterinary Medicine Timisoara by the following methods:

- Qualitative method - identification of the parasitic load of the whole digestive tract with light eggs of nematodes, cestodes, protozoan oocysts
- Polyvalent method (of successive washes) - identification of the presence of trematode eggs

- Larvoscopic method - highlighting parasitism with pulmonary nematodes
- Necropsy examination - according to the technical instructions of necropsy (Fig. 2. a, b, c, d) (9).



a. b. c. d.

Fig. 2. a, b, c, d. Necropsy examination

Results and discussions

The results of the coprological examination performed by the flotation method revealed the presence of the following parasitic elements: morulated eggs (gastrointestinal nematodes, *Nematodirus* spp.) in 18 out of 28 samples examined (64.29%) (Fig. 3., Fig. 4).

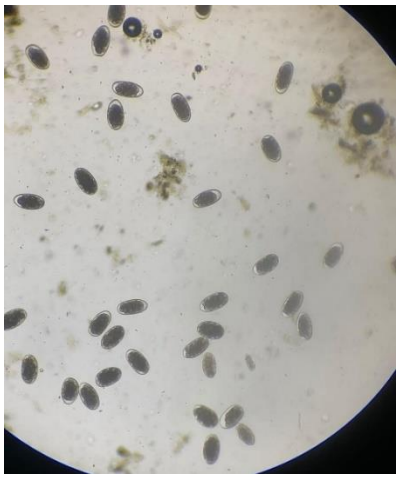


Fig. 3. Nematodes eggs

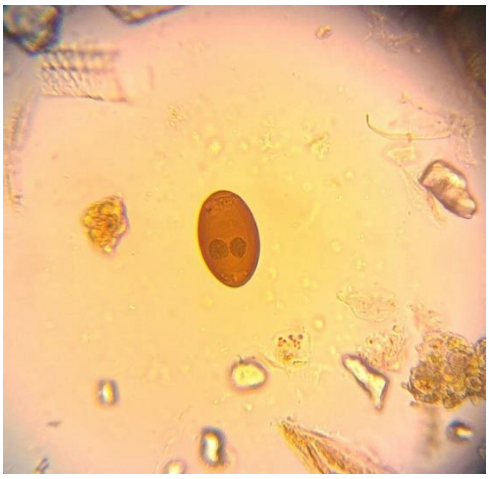


Fig. 4. *Dicrocelium* spp. egg

The results of the coprological examination performed by the sedimentation method revealed the presence of parasitic elements belonging to the genus *Dicrocelium*.

Pulmonary nematode larvae were not evident in the Baermann/Vajda examinations.

The results of the necropsy examination of the gastrointestinal mass revealed the presence of different genres of gastrointestinal nematodes (GIN) in 18 out of 28 samples examined (64.29 %) with the following localization: in abomasum – *Haemonchus*, in the small intestine – *Nematodirus,* and in the large intestine – *Oesophagostomum* (Fig. 5).

Only the trematode *Dicrocelium lanceolatum* was present in the liver of the examined fallow deer (Fig. 6).

The identification of digestive endoparasites in a considerable percentage reveals their impact on the health of the fallow deer but also warns about the possible risk of infestation of the environment and implicitly that of other possible host animals (5, 7, 8, 10, 15).

Survival strategies of parasites and the impact of endo-parasitism on wildlife are subjects of several studies (3, 6, 11, 12, 13, 14, 16, 19).

Popovici et al. (2022) examined fallow deer dropping collected from Arad County hunting grounds by coprological methods and were identify the presence of gastrointestinal nematodes eggs (66.7 %), *Gongylonema* spp. eggs (17%), cestode oncospheres (3.3%), and the presence of *Paramphistomum* spp. eggs (10%) (18).

Fig. 5. Gastrointestinal nematod

Fig. 6. *Dicrocelium lanceolatum* - adults

A study conducted in the south of Northrhine-Westfalia, Germany on a herd of 64 fallow deer reveals a prevalence of 19 species of gastrointestinal nematodes and one lungworm species. The prevalence of nematodes in the abomasum, small intestine, and large intestine was 93.8 %, 57.8 %, and 87.5 %, respectively (20).

Conclusions

We identified the presence of gastrointestinal nematodes (64.29 %), and the trematode *Dicrocelium lanceolatum* (10.7 %) in fallow deer samples collected from Bihor County hunting grounds and examined by coprological methods and necropsy.

The increased prevalence of endoparasitism in fallow deer with the risk of infestation of the domestic ruminants indicates the existence of an epidemiological context favorable to the development of parasitic elements in Bihor County. This is a warning for domestic animal breeders, with possible repercussions on herd health and decreased production.

Acknowledgments

This scientific paper was carried out in the Laboratory of Parasitology and Parasitic Diseases at the Faculty of Veterinary Medicine Timisoara, Laboratory within the Animal Hygiene and Pathology Research Center**/** ULS Timisoara.

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STUDY REGARDING THE AGE INFLUENCE ON SERUM PHOSPHORUS LEVEL IN FELINE CHRONIC KIDNEY DISEASE

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Summary

Chronic kidney disease (CKD) is the progressive loss of kidney function and its prevalence has been estimated to be between 1% and 3% in felines. However, its occurrence increases with age, especially in the geriatric feline population (about 80%) CKD is a prevalent clinical condition characterized by elevated phosphorus (P) retention and protein catabolites in felines, also a common cause of morbidity and mortality in felines. The purpose of this study is to evaluate from biochemical point of view (the level of serum phosphorus, urea and creatinine), patients of different ages who present symptoms that lead to the diagnosis of chronic kidney disease and to investigate the possible correlation between feline aged 10 years or aging over 10 years. The study was performed within the University Emergency Hospital Prof. univ. Dr. Alin Bîrțoiu, Bucharest on a number of 18 felines with chronic kidney disease, 10 of these, over 10 years old, being considered eloquent for our study. The clinical examination was performed on each patient separately, by general methods, as well as by paraclinical methods, such as: biochemical and hematological examination, urine sediment examination, symmetric dimethylarginine (SDMA), ultrasound examination. From the total number of patients included in this study (n=10), one feline, an 11-year-old patient in uremic coma (n=1) presented the highest value of serum phosphorus and the others 9 patients (n=9), between the ages of 11 and 15, presented serum phosphorus values above 7.35 mg/dl. From the total of10 patients included into the study (n=10), 8 were male (80%) and only two female (20%), all patients belonged to European race (100%).

Keywords: serum phosphorus, chronic kidney disease, geriatric patients, felines.

Chronic kidney disease (CKD) refers to the gradual loss of the kidney function over time. The kidney function's impairment can result in a wide variety of pathologies in feline (1, 3, 5). Healthy kidneys perform a number of essential physiological functions, including blood filtration and urine production (4,6). Chronic kidney disease represents a common diagnosis in geriatric feline patients. According to the specialty literature, the prevalence of chronic kidney disease increases with 30% to 40% in patients over 10 years old (8, 10, 12).

A severe kidney injury, a severe infection (including feline infectious peritonitis, feline immunodeficiency virus, and pyelonephritis), the ingestion of a toxic substance (such as antifreeze or lilies), or certain medications could represent the main causes of the disease in some felines (2, 7, 11).

The specialty literature also mentions that polycystic kidney disease (a specific type of renal kidney disease) and amyloidosis, a rare organ disease seen in breeds such as the Persian and Abyssinian, can be inherited in some cases (13, 15).

CKD may also be caused by underlying immune-mediated diseases, stroke-like events, clotting disorders, and even lymphoma (17).

Hyperphosphatemia is an electrolytic disorder characterized by abnormally elevated blood levels of phosphate. It can occur at any age, but, according to specialty literature, it has a higher prevalence in felines with various kidney pathologies (18, 19).

The phosphate retention is a major factor in the progression of chronic kidney disease in many species and it is well known that hyperphosphatemia is associated with a substantial risk of mortality in patients with terminal-stage of renal disease (9, 14).

Serum phosphorus concentration is dependent on the dietary phosphorus intake, gastrointestinal absorption across the duodenum and jejunum, translocation into intracellular sites and its urinary excretion (16, 20).

In addition to the specific symptoms of chronic kidney disease in cats, it is also possible to observe the specific symptoms of hyperphosphatemia, such as: nausea or vomiting, weakness, “rubber jaw” (the excess of phosphorus may cause tooth loss and soft jaw bones), excessive thirst, muscle tremors (18).

Materials and methods

This study was conducted over a two-year period (2020-2022) at the University Emergency Hospital Prof. Univ. Dr. Alin Birțoiu in Bucharest and at the Vietatis – The Vets Clinic, Bucharest, on a total number of 20 feline patients suspected of chronic kidney disease. A group of ten patients, older than 10 years of age, were selected for this research.

Each patient was examined by clinical and paraclinical methods, using both general and specialized techniques, such as biochemical investigations (for dosing the creatinine and phosphorus blood levels). For the biochemical examination were used Skyla, IDEXX and Spotchem SP devices.

Results and discussions

The physiological interval for serum phosphorus is between 2.7 and 6.5 mg/dL in this species. The advanced age and the correlation with an inadequate diet (low-quality protein and an elevated level of phosphorus), phosphorus levels increase, resulting in hyperphosphatemia.

In the first stage of chronic kidney disease, this parameter is not significantly increased, but as chronic kidney disease progresses (up to stage 3 or even the final stage - stage 4), serum phosphorus can reach 19 mg/dL, as it was the case of a 10-year-old patient who presented to the clinic in uremic coma.

First step of our study consisted in establishing the CKD stage, in each of the ten patients, based on their creatinine level. Therefore, considering the fact that, according to speciality literature (4, 9), the first stage of chronic kidney disease is

characterized by creatinine levels bellow 1.6 mg/dL, while the second stage is characterized by creatinine levels of 1.6 to 2.8 mg/dL, none of our studied patients fit in these two stages, as they presented at the clinic with higher creatinine levels.

The third stage of CKD implies creatinine values between 2.9 and 5 mg/dL, therefore, according to the data presented in Table 1 and the graph (Fig. 1), 3 of our patients belonged to this category, while the other seven patients presented forth stage CKD, with creatinine levels exceeding the value of 5 mg/dL.

Table 1

The CKD staging based on the creatinine means levels for each studied patient

PATIENT'S NO.	PATIENT'S AGE AND RACE		CREATININE LEVEL *(mg/dL)*	CREATININE AVERAGE VALUES/AGE GROUP	CKD STAGE
1	10 y.o.	European	19.01	19.01	**IV**
2	11 y.o.	European	4.26	4.26	**III**
3	12 y.o.	European	8.34	6.92	**IV**
4		Scottish Fold	5.5		**IV**
5	13 y.o.	European	10.2	10.2	**IV**
6	15 y.o.	British Short Hair	3.45	4.76	**III**
7		British Short Hair	3.64		**III**
8		European	7.2		**IV**
9	18 y.o.	European	8.8	8.05	**IV**
10		European	7.3		**IV**

The average creatinine values for the 12-years-old patients was 6.92 mg/dL, being all classified in the fourth stage of chronic kidney disease.

For patients aged 15 years old, the average of creatinine values was 4.76 mg/dL, 2 of them being classified in the third stage of chronic kidney disease (creatinine < 5.5 mg/dL).

For the last age group, 18 years old, the average creatinine values was 8.05 mg/dL, being classified, therefore, in the fourth stage of chronic kidney disease.

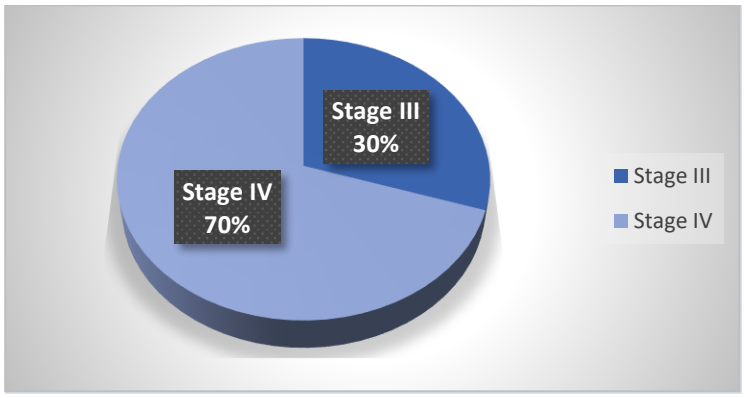

Fig. 1. The CKD stage, based on the mean creatinine values, for the studied group

After classifying the patients based on the CKD stage, the second, and main part of our study consisted in determining the serum phosphorus level of each patient and correlating it with the CKD stage of the patients, but also with the age (Table 2), as we previously stated, the age being an important factor in the occurrence of phosphorus imbalances in the body.

Table 2

The average serum phosphorus level of each studied patient, correlated with age and CKD stage

No.	Patient's age	CKD stage	Phosphorus level *(mg/dL)*	Phosphorus average values/age group
1	10 y.o.	IV	27.21	**27.21**
2	11 y.o.	III	8.2	**8.2**
3	12 y.o.	IV	15.2	**11.5**
4		IV	7.8	
5	13 y.o.	IV	18	**18**
6	15 y.o.	III	7.35	**7.53**
7		III	6.76	
8		IV	8.5	
9	18 y.o.	IV	10.7	**9.7**
10		IV	8.7	

Based on the data presented in Table 2, all of the studied patients presented hyperphosphataemia.

The highest values of the phosphorus level 27.21, 18 and 15.2 mg/dL were observed in patients with IVth stage CKD, while the 3 patients with IIIrd level CKD presented the lowest phosphorus values, all bellow 8 mg/dL: 8.2, 7.35 and, respectively, 6.76 mg/dL.

As concerning the correlation between the phosphorus level and age, the average phosphorus value for the 12-years-old patients was 11.5 mg/dL, for patients aged 15, the average of phosphorus value was 7.53 mg/dL and for the last age group, 18 years old, the average phosphorus value was 9.7 mg/dL.

We also did a comparative analysis between the phosphorus level and the creatinine level for the 3 age groups (12, 15 and 18 years old) – Fig. 2.

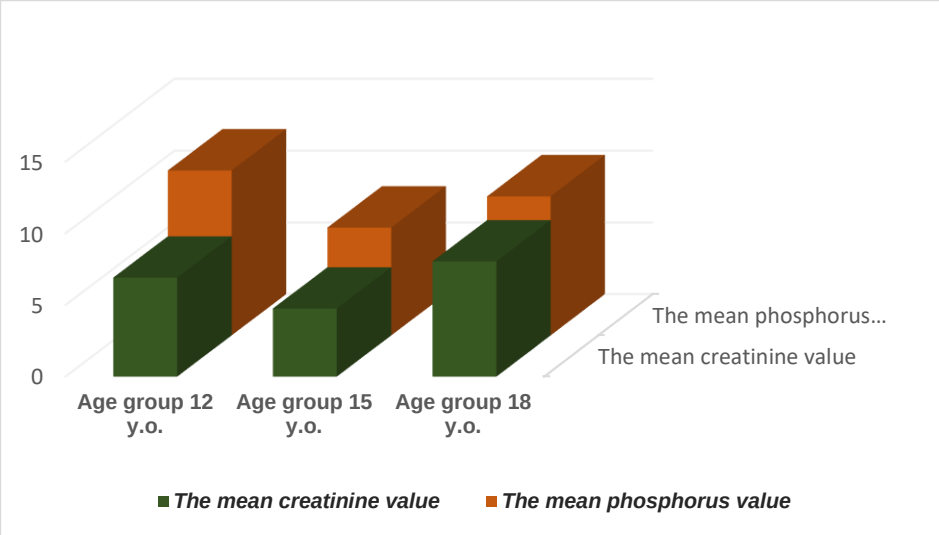

Fig. 2. The average values of creatinine and phosphorus for each age group

As presented in the graph in Fig. 2, the most obvious positive correlation between the creatinine level and the phosphorus level is found in age group 18 years old.

Conclusions

The advanced age, especially over 10 years represents a very important factor in the occurrence of chronic kidney disease in felines and in its progression.

The phosphorus values are increased in all studied patients, all presenting hyperphosphataemia due, most likely, to the advanced age.

The phosphorus mean levels in the studied patients are directly and tightly correlated with the stage of the chronic kidney disease, as the patients in the fourth stage of CKD presented severe hyperphosphatemia, while patients in the third stage of CKD presented lower phosphorus levels.

Acknowledgement

The present research was carried out as part of an extensive study, the preliminary results being part of the PhD Thesis: *"Study on correlations between serum phosphorus level and different stages of feline chronic kidney disease".*

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MORPHOLOGICAL PARTICULARITIES OF THE SKULL IN THE SOUTH AMERICAN SEA LION (*OTARIA FLAVESCENS S. OTARIA BYRONIA*) – CASE STUDY

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Summary

The South American sea lion - *Otaria flavescens* or *Otaria byronia*, is part of the Family *Otariidae*, Order *Carnivora*, Genus *Otaria*. The international scientific community accepts both variants of the scientific name. Considering that the skull's morphology is important in the classification and differentiation of marine mammals, this study aimed to describe the morphological particularities of the skull of the South American sea lion, an exotic species for Europe found only in zoos and dolphinariums. Data in the scientific literature regarding the skull morphology of this species are limited. The study was conducted on one skull belonging to a female South American sea lion from the collection of the Anatomy discipline at the Faculty of Veterinary Medicine of Bucharest. Among the most important aspects observed, we can list: the presence of a high, thin, and sharp rectilinear external sagittal crest that, in the aboral part of the frontal bone, divides into two unequal temporal lines; the absence of the supraorbital foramen; the absence of the lacrimal bone; the presence of a long nasopharyngeal canal; the presence of the hypoglossal canal that crosses the lateral portion of the occipital (with the entrance on the internal face of the condyle and the exit located on the ventral face of the basioccipital); the angular process of the mandible with a blade-like appearance and orientated medio-aborally.

Keywords: South American sea lion, morphology, skull.

The South American sea lion - *Otaria flavescens s. Otaria byronia* is part of the Family *Otariidae*, Order *Carnivora*, Genus *Otaria*. The international scientific community accepts both variants of the scientific names. However, in 2012 Berta and Churchill concluded that *O. byronia* is "the oldest available name with certainty applicable", also endorsed by the Society for Marine Mammalogy (Committee on Taxonomy 2014). However, South American scientists use the name *Otaria flavescens* (17).

In the specialized literature, from Romania, regarding the morphological particularities of the skull in carnivores, skulls of wild cats (jaguar, cheetah, puma, tiger, bear, wolf, fox, jackal) were described (1, 7, 8, 10, 11, 12). In contrast, the skulls of aquatic mammals in the Family *Carnivora* have yet to be studied in detail.

The anatomical studies in specialized literature carried out on the skulls of this species were focused on the changes produced during its phylogenetic evolution; for example, changes in the dentition, the orientation and arrangement of bones and bone formations (positioning of the maxillary tuberosity, length of the tympanic bone, individualization of the cochlear canal). Comparative morphometric studies were also carried out between different species of pinnipeds (2, 3, 5), as well as studies on the ontogeny and sexual dimorphism of the auditory region in *O. byronia*, in which it was observed that the average shape of the tympanic bulla in this species has a subtriangular outline, with variations between sexes and different ages (9).

We can also cite some studies on the morphological particularities of the skull in seals, both within the same family (13) and in relation to other carnivores, observing significant differences at the level of the palatine processes of the maxilla, the nasal processes, the zygomatic process of the frontal, tympanic bulla, condyloid process and lacrimal bone (4, 6, 14, 15).

Materials and methods

This study used a skull of an adult female South American sea lion, *Otaria flavescens s. Otaria byronia*, from the Anatomy discipline's collection. The morphological characteristics of the skull bones were described, and the most exciting aspects were photographed. The description and identification followed Nomina Anatomica Veterinaria (N.A.V.) 2017.

Results and discussions

The sea lion's skull appears elongated, with the viscerocranium more developed than the neurocranium.

The dorsal face of the neurocranium is crossed in the median plane by a rectilinear, high external sagittal crest with a thin and sharp edge. In the aboral extremity of the frontal, the external sagittal crest divides into two unequal temporal lines. The temporal lines are well highlighted in the aboral extremity, gradually fading towards the zygomatic process of the frontal.

The external occipital protuberance is lower in height than the nuchal crests and appears chamfered. The nuchal crests, arranged obliquely dorsolateral, have the appearance of blades with a sharp dorsal edge.

The zygomatic process of the frontal, located latero-aboro-ventrally, ends with a spine-like extension, directed aboro-ventral, and does not present the supraorbital foramen. Between the rostral portion of the parietals and the aboral part of the frontals, the area narrows, and the aboral extremity of the frontals has a convex external surface in a dorsoventral sense. The rostral extremity of the frontal is bifid. Between the two extensions, the aboral extremity of the nasal penetrates deeply to form the frontonasal suture (Fig. 1). The orbit is high, with the upper edge rounded and deeply excavated. This margin is like a wide incision delimited by the

zygomatic process of the frontal and the preorbital apophysis. The preorbital apophysis has the appearance of a rough ridge, wider at the base and narrower in the dorsal plane (Fig. 1).

The nasal bones are narrow and pointed at the aboral extremity, while their rostral extremity is widened and divided into two portions by a small incision.

The entrance to the nasal cavities is broad, allowing the rostral extremities of the simple dorsal nasoturbinate and the branched ventral maxilloturbinate.

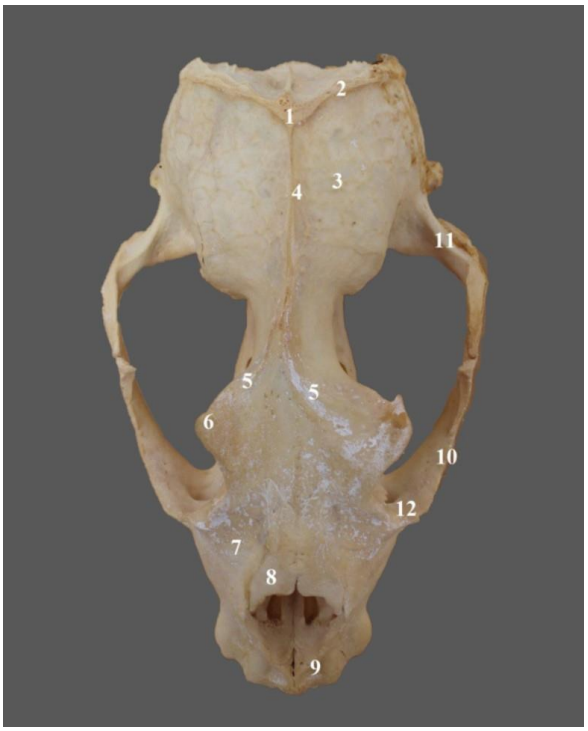

Fig. 1. South American sea lion skull (*Otaria flavescens s. Otaria byronia*) – dorsal face

1.External occipital protuberance; 2. Nuchal crest; 3. Parietal; 4. External sagittal crest; 5. Temporal lines; 6. Zygomatic process of the frontal; 7. Maxilla; 8. Nasal; 9. Incisive; 10. Zygomatic; 11. The zygomatic process of the temporal; 12.Preorbital apophysis.

The incisive has a narrow nasal process. Above the first incisors, at the suture site between the two incisive bones, is a nipple-like tuberosity.

The vomer, located inside the viscerocranium, has a wide and deep groove in which the ventral edge of the nasal septum is fixed (Fig. 2). The ridge of the vomer is fixed to the floor of the nasal cavity only in the rostral third. In the middle and aboral

thirds, there is a vast communication space between the two nasal cavities, the width of the space increases in the oroaboral direction. The lacrimal bone is absent.

The lateral face of the skull has the greatest width at the level of the aboral extremity.

The parietal bones are convex from one side to the other, and the zygomatic processes detach from the lateral face of the temporal bone in a lateral-rostral direction. This zygomatic process has a relatively triangular appearance in the aboro-lateral portion, and the lateral-dorsal-rostral portion is elongated but flattened latero-medially. The aboral face of the zygomatic process presents a prominent spine on the ventral edge, directed ventromedial.

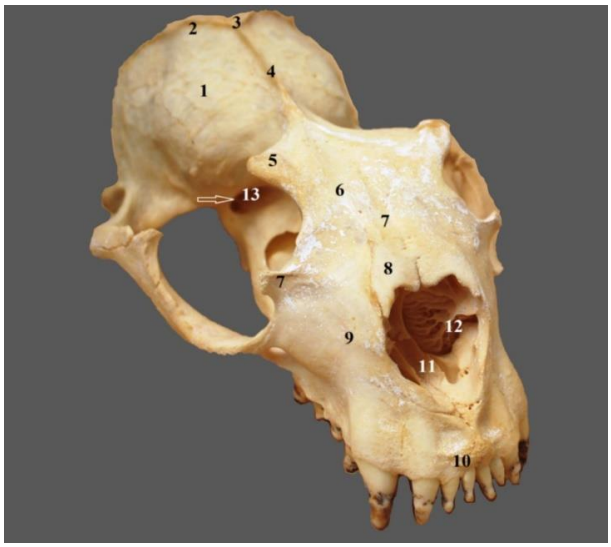

Fig. 2. South American sea lion skull (*Otaria flavescens s. Otaria byronia*) – dorsolateral face

1. Parietal; 2. Nuchal crest; 3. External occipital protuberance; 4. External sagittal crest; 5. Zygomatic process of the frontal; 6. Frontal; 7. Nasal processes of the frontal; 7'. Preorbital apophysis; 8. Nasal; 9. Maxilla; 10. Incisive tuberosity; 11. Vomer; 12. Ventral nasal horn; 13. Anterior alar foramen.

The zygomatic process of the temporal shows a well-defined temporal line on its dorsal surface.

The zygomatic bone ends bifid aborally and presents a reduced dorsal process, called the postorbital process, and a ventral, elongated latero-ventro-aboral process, called the temporal process (Fig. 3 and 4).

The infraorbital canal can be observed at the base of the zygomatic bone, very short, delimited rostrally by the infraorbital foramen and aborally by the maxillary

foramen. The posterior palatine foramen is halfway up the upper border of the perpendicular process of the palatine. From this level, the palatine canals start in the rostral direction and open on the ventral side of the palatine processes of the maxilla through the anterior palatine foramen. The palatine grooves start dorsally from the anterior palatal foramen, well represented in their initial portion.

A wide incision can be observed between the preorbital apophysis and the upper edge of the zygomatic bone.

The maxilla is short but broad, showing an obvious tuberosity at the aboral extremity. An elongated and deep fossa is present ventral to the tuberosity. A small tubercle can be observed at the base of the frontal process of the maxilla.

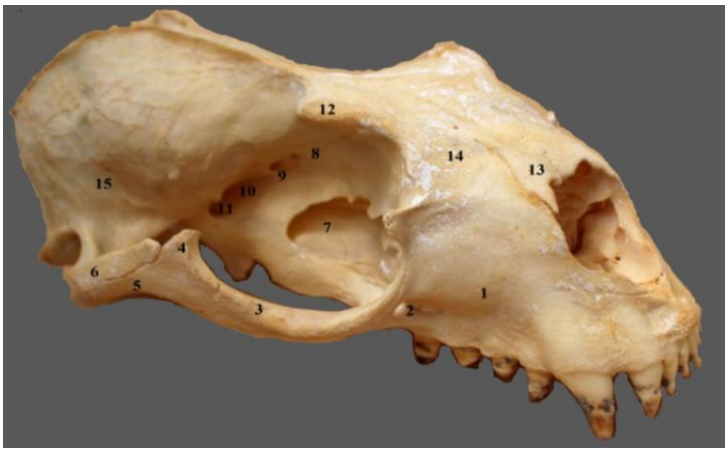

Fig. 3. South American sea lion skull (*Otaria flavescens s. Otaria byronia*) – laterodorsal face

1. Maxilla; 2. Infraorbital foramen; 3. Zygomatic; 4. The dorsal (postorbital) process of the zygomatic; 5. Ventral (temporal) process of the zygomatic; 6. The zygomatic process of the temporal; 7. Foramen of the medial face of the orbit; 8. Ethmoid foramen; 9. Optical foramen; 10. Orbitorotudum foramen; 11. Anterior alar foramen; 12. Zygomatic process of the frontal; 13. Nasal; 14. Frontal process of the maxilla; 15. Temporal.

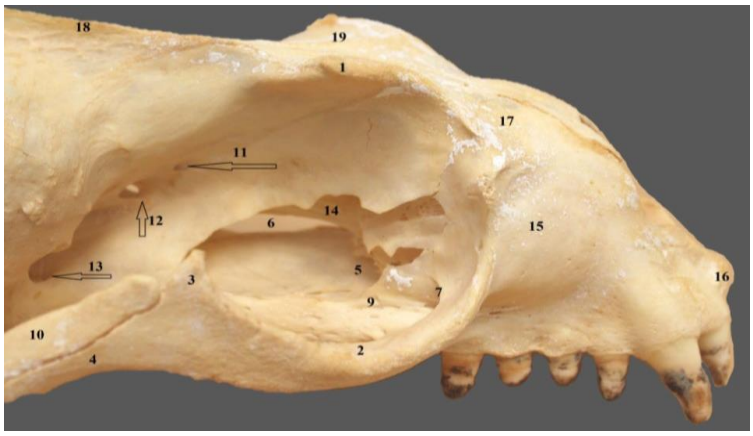

Fig. 4. South American sea lion skull (*Otaria flavescens s. Otaria byronia*) – lateral face (rostral extremity of the skull)

1. Zygomatic process of the frontal; 2. Zygomatic; 3. The dorsal (postorbital) process of the zygomatic; 4. Ventral (temporal) process of the zygomatic; 5. Foramen of the medial face of the orbit; 6. Nasopharyngeal canal; 7. Maxillary foarmen; 9. Anterior palatine foramen; 10. The zygomatic process of the temporal; 11. Ethmoid foramen; 12. Optical foramen; 13. Anterior alar foramen; 14. Ridge of the vomer; 15. Tuberosity of the maxilla; 16. Incisive tuberosity; 17. The preorbital process; 18. External sagittal crest; 19. Frontal.

In the orbital hiatus open: the ethmoid foramen, the optic canal, the orbitorotundum foramen, and the anterior alar foramen (Fig. 5). The anterior alar foramen communicates through a long alar canal with the posterior alar foramen. The temporal fossa is deep and elongated.

In the aboral extremity, on the sides of the viscerocranium, is a vast space, delimited by the frontal, sphenoid, palatine, and maxillary bones, that allows communication with the nasal cavities. This space constitutes the foramen of the medial face of the orbit.

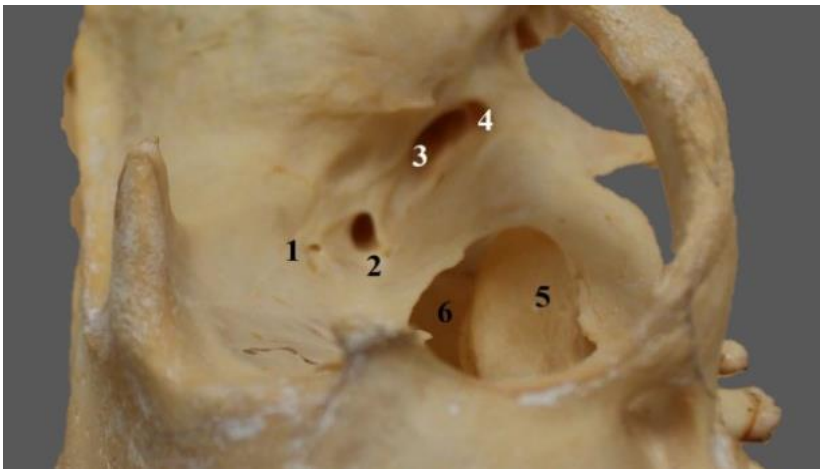

Fig. 5. South American sea lion skull (*Otaria flavescens s. Otaria byronia*) – lateral face (orbital hiatus)
1. Ethmoid foramen; 2. Optical foramen; 3. Orbitorotundum foramen; 4. Anterior alar foramen; 5. Foramen of the medial face of the orbit; 6. Nasopharyngeal canal.

The nasal conchae are not well defined in the posterior extremity, the two nasal cavities having a joint opening with the pharynx through the nasopharyngeal canal, wide and located in the aboral half of the floor of the nasal cavities (Fig. 6).

Behind the base of the zygomatic process of the temporal, the external acoustic meatus can be observed, reduced with the appearance of an oval-shaped foramen.

A tympanic notch can be seen above the external acoustic meatus and an prominent mastoid process behind it.

The ventral face of the skull presents biconvex occipital condyles at the aboral extremity.

The basioccipital is short and wide, with a rectangular appearance. On the aboral part is the opening of the hypoglossal nerve canal, represented by the hypoglossal foramen. In the middle part of the basioccipital, there is an obvious pharyngeal tubercle, which continues to the rostral edge of the basioccipital with a thin, sharp, and high ridge. On each side of the ridge, two wide pharyngeal fossae are evident, arranged obliquely, and flanked rostrally by a prominent muscular tubercle (Fig. 7 and 8).

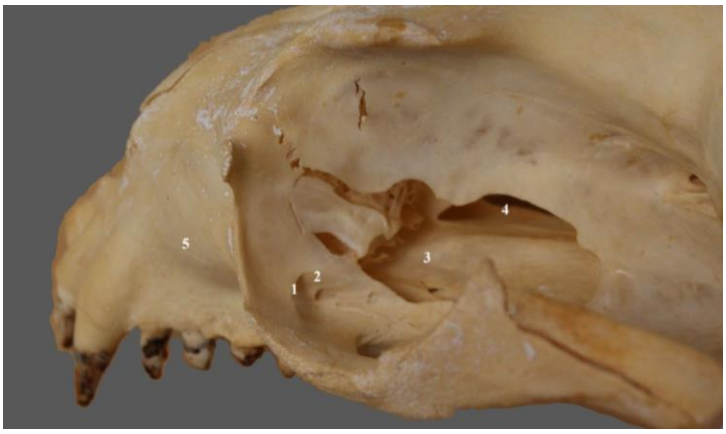

Fig. 6. South American sea lion skull (*Otaria flavescens s. Otaria byronia*) – lateral face (rostral extremity of the skull)
1. Maxillary foramen; 2. Anterior palatine foramen; 3. The palatine process of the maxilla; 4. Nasopharyngeal canal; 5. Tuberosity of the maxilla.

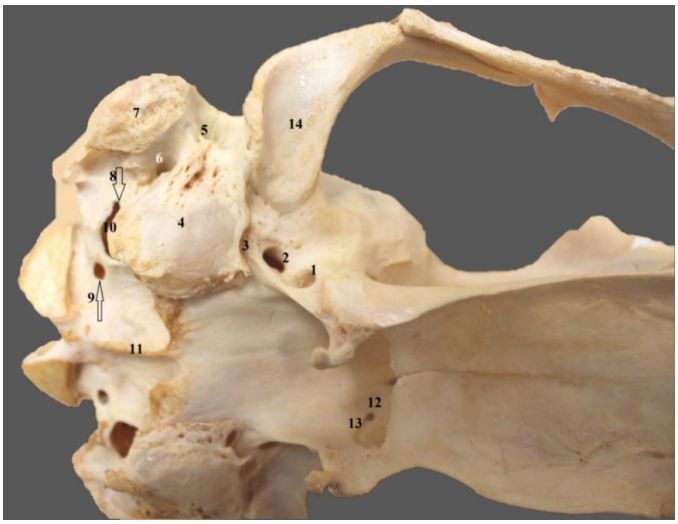

Fig. 7. South American sea lion skull (*Otaria flavescens s. Otaria byronia*) – ventral face
1. Aboral alar foramen; 2. Oval foramen; 3. Spinous foramen; 4. Tympanic bulla; 5. External acoustic meatus; 6. Stilomastoid foramen; 7. Mastoid process; 8,10. Jugular foramen; 9. Hypoglossal foramen; 11. Pharyngeal tubercle; 12. Vascular foramen; 13. Pterygoid grooves.

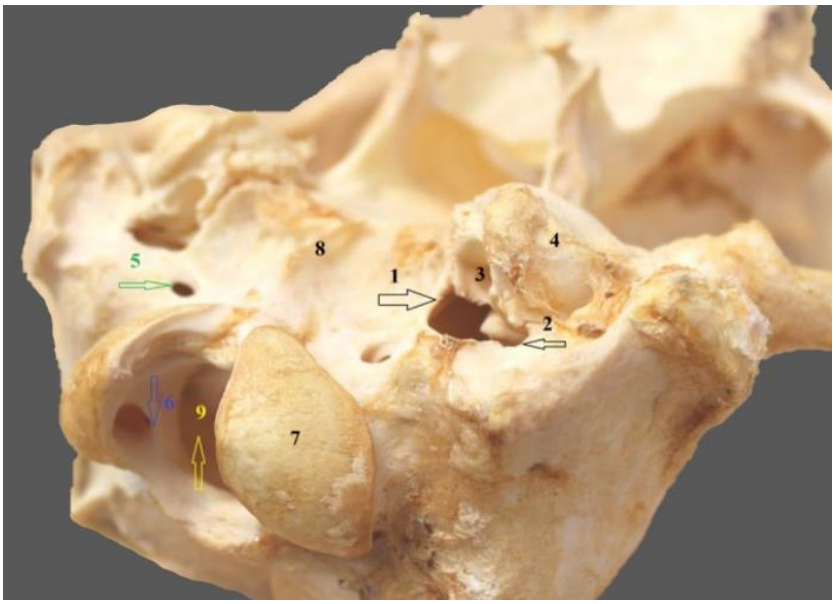

Fig. 8. South American sea lion skull (*Otaria flavescens s. Otaria byronia*) – ventro-aboral face

1. Jugular foramen; 2. The incisions on the aboral edge of the basisphenoid; 3. Posterior carotid foramen; 4. Tympanic bulla; 5. Hypoglossal foramen; 6. The entrance foramen in the canal of the hypoglossal nerve; 7. Occipital condyles; 8. Pharyngeal tubercle; 9. Foramen magnum.

At the base of the mastoid process is the stilomastoid foramen.

The tympanic bullae are evident, elongated, and widened medially. The posterior carotid foramen can be seen on the medial and aboral sides of the tympanic bulla. The posterior carotid foramen communicates rostrally, through a long carotid canal, with the anterior carotid foramen located in the rostro-latero-ventral extremity of the tympanic bulla.

A thin bony plate separates the anterior carotid foramen from the spinous foramen, which is located medially from the first one. The oval foramen is present on the ventral side of the sphenoid, rostro-lateral to the anterior carotid foramen (Fig. 9).

The paracondylar processes are very small, and at their base is the jugular forame.

The muscular process is minimal. It looks like a spine, and dorsal to it is the musculotubal canal, short and narrow.

On the ventral side of the zygomatic process of the temporal is the articulation surface for the mandible, represented by a strongly excavated and

transversely elongated articular cavity and, behind it, a reduced retro-articular process. Aboral to this process, a small retroarticular foramen is observed.

The pterygopalatine ridges are apparent. The hamulus of the pterygoid is developed and directed aborally (Fig. 10).

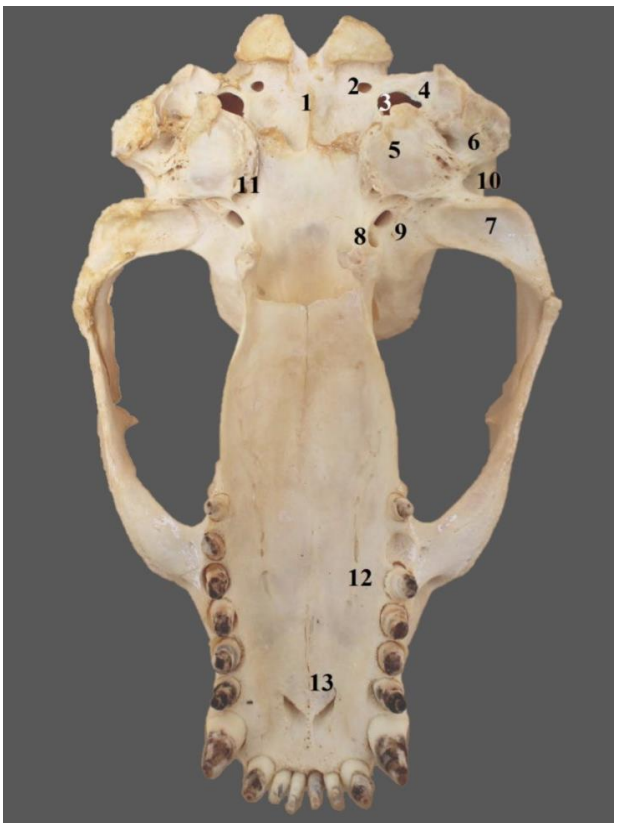

Fig. 9. South American sea lion skull (*Otaria flavescens s. Otaria byronia*) – ventral face

1. Pharyngeal tubercle; 2. Hypoglossal foramen; 3. Jugular foramen; 4. Paracondylar process; 5. Tympanic bulla; 6. Stilomastoid foramen; 7. Joint cavity; 8. Posterior alar foramen; 9. Oval foramen; 10. External acoustic meatus; 11. Anterior carotid foramen; 12. Palatine grooves; 13. Palatal fissure.

The horizontal blade of the palatine is very long, representing the aboral third of the palatine vault. The hard palate is the widest in its middle third. Palatal fissures can be observed at the relatively triangular rostral extremity of the hard palate, with the base arranged rostrally and the apex aborally.

The palatal processes of the incisive bones are arranged between the palatal processes of the maxilla and are quite short.

The orbital portion of the palatines is high.

The nuchal face of the skull has a triangular aspect, slightly oblique, with the base located ventrally and the apex dorsal.

The occipital protuberance is reduced, and the nuchal crests are high in their dorsal third.

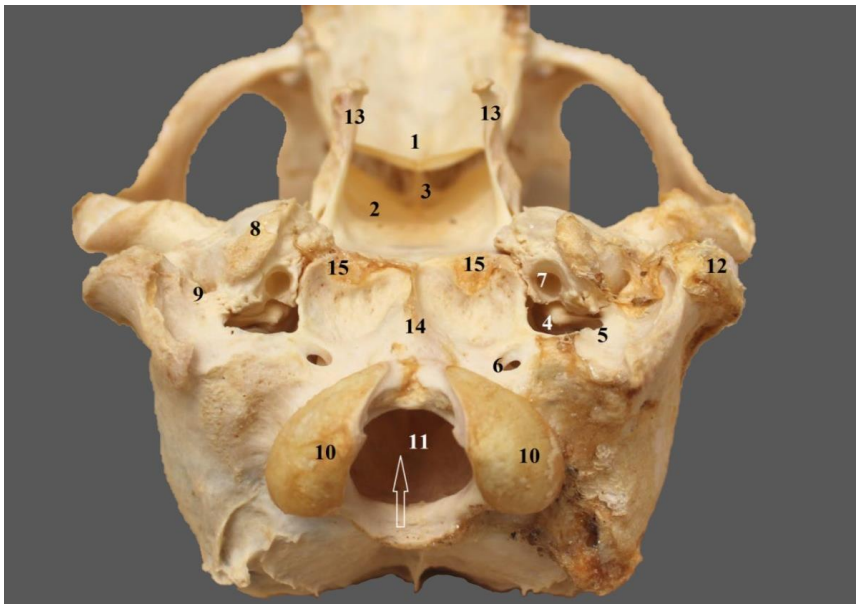

Fig. 10. South American sea lion skull (*Otaria flavescens s. Otaria byronia*) – ventral face

1. The horizontal processes of the palatine; 2. Wing of the presphenoid; 3. Vomer; 4. Jugular foramen; 5. Paracondylian processes; 6. Hypoglossal foramen; 7. Aboral carotid foramen; 8. Tympanic bulla; 9. Stilomastoid foramen; 10. Occipital condyles; 11. Foramen magnum; 12. Mastoid process; 13. Pterygoid hooks; 14. Pharyngeal tubercle; 15. Sphenobasioccipital tubercles.

On the nuchal face, the external occipital crest descends from the level of the external occipital protuberance towards the occipital foramen. This crest is extremely developed in the middle third, reduced in the upper third, and absent in the lower third. Numerous rugosities of muscular insertion exist on both sides of the external occipital crest.

The occipital condyles, biconvex, located at the ventral extremity of the nuchal face, are positioned slightly obliquely latero-medially, delimiting a wide,

circular occipital foramen, foramen magnum. The endocranial opening of the hypoglossal nerve canal can be observed on the condyles' inner face. Above each condyle is an elongated and slightly oblique dorsal condylar fossa (Fig. 11). The paracondylar processes are significantly reduced.

The mandible is a paired bone. The horizontal portion of the mandible has a concave-convex ventral edge in the oro-aboral direction. It ends with an elongated aboral tuberosity arranged at the level of the recurved angle of the mandible.

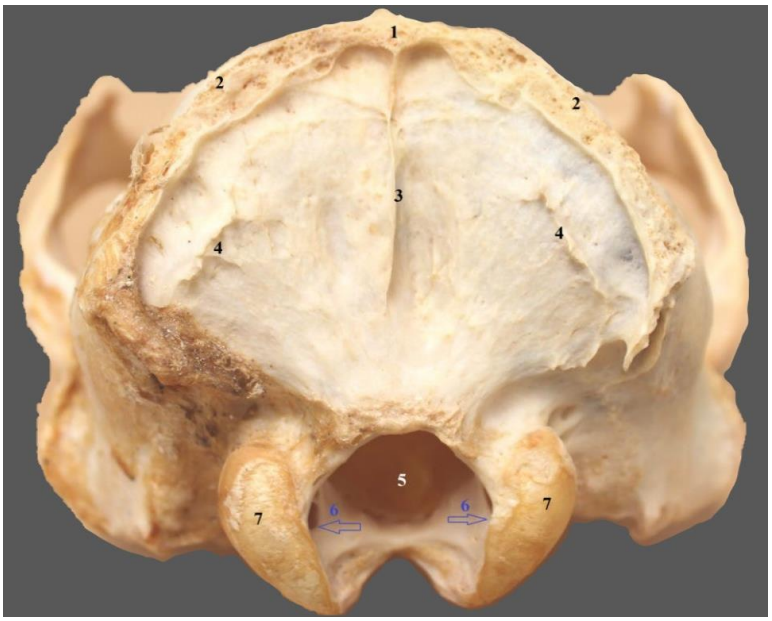

Fig. 11. South American sea lion skull (*Otaria flavescens, s. Otaria byronia*) – nuchal face

1. External occipital protuberance; 2. Nuchal crests; 3. External occipital crest; 4. Rugosities of muscle insertion; 5. Foramen magnum; 6. The entrance foramen in the canal of the hypoglossal nerve; 7. Occipital condyles.

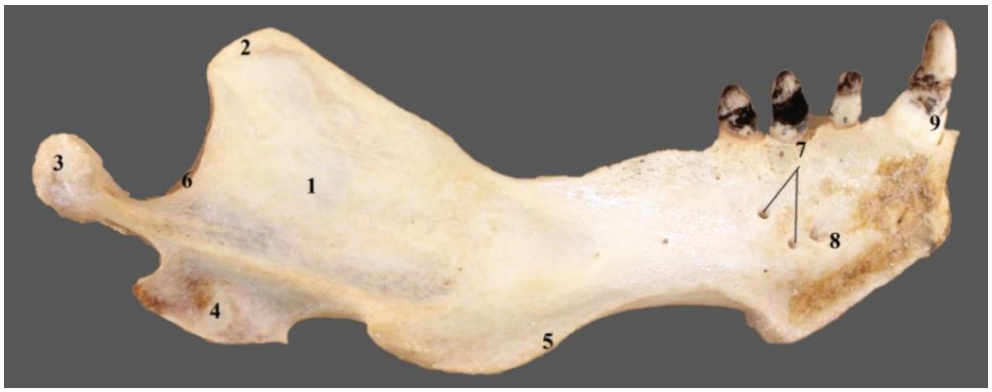

Fig. 12. South American sea lion mandible (*Otaria flavescens, s. Otaria byronia*) – lateral face

1. Masseterine fossa; 2. The coronoid process; 3. The condylar process; 4. Angular process; 5. The ventral edge of the horizontal branch of the mandible; 6. Corono-condylian incision; 7. Accessory mental foramina; 8. Mental foramen; 9. Mental foramen of the rostral extremity of the mandible.

The vertical branch of the mandible is oblique, the coronoid process is drawn far aborally, and its rostral edge is oblique, increasing in height oro-aborally, looking like a thin and sharp blade.

The angular process detaches from the aboral extremity of the mandible's caudal edge of the recurved branch, with the appearance of a medio-aboral drawn blade located at a distance from the mandibular angle. (Fig. 12)

The condylar process appears like a slightly excavated blade on the dorsal side towards the lateral side and is drawn in an aboral direction. The articulation surface is slightly convex dorso-ventrally and concave oro-aborally.

The coronoid process has a rounded upper edge and is drawn aborally.

The masseterine fossa is excavated and extends to the level of the coronoid process. The smaller pterygoid fossa is excavated in the middle and distal third. At the base of the pterygoid fossa is the mandibular foramen representing the mandibular canal entrance.

On the rostral face of the body of each mandible, there is a rostral mental foramen. On the lateral side of the body of the mandible, under the first premolar, there is the aboral mental foramen, and behind it are two accessory mental foramen.

Conclusions

The external sagittal ridge is high, rectilinear, and in the aboral part of the frontal, it divides into two unequal temporal lines. The zygomatic process of the frontal, ends with a spine-like aboro-ventral extension and does not present the supraorbital foramen.

The rostral extremity of the frontal is bifid, and the medial part represents the nasal process of the frontal. The orbit, in the rostral plane, presents a preorbital apophysis with the appearance of a rough ridge, wider at the base and narrow in the dorsal plane.

The entrance to the nasal cavities is wide, and the dorsal and ventral nasal turbinates are visible. The lacrimal bone is missing.

At the aboral extremity, the zygomatic bone ends bifid, with a reduced dorsal process, the postorbital process, and an elongated latero-ventro-aboral ventral process, the temporal process.

The ethmoid foramen, the optic canal, the orbitorotundum foramen and the anterior alar foramen open in the orbital hiatus. The anterior alar foramen communicates through a long alar canal with the posterior alar foramen.

On the sides of the viscerocranium, there is a wide space delimited by the frontal, sphenoid, palatine, and maxillary bones, allowing communication with the nasal cavities and forming the foramen of the medial face of the orbit.

The pharyngeal tubercle continues to the rostral edge of the basioccipital with a thin, sharp, and high ridge.

An external occipital ridge, highly developed in the middle third, can be observed on the nuchal face. Above each condyle is an elongated and slightly oblique dorsal condylar fossa.

An elongated aboral tuberosity is present at the level of the recurved angle of the mandible. The angular process has a blade-like appearance, drawn medio-aborally. A rostral mental foramen is at the extremity of each mandible's body.

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USE OF CONTRAST ENHANCED CT IN FELINE AORTIC THROMBOEMBOLISM: A CASE REPORT OF A 2-YEAR-OLD BRITISH SHORTHAIR CAT

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Summary

Feline aortic thromboembolism (FATE) is a syndrome that occurs secondary to embolization of thrombi of cardiac origin with any possible localization, although the aortic bifurcation is the most commonly affected site. The most common causes that lead to FATE are hypertrophic cardiomyopathy (HCM) and unclassified cardiomyopathy (UCM); dilated cardiomyopathy (DCM) rarely leads to this syndrome. Certain cat breeds - the British shorthair included - are at risk for developing FATE due to predisposition to these cardiac conditions. Presentation is usually acute with some of the most common complaints being sudden collapse, hemiparesis/paresis and vocalizing due to severe pain. Coexisting cardiac conditions are frequently present. Diagnostic tools vary, although findings on physical examination and history are usually suggestive; imaging studies may be useful in some cases. The patient presented here had signs specific for a saddle thrombus. Severe HCM and dilation of the left atrium were diagnosed using echocardiography. CECT (contrast enhanced computed tomography) was useful in localizing the thrombus, due to its uncommon localization. Taking into account the severity of this condition, high probability of recurrence, prolonged and partial recovery, owners elected euthanasia.

Keywords: FATE, British shorthair, echocardiography, contrast enhanced CT.

FATE is clinical syndrome caused by embolization of cardiac thrombi, with aortoiliac bifurcation being the most common localization, eventhough embolization can occur in any part of the vascular system, with the result being ischemic necrosis of the affected organs/tissues. HCM is one of the most common causes leading to thromboembolism (9, 17**)**. Hyperthyroidism, unidentifiable underlying pathologies combined as well as conditions leading to hypercoagulable states (eg neoplasia, nephropaties) (17).

Certain cat breeds may be predisposed, such as Maine Coons, Ragdol, British shorthairs, Persians, Burmese, Siamese, and well as nonpedigree cats etc (1). American shorthair and Norwegian were also reported (17)

In one study, males were slightly over-represented (1).

A history of heart disease may also be known, as well as wether the patient is hyperthiroid (1).

Clinical signs typically are suggestive: acute onset, with cold and pale extremities, absent peripheral pulse, neurological signs typical of lower motor neuron disease combined with neuromuscular pain (1); tetraparesis has also been reported

(2).

Imaging studies may be of help in diagnosing this condition, although clinical signs are usually sufficient (17).

Differential diagnosis should be made against other pathologies leading to acute limb dysfunction, spinal and limb trauma, intervertebral disc disease etc (17).

Elevated muscle enzymes, hyperkalemia and hyperglycemia may be identified (17).

Ultrasonography – color-flow Doppler – may help in identifying the thrombus or lack of blood flow. Contrast angiography can reveal occlusion of blood vessels, but is not recommended. Cardiac disease, as well as intracardiac thrombi (usually located in the left atrium), may be confirmed by use of echocardiography (17).

Postmortem examination may reveal the site of the thrombi in some cases if performed immediately; cardiac disease, atrial thrombosis and ischemic muscles can also be identified (17).

Patients should be hospitalized and discharged when pain and the other clinical signs have been resolved (17).

Pain control, control of hyperkalemia, oxygen therapy and congestive heart failure (CHF) therapy should be implemented. Fluid therapy should be used with caution. Thrombolytic therapy may lead to high mortality due to reperfusion injuries (17).

Surgical embolectomy may be considered in cases with acute onset of bilateral aortic thromboembolism (15).

Materials and methods

The patient was examined in the Faculty of Veterinary Medicine from Timişoara, in several clinics.

Initially, the patient – a 2-year-old indoor male British shorthair cat - was seen in the Surgery Clinic. Following examination, a radiographic study was recommended to rule out vertebral and limb trauma.

For the radiographic study, a Siemens Multix Swing was used, with the patient non-sedated (Fig. 1).

Given the clinical signs and a high suspicion of FATE, a cardiac consult was also recommended and was performed in the Cardiology Unit / Internal Disease Clinic, where a Contec 300 GA electrocardiograph unit (with thermic printer and 12 derivations) and a model X Vision My Lab 70 Vet stationary ultrasound (sectorial, microliniary and liniary probes, with multiple frequencies and cardiac consult module) were used.

Furthermore, a CECT using a Siemens Somatom Definition AS 64 slice CT scanner (Fig. 2), was also performed, with the patient anesthetised using a combination of medetomidine (20-40 μg/kg) and propofol (0.5 – 1.5 mg/kg). Recovery was obtained with atipamezol (at 5 times the dose of medetomidine).

Fig. 1. Siemens Multix Swing (original)

Fig. 2. Siemens Somatom Definition AS 64 (original)

Multiplanar reconstruction (MPR) at 0.6 mm slice thickness and 3D volume rendering technique (VRT) were used. Ultravist 370 (Iopromide, Bayer), at a dose of 1 ml/kg was the contrast medium of choice.

Results and discussions

The median age of patients at the time of presentation is 12-years-old (range 1–21 years) (17); this particular patient was 2-years-old, with no known cardiac disease.

The clinical signs with which the patient presented consisted of severe

tachypnea (over 70 breaths per minute), dyspnea, open-mouth breathing (Fig. 3), paraplegia and cyanotic foot pads of hind limbs (Fig. 4).

Fig. 3. Open-mouth breathing, tachypnea and dyspnea (original)

Fig. 4. Hind limb with cyanotic foot pads (right) and front limb (left) for comparison (original)

In one study, pale paws were more common than cyanosed paws (86.7% versus 13.3%) (6).

The rectal temperature in this patient was lower than 32˚C. Non-surviving cats have a lower mean rectal temperature as compared to that of survivors (1).

The radiographical study revealed severely enlarged cardiac silhouette, pulmonary edema (alveolar pulmonary pattern) and dilated esophagus and stomach due to open-mouth breathing and aeropahgia (Fig. 5).

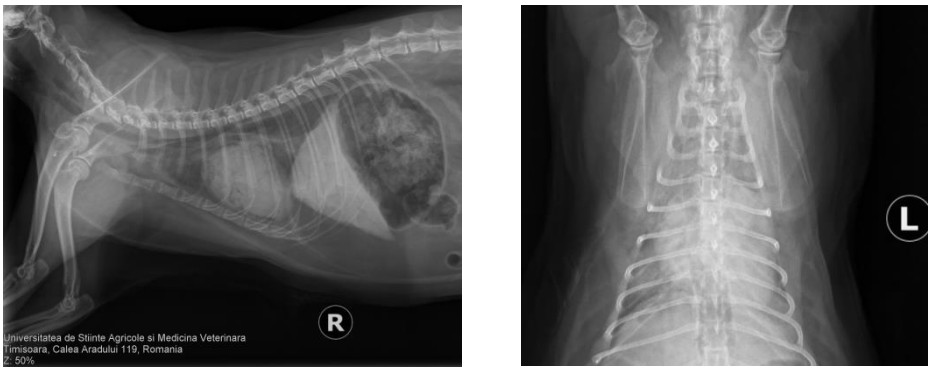

Fig. 5. Right lateral view (left) and dorsoventral view (right) of the thoracic cavity and cranial abdomen revealing an enlarged cardiac silhouette, pulmonary edema and signs of aerophagia (original).

The cardiac silhouette may not always appear enlarged on radiographs, especially when the cardiac hypertrophy is concentric (9).

In this case, the cardiac silhouette is seen to occupy 3.5 intercostal spaces, with the normal range in healthy cats being 2 – 2.5 intercostal spaces (**7**).

The mixed interstitial-alveolar pulmonary pattern displayed here could be caused by pulmonary thromboembolism, although this is less common (14). Most likely, in our patient, the pulmonary pattern was most likely caused by pulmonary edema.

The vertebral heart score (VHS) is an objective measuring system that uses the long and short axes of the heart on lateral x-rays, added up, and then scaled against the thoracic segment of the vertebral column (11). The normal range for the VHS in cats is reported as ranging from 6.7 – 8.1 (18), while other authors report a normal normal value of 7.5 ± 0.3v (11). A value of the VHS greater than 8.2v is indicative of cardiomegaly (9).

In our patient, the VHS was 8.9v indicating severe cardiomegaly (Fig. 6).

No signs of spinal injury or trauma were noted on the radiographs taken.

Electrocardiography – using simple bipolar derivations I, II, II and unipolar derivations aVR, aVL and aVF – revealed the following: a heart rate of 120 beats per minute (BPM), synus rythm, left axial deviation, P= 0.04s/0.2 mV, PR= 0.08 s, QRS= 0.03s/0.6 mV and ST=- 0.3 mV (Fig. 7).

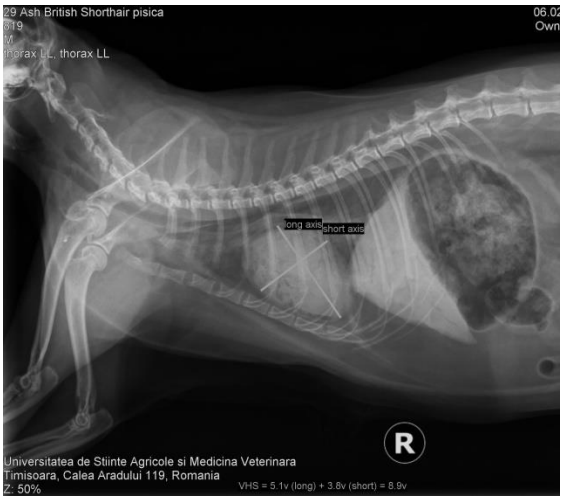

Fig. 6. Right lateral view of the thoracic cavity with the VHS 8.9v (original)

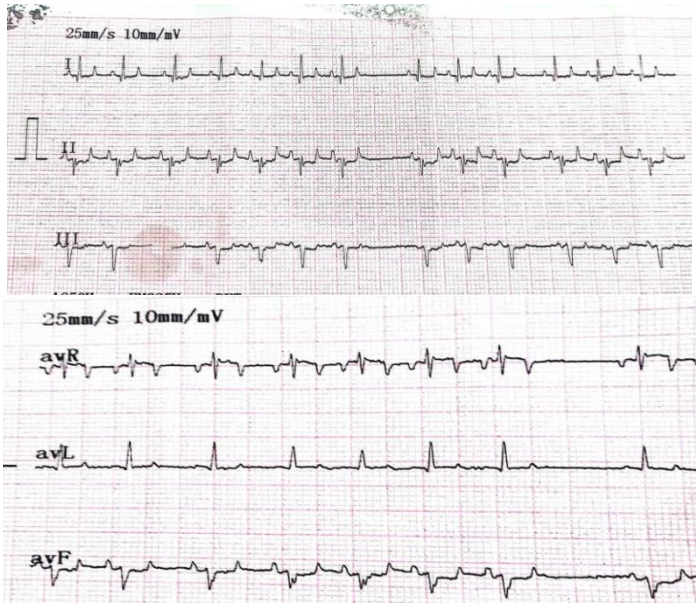

Fig. 7. Electrocardiogram showing hypertrophic cardiomyopathy (original)

Taken into account the values of the electrocardiographic parameters recorded, we can affirm the existence of synus arithmia, due to sinoatrial block and

left bundle branch block, by a negative QRS complex in D3 and aVF derivations, and widening of these in aVF unipolar derivation.

Also, subleveling of the ST segment – subendocardic ischemia – is due to intermediary adaptive cardiac hypertrophy, which is seen in heart failure. This is in conjunction with the cardiorespiratory signs the patient was displaying.

Prolonged QRS interval, 3rd degree atrio-ventricular block are indicative of HCM in cats (8).

From an echocardiografic stand point – B module – a severe cardiomegaly of the left atrium is seen, with the AS/Ao ratio being greater than 1,6 cm (Fig. 8).

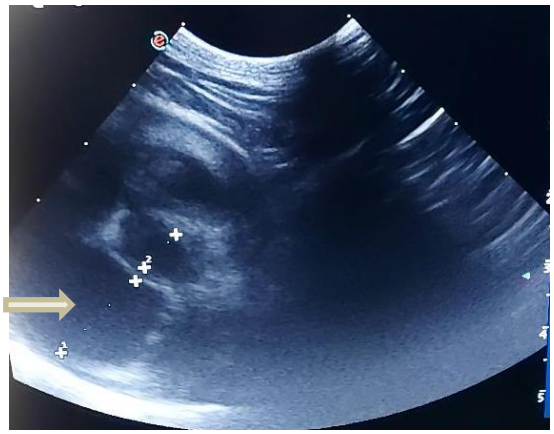

Fig. 8. B module echocardiography at the level of the left atrium (LA) and aorta (Ao) (original)

In one study, HCM was recorded in 30% of cats with FATE (6).

B+M module echocardiography revealed a thickening of the free wall of left ventricle and of the interventricular septum, greatly reducing the left ventricular cavity, which leads to an increased right intra-atrial pressure and with this an enlargement of the right atrium, increasing the risk for a thromboembolism (Fig. 9).

Ultrasound of the abdomen, targeting the abdominal aorta, performed using a high frequency liniar probe, revealed an uneven lumen, with hypo- and hyperechogenic areas, this leading to a suspicion of a thrombotic area, in conjuncture with the clinical signs (Fig. 10).

CECT - MPR was useful in determining the exact location of the thrombotic area, by observing exactly where the contrast medium filled abdominal aorta was ocluded by an elongated, irregular, intraluminal mass (Fig. 11).

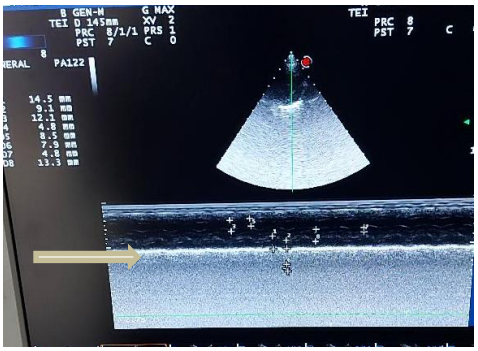

Fig. 9. B+M module echocardiography at the level of the left atrium (LA) and interventriculat septum (IVS) (original)

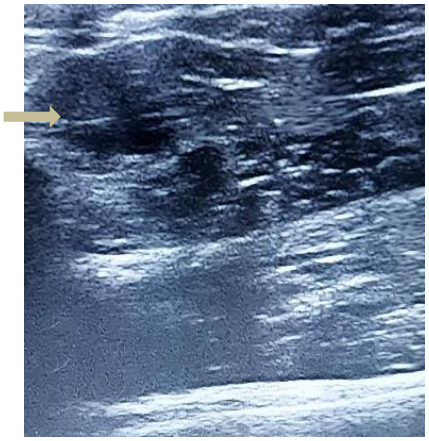

Fig. 10. Module B echocardiography at the level of the abdominal aorta

This is an abnormal localization for an aortic thrombus, since most thrombi are located at the level of aortoiliac bifurcation (17).

VRT is also helpful in creating a three-dimensional view of the patient's vasculature revealing exactly where the thrombus was located and may help with planning surgery (Fig. 12).

Fig. 11. Sagital view of the abdomen. Ventral to the 3rd lumbar vertebra, the contrast medium filled abominal aorta has an abrupt interruption due to an elongated intraluminal mass (small arrows), continuing from the level of the 5th lumbar vertebra with a very narrow lumen, revealing a decreased bloodflow caudaly (original)

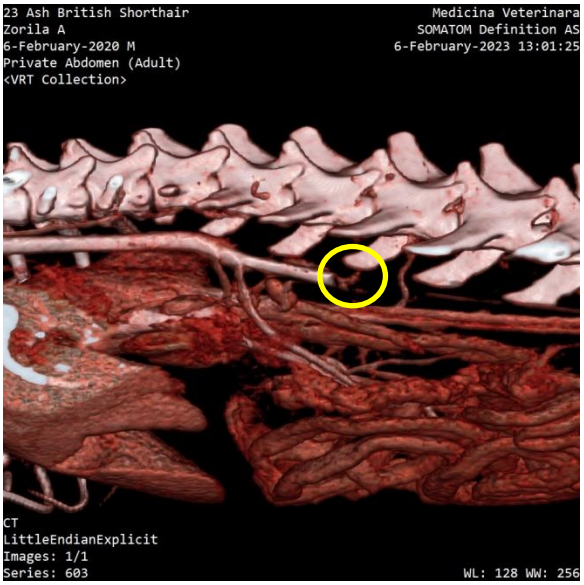

Fig. 12. VRT CECT revealing the abrupt stop in the contrast flow in the abdominal aorta (yellow circle) (original)

The vertebral column and spinal cord were also free of any anomalies.

CT is also useful when evaluating patients for pulmonary thromboembolism, being the modality of choice (4).

Magnetic Resonance Imaging is also of use and may reveal presence of large vessel thrombosis, including the abdominal aorta (16).

Management of arterial thromboembolism include pain control (5), rheolytic thrombectomy (3), aspirin (5), clopidogrel (5); CHF if present requires treatment with furosemide (5).

Long-term therapy should take into consideration the underlying conditions (eg CHF, HCM) (17).

Reccurence of FATE is common, with following episodes proving to be fatal (1). Long-term mortality with feline arterial thromboembolism is mainly due to the underlying severe cardiac pathologies (12).

In one study regarding feline arterial thromboembolism, significant differences were found between survivors and non-survivors regarding body temperature, heart rate blood levels of phosphorus, number of affected limbs and motor function (13).

Prophylactic therapy using aspirin can be considered in patients surviving the initial episode, although in one study 17% of cats had an episode of re-embolisation (10).

Euthanasia performed at presentation is common (1).

Conclusions

FATE is an acute condition, often caused by severe underlying cardiac disease, with euthanasia being commonly pursued.

In predisposed cat breeds, screening for conditions leading to FATE could be pursued for early detection.

If surgery is planned, CECT may be used to identify the exact location of the thrombus.

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STUDY REGARDING THE PHARMACOLOGICAL AND DIETARY TREATMENT IN CANINE MALABSORPTION SYNDROM

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Summary

Canine malabsorption syndrome includes a group of chronic enteropathies characterized by intestinal absorption deficiency due to mucosal damage and increase of intraluminal osmotic pressure, which can leads to chronic diarrhea. Numerous chronic enteropathies associated with the malabsorption syndrome include small intestinal inflammatory diseases and exocrine pancreatic insufficiency. The aim of this study is to establish a conclusive diagnosis of malabsorption syndrome, based on symptomatology and hematological, biochemical exams and then to follow the response to a combined dietary/immunosuppressive pharmacological treatment. The study was performed over a period of two years, on a number of 18 dogs with malabsorption syndrome (12 males and 6 females of various breeds, ranging from 2-6 years of age). All patients present chronic diarrhea (characterized by pale-colored stools with steatorrhea) which persisted for more than 4 weeks. In addition, all the dogs suffered weight loss, despite of the polyphagia reported by the owners. Hematological and biochemical exams (urea and creatinine, hepatic ALT-performance, total serum protein, albumin levels and albumin/globulin ratio) were performed. Hematological parameters of all individuals were within normal, physiological limits. The 18 dogs with malabsorption syndrome were divided into two groups, based on the presence or absence of hypoproteinemia (serum protein levels below 4.5 g/dL). Group 1 is represented by 10 dogs in which the proteinemia was within normal limits and all the individuals from this group received a treatment based on Prednisone and group 2, consists of 8 dogs with hypoproteinemia, which received a treatment based on metronidazole and prednisone. The doses were gradually reduced and the intervals at which they were administered were adjusted. Canine malabsorption syndrome can be treated by means of a combination of dietary therapy (low-fat, low-fiber, highly digestible diet) and pharmacological treatment with immunosuppressive agents.

Keywords: malabsorption, dietary therapy, pharmacological treatment, dogs.

Chronic diarrhea is a frequent cause of consultation in routine, small animal practice (6, 10). A dog with chronic diarrhea must be correctly diagnosed in order to prescribe the most appropriate pharmacological treatment, as well as dietary treatment. The malabsorption syndrome in dogs includes a group of chronic enteropathies (small intestinal inflammatory diseases, exocrine pancreatic insufficiency, intestinal lymphangiectasia), characterized by faulty intestinal absorption due to mucosal damage and increase of intraluminal osmotic pressure, which can leads to chronic diarrhea (8, 12). Malabsorption syndrome may also lead to chronic vomiting, usually lose weight, in despite of increased food intake, chronic and intermittent episodes of abdominal pain (1, 14, 19).

To assure a successful outcome in dogs with chronic enteropathies, which can lead to a malabsorption syndrome, is recommended a combined dietary and pharmacological therapy (5, 15, 18). To ameliorate the symptomatology, dietary treatment should be focused on the use of highly digestible diets to reduce osmotic pressure within the intestinal lumen, and also on low-fat and low-fiber diets (17, 21). In the same time, for possible protein losses, which may occur as a result of mucosal damage, diets including highly digestible proteins are recommended (16, 20).

Together with paraclinical examinations, which have recently become more efficient and accurate in establishing the diagnosis of malabsorption syndrome, the clinical exam had and continues to have great importance in establishing the diagnosis and consequently, the treatment (4, 11, 13).

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), on a number of 18 dogs with malabsorption syndrome (12 males and 6 females of various breeds, ranging from 2-6 years of age). All patients experienced chronic small intestinal diarrhea (pale-colored stools with steatorrhea) which persisted for more than 4 weeks. In addition, all the dogs suffered weight loss, in despite of the polyphagia reported by the owners.

The main selection criteria were based on the presence or absence of hypoproteinemia (serum protein levels below 4.5 g/dL).

Group 1 was represented by 10 dogs in which the proteinemia was within normal limits and all the individuals from this group received a treatment based on Prednisone (2 mg/kg body weight/12 h for 10 days, 1 mg/kg body weight /12 h for 10 days, 0.5 mg/kg body weight/12 h for 10 days). The doses were gradually reduced and the intervals at which they were administered were adjusted.

Group 2, consists of 8 dogs with hypoproteinemia, which received a treatment based on metronidazole (10 mg/kg body weight/8 h for 21 days) and prednisone (2 mg/kg body weight /12 h for 10 days, 1 mg/kg body weight /12 h for 10 days and 0.5 mg/kg body weight /12 h for 10 days).

All dogs (n=18, groups 1 and 2) followed a dietary treatment consisting of easily digested, low-fat, low-fiber carbohydrates and proteins.

Hematological and biochemical exams (urea and creatinine, hepatic ALT-performance, total serum protein, albumin levels and albumin/globulin ratio) were performed (2, 3, 9).

Results and discussions

Routine blood biochemistry values for each of the 18 subjects with malabsorption syndrome (group 1 – with normal serum protein levels and group 2 – with hypoproteinemia), are presented in Table 1-2.

Table 1

Biochemical parameters values in dog with malabsorption syndrome – *group 1* - with normal serum protein levels

Case no.	**CREA** (mg/dL)	**BUN** (mg/dL)	**Total** serum protein (g/dL)	**Albumin** (g/dL)	**Ratio Albumin/globulin**
1	0.7	31	6.4	3.1	0.9
2	0.9	39	6.2	3.0	0.9
3	1.0	35	6.0	3.0	1.0
4	0.7	38	6.0	2.9	0.9
5	0.8	32	6.2	3.1	1.0
6	0.7	35	6.4	3.1	0.9
7	0.8	36	5.8	2.9	1.0
8	0.7	34	6.2	3.0	0.9
9	0.9	37	6.2	3.0	0.9
10	0.8	33	6.1	2.9	0.9
X±SD	**0.8±0.1**	**35±3.4**	**6.1±0.2**	**2.9±0.07**	**0.92±0.04**

Biochemical parameters and their arithmetic means and standard deviations in all dogs with malabsorption syndrome (from group 1), were within physiological limits (Table 1).

In our opinion, the hypoproteinemia observed in group 2, was due to changes in epithelial permeability, rather than to post- mucosal damage, because in all individuals, after initiation of pharmacological and dietary treatment, a favorable clinical evolution is observed, which, from histological point of view, means the recovery of the intestinal mucosa.

After the administration of the treatment, the dogs from group 1, present a significant increase in serum proteins. In relation to initial values, serum proteins increased an average of 2.5±1.7% after first 10 days of pharmacological treatment with immunosuppressive agents, and 4.7±1.8 % after 30 days of treatment.

In the group of dogs with malabsorption syndrome associated with hypo-proteinemia (group 2), the greatest increase of serum proteins was observed during

the first 30 days of treatment, after simultaneously administration of the two immunosuppressive drugs (prednisone and metronidazole).

Table 2

Biochemical parameters values in dog with malabsorption syndrome – *group 2* - with hypoproteinemia

Case no.	**CREA** (mg/dL)	**BUN** (mg/dL)	**Total** serum protein (g/dL)	**Albumin** (g/dL)	**Ratio Albumin/globulin**
1	0.7	27	3,8	1,8	0.9
2	0.9	39	3,9	1.8	0.8
3	0.8	30	3,8	2.0	1.1
4	0.7	36	4.0	1.9	0.9
5	0.7	34	4.2	2.2	1.1
6	0.9	32	3.9	1.8	0.8
7	0.8	31	4.4	2.3	1.0
8	0.8	35	3.2	1.5	0.8
X±SD	**0.8**±0.1	**33**±0,7	**3.8**±0.4	**1,8**±0,3	**0,96**±0.1

So, the patients from group B, present 10 days after treatment began, an increase of serum protein levels with an average of 27.1±11.1%, and an average of 54±15.5% after 30 days of treatment with immunosuppressive drugs, which means the normalization of mucosal permeability and in the same time the restoration of intestinal absorption (Table 3).

Is important to mention that, the favorable evolution in all 18 dogs taken in this study, was due to the combination of the dietary treatment and pharmacological treatment.

Table 3

Dynamic of serum proteins after dietary and pharmacological treatment, in relation to initial protein values

GROUP no 1 – dog with malabsorption syndrome - with normal serum protein levels					
Case	Total serum protein				
	Initial (g/dL)	**10 days** (g/dL)	**%**	**30 days** (g/dL)	**%**
1	6.4	6.6	**3.1**	6.6	**3.1**
2	6.2	6.2	**0**	6.4	**3.2**
3	6.0	6.2	**3.3**	6.4	**6.6**
4	6.0	6.0	**0**	6.4	**6.5**
5	6.2	6.4	**3.2**	6.4	**3.2**
6	6.4	6.8	**6.2**	6.9	**6.2**
7	5.8	6.0	**3.4**	6.2	**6.9**
8	6.2	6.4	**3.2**	6.6	**6.4**
9	6.2	6.2	**0**	6.4	**3.2**
10	6.1	6.2	**3.3**	6.2	**3.3**
X±SD	**6.1±0.2**	**6.3±0.2**	**2.5±1.7**	6.4**±0.2**	**4.7±1.8**
GROUP no 2 dog with malabsorption syndrome - with hypoproteinemia					
Case	Total serum protein				
	Initial (g/dL)	**10 days** (g/dL)	**%**	**30 days** (g/dL)	**%**
1	3.8	4.9	**27**	6.0	**54**
2	3.9	4.8	**20**	5.8	**45**
3	3.8	4.2	**10.4**	6.1	**58**
4	4.0	5.6	**40**	5.8	**45**
5	4.2	5.2	**23.8**	5.8	**38.1**
6	3.9	4.8	**23**	6.2	**59**
7	4.4	5.4	**22.6**	6.4	**45.5**
8	3.2	4.8	**50**	6	**87.4**
X±SD	**0.8±0.1**	**4.9±1.9**	**27.1±11.1**	**6.01±0.2**	**54±15.5**

Conclusions

The malabsorption syndrome in dogs, can be treated by a combination of dietary therapy (low-fat, low-fiber, highly digestible diet) and pharmacological treatment (administering immunosuppressive drugs, like prednisone and metronidazole).

In canine malabsorption syndrome, the combinations of prednisone and metronidazole potentiate their immunosuppressive action.

In all patients after the treatment, the clinical evaluation can be done based on the clinical signs.

In those patients who had initially presented malabsorption syndrome associated with hypoproteinemia, the clinical evaluation can be completed with the monitoring of the serum protein level evolution, because the recovery of serum protein levels, means the normalization of mucosal permeability and in the same time the restoration of intestinal absorption.

Acknowledgement

The research was carried out as part of an extensive study, the preliminary results being part of the PhD Thesis *"Study regarding the optimization of the clinical-diagnostic and therapeutic approach in maldigestion and malabsorption syndromes".*

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ANALYSIS OF CHEMICAL COMPOSITION IN SOW'S MILK

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Summary

In this paper, the chemical composition of sow milk from birth to 28 days was analyzed, quantitatively and qualitatively, in order to conclude if there it is possible to intervene in any way in the technology of breeding, that can reduce piglets' morbidity and mortality. Collecting milk from the sow is more difficult than from the cow, and from the seventh day was very difficult, therefore samples from several sows were needed to get enough milk for one determination. From each sample, protein, fat and lactose were determined using standard methods. It was found an increase in protein synthesis by the 14th day, followed by a decrease. The milk analysis revealed relatively higher levels of fat (7.5% vs. 6.5%), lower lactose levels (5% vs. 6%) and similar protein expression (5%), when compared to colostrum.
Keywords: sow, colostrum, determination, sampling, husbandry.

Milk is the primary source of nutrients for suckling piglets. During the first 24-48 hours after birth, sows produce colostrum, a thick and yellowish secretion that contains higher concentrations of immunoglobulins (2, 3, 14), growth factors, and other bioactive compounds compared to milk. Colostrum plays a crucial role in the transfer of passive immunity from the sow to the piglet, protecting the neonate against infectious diseases. In addition to its immunological properties, colostrum is also a rich source of nutrients, providing the piglet with essential amino acids, fatty acids, and carbohydrates (1, 5). The composition of colostrum and milk, however, differs significantly in terms of their protein, fat, and lactose content.

Piglets fed to highly productive sows grow on average around 250 g per day between birth and weaning, but this growth rate is well below their biological potential. It has been known for decades that captive-reared pups can grow over 450 g/day with ad libitum use of milk replacer, and perhaps today's modern genotypes can grow even faster. Evolutionarily speaking,high-fath fat content of mother's milk is most beneficial for piglets to increase body fat content (10), thereby improving piglet survival.

However, several authors (17, 13, 20) have suggested that the milk protein concentration or milk protein to energy ratio is too low to support maximal growth in piglets with lean genotypes. Increasing the concentration of feed protein in sow diets leads to higher concentrations of milk casein and milk protein, but it is unclear what concentration of milk protein is optimal for piglet growth (5).

Materials and methods

In the second part of this experiment, milking of sows was attempted to determine the chemical composition of the colostrum, then of the milk. Compared to the cow, the sow is not milked by pressing the udder (regardless of the form of pressing) on the one hand, and on the other hand due to its size. The sow is milked imitating the suckling of the piglet, that is, a massage of the teat, followed by the ejection of the milk. During the colostral period, the sow accepts the "substitution" the piglet by human hands, but after five days it is very difficult to obtain a quantity of milk which corresponds to an analysis of the addition. 10 milk samples were collected during the colostral period, on the seventh, 14th, 21st and 28th days.In order to obtain a sufficient amount of milk, samples were collected from several sows in the same lactation phase. Protein, fat and lactose were determined from these samples. During the colostral period, since they could be collected more easily, the samples were collected: at farrowing at six o'clock; at 12 hours; at 18 hours and 24 hours postpartum. Twenty sows (Landrace x Yorkshire) were used in the present study.

The sows were housed in individual farrowing crates and were fed a commercial gestation diet throughout pregnancy. Within 12 hours after farrowing, colostrum samples were collected from each sow by hand milking. Subsequently, milk samples were collected at 24 and 48 hours after farrowing. The samples were collected in sterile containers and stored at -20°C until analysis.The protein, fat, and lactose content of the samples were analyzed using the Kjeldahl method, Gerber method, and Lactostar method, respectively. The results were expressed as percentages (%). Data were analyzed using one-way ANOVA followed by Tukey's multiple comparison test. $P < 0.05$ was considered significant.

Results and discussions

The results showed that the percentage of protein was significantly higher in colostrum (15.3 ± 1.8%) than in milk at 24 hours (5.9 ± 0.8%) and 48 hours (5.5 ± 0.6%) postpartum ($P < 0.05$). The percentage of fat, on the other hand, was significantly lower in colostrum (3.8 ± 0.6%) than in milk at 24 hours (8.6 ± 1.0%) and 48 hours (7.4 ± 0.8%) postpartum ($P < 0.05$). The percentage of lactose was also significantly higher in colostrum (3.7 ± 0.3%) than in milk at 24 hours (2.6 ± 0.2%) and 48 hours (2.8 ± 0.2%) postpartum ($P < 0.05$).

Our results showed that the chemical composition of milk and colostrum in sows varied significantly throughout lactation. Colostrum had higher levels of protein, fat, and immunoglobulins than regular milk (7, 8, 9). The protein content in colostrum (Table 1) was about 15% on average, while regular milk contained around 5% protein. The fat content in colostrum was about 8%, while regular milk contained about 6%. The lactose content in milk increased as lactation progressed (Table 3, 4), while the mineral content remained relatively constant.

Table 1

Colostrum protein

No. Crt.	After farrowing	At 6 o'clock	At 12 hours	At 18 hours	In 24 hours
1	15	13	8.5	7.3	6.4
2	15.2	13.3	8.3	7.1	6.4
3	14.9	13.5	8.2	7.3	6.3
4	14.8	12.8	8.3	7.2	6.2
5	14	12.9	8.5	6.8	6.2
6	15.5	13	8.2	6.9	6.1
7	15.3	13.5	8.4	7.1	6.4
8	14.9	13.1	8.1	7.3	6.5
9	15	13.4	8.7	7.2	6.6
10	15.2	12.8	8.5	7.3	6.4

We also found that the nutrient content of milk and colostrum varied depending on the parity and nutrition of the sow. Our results showed that the percentage of proteins in colostrum varied significantly depending on the time postpartum.

The colostrum samples collected within the first 24 hours postpartum had the highest percentage of proteins. The percentage of proteins decreased gradually over time. The high percentage of proteins in colostrum within the first 24 hours postpartum is crucial for providing piglets with essential amino acids and immunoglobulins for their growth and development. The decrease in protein percentage over time is likely due to the transition from colostrum to regular milk production.

Regular milk contains lower levels (Table 2) of proteins but still provides adequate amounts of essential amino acids for piglet growth and development. The variations in protein percentage in colostrum among sows may be due to differences in breed, parity, and nutrition, highlighting the need for proper management and nutrition of sows to ensure optimal colostrum quality (6, 15, 19).

From the data gathered and compiled, we analysed firstly, just the percentages of protein found in milk and colostrum. It can be seen comparing the percentage from farrowing and at 28 days an extreme decrease (with a mean of 14.98 after farrowing and 4,27 at 28 days. The results showed that the percentage of lactose was significantly higher in colostrum (4.4 ± 0.3%) than in milk (3.2 ± 0.2%) postpartum ($P < 0.05$) (Table 3). There was no significant difference in lactose content between milk samples collected ($P > 0.05$).

Table 2

Milk protein

No. crt.	In 7 days	At 14 days	At 21 days	At 28 days
1	8.5	6.2	5.2	4.4
2	8.3	5.9	5.1	4.1
3	8.6	5.9	5.3	4.2
4	8.6	6.4	5.2	4.4
5	8.5	6.1	5.3	4.1
6	8.2	6.2	5.1	4.3
7	8.1	6.3	4.9	4.2
8	8.3	6.2	5.4	4.2
9	8.7	6.1	5.1	4.4
10	8.5	6.2	5.2	4.4

Table 3

Lactose in colostrum

No. crt.	After farrowing	At 6 o'clock	At 12 hours	At 18 hours	In 24 hours
1	3.3	3,4	4.3	4.4	4.4
2	3.2	3,4	4.1	4.3	4.5
3	3.1	3.5	4.3	4.5	4.3
4	3.3	3.2	4.5	4.6	4.4
5	3,4	3,4	4.3	4.1	4.2
6	3.5	3,4	4.2	4.3	4.5
7	3.1	3.5	4.2	4.4	4.4
8	3.3	3.1	4.1	4.3	4.3
9	3.2	3,4	4.2	4.4	4.5
10	3.3	3.2	4.3	4.5	4.4

Table 4

Lactose milk

No. crt.	In 7 days	At 14 days	At 21 days	At 28 days
1	3.2	5.3	5.8	5.4
2	3.5	5.2	5.5	5.8
3	3.6	5.5	5.6	5.6
4	3.2	5.3	5.6	5.4
5	3.5	5.3	5.2	5.3
6	3.1	5.2	5.3	5.4
7	3.5	5.4	5.5	5.6
8	3.2	5.3	5.8	5.3
9	3.4	5.2	5.9	5.3
10	3.2	5.2	5.8	5.4

The fat content in milk is higher than colostrum (Table 5,6). Colostrum contains approximately 5-6 % fat, while regular milk contains approximately 7-8% fat. The high-fat content in colostrum is essential for providing piglets with the necessary energy for their growth and development. The fat content in milk and colostrum is affected by the breed, parity, and nutrition of the sow. Sows fed a high-fat diet may produce milk with a higher fat content (11, 18). There was no significant difference in fat content between milk samples collected at 24 and 48 hours postpartum ($P > 0.05$).

Table 5

Colostrum fat

No. crt.	After farrowing	At 6 o'clock	At 12 hours	At 18 hours	In 24 hours
1	5	4.5	4.3	5.2	5.5
2	3.2	3.4	4.1	4.3	4.5
3	3.1	3.5	4.3	4.5	4.3
4	3.3	3.2	4.5	4.6	4.4
5	3.4	3.4	4.3	4.1	4.2
6	3.5	3.4	4.2	4.3	4.5
7	3.1	3.5	4.2	4.4	4.4
8	3.3	3.1	4.1	4.3	4.3
9	3.2	3.4	4.2	4.4	4.5
10	3.3	3.2	4.3	4.5	4.4

Table 6

Milk fat

No. crt.	In 7 days	At 12 days	At 18 days	At 24 days
1	8.01	7.5	6.6	6.4
2	8.2	7.4	6.3	6.4
3	8.1	7.1	6.4	6.3
4	8.05	7.4	6.5	6.2
5	7.95	7.3	6.6	6.4
6	8.03	7.5	6.7	6.3
7	8.2	7.5	6.5	6.5
8	8.1	7.2	6.4	6.2
9	8.03	7.6	6.3	6.3
10	8.05	7.4	6.5	6.3

It has been shown that litter size does not change the total milk production of the sow using the weigh-suck-weigh estimation method (12, 16). This allows the conclusion that the nutrient intake of piglets in large litters may be restricted, as they receive less milk but of similar quality. Therefore, providing milk replacers to newborns from prolific sows could be an effective way to support survival rate and weaning weight.

These researches were conducted because it was hypothesized that there is a higher level of macronutrient components in sow's milk, along with an increase in reproductive indices. It was found that the values of milk macronutrients do not increase significantly in relation to the improved reproductive performance of sows, and the current values of protein, fat and lactose in colostrum are similar to those of 30 years ago (16% protein, 3 % lactose and 5% fat).

Conclusions

As a general conclusion we can say that the study showed that even though it can be seen a slight difference between the colostrum and normal milk collected, it does not show a significant impact of the colostrum being fed to piglets as quality feed, but the amount that it is given its much more important due to the permeability of the intestine. It can be seen that not the composition of the colostrum is important for the development of the piglets but, the moment when they are fed.

Acknowledgements

This work appeared within the project: Program for increasing performance and innovation in doctoral and postdoctoral excellence research - PROINVENT, Contract no. 62487/03.06.2022 POCU/993/6/13 - SMIS code:153299.

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HANTAVIRUS INFECTION IN EUROPE: THE IMPLIED STRAINS AND THEIR EPIDEMIOLOGY – A SHORT REVIEW

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Summary

Rodents make up about 40% of all mammal species and can be found in various habitats around the world, except for Antarctica. They are known to play an essential role in transmitting various diseases with zoonotic potential. Throughout history, there have been numerous infectious episodes, starting from the Bubonic Plague pandemic, produced by *Yersinia pestis*, transmitted to humans through fleas previously fed on rats, and up to the viroses and bacterioses that currently manifest themselves globally. This category includes Typhus (infections caused by *Rickettsia* species), Rat-bite fever, Yersiniosis, Leptospirosis, Tularemia, as well as Tick-borne encephalitis, Hantavirus, Hepatitis E, Borna virus, Lassa fever, Omsk hemorrhagic fever or even Cowpox. In 1978, the causative agent of Korean hemorrhagic fever was isolated from infected small field rodents, *Apodemus agrarius*, near the Hantan River in South Korea. The virus was named the Hantaan virus after the nearby river. Its electron microscope images soon revealed that the virus was a new member of the *Bunyaviridae* family. It has been observed that hantaviruses, unlike other members of this family, do not have an arthropod vector and cause a persistent infection in the population of their specific rodent hosts. The genus includes the viruses that cause hemorrhagic fever with renal syndrome (HFRS) in Europe and eastern Asia and hantaviral cardiopulmonary syndrome (HCPS) in the Americas. Currently, the genus includes more than 21 species and exceeds 30 genotypes, expanding to multiple territories in the world. A good knowledge of the epidemiology, symptomatology, but above all, of the main species of rodents that can be involved in the transmission of zoonoses, plays an important role in preventing potential outbreaks of infection and narrowing the area of diseases. While most countries in Europe report Hantavirus infections in humans, such data is very limited in Romania due to the lack of studies or official reports. In this review, we aim to update the data on the spread of Hantaviruses in Europe.

Keywords: rodents, zoonoses, hantavirus.

Hantaviruses have evolved for millions of years through their natural reservoirs represented by rodents and insectivores. Natural reservoirs include both *Cricetidae* rodents (subfamilies *Arvicolinae, Neotominae* and *Sigmodontinae*) and *Muridae* rodents (subfamily *Murinae*). *Cricetidae* rodents include individuals widespread in the northern hemisphere and American territory. *Muridae* rodents include mice and rats distributed throughout the rest of the globe (13, 22, 24).

Hantavirus phylogeny closely follows that of their rodent hosts, suggesting long-term coevolution, although there has been evidence of occasional changes in hosts. The phylogenetic tree suggests that the interbreeding of hantaviruses with hosts from four different rodent subfamilies appears to influence their ability to cause

a specific clinical manifestation in humans (5, 27). For example, it is known that most viruses affecting the *Neotominae* and *Sigmodontinae* subfamilies cause severe HCPS with a high mortality rate (40-50%). These viruses are widespread in North and South America in different species of *Neotominae* and *Sigmodontinae* rodents. The hantaviruses which evolved alongside *Murinae* rodents cause severe HFRS, which primarily affects kidney function, resulting in a rate of mortality of 0-15% (13, 16).

Although the disease caused by viruses found in *Murinae* has a lower mortality rate, it still poses a significant threat to human health because of the disease severity and the ability of viruses to cause epidemics. Of the hantaviruses spread by *Arvicolinae*, only Puumala virus (PUUV) causes a mild form of the disease in humans, often referred to as a nephropathy epidemic, with a mortality rate of less than 1% (1, 2). Interestingly, most other members of this subfamily are not pathogenic to humans. Until recently, the only exception that did not have a confirmed link to rodents is Thottapalayam virus (TPMV), which has been isolated from the Asian house shrew (*Suncus murinus*), captured in 1964 during a survey for surveillance of Japanese encephalitis virus in southern India (13, 25).

Literature up-to-date key information

- **RODENT SPECIES OF INTEREST**

Important rodent species of the *Muridae* family with a wide distribution in Europe include, in addition to rats and house mice, members of the *Apodemus* genus:

(a) The collared mouse (*Apodemus flavicollis*) (Fig. 1A) is widespread in European forests, especially at their edges.

b) The wood mouse (*Apodemus sylvaticus*) (Fig. 1B) also lives in forests, but also in meadows.

c) The striped field mouse (*Apodemus agrarius*) (Fig. 1C) lives in forest margins and woodland areas. This rodent is found in two separate populations: in central Europe and Eastern Europe to Russia and China, as well as in parts of Southeast Asia (21).

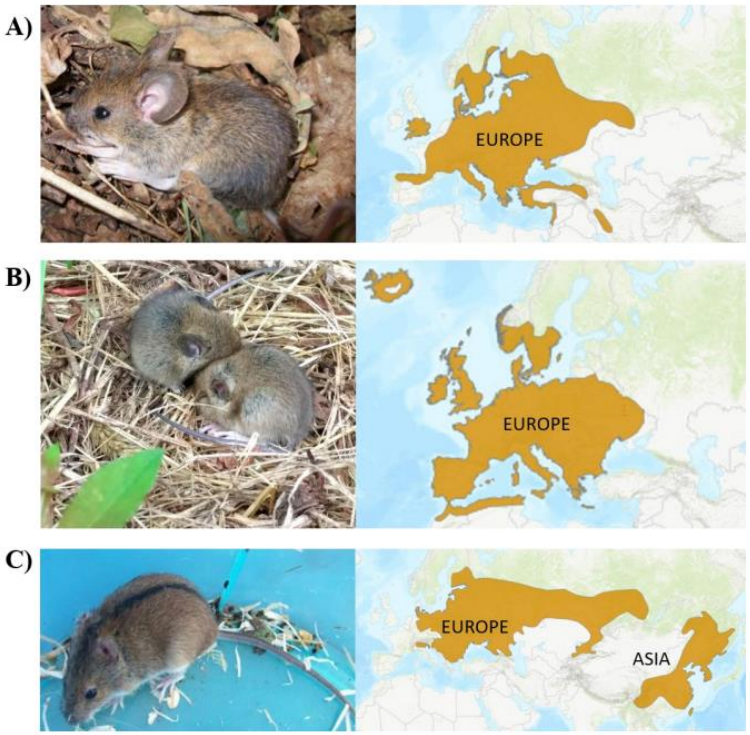

Fig. 1.The main rodent species of the *Muridae* family found in Europe (12)

In addition to the *Muridae* family, the *Cricetidae* family with the *Arvicolinae* subfamily is of great importance as it carries a large number of zoonotic agents. The *Microtus* genus consists of about 60 species, of which about 20 species can be found in Europe. Some species of mice and rats in this family are restricted to small geographical areas, such as *Microtus lusitanicus* (Fig. 2A) in Portugal and north-western Spain and *Microtus subterraneus* in central Europe (Fig. 2B) (21, 29).

Other species, such as the field mouse (*Microtus arvalis*) (Fig. 2C) and *Microtus agrestis* (Fig. 2D) are found in larger geographical areas such as Europe and parts of Asia. In particular, the tundra mouse (*Microtus oeconomus*) can be detected in areas of three continents (Asia, Europe and North America) (Fig. 2E) (21). The mouse (*Myodes glareolus*) (Fig. 2F) is found in most parts of Europe, up to western parts of Russia, living in mixed and coniferous forests and preferring vegetation from the ground level (21).

Field mouse population cycles peak every two to five years, with a spread of up to 2000 individuals per hectare (4). This leads to both massive agricultural damage, but also an increased risk of pathogenic transmission, as reported for

previous tularemia outbreaks in Spain (14, 21, 22) and outbreaks of leptospirosis in Germany that have been associated with this species (9, 11, 20).

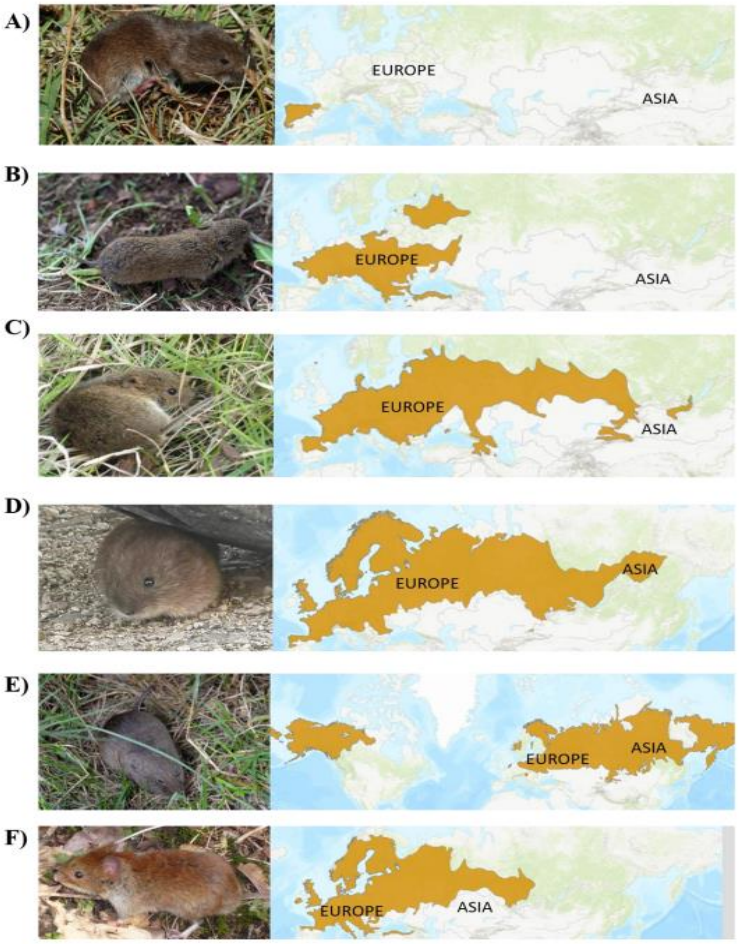

Fig. 2. The main rodent species of the *Cricetidae* family found in Europe (12)

The virus is spread through the urine, faeces and the saliva of infected animals. Knowing that the virus is transmitted by bite and aerosols from saliva, it has been assumed that there is an additional route of transmission between reservoir hosts, since saliva was detected with high viral loads. Hantavirus infection in rodents is thought to be asymptomatic, but changes in health status have been observed, such as decreased survival of PUUV-infected individuals in winter. Outside the body,

the virus can persist for up to several weeks, depending on outdoor conditions such as temperature and intensity of UV radiation (3, 19, 23).

- **HANTAVIRUS INDUCED SYNDROMES**

Hantavirus infection in humans can lead to two clinical syndromes: HFRS or HCPS caused by hantaviruses of the Old World or New World, respectively. The differences between the diseases are indicated by the fact that the vascular system is predominantly affected, but with specific sites: renal capillaries at the medullary level in HFRS and pulmonary capillaries in HCPS. On the other hand, the initial symptoms of hantavirus infections are similar, including sudden onset of high fever, general malaise, myalgia and influenza-like symptoms (3, 15, 19, 28).

The incubation period of hantaviruses is relatively long: 2-4 weeks. The onset of the disease is abrupt, with flu-like symptoms progressing rapidly to - in some cases - severe or even fatal disease (19). What happens in the infected individual during the incubation period and why symptoms start so suddenly is largely unknown. Hantavirus RNA has been found in the serum of patients up to three weeks before the onset of illness, and the virus was isolated from serum before.

1. Haemorrhagic fever with renal syndrome (HFRS)

The main reservoir is represented by rodents of the *Muridae* family, which develop inapparent but persistent infections. In the natural reservoir, infection is apparently asymptomatic (19). In rodents, after the virus enters into the body, replication occurs in the vascular endothelium of internal organs: salivary glands, the lymph nodes, kidneys, liver, spleen, and the intestines. The virus is shed through saliva, urine, faeces, contaminating the environment and the receptive host - humans. Transmission of the virus between rodents occurs horizontally. After the viremia period, neutralising antibodies appear and persist throughout the rest of the animal's life. Apart from rodents, the virus has only been reported in humans. They become infected via the respiratory route, by inhaling aerosols from the excretions of infected mice and after being bitten. The outbreak is often seasonal and depends on fluctuating rodent densities and high-risk human activities that determine exposure to contaminated materials (19, 23).

The clinical presentation of HFRS ranges from subclinical, mild and moderate to severe depending on the causative agent of the disease. A case of PUUV infection can be severe, an individual infection with HTNV can be mild, and infections are usually subclinical. The typical course of HFRS can be divided into five distinct stages: febrile, hypotensive, oliguric, diuretic and convalescent (30).

These phases are best distinguished in severe forms of disease caused by HTNV and DOBV. After an incubation period of 2 to 4 weeks, the disease begins suddenly with fever chills, headache, backache, abdominal pain, nausea and vomiting. Drowsiness, and visual disturbances (blurred vision) are frequently reported. This febrile phase usually lasts for 3 to 7 days. Towards the end of this phase, conjunctival haemorrhages and spots appear on the palatal vault (30). The hypotensive phase can last from a few hours to 2 days. In severe cases,

hypotension, even shock, can develop rapidly and one-third of HFRS deaths are associated with irreversible shock at this stage. Thrombocytopenia and leukocytosis are characteristic of this phase and if severe haemorrhagic disease occurs, its onset may be seen at this stage. Haemorrhagic manifestations may take the form of skin and mucous membrane spots, emaciations, hematemesis, epistaxis, hematuria, melena and fatal intracranial hemorrhages (19, 30).

In the oliguric phase, which lasts 3-7 days, blood pressure returns to normal parameters in while kidney function transiently declines, leading to oliguria or even anuria, proteinuria, abnormal urinary sediment, including microscopic haematuria and azotemia. During the oliguric phase, which is usually accompanied by abdominal or back pain, patients with severe symptoms should be treated by haemodialysis. Half of all deaths occur in this phase. The laboratory tests frequently show elevated serum creatinine and urea levels (19, 30).

In the diuretic phase, renal function begins to recover and urine output increases. The onset phase indicates a favourable prognosis for the patient. It may last for days or weeks in patients who eliminate several litres of urine per day (18, 29). The convalescent phase, characterized by recovery of clinical and laboratory markers, is usually prolonged, lasting up to 6 months. Recovery is usually complete and further complications are rare but may include chronic renal failure and hypertension. In children, the clinical picture closely mimics adult symptoms, but is often less severe. However, abdominal manifestations are reported more often (19, 30).

Functional failure, is generally less evident than in HFRS caused by several hantaviruses, with oliguria or anuria occurring in less than half of patients. Overall, as the clinical course of PUUV infection is often uncharacteristic and more like a febrile illness accompanied by abdominal pain, often not diagnosed correctly (5, 6, 7).

2. Hantavirus cardiopulmonary syndrome (HCPS)

The clinical course of HCPS consists of three stages: prodromal, cardiopulmonary and convalescence, and clinical manifestations can range from mild hypoxaemia to cardiogenic shock, chills, myalgia, nausea, headache, vomiting, abdominal pain and diarrhoea. This is followed by a rapid progression to the cardiopulmonary phase with sudden onset manifested by progressive coughing, dyspnoea and tachycardia. Patients develop acute pulmonary oedema and hypotension. Bilateral infiltrates develop rapidly, sometimes being associated with pleural effusions, causing respiratory failure, thus requiring mechanical ventilation. In some cases, this stage is complicated by cardiogenic shock, lactacidaemia and massive haemoconcentration (7, 19).

Patients may die within hours of admission. Those who survive the acute phase of the disease enter the diuretic stage, which is accompanied by the resolution of the pulmonary oedema. Although convalescence is slow and patients often manifest weakness, fatigue and intolerance to physical exertion, recovery is generally complete without sequelae (7, 19).

Although kidney disease is usually attributed to HFRS and HCPS lung disease, research from the medical field has led to the conclusion that both syndromes partially overlap. Issues of reported cases of HFRS with lung disease and HCPS with kidney disease and/or haemorrhagic are increasing (7, 19).

- **HANTAVIRUS EPIDEMIOLOGY**

The geographical distribution of hantavirus hosts reflects the epidemiology and distribution of infection. Hantavirus infection in humans is considered to be an infection causing two types of serious disease, HFRS and HCPS. The main route of infection for both syndromes is inhalation of the virus. In general, people become infected with hantavirus by the direct contact with infected rodents or their droppings in aerosolised form. However, there are reports documenting the spread of Andes virus (ANDV) from humans to humans. HFRS is caused by Old World hantaviruses and most cases have been reported in Eastern Asia (China, Korea and eastern Russia) and Europe (including the European part of Russia) (4, 19). Annually, more than 100,000 cases of HFRS are reported in China alone, and more than 900 cases are reported in Korea and Eastern Russia. (12) In Europe, most HFRS cases are reported in Russia, Finland and Sweden (19, 24). The majority of HFRS patients are men aged between 20 and 50. The mortality rate for HFRS depends on the type of virus and generally ranges from 0.1% to 10%. Patients with HRFS mainly come from rural areas, where rodent hosts are very widespread (5, 27). HCPS outbreaks in North America are associated with the increasing population of the natural reservoir (*Peromyscus maniculatus*). HCPS has also been reported in other countries in South America and the Caribbean Peninsula, along with Argentina, Brazil, Chile, Bolivia, Paraguay, Uruguay and Panama (7, 10).

Regarding Romania, the literature mentions the existence of 27 clinical cases of HFRS, reported between 1956 and 1977. Subsequently, the disease seems to have died out, until the National Reference Laboratory was able to analyse 6 suspected cases of leptospirosis dating from 2005-2007, thus allowing the diagnosis of HFRS (15). Afterwards, the European Centre for Disease Prevention and Control (ECDC) reported a total number of 18 cases of Hantavirus infection only in Romania, between 2016 and 2020 (Table 1) (31). Moreover, the same platform signals the disease occurring more in men than in women. The professional environment that implies contact with rodents is mainly occupied by men rather than women, which explains the cases' occurrence.

According to the table, the reported cases are expanded on the whole European continent, but most of these occurred in countries that have a large forest area (i.e., Sweden, Germany, Finland), and in the ones located in the Eastern region, where economic issues play a major role in daily life. Because all of Romania's neighboring countries have reported Hantavirus infections in humans, it is important to maintain proper hands and working environment hygiene, especially for the workers who have a higher risk of disease (farmers, lumberjacks, hunters, foresters etc.). (31)

Table 1

Number of Hantavirus cases reported in Europe between 2016-2020

Country / Year	2016		2017		2018		2019		2020	
	No.	Rate	No.	Rate	No.	Rate	No.	Rate	No.	Rate
Austria	30	0.3	90	1.0	24	0.3	276	3.1	30	0.3
Belgium	38	0.3	123	1.1	85	0.7	57	0.5	9	0.1
Bulgaria	10	0.1	8	0.1	7	0.1	6	0.1	1	0.0
Croatia	31	0.7	389	9.4	18	0.4	191	4.7	17	0.4
Cyprus	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
Czechia	10	0.1	17	0.2	4	0.0	15	0.1	5	0.0
Denmark	No data	No rate	No data	No rate	No data	No rate	No data	No rate	No data	No rate
Estonia	11	0.8	26	2.0	15	1.1	26	2.0	17	1.3
Finland	1663	30.3	1246	22.6	999	18.1	1256	22.8	1164	21.1
France	58	0.1	236	0.4	55	0.1	131	0.2	26	0.0
Germany	282	0.3	1731	2.1	235	0.3	1535	1.8	229	0.3
Greece	1	0.0	2	0.0	3	0.0	1	0.0	1	0.0
Hungary	7	0.1	16	0.2	6	0.1	13	0.1	4	0.0
Iceland	No data	No rate	No data	No rate	0	0.0	0	0.0	0	0.0
Ireland	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
Italy	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
Latvia	8	0.4	4	0.2	3	0.2	5	0.3	3	0.2
Liechtenstein	No data	No rate	No data	No rate	No data	No rate	No data	No rate	No data	No rate
Lithuania	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
Luxembourg	1	0.2	15	2.5	0	0.0	8	1.3	0	0.0
Malta	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
Netherlands	2	0.0	6	0.0	1	0.0	0	0.0	0	0.0
Norway	10	0.2	26	0.5	21	0.4	11	0.2	12	0.2
Poland	8	0.0	14	0.0	11	0.0	9	0.0	3	0.0
Portugal	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
Romania	**0**	**0.0**	**12**	**0.1**	**1**	**0.0**	**4**	**0.0**	**1**	**0.0**
Slovakia	6	0.1	53	1.0	88	1.6	94	1.7	50	0.9
Slovenia	12	0.6	76	3.7	12	0.6	252	12.1	14	0.7
Spain	0	0.0	1	0.0	0	0.0	0	0.0	0	0.0
Sweden	92	0.9	158	1.6	243	2.4	155	1.5	61	0.6
UK	No data	No rate	No data	No rate	No data	No rate	3	0.0	No data	No rate
EU-EEA	**2280**	**0.5**	**4249**	**1.0**	**1831**	**0.4**	**4048**	**0.8**	**1647**	**0.4**

Source: ecdc.com (31)

➢ DIAGNOSIS FOR THE HANTAVIRUS INFECTION

The diagnosis of HFRS and HCPS is based on clinical and epidemiological data, as well as on laboratory tests. Symptoms that should alert the doctor about a possible hantavirus infection are high fever, headache, abdominal and back pain, analysis of laboratory showing leukocytosis, thrombocytopenia, elevated serum creatinine, proteinuria and hematuria (20, 19). However, it is almost impossible to diagnose hantavirus infection only from clinical signs, especially in cases with mild and moderate clinical symptoms, because the early signs of the disease are not specific. Laboratory diagnosis of infections with acute hantavirus is based on serology because virtually all patients have IgM and usually IgG antibodies are present in the serum at the onset of symptoms. The most frequently used serological

test is the indirect ELISA. Indirect immunofluorescence assays are also regularly used for diagnosis but have lower specificity. Besides, rapid tests that provide the result within 5 minutes, easy to use, immunochromatographic, for IgM antibodies were developed and are commercially available (19).

Hantavirus infection can also be confirmed by detecting the hantavirus genome in samples of blood or serum by RT-PCR. Both classical and quantitative RT-PCR are used for viremia detection. Although the presence of viremia varies, viral RNA can usually be detected if it is a sample obtained in the acute phase of the disease. It has also been suggested that higher viremia is found in more serious hantavirus infections (DOBV, SNV, ANDV) comparatively with the milder infections caused by PUUV. Simultaneously with the detection of RNA virally, hantavirus infection could be confirmed even before the presence of specific antibodies (17).

When testing for the detection of the virus in rodents, it is recommended that the collected samples are of blood and thoracic fluid. Furthermore, these may be tested through ELISA for a rapid result. For a more precise diagnosis, RT-qPCR or conventional PCR followed by a Nested-PCR assay should be applied on lung, kidney and urinary bladder samples previously collected from the rodents.

- **PROPHYLAXIS**

Preventive measures are mainly based on rodent control, reducing their shelter and food sources from the proximity of human dwellings, eliminating contact with potentially contaminated areas (19, 20).

In Asia, the Republic of Korea, Hantavax® has been used for several years. The vaccine is derived from formalin-inactivated HTNV-infected mouse brain, but frequent doses are required to ensure immunity. In China, several vaccines have been produced and various formalin-inactivated animal tissues have been used, but none have been EMA approved (8, 26).

Thus, only bimolecular vaccines against HFRS were tested in humans, the first being the recombinant vectored vaccine expressing the M segment of HTNV, while the second included plasmid DNA. The advantage of DNA vaccines is that they provide an easy way to obtain multivalent vaccines and are capable of inducing long-lasting humoral and cellular immunity. Such a vaccine, based on an M segment of HTN and PUUV, is currently available in phase I clinical trials in the US to determine safety, tolerability and immunogenicity (8, 26).

Conclusions

Rodent-borne infections belong to the group of emerging zoonotic diseases. At the same time, special attention should be paid to all viral or bacterial diseases, which can be transmitted from rodents to humans. In recent years, the understanding and recognition of these have greatly improved worldwide.

The extent and magnitude of hantavirus outbreaks have increased. This could be explained by improved clinical awareness, the development of specific diagnostic tests, research on natural reservoirs and changing climatic conditions. Although in the case of the hantavirus genus, some variants have only recently been discovered, it has a long history, but environmental changes may affect the geographical distribution, abundance and dynamics of carrier rodent species and therefore the epidemiology of hantavirus. Although it is currently only speculated how widespread and how significant the climate and environmental chages truly are, rodent-borne zoonoses will remain a threat to public health.

Until official up-to-date reports are to be offered to the public, further research is needed on pathogenesis, diagnosis, development of antimicrobial and antiviral drugs and vaccines, in order to prevent and control rodent-borne infections. Thus, we aim to study more the occurance of rodent-borne zoonoses in Romania, through officially reported database, but also using laboratory testing on rodent and human samples.

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THE EFFECT OF WHITE CLOVER (*TRIFOLIUM REPENS L.*) AND NITROGEN FERTILISATION ON BIOMASS PRODUCTION, MORPHOLOGY, CHEMICAL COMPOSITION AND WEED INFESTATION OF WILLOW (*SALIX VIMINALIS L.)* USED FOR GRAZING SHEEP

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Summary

This study aimed to determine the effect of nitrogen fertilization and the use of white clover on biomass production on land, where willows were naturally grown. The morphology and chemical composition of the biomass was assessed in order to determine if the land can be used as a grazing area for sheep or the green mass ca be converted into animal feed. The experiment started in 2018 at SCDCOC Caransebeș and was carried out until 2022, on the part of the pasture where willow plants naturally appeared. It was found that the number and dry mass of willow stalks reported at 1m2 and their fresh weight yield was lower where the clover was sown. It was found that the willows were taller after fertilization with nitrogen, and the yield expressed in Dry matter and their diameter did not depend on the method of cultivation. Fertilization with nitrogen led to a higher ash content and clover content where the willow stalks were larger, and higher nitrogen values were obtained compared to the pure crop. The ratio of DM of willows and DM of clover to nitrogen fertilization was 3.97:1 and 3.91:1 for the control. It can be concluded that in the first years after the sowing of the white clover, the presence of willows can be a good alternative to improve the nitrogen level in the soil, thus the pasture can be converted into high-quality forage for sheep.

Keywords: grazing, sheep, analysis, pasture.

The increasing demand for renewable energy sources has led to growing interest in the use of biomass for energy production. White clover is a fast-growing legume that has been identified as a potential source of biomass for energy production. However, little research has been conducted on the optimal management practices for white clover grown for biomass production (17, 18, 21). Grazing animals, more precisely sheep, leads, over time, to the erosion of the soil both by grazing close to the ground and by passing sheep on that surface. To this is added grazing in rainy weather, when the soil is soft and it can end up plucking edible plants and growing weeds. That is why it is necessary to rotate crops and, more recently, to return all planting timber with a high growth rate, which is a determined factor in ensuring a safer global energy future (1, 17). In addition, the existence of these plants reduces soil erosion (2, 3, 20) and leads to an increase in humus content and improvement of soil structure (4, 5). Energy crops reduce carbon dioxide emissions both by replacing fossil fuels and by immobilizing organic carbon in the

soil (6–10). Growing these plants can prevent deforestation (10). In the near future, biomass from these plants may become more important and beneficial (11, 15). Of all the species cultivated for energy purposes, cultivated in the largest area of Europe, the willow is in the first place (6, 11, 12). Due to the numerous environmental benefits, the willow-growing area is expected to grow rapidly in the coming decades (13). This plant is characterized by large increases in biomass (14), it can be easily propagated by cuttings (13) and due to its natural appearance in Romania, this plant is perfectly adapted to the climatic conditions of this country. The aim of this study was to investigate the growth of white clover for biomass production under different fertilization levels and cutting frequencies.

Materials and methods

Places of study

The study was conducted at SCDCOC between 2018 and 2022. The land was a part of the outskirts of the Salbăgel pasture, 10 km from Sacu, Caraş-Severin County, located at an altitude of 154m. Soil conditions Experiments in the field were conducted on a soil slightly defined as very light alluvial soil, on loose sand and sandy gravel. Soil samples (from each plot) were analyzed each year. The samples were taken at a depth of 0–30 cm at the beginning of the growing season. The soil is visibly alkaline, the phosphorus content varies from medium to large, and potassium from high to very high, and the mg content is medium.

Weather conditions

The research was carried out on the pasture from Tucico, near Sălbăgel, Caras-Severin County. The average annual air temperature was 8.6°C, the average temperature of the growing season (IV-IX) was 14.7°C. The groundwater level was 85 cm below ground level. The average annual temperature during the study was higher than the long-term trend. The monthly and annual average rainfall amounts varied. The lowest rainfall was reported in 2021.

We established six experimental plots, each measuring 1m x 1m. Three of these plots were planted with willow seedlings, and three were planted with white clover seedlings. The plots were arranged in pairs, with one willow plot and one clover plot in each pair. The pairs were randomly assigned to three different soil types: sandy, loamy, and clayey. Soil samples were taken from each plot at the beginning of the study, and analyzed for pH, nutrient content, and organic matter content. The plots were watered and weeded as necessary throughout the study period. Measurements were taken at regular intervals throughout the study period to assess the growth and health of the plants. We measured the height and diameter of each plant, as well as the number of leaves and flowers produced. We also measured soil moisture content and temperature at regular intervals, using soil moisture sensors and thermocouples. We measured the height and diameter of each plant, as well as the number of leaves and flowers produced. We also measured the dry weight of the plants at each harvest and analyzed the nutrient content of the

plants. White clover seeds were sown in early spring, and the plants were fertilized with either 0, 50, or 100 kg/ha of nitrogen fertilizer.

The plots were arranged in a randomized complete block design, with four replicates of each treatment. The plots were mowed at either a high frequency (every three weeks) or a low frequency (every six weeks) throughout the growing season. Measurements were taken at regular intervals throughout the study period to assess the growth and biomass production of the plants. We measured the height and diameter of each plant, as well as the number of leaves and flowers produced. We also measured the dry weight of the plants at each harvest and analyzed the nutrient content of the plants. Experimental lots of white clover were established in four different fields, each with varying levels of weed infestation. We measured the biomass production and nutrient content of the white clover, as well as the weed biomass and species composition in each field. We also assessed the impact of weed infestation on animal grazing systems, by monitoring the weight gain and health of grazing animals in each field.

Results and discussions

Table 1

Values of biomass over the growing period

Growing period (days)	Biomass yield (kg/ha)	Protein content (%)	Energy content (MJ/kg)
30	**1500**	**12.3**	**15.6**
60	**2500**	**15.6**	**16.8**
90	**3500**	**18.2**	**18.5**

In agricultural systems, white clover is often used as a forage crop for animal grazing due to its high protein content and palatability (23). However, weed infestations can significantly impact the productivity and quality of the forage, leading to reduced animal performance and economic losses for farmers (16).

In this study, we aimed to investigate the impact of different levels of weed infestation on experimental lots of white clover, and how this impacted animal grazing systems. The results of our study suggest that white clover can be a viable option for biomass production, but that careful management is necessary to optimize yields. The fact that higher levels of nitrogen fertilizer led to an increase in biomass production highlights the importance of soil fertility management for maximizing yields. However, the fact that excessive nitrogen fertilizer led to a decrease in the quality of the biomass suggests that there is a trade-off between yield and quality. The fact that cutting frequency also had a significant impact on biomass production suggests that careful management of harvest timing is also important for maximizing yields.

Our results suggest that a balance between high-frequency and low-frequency cutting may be optimal, as this can maximize both biomass yield and quality.

Table 2

Density of plants

Weed Density (plants/m²)	White Clover Biomass Yield (kg/ha)
0	4500
25	3900
50	3200
75	2400
100	1700

Our results suggest that both willow and white clover compete for resources such as water, light, and nutrients. However, the extent of this competition varied depending on the soil type and other environmental factors. In general, willow tended to outcompete white clover in clayey soils, while white clover tended to outcompete willow in sandy soils. In loamy soils, the competition between the two species was more evenly balanced (9). We also found that temperature and rainfall patterns influenced the outcome of the competition, with willow performing better in cooler and wetter conditions, and white clover performing better in hotter and drier conditions.

The results of our study suggest that competition between willow and white clover is influenced by a complex interplay of factors, including soil type, temperature, and rainfall patterns. The fact that willow tends to outcompete white clover in clayey soils may be due to its ability to tolerate waterlogged conditions, while the fact that white clover tends to outcompete willow in sandy soils may be due to its ability to fix nitrogen from the air. The fact that temperature and rainfall patterns also influence the outcome of the competition may be due to the different physiological requirements of the two species (15, 23). Overall, our study highlights the importance of considering multiple environmental factors when studying competition between plant species.

Table 3 shows the biomass of white clover and weeds over a two-year period. As time progresses, the biomass of white clover increases while the biomass of weeds also increases. However, the rate of increase for white clover is higher than that of weeds, indicating that white clover can outcompete weeds over time. Cutting frequency also had a significant impact on the growth and biomass production of white clover. Plants that were cut more frequently produced less biomass per cut, but had a higher total biomass yield over the course of the growing season. Plants that were cut less frequently produced more biomass per cut, but had a lower total biomass yield over the course of the growing season.

Table 3

The biomass of white clover and weeds

Time (months)	White Clover Biomass (kg/ha)	Weed Biomass (kg/ha)
0	**0**	**0**
3	**1500**	**500**
6	**2500**	**1000**
9	**3500**	**2000**
12	**4500**	**3000**
15	**5000**	**3500**
18	**5500**	**4000**
21	**6000**	**4500**
24	**6500**	**5000**

The results of our study highlight the importance of weed management in white-clover forage systems. Weeds can significantly impact the productivity and quality of white clover crops (8, 19), leading to reduced animal performance and economic losses for farmers. The fact that weed species composition varied between fields suggests that different weed management strategies may be necessary depending on the specific weed species present. The impact of weed infestation on animal grazing systems also has important implications for farmers (7, 20, 22). Poor animal performance can lead to reduced profits and increased veterinary costs, highlighting the need for effective weed management strategies to maintain animal health and productivity.

Table 4

Growing rates of experimental clover

Time (weeks)	Year 1 Growth Rate (cm/week)	Year 2 Growth Rate (cm/week)
0	0.5	0.6
6	0.8	0.9
12	1.2	1.3
18	1.6	1.7
24	2.0	2.1
30	2.4	2.5
36	2.8	2.9

Table 4, shows the growth rate of white clover over two years, with measurements taken every 6 weeks. The growth rate of the second year is higher than the first year, which indicates that the white clover is adapting well to the environment. However, the growth rate in the final 6 weeks of the second year is

significantly higher than the previous measurements, which indicates an abnormal growth rate. This could be due to a variety of factors, such as weather conditions or nutrient availability. Further investigation is necessary to determine the cause of this abnormal growth rate.

Conclusions

Our study provides important insights into the nature of competition between willow and white clover, and the factors that influence the outcome of this competition. The results suggest that competition for resources such as water, light, and nutrients is a key driver of the dynamics of these two species and that this competition is influenced by a variety of environmental factors. The results suggest that careful management of soil fertility and harvest timing is necessary to optimize yields and that there may be a trade-off between yield and quality. Also, highlights the importance of weed management in maintaining the productivity and quality of white clover crops, and the need for effective weed management strategies to maintain animal health and performance.

The findings of this study may help to inform the development of sustainable and efficient forage systems for animal grazing.

Acknowledgments

This work appeared within the project: Program for increasing performance and innovation in doctoral and postdoctoral excellence research - PROINVENT, Contract no. 62487/03.06.2022 POCU/993/6/13 - SMIS code: 153299.

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OCCURRENCE OF VERO-TOXIGENIC PRODUCING *E. COLI* (VTEC) IN SOME COMMERCIAL LIVESTOCK FARMS IN KANO STATE, NIGERIA

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Summary

Vero-toxin-producing *E. coli* (VTEC) have gained increasing global concern as food-borne pathogens and are the only diarrhoeagenic *E. coli* pathogroup with an ascertained zoonotic origin, with ruminants being regarded as the main animal reservoir. This study aimed at determining the occurrence of VTEC in some commercial livestock farms in Kano State, Nigeria. A total of 240 samples were collected from the three Agro-climatic zones in Kano State with 80 samples per zone. The samples were processed in accordance with the International Standards Organisation reference method (ISO 16654) for the isolation of *E. coli* ,195 (81.3%) samples were suggestive of *E. coli* on Eosin Methylene Blue Agar (EMB). The Isolates were further screened biochemically [42 (17.5%) isolates are positive] and on CT Smac agar for selection of *E. coli* 0157 were 30 (12.5%) isolates were suggestive of *E. coli* 0157. These isolates were further screened using Latex Agglutination Test, where 24 (10%) isolates were confirmed to be *E coli* 0157. This study showed that Cattle from commercial livestock farms shed *E. coli* 0157 in their feaces. *E. coli* 0157 is widely distributed across commercial livestock farms.

Keywords: *E.coli*, vero-toxin, livestock, Nigeria.

Verocytotoxin-producing *Escherichia coli* (VTEC) are members of a set of pathogenic *Escherichia coli* strains and are significant food borne pathogen associated with serious disease outbreak globally (7, 13). The outbreak of VTEC was first reported in 1982 in U.S.A, from individuals with severe abdominal cramps and bloody diarrhea after being eating hamburgers in a food restaurant (18).Verocytotoxin-producing *Escherichia coli* are the major zoonotic food-borne organisms causing several illnesses in both humans and animals (8). And it has been associated with life threatening conditions among human such as haemolytic uremic syndrome (HUS), thrombotic thrombocytopenic purpura (TTP), and haemorrhagic colitis (HC) (10).

VTEC can be divided into sub-groups based on the presence of biochemicals called O antigens on the cell surface and further sub-divided into serotypes (also called serovars) based on the presence of different H flagella and K capsular antigens (19). The bacterium has the ability to produce a potent cytotoxins called Shiga toxins (*Stx*), which are the most virulence factor during pathogenesis of diseases caused by the VTEC (12). The *stx2* is more significant than *stx1* in terms of causing severe diseases in human and importantly linked with increased risk of haemolytic uremic syndrome in Verocytotoxin-producing *E. coli* infection (17).

Ruminants are the major source of VTEC with cattle as the principal reservoir (10, 12). Non ruminant animals such as pig and pigeons are also reported to becomes reservoirs of VTEC, and these may harbour and shed VTEC while asymptomatic (2). However, people are vulnerable to HUS after direct contact with faeces or consumption of animal products that are contaminated such as meat, milk and milk products (10).

Enterohaemorrhagic *Escherichia coli* (EHEC) is the most important subset of verotoxin producing *E. coli* (VTEC) and can cause a broad spectrum of clinical manifestations in humans ranging from asymptomatic infections to mild diarrhoea or haemorrhagic colitis which occasionally progresses to haemolytic uraemic syndrome (HUS), an important cause of acute renal failure in children and morbidity and mortality in adults (14). EHEC outbreaks are more often reported from industrialized countries than from developing countries, because surveillance and reporting systems are in place in industrialized countries and absent in most developing countries (3). There is also paucity of information regarding the epidemiology of *E. coli* O157:H7 in developing countries (1).

The study determined the occurrence of Vero-toxin producing *E. coli* (VTEC) in some commercial livestock farms in Kano State through cultural isolation, biochemical characterization and latex agglutination test from feacal sample. It also determined the occurrence of E. coli 0157 between different breeds and sex of animals in commercial livestock farms in Kano State.

Materials and methods

Study area

All samples were collected from within Kano state. Kano State is the commercial center of Northern Nigeria and is the second largest city in Nigeria after Lagos. According to the 2006 census, Kano is the most populous state in Nigeria, with about 9,383,682 million people. There are two distinct seasons; wet season (May - September) and dry season (October-April). The temperature ranges between 21- 39 0C (22). Annual rainfall ranges between 787 and 960mm (Kano State Agricultural and Rural Development Authority (21). It is situated in Sudan savanna zone within latitude 13053'N and 10025'N and longitude70 0'E and 10053'E. Farming is the main occupation of its people, who are predominantly Hausa/Fulani engaged in production of crops like millet, sorghum, maize, cowpea,

groundnut, pepper, onion, etc., and rearing animals like cattle, sheep, goat, poultry, etc. (9). KNARDA has divided Kano State into three administrative division called Agricultural Development Programme (ADP) zones namely, zone I, zone II and zone III.

Study Design

A cross sectional Study was utilized to collect feacal samples from some livestock farms within the State. The farms were selected using simple random sampling method from the three agricultural zones in the State namely Zone I (Rano Zone), Zone II (Danbatta Zone) & Zone III (Gaya Zone). A total of 240 samples were collected from the three Agro-climatic zones in Kano State with 80 samples per zone.

Systematic random method was used to obtain the samples from the animals on the farm with every fourth animal being selected. The faecal samples were taken from each of the sampling animals via rectal scooping of the faeces using a sterile polythene bag, and were transported to the Department of Veterinary Public Health and Preventive Medicine Laboratory, Ahmadu Bello University Zaria inside ice packed cool box for analysis.

Isolation and identification of *Escherichia coli*

The procedure was in accordance with the International Standards Organisation reference method (20) for isolation of *E. coli*.

Primary culture – 10g of the sample was suspended on to 90mls of 0.1% of peptone water and then homogenise. Then 10mls of the homogenised sample was inoculated on 90mls of tryptone soy broth then incubated at 37^{o}c for 24hrs for enrichment. A loopful of the overnight culture was streaked on Eosin Methylene Blue (Oxoid, U.K.) then incubated at 37^{o}c for 24hrs for the detection of *E. coli* through production of greenish metallic sheen colonies.

Secondary culture –The well- separated colonies from above were picked up and inoculated on nutrient agar (Oxoid, U.K.) slants and incubated at 37°C then stored at 4^{o}Cfor further identification.

Biochemical test

Colonies growing on nutrient agar slants were subjected to further biochemical tests namely; Simmon citrate, Urea, Triple Iron Sugar (TSI), sulfate, Indole, Motility (SIM), Methyl Red (MR), Vogesproskeur (VP). Various reactions of the tests such as color change, motility and gas formation were used to interpret results as either positive or negative after 24 hours incubation. These tests were carried out as described in the methodology of (6).

Positive isolates were also be further characterized using sugars; Glucose, Xylose, Lactose, Mannitol, Sorbitol and Sucrose.

Isolates that were identified biochemically as *E. coli* were further screened on Cefixime Tellurite Sorbitol MacConkay agar (CT-SMAC, Oxoid Basingstoke, UK) by incubating at 37^{o} C for 24 hours.

Latex agglutination test

Colonies that appeared colorless on CT-SMAC (nonsorbitol fermenters) were presumed to be *E. coli* O157 and were preserved on Nutrient agar slants for further confirmation using Latex agglutination test (pro-Lab Diagnostics, Richmond Hill, Canada).

Data Analysis

Data were presented in forms of charts and tables. SPSS version 20.0 was used where analysis such as chi-square (χ^2) was used to show association between categorical variables

Results and discussions

Out of the 240 samples ,195 (81.3%) of the isolates were suggestive of *E. coli on Eosine Methylene Blue (EMB) agar*. On further biochemical test 42 (17.5%) were suggestive of *E. coli*. Thirty isolates (12.5%) were suggestive of *E. coli* 0157 on CT smac. These isolates were further screened using Latex Agglutination Test where 24 (10%) isolates were confirmed to be *E coli* 0157: H7 (Table 1). These showed high occurrence of *E.coli* O157: H7 (10% of isolates). High occurrence of *E.coli* O157: H7 was also reported by (11) of 8.7% from healthy cattle in Spain. Similarly, In Egypt, (16) reported prevalence 6.7% from raw milk, rectal swabs from apparently healthy and diarrhoeic calves and stool samples of children. Also researchers such as, (4, 5) reported occurrence of 33.9% and 36.7% of VTEC respectively. VTEC shed in faeces of ruminants can contaminate the environment, water sources, and cause diarrhoeal related infections when this contaminated water is used as drinking water without treatment (15). Evidence has shown that contact with animal faeces is a risk factor for sporadic *E. coli* O157:H7 infection (9).

Table 1

Isolation of *E. coli* O157: H7

Test	Total number Sampled	Number Positive	%
EMB	240	195	81.3
Biochemical	240	42	17.5
CT Smac	240	30	12.5
Latex Agglutination	240	24	10.0

The prevalence of *E. coli* O157: H7 across the Agro climatic zones was 11.3% for Zone I & II, and 7.5% for Zone III. The relationship between the prevalence and location of the Zones o is not statistically significant (Table 2). Occurrence of

VTEC was higher in Zone I and Zone II. These are areas with more intensification of livestock farming, intervention programs and size capacity of than those of Zone III. This may pose hazard and increased risk of exposure to the human beings residing in the areas investigated.

Table 2

Distribution of *E. coli* O157: H7 across the Agro-climatic Zones

Zones	Total Sampled	Number Positive (%)
Zone I	80	9 (11.3%)
Zone II	80	9 (11.3%)
Zone III	80	6 (7.5%)
Total	240	24 (10%)

χ^2 = 0.833, df =2, p value =0.6590

The occurrence of *E. coli* O157: H7 among the various breeds was; White Fulani 10%, Bokolo 13.3%, Crosses 5.7% and Friesians 13.3%. But the relationship observed in this study was not statistically significant (Table 3). The occurrence was higher in Bokolo and Friesians than that of crosses and white Fulani. The Bokolo and Friesians are mostly kept in conventional and high capacity farms. By implication, there might be more chances of spread of the organism among the breeds on these farms.

The sex distribution of *E. coli* O157: H7 was observed as Males 7.9% and Females 25.7% and the differences between is statistically significant (Fig. 1)

The occurrence is higher in female animals than male animals. Since traditionally female animals are kept for longer periods than male animals, there **will be prolonged period of shading of this organism in the environment**

Table 3

Breed Distribution of *E. coli* O157: H7

Breed	Total Sampled	Number Positive (%)
White Fulani	80	8 (10%)
Bokolo	60	8 (13.3%)
Crosses	70	4 (5.7%)
Friesians	30	4 (13.3%)
Total	240	24 (10.0%)

χ^2 = 2.540, df =3, p value =0.4680

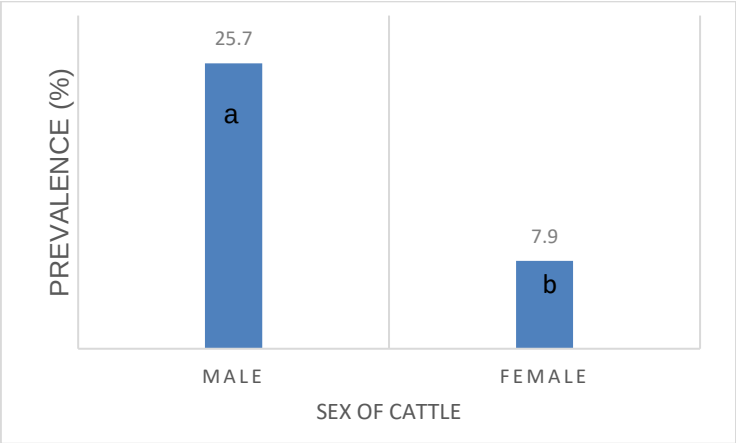

Fig. 1. Sex distribution of VTEC isolated from cattle faeces
a, b: p = 0.002

Conclusions

This study has established that Cattle from the commercial livestock farms in Kano state shed *E. coli* O157: H7 in their feces, *E. coli* O157: H7 is widely distributed across commercial livestock farm. There's is need for public enlightenment about preventive and control measures of spread of Pathogenic *E. coli* in farm and environment; Further studies are required to ascertain the genes of *E. coli* O157: H7 circulating on these farms.

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