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CONTENTS

ETIOLOGY OF REGENERATIVE ANEMIA IN DOGS AND CATS

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Abstract

The study was conducted between March 2020 – December 2022 on 129 dogs and 63 cats, different breeds and age, both males and females, clinically diagnosed with anemia. All individuals were subjected to a full hematology test including blood analysis and blood smear assessment where regenerative reaction was confirmed in 22 dogs and 9 cats.
To understand the ability of the hematogenous bone marrow to respond and develop new red blood cells (RBCs) and the effectiveness of the therapy is important to recognize the main causes of anemia that lead to the destruction, loss or deficit in production. The research highlighted post-hemorrhagic anemia as the most important cause of regenerative reaction in dogs (68,2%) while in cats, hemolytic anemia was the primary reason of reticulocytosis (55,6%).
Correctly identifying the type of anemia in terms of hematogenous bone marrow responsiveness is of utmost importance in tailoring the treatment, preventing complications or monitoring the progress of the patient and one of main tools used to assess and differentiate between the RBCs disorders

Key words: anemia, reticulocytes, dogs, cats, hemolysis

Regenerative anemia, characterized by a reduction in red blood cell mass with a concurrent increase in the production of immature erythrocytes, represents a common hematologic disorder in domesticated dogs and cats (Glogowska E. and Gallagher P.G., 2015). This condition, often associated with a range of underlying pathologies, poses a significant clinical challenge to veterinary practitioners and warrants comprehensive investigation to enhance diagnostic accuracy and refine therapeutic approaches. Despite its prevalence, a precise understanding of the multifactorial causes and contributing factors that drive regenerative anemia in these companion animals remains incomplete.

The pathogenesis of regenerative anemia is intricately linked to a dynamic interplay of factors encompassing both intrinsic and extrinsic components of erythropoiesis (Weiss D.J. *et al*., 2019). Intrinsic factors pertain to disruptions within the erythroid lineage itself, while extrinsic factors involve various diseases, toxins, or conditions affecting the bone marrow microenvironment or triggering anemia-inducing systemic responses (Glogowska E. and Gallagher P.G., 2015). Although several studies have endeavored to elucidate the mechanisms governing regenerative anemia in dogs and cats, there exists a need for a comprehensive synthesis of current knowledge and a critical evaluation of emerging research findings.

This article aims to address this knowledge gap by systematically reviewing and analyzing the main causes of regenerative anemia in dogs and cats. Furthermore, a nuanced understanding of these etiological factors is imperative for the development of targeted diagnostic protocols and therapeutic interventions, ultimately improving the clinical management of regenerative anemia in canine and feline populations

MATERIAL AND METHOD

The study was conducted over a 2-year period between March 2020-December 2022 at the Faculty of Veterinary Medicine in Iasi. The study was performed on 129 dogs and 63 cats, different breeds and age, both males and females. Clinical examination preceded hematological investigation for all cases and specifically diagnosed the included subjects with anemia. Hematology was performed using Abaxis Vetscan HM5 automated hematological analyzer. Blood samples were collected either from the external saphenous vein or the jugular vein. The vacutainers contained EDTA as anticoagulant substance and the samples were analyzed immediately after collecting them. For each case have been determined the following parameters: red blood cells (RBCs), packed cell

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volume (PCV), hemoglobin, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC). All parameters provide full assessment on red blood elements integrity.

For all the subjects a May Grunwald Giemsa stained blood smear was prepared to assess qualitative changes of the RBCs. Also, another blood smear stained by Brilliant Cresyl Blue method to quantitatively determine the reticulocytes within the smear.

RESULTS AND DISCUSSIONS

Regenerative anemia is a common hematologic disorder in domesticated dogs and cats, and understanding its etiology is crucial for effective diagnosis and treatment. The results of our study shed light on the primary causes of regenerative anemia in these two species.

Infectious diseases and immune-mediated processes represent significant contributors to regenerative anemia in both dogs and cats. The study revealed that infections, especially those caused by hemotropic pathogens like Mycoplasma spp. and feline leukemia virus (FeLV), played a substantial role in the etiology of regenerative anemia in cats (Hartmann K., 2011).

Hemolytic anemia emerged as the primary cause of regenerative anemia in cats, accounting for 55.6% of cases (table 1). Hemolytic anemia in cats can result from various underlying etiologies, including immune-mediated hemolysis, infectious diseases, and genetic predispositions (Giger U., 2020). The high proportion of hemolytic anemia in cats aligns with the feline predisposition to immune-mediated disorders, particularly immune-mediated hemolytic anemia (IMHA) (Weinkle T.K. *et al.*, 2008). IMHA is characterized by the destruction of RBCs by the immune system, leading to a regenerative response as the bone marrow attempts to replace the lost RBCs.

Feline leukemia virus (FeLV) and feline immunodeficiency virus (FIV) infections can also contribute to hemolytic anemia in cats (Hartmann, 2011). These retroviral infections can lead to immune suppression and associated opportunistic infections, as well as direct bone marrow suppression, impairing erythropoiesis and promoting the development of regenerative anemia.

Furthermore, certain breeds of cats may be genetically predisposed to hemolytic anemias. For example, Abyssinian cats have been reported to have a higher incidence of pyruvate kinase deficiency, an inherited enzyme deficiency that can lead to hemolytic anemia (Giger U., 2020). Understanding these genetic predispositions is vital for early diagnosis and management in affected breeds.

Table 1

Main causes of regenerative anemia in dogs and cats

Cause	Dogs		Cats	
	No. of cases	%	No. of cases	%
Acute post-hemorrhagic anemia	9	40.9%	2	22.2
Chronic post-hemorrhagic anemia	6	27.3	2	22.2
Hemolytic anemia	6	27.3	5	54.6
Unknown origin	1	0.5	0	0.0
Total subjects	**22**		**9**	

The substantial prevalence of post-hemorrhagic anemia in dogs found in this study aligns with previous reports in the veterinary literature (Brunori L. *et al.*, 2023). This form of anemia occurs due to acute blood loss resulting from trauma, surgery, or internal bleeding, leading to a rapid reduction in red blood cell (RBCs) mass. Dogs, as active and sometimes adventurous animals, are more susceptible to injuries and accidents that can result in significant blood loss. Additionally, certain breeds with a predisposition to bleeding disorders, such as Doberman Pinschers and Greyhounds, may contribute to the high incidence of post-hemorrhagic anemia (Dow S.W. *et al.*, 2016).

The regenerative response observed in dogs with post-hemorrhagic anemia can be attributed to the erythropoietic compensatory mechanism within the bone marrow. Erythropoietin, a hormone produced by the kidney in response to hypoxia, stimulates the production of RBCs precursors (reticulocytes) to compensate for the acute loss of mature RBCs (Glogowska E. and Gallagher P.G., 2015). This compensatory mechanism is reflected in the elevated reticulocyte count often observed in these cases (Weiss D.J. *et al*., 2019).

The results for the reticulocytes count in dogs with acute and chronic post-hemorrhagic anemia reveal higher numbers for the acute processes compared to the chronic ones due to the ability of the hematogenous bone marrow to initially compensate the RBCs loss.

The observed difference in the main causes of regenerative anemia between dogs and cats may be attributed to several factors, including species-specific vulnerabilities and environmental influences. Dogs, as more active animals with a higher likelihood of trauma-related injuries, may naturally be at greater risk of post-hemorrhagic anemia (Dow S.W. *et al.*, 2016). In contrast, cats may be more susceptible to immune-mediated disorders due to their known predisposition and the potential influence of infectious diseases like FeLV and FIV (Hartmann K., 2011).

It is worth noting that advances in diagnostic techniques and increased awareness among veterinary practitioners may also contribute to variations in the reported causes of regenerative anemia over time. Improved diagnostic capabilities, including serological tests for infectious diseases and genetic screening for breed-specific disorders, enable more accurate identification of underlying etiologies (Malancus R., 2019).

Understanding the predominant causes of regenerative anemia in dogs and cats has critical clinical implications. Veterinarians should consider these findings when evaluating and diagnosing anemic patients, as they can guide initial diagnostic tests and treatment strategies.

CONCLUSIONS

The present study provides valuable insights into the primary causes of regenerative anemia in dogs and cats. Post-hemorrhagic anemia prevails in dogs, likely due to their propensity for injuries and accidents, while hemolytic anemia is the leading cause in cats, influenced by their predisposition to immune-mediated disorders and retroviral infections. Recognizing these differences in etiology is essential for veterinary practitioners to enhance their diagnostic accuracy and tailor treatment strategies to effectively manage regenerative anemia in these companion animals.

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FACTORIAL ANALYSIS OF SOME INDICATORS IN CHRONIC OSTEO-ATICULAR MODEL OF RABBITS

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Abstract

The using the of animals in the procedure of research project are strictly regulated by EU and Romanian low. Currently, the assessment of pain, suffering or distress in animals used in procedures is based on the physiological responses and behavioral changes that the animal exhibits. In long time models is better to take into consideration more quantifiable variables. The aim of the paper was to study some variables such us body mass, feed conversion, average daily gain, feed intake or feed rests associated with chronic osteo-articular rabbit model (OA) in rabbits. A number of 30 (3-31/2 month old) rabbits in 4 groups (non OA, OC-control, OA-treatment 1 and OA-treatment 2) where observed for 8 weeks period. By the trial period, the initial ($F=14.648$ at $P<0.000$) and final body weight ($F=17.141$ at $P<0.000$) and average daily gain ($F=3.596$ at $P=0.029$) were associated with the OA, also group x weight interactions [$F= 2.692$ at $p = 0.026$] was found. The main effect of time was statistically significant ($F=11.210$ at $p=0.000$) on ADG and the interaction group x time was also effective ($F=2.244$ at $p=0.009$); the interaction was also significant for interaction group x feed consumption ($F= 2.325$ at $p = 0.004$). Generally, the results of the study were clearly influenced by treatments and sometimes by the environmental conditions and the interactions between factors in a multivariate analysis but repeated measuring of body mass (weekly) is enough for following the welfare of rabbits in chronically OA animal models.

Key words: osteo-articular, rabbit model, (OA) body mass.

The using of the animals in procedures of research project are strictly regulated by EU under Directive 2010/63/EU on the protection of animals used for scientific purposes, national rules (Low 43 / 2014 regarding the protection of animals used for scientific purposes and ANSVSA order no. 97/2015 for the approval of the Veterinary Sanitary Norm regarding the veterinary sanitary authorization procedure of units that use, breed and supply animals used for scientific purposes, for the approval of the Veterinary Sanitary Norm regarding the veterinary sanitary authorization procedure of projects involving the use of animals in procedures).

The Experimental Units of *Horia Cernescu* Research Unit are a authorised research infrastructure for using animal in the procedure of research projects under FELASA recommendations, SOPs, clinical observation or Welfare Committee controls together with principal investigator. Currently, the assessment of pain, suffering or distress in animals used in procedures is based on the physiological responses and behavioral changes that the animal exhibits. *(Hutu, 2018; Mota-Rojas et al, 2020, Benato et, al 2019).* Beside all of the SOP's, pain score charts, in long period procedures, some associated variables with less suffering have to be use.

The aim of paper is to study some variables (body mass, feed conversion, average daily gain, feed intake or feed rests) associated with chronic osteo-articular animal model (OA) in order to quantify the effects of time, treatments and housing on animals or to take the decision of end point of study in an irrefutable way for the study and principal investigator *(Yoshioka et al, 1996, Pelletier et al, 2015).*

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MATERIAL AND METHODS

A number of 30 New Zealand micro-chipped females rabbits, aged 3 - 31/2 months, were used in a osteoarthritis study. The study took place over a period of 8 weeks in the Experimental Units of the University of Life Science "King Michel I" from Timisoara under the Ethical Statement no. 87 07.05.2018 and Project authorization no. 002 25.06.2018.

The animals were divided into 4 groups: A non OA (3 rabbits), B –control OA (no treatments-9 rabbits), C – OA- treatment 1 (9 rabbits) and D – OA-treatment 2 (9 rabbits). After the accommodation period, in the first week of study the animal model was performed, followed in 2nd week by first intra-articular treatment and in 7th week by last treatment.

During the trial, the clinical signs, telemetry temperature *(Huțu et al, 2018)*, pain scoring *(Miller et. al 2022),* the pressure of legs was strictly monitored.

The rabbits were kept individual in four different types of cages (LxlxH): standard (S) cages (713×716×476 mm, Techniplast®) with plastic floor with holes, cats (C) and dog (D) stainless steel cages (1490x640x1580 mm) with steel floor with holes and Guinea Pig (GP) doubled cages (846×610 ×256+256, Techniplast®) with plastic floor with square holes, in three rooms: rabbits room (14.69 m^3) , guinea pig room (10.52 m^3) and rats room (11.35 m^3) .

The environment temperature and humidity were continuously monitored (every half an hour) by multi-functional wireless digital device Weather Station PCE FWS 20. The lighting program was 14 hours light /10 hours dark.

Each rabbit received daily 160 g of pelleted feed (*Davidson, 1975)* and water *ad libitum*. The fodder residues were weighed every week, on the same day being noted for each individual animal. The actual intake was calculated by multiplying the amount of feed administered daily (160 g) by 7 (days of the week), from which the remaining amount of feed was subtracted

To calculate the average daily gain (ADG), the rabbits were weighed every week *(Zawislak et al. 2015).*

The initial weight was subtracted from the final weight and thus the total week gain was obtained – for ADG, the total gain was divided by 7. The weekly feed consumption was established after eliminating the unconsumed feed from weekly consumption (regularly, 160 g intake x 7 days). The feed efficiency was calculated by dividing the feed consumption to body mass from each week during the trial period.

The statistical tests used were: ANOVA, *t*-test, GLM Analisys (Test of Equality of Covariance, Mauchly's Test, followed by Greenhouse-Geisser and / Huynh-Feldt) with reapeted measures using *SPSS Statistics for Windows, Version 17.0. (Chicago: SPSS Inc. USA).* A P-value of <0.05 was considered to be statistically significant.

RESULTS AND DISCUSSIONS

The treatments of the groups A-D, have had impact on the variables such as body mass (initial mass F=7.105 at P=0.000 and final F=7.790 at P=0.000), ADG (F=2.880 at P=0.037), rest of feed (F=3.277 at P=0.022) and feed consumption (F=6.169 at P=0.000).

The body mass was higher in group B (OA model without treatment - 3472.64±32.47g) and A (rabbits without OA - 3367.50±91.36 g) and lower in groups with treatments; C (3255±28.45 g) and D (3289.29±39.78 g). Also, the ADG was higher in group A (95.04±21.26) and B (54.03±11.18 g) and lower in groups C (50.69±11.18 g) and D (19.01±18.15 g).

During the trial period, which lasted for 8 weeks, the initial (F=14.648 at P<0.000) and final body weight (F=17.141 at P<0.000) and average daily gain (F=3.596 at P=0.029) were associated with the OA. The body mass had an increasing trend between initial 3,165.33±43.85 g to 3,690±43.01 g and final mass in week 8. The interactions of treatments (groups) with body mass, during 8 weeks (*Figure 1* by GLM method), was significant. The Mauchly's Test demonstrated that the sphericity assumption was not met (p=0,000). The repeated measure with Greenhouse-Geisser method of correction indicated that there were significant differences during the trial in weight [F= 105.077 at p = 0.000] and lot x weight interactions [F= 2.692 at p = 0.026].

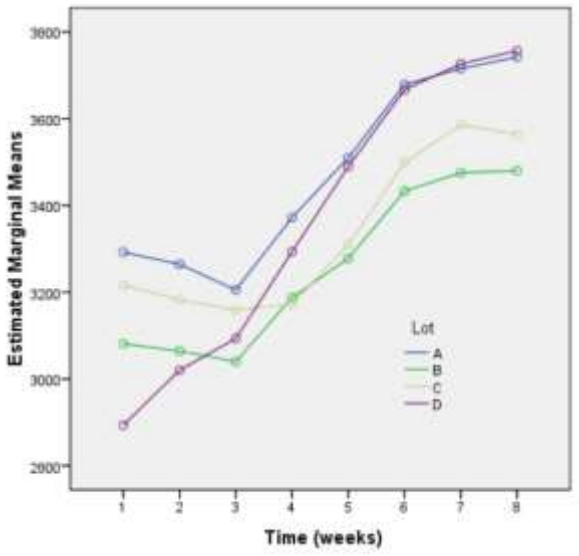

Figure 1. **Estimated marginal means of weight**

For entire study period the ADG was 46.97±7.55. The ADG was negative in the first two

weeks *(Figure 2).* In the first week the OA model was perform and ADG was -7.30±17.74 g and in the second week the first intra-articular injection was perform and ADG was -25±15.83 g. From the third week it starts to grow (109.33±24.99 g in 3th week, 101.67±31.55 g in the 4th week, in 5th week, 58.73±22,152 g, in week 6 it was 54.83±12,354 g and 76.90±9.90 g in week 8). In the 7th week, the week of second intra-articular treatment the ADG was 6.55±12.47 g. The Box's test of equality of covariance indicates that the assumptions of homogeneity of covariance was met (p=0.103). The multivariate test demonstrated that the main effect of time was statistically significant Wilks' Lambda = 0.195, F(7,19)=11.210 at p=0.000 on ADG. This effect was qualified by any time x lot interactions, Wilks' Lambda = 0.170, F(21,55.1)=2.244 at p=0.009.

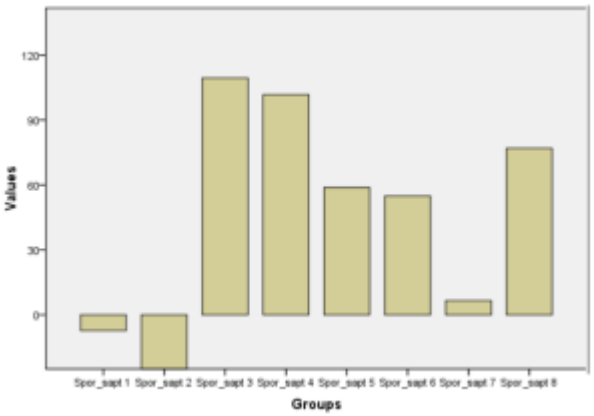

Figure 2. **Average daily gain distribution**

The cage type was associated with the body mass (F=9.716 at P=0.000 for initial body mass and F=11.315 at P=0.029 for final mass) and feed consumption (F=14.589 at P=0.000); the plastic floors of cages increased the food consumption (1178.06±19.28 g of pellets for S cages and 1248.33±28.36 g of pellets for GP cages).

Weekly feed consumption follows the same distribution like body mass: it was higher in group A (1248.33±28.36g) and B (1178.06±19.28 g) and lower in groups C (1135.42±17.01 g) and D (1087.68±26.63 g). The Mauchly's Test demonstrated that the sphericity assumption was not met (p=0,000). The repeated measure with Huynh-Feldt method (epsilon 0.776) of correction indicated that there were not significant differences in feed consumption during the 8 weeks of trial [F= 1236 at p = 0.294] but the interaction lot x feed consumption was significant [F= 2.325 at p = 0.004].

The rest of the feed accumulated in a week period was lowest in group A (-56.67±13.24 g) and highest in group D (-104.49±12.554 g) but the study was not powerful enough to find the significant factors associated.

One of the rabbits had to be euthanatized because of dramatically losses of body weight. In absence of any clinical signs of illness / Welfare Committee recommendation, when the body weight start do decrease, the lower action limit (LAL) by body weight was proposed and established at X±1.96x√2xSD for two consecutive weeks (measurements). After the necropsy we conclude that the peritonitis was the main cause of the death; the cause was not associated with the treatments which were intra-articular injections of tested products.

Generally, the results of the study were clearly influenced by treatments and sometimes by the environmental conditions and the interactions between factors in a multivariate analysis. Future statistics are need for establishing the end point of the study/animals for the cases which are outside of the limits in order to help the decision of Welfare Committee or the principal investigator.

CONCLUSIONS

Variation of the body mass is a good indicator reflecting the quality of life of animals in OA models. By the repeated measure design, the effect of time can be easily observed. Performing multivariate analysis, the effects of treatments or other variables can be measured in order to take the correct decision for welfare of animals.

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RESEARCH REGARDING THE RESISTANCE PHENOTYPES OF BACTERIA ISOLATED FROM DOGS WITH RESPIRATORY TRACT INFECTIONS

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Abstract

The resistance phenotypes to animal pathogenic bacteria (both Gram-positive and Gram-negative bacteria) are increasing in frequency due to the use of antibiotic-based veterinary medicinal products in both in farm animals and pets. The research aimed to establish phenotypically the antibiotic resistance in bacterial strains isolated from dogs with various respiratory tract infections. Both susceptible Gram-positive and Gram-negative isolated strains had the highest frequency to enrofloxacin (82.45% and 81.81%). Gram-positive resistant strains had the highest frequency to penicillin G (70.18%), while Gram-negative strains had the highest frequency of resistance to lincomycin. The results confirm the marked increase of resistance phenotypes in both Gram-positive and Gram-negative strains to a wide range of antimicrobial substances, frequently used in the therapy of infectious diseases in dogs.

Key words: bacteria, dogs, resistance phenotypes, respiratory tract

Antimicrobial resistance in bacteria represents a very topical problem in veterinary and human medicine, since is considered a phenomenon with pronounced zoonotic risk. The resistance phenotypes to animal pathogenic bacteria (both Gram-positive and Gram-negative bacteria) are increasing in frequency due to the use of antibiotic-based veterinary medicinal products in both in farm animals and pets (Aarestrup, F., M., 2006; Guardabassi, L., Courvalin, P., 2006; Markey, B., *et al*, 2013; Riedel, S., *et al*, 2019; Schwarz, S., *et al*, 2006, Viţălaru, A., B., 2020).

The expansion of multiple antibiotic resistance, in bacterial species pathogenic for animals and humans, led to extensive phenotypic and genotypic studies to clarify, as deeply this phenomenon. Thus, it was demonstrated that antibiotic resistance is genetically encoded, supported by many resistance genes present in the bacterial chromosome and in mobile genetic elements (plasmid R, intergons, transposons). Through it, the genes can be transferred between strains of the same bacterial species (intraspecific transmission), as well as between strains belonging to other bacterial species, respectively interspecific transmission (Aarestrup F., M., 2006; Arber, W., 2014; Guardabassi, L., Courvalin, P., 2006; Schwarz, S., *et al*, 2006).

In recent years, the antibiotic resistance of a large number of bacterial germs has become a global threat to public health. Among the bacteria that represent the greatest threat to human health, due to the increase in antibiotic resistance, there are several Gram-positive bacteria, such as *Staphylococcaceae* or *Streptococcaceae* family, as well as Gram-negative bacteria, included in *Enterobacteriaceae* family, especially *Escherichia coli*, *Salmonella* spp. or *Klebsiella* spp. (Cummings, K., *et al*, 2015; Guardabassi, L., Courvalin, P., 2006; Li, Y., *et al*, 2021; Schwarz, S., *et al*, 2006; Mavrides, D. E., *et al*, 2021).

The research aimed to establish phenotypically the antibiotic resistance in bacterial strains isolated from dogs with various respiratory tract infections.

MATERIAL AND METHOD

The samples with pathological material were obtained from dogs with various diseases of the respiratory tract (cough, tachypnea, dyspnea or various secretions), and the sampling was carried out before starting the therapy with antimicrobial substances, or in case it was started, 48 hours after its interruption. Therefore, a total of 70 samples were collected from dogs.

For the isolation of primary cultures, samples with pathological material were inoculated in nutrient broth and incubated at a temperature of 37°C, under aerobic conditions, for 18-20 hours. Next, to identify the bacterial species, inoculations were made on CHROMagar Orientation medium, a

chromogenic, non-selective culture medium used for the direct qualitative detection of some pathogenic bacteria.

After incubation, the plates were examined, respectively the cultural characters of the colonies were observed, and the genera and bacterial species were preliminarily identified. To confirm the isolated colonies on this medium, inoculations were made on other media, such as Chapman, 7% defibrinated sheep blood agar, Levine, acetamide agar and MacConkey.

Gram-stained smears were made from the characteristic isolated colonies, to examine the morphological characters and confirm the genera or species. Subsequently, inoculations were made on 7% defibrinated sheep blood agar to obtain fresh strains in pure cultures, used for the susceptibility testing to antimicrobial substances.

All isolated and identified bacterial strains were tested for susceptibility to the following antimicrobial substances: aminoglycosides - streptomycin (S), kanamycin (K), gentamicin (GM); *β*- lactams - ampicillin (AMP), amoxicillin with clavulanic acid (AMC), penicillin G (P); cephalosporins - cefadroxil (CDX), cephalexin (CN), cefquinome (CFQ); phenicols - chloramphenicol (C); lincosamides - lincomycin (L); macrolides - clindamycin (CD), erythromycin (E); quinolone - enrofloxacin (ENR); sulfonamides - trimethoprim/sulfamethoxazole (SXT); tetracyclines - tetracycline (TE), doxycycline (DO).

These 17 antimicrobial substances, used to determine the resistance profile, were chosen according to: form and mode of administration, therapeutic characteristics and effectiveness, degree of absorption, as well as availability.

The susceptibility testing was done with the disk-diffusimetric method (Kirby-Bauer method) using the Mueller-Hinton medium, respectively biodiscs with the antimicrobial substances mentioned above, kept tightly closed and refrigerated at 4-8˚C. Thus, a total number of 90 isolated strains, included 8 bacterial species, were tested.

Comparisons between the prevalence of antibiotic resistance Gram positive and negative strains were performed using the Chi-square test at a level of significance set at $p<0.05$.

RESULTS AND DISCUSSIONS

From the primary cultures, obtained in nutrient broth, inoculations were made, with the bacteriological loop, by dispersion on the CHROMagar Orientation medium. A total of 90 bacterial strains (from a total of 60 positive samples) and a number of 10 sterile samples were isolated. Thus, the strains could be classified into three Gram positive genera (*Enterococcus*, *Staphylococcus* and *Streptococcus*), respectively into four Gram negative genera (*Escherichia*, *Klebsiella*, *Proteus* and *Pseudomonas*).

To confirm the species, inoculations were carried out on special media mentioned previously, and after smears stained by the Gram method. The results showed germs with shape and characteristic grouping of each species, stained Gram positive, respectively Gram negative.

Thus, based on the bacteriological and bacterioscopic examinations carried out, respectively based on the morphological and cultural characters developed by the inoculated bacteria, 90 bacterial strains were isolated, classified into 8 species (*table 1*).

Table 1

Bacterial species isolated from dogs

Crt. no	Sample	Identified bacterial species	No. of strains	
			No.	%
	Pharyngeal exudate	**Gram positive species**		
1.		*Enterococcus spp.*	20	22.23
2.		*S. aureus*	26	28.89
3.		*Staphylococcus spp.*	8	8.88
4.		*Streptococcus spp.*	3	3.33
		Gram negative species		
5.		*E. coli*	24	26.67
6.		*Klebsiella spp.*	5	5.55
7.		*Proteus spp.*	2	2.22
8.		*P. aeruginosa*	2	2.22
TOTAL			**90**	**100**

According to results, 90 bacterial strains were isolated from the pathological material samples taken from the pharyngeal exudate of dogs. Thus, a total number of 57 Gram positive species were isolated, namely 20 strains of *Enterococcus* spp., 26 strains of *S. aureus*, 8 strains of *Staphylococcus spp.*, 3 strains of *Streptococcus* spp. Gram negative species were isolated in a number of 33 strains, respectively 24 strains of *E.*

coli, 5 strains of *Klebsiella* spp., two strains of *Proteus* spp. and two strains of *P. aeruginosa*.

The results obtained regarding the susceptibility testing to antimicrobial substances were done according to Gram positive and Gram negative bacterial species, but also according to the class of antibiotics.

Thirteen antimicrobial substances for Gram positive strains and fourteen antimicrobial substances for Gram negative strains, from several classes, were used to identify the resistance phenotypes.

In case of isolated Gram positive species, the results obtained revealed that sensitive strains had a frequency between 29.82% for penicillin G and 82.45% in the case of enrofloxacin. The resistant strains had a frequency between 17.55% for enrofloxacin and 70.18% for penicillin G. All the interpretations were made according to EUCAST 2022 recommendations (*table 2*).

Table 2

The results obtained regarding the susceptibility testing of Gram positive strains

Antibiotic	C (µg)	No. of strains (57)			
		S		R	
		No.	%	No.	%
Kanamycin	30	33	57.89	24	42.11
Gentamicin	10	29	50.87	28	49.13
Ampicillin	10	25	43.86	32	56.14
Amoxicillin + clavulanic acid	20-10	24	42.11	33	57.89
Penicillin G	6	17	29.82	40	70.18
Cephalexin	30	34	59.65	23	40.35
Cefadroxil	30	35	61.40	22	38.60
Chloramphenicol	30	34	59.65	23	40.35
Clindamycin	2	21	36.84	36	63.16
Erythromycin	15	30	52.63	27	47.37
Enrofloxacin	5	47	82.45	10	17.55
Tetracycline	30	31	54.38	26	45.62
Doxycycline	30	31	54.38	26	45.62

Legend: C = concentration S = sensitive strains; R = resistant strains

According to the class of antimicrobial substances, for the group of Gram-positive strains, the results were different. Thus, from the **aminoglycosides** group, the antibiotic resistance testing was made for kanamycin and gentamicin. The resistance of the isolated strains had a similar frequency to the two selected antimicrobial substances, respectively 49.13% to gentamicin and 42.11% to kanamycin.

In case of **β-lactams**, for the isolated Gram-positive bacteria were selected the most antibiotics, to which the resistance phenotypes were determined, namely ampicillin, amoxicillin with clavulanic acid and penicillin G, considering that β-lactams are recommended in the treatment of infections caused by both Gram-positive and Gram-negative bacteria.

Therefore, antibiotic resistance had the highest frequency to penicillin G (70.18%), followed by amoxicillin with clavulanic acid (57.89%) and ampicillin 56.14%. Regarding the frequency of susceptible strains, the highest frequency was for the strains susceptible to ampicillin (43.86%), followed by amoxicillin with clavulanic acid (42.11%) and penicillin G (29.82 %).

From the **cephalosporins** group, two antimicrobial substances, namely cephalexin and cefadroxil, were selected for the identification of resistance phenotypes. Following the results, was observed that the frequency of resistant strains was higher to cephalexin (40.35%) than to cefadroxil (38.60%), but lower than that of sensitive strains to both antimicrobial substances.

In case of **phenicols** group, where resistance phenotypes were made only to chloramphenicol, it was observed that the frequency of antibiotic sensitivity was higher than that of antibiotic resistance.

Two antibiotics were selected from the **macrolide** category, namely erythromycin (indicating the inducible resistance to 14-atom macrolides) and clindamycin (indicating the inducible resistance to 16-atom macrolides). Of the total number of Gram positive strains isolated, the resistant strains had a frequency of 63.16% to clindamycin and 47.37% to erythromycin.

The antibiotic resistance to the **quinolone** group, was done only for enrofloxacin Thus, analyzing the results obtained, was observed that the frequency of Gram positive strains sensitive to

enrofloxacin was the highest, respectively 72.09%, which suggests a reduced use of this antimicrobial substance in the therapy of infections in dogs.

The resistance phenotypes to **tetracycline** antibiotics was established for two antimicrobial substances, namely tetracycline and doxycycline. Therefore, the results revealed the same frequency, in the case of the two antimicrobial substances, the frequency of sensitive strains (54.38%) being slightly higher than that of resistant strains (45.62%).

For the isolated Gram negative strains, the resistance phenotyopes were establised for 14 antimicrobial substances, from nine classes, and the frequency of resistant strains was between 18.19% for enrofloxacin and 81.81% for lincomycin (*table 3*).

Table 3

The results obtained regarding the susceptibility testing of Gram negative strains

Antibiotic	C (µg)	No. of strains (33)			
		S		R	
		Nr.	%	Nr.	%
Streptomycin	10	9	27.27	24	72.72
Gentamicin	10	20	60.61	13	39.39
Ampicillin	10	11	33.33	22	66.67
Amoxicillin + clavulanic acid	20-10	14	42.42	19	57.58
Penicillin G	6	9	27.27	24	72.72
Cephalexin	30	23	69.70	10	30.30
Cefquinome	30	25	75.75	8	24.25
Chloramphenicol	30	21	63.63	12	36.37
Lincomycin	15	6	18.18	27	81.82
Clindamycin	2	10	30.30	23	69.70
Erythromycin	15	16	48.48	17	51.52
Enrofloxacin	5	27	81.81	6	18.19
Doxycycline	30	14	42.42	19	57.58
Trimethoprim/ Sulfamethoxazole	30	12	36.36	21	63.64

Legend: C = concentration S = sensitive strains; R = resistant strains

From the group of **aminoglycosides**, the identification of resistance phenotypes was made for streptomycin and gentamicin and the results were the following: the frequency of resistant Gram negative strains was higher for streptomycin (72.72%) compared to gentamicin (39.39%), which suggests greater use of this antimicrobial substance in infections caused by Gram-negative bacteria.

In case of the **β-lactams** group, the same three antimicrobial substances were also selected for testing the Gram-negative strains. Thus, compared to the resistant Gram positive strains, the Gram negative ones had the highest frequency against penicillin G (72.72%), which indicates the wide use of this antimicrobial substance in the therapy of infections in dogs, regardless of the category of bacteria that produced those infections. However, the frequency of resistant Gram negative strains was made to ampicillin (66.67%), respectively to amoxicillin with clavulanic acid (57.58%).

For the isolated Gram negative strains, from the **cephalosporins** group, two antimicrobial substances were selected, namely cephalexin and cefquinome, which had a relatively close frequency of resistant strains (30.30% to cephalexin and 24.25% to cefquinome), but much lower, compared to the frequency of strains sensitive to these two antibiotics.

In the case of the **phenicols** group, resistance phenotypes were made only to chloramphenicol, where the frequency of susceptible strains was higher (63.63%) than that of resistant strains (36.37%).

Also, in the case of the **lincosamide** group, testing the antibiotic resistance was done only to lincomycin, the obtained results indicating the highest frequency of resistant Gram negative strains and, therefore, a low frequency of sensitive strains.

From the **macrolides** category, the same two antibiotics, erythromycin and clindamycin, were selected, also used for the identification of resistance phenotypes of Gram positive strains. However, the Gram-negative strains had a higher frequency of antibiotic resistance to clindamycin (69.70%) compared to erythromycin, where the frequency was 51.52%.

As in the case of Gram positive strains, the sensitive Gram negative strains also had the highest frequency of 81.81% to enrofloxacin, the antimicrobial substance used from the **quinolone** group, while the resistant strains had a frequency of only 18.19%.

The resistance phenotypes for the Gram negative strains, from the **tetracycline** group, were established for doxycycline and had a higher frequency than that of sensitive phenotypes towards this antibiotic.

From the **sulfonamides** group, the trimethoprim with sulfamethoxazole was selected, for the identification of resistance phenotypes, with the following values: a frequency of 63.64% for the resistant Gram negative strains, respectively a frequency of 36.36% in the case of sensitive strains.

Statiscally, was noticed that there is no association ($p>0.05$) between Gram positive strains, respectively Gram negative strains and the behavior towards antibiotics that were common to the two categories of isolated strains: GM X^2 (1, N=90) = 0.79, AMP X^2 (1, N=90) = 0.96, AMC X^2 (1, N=90) = 0.0009, P X^2 (1, N=90) = 0.0003, CN X^2 (1, N=90) = 0.90, C X^2 (1, N=90) = 0.13, CD X^2 (1, N=90) = 0.39, E X^2 (1, N=90) = 0.14, ENR X^2 (1, N=90) = 0.005 and DO X^2 (1, N=90) = 1.19.

Multi-resistant strains have an increasing frequency and the identification of these resistance phenotypes to the antibiotics used in therapy is a very important aspect, as it indicates the continuous expansion of this phenomenon through the two-way animal-human epidemiological circuit. Thus, numerous research teams focus on the identification of multi-resistant strains, both Gram positive and Gram negative, as well as their portage from animal to human and vice versa (Bertelloni, F., *et al,* 2021; Marchetti, L., *et al*, 2021*;* Moon, D.-C., *et al*, 2022; Murray, A., K., *et al,* 2019).

For example, the study by Roca L. et al. aimed to determine the antibiotic resistance in strains of pathogenic bacteria isolated from dogs. Thus, the susceptibility to antibiotics of the isolated strains was determined by the disc-diffusimetric method, on a total number of 81 bacterial strains, the most common species being *Staphylococcus intermedius, Pseudomonas aeruginosa* and *Escherichia coli*. The results indicated the resistance of these species to some antibiotics, respectively: *S. intermedius* was resistant to trimethoprim/sulfamethoxazole (31%) and enrofloxacin (23%), *P. aeruginosa* was resistant to cephalexin (86%) and clindamycin (76 %) and *E.coli* was resistant to clindamycin (78%) and trimethoprim/sulfamethoxazole (75%). Therefore, Gram-negative species demonstrated the highest frequencies of resistance (*Roca, L., et al, 2017).*

A study done by Pedersen K. et al., on samples from dogs, provided data on the emergence of antibiotic resistance in important pathogens. Resistance to cephalosporins and amoxicillin with clavulanic acid was decreased for almost all bacterial species examined, except for *P. aeruginosa*. Of the isolated *S. intermedius* samples, 60.2% were resistant to penicillin, 30.2% to fusidic acid and 27.9% to macrolides and in *E. coli* samples, the highest resistance was reported to ampicillin, sulfonamide, tetracyclines and streptomycin (Pedersen, K., *et al*, 2007).

Daodu O. B. et al. investigated the antibiotic resistance profile of 41 strains of *E. coli* from 173 samples collected from the respiratory tract of clinically healthy dogs. Thus, antibiotic resistance had the following values: amoxicillin with a frequency of 53.7% of resistant strains, chloramphenicol with a frequency of 22%, respectively gentamicin with a frequency of 29.3%. Likewise, on another study, on the antibiotic susceptibility of bacteria isolated from 502 dogs with respiratory symptoms, , Rheinwald, M. et al. identified *E. coli* strains with an antibiotic resistance to enrofloxacin (72.5%), to gentamicin (70%), to cephalexin (50.06%), to amoxicillin/clavulanic acid (39.1%), to trimethoprim/sulfomethoxazole (47.5%), to doxycycline (27.5%), respectively to ampicillin (32.4%) (Daodu, O., B., *et al*, 2016; Rheinwald, M., *et al*, 2014).

In the research carried out by Qekwana D. N. et al., on 157 dogs with lower respiratory tract infections, the authors identified 162 bacterial strains. Almost all isolated strains (99.5%) showed resistance to at least one antibiotic and 64.7% were multi-resistant, with resistance to penicillin G (90.9%), lincomycin (100%), tylosin (75.8%), lincospectin (73.7%), ampicillin (72.5%) and kanamycin (68.4%) (QEKWANA, D. N., *et al*, 2020).

Therefore, the results obtained regarding the resistance phenotypes to Gram positive and Gram negative strains isolated from dogs with different respiratory tract diseases underline the importance of identifying these strains, which may have a zoonotic character. Thus, pets can act as a real microbial reservoir for humans, especially their owners, but also vice versa, from humans to pets, demonstrating this complex epidemiological circuit existing in both Gram-positive and Gram negative bacterial species.

CONCLUSIONS

Both susceptible Gram-positive and Gram-negative isolated strains had the highest frequency to enrofloxacin (82.45% and 81.81%).

Gram-positive resistant strains had the highest frequency to penicillin G (70.18%), while

Gram-negative strains had the highest frequency of resistance to lincomycin.

The results confirm the marked increase of resistance phenotypes in both Gram-positive and Gram-negative strains to a wide range of antimicrobial substances, frequently used in the therapy of infectious diseases in dogs.

In conclusion, the abusive use of antimicrobial substances cannot be recommended for the treatment of the most common respiratory tract infections in dogs, and their selection must be based on the results of susceptibility tests.

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SEROEPIDEMIOLOGICAL AND ANATOMOPATHOLOGICAL INVESTIGATIONS ON FELINE PANLEUKOPENIA IN NORTHEASTERN ROMANIA

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Abstract

The objectives of this study are as follows: to carry out seroepidemiological and hematological investigations on feline parvovirus infections in the north-eastern region of România and, on the other hand, to study the clinical and anatomopathological aspects in parvovirus infections in cats, following the prevalence of feline paleukopenia cases in the counties of Suceava, Botoşani, Iaşi and Vaslui, in România. To carry out the study, clinical examination, rapid commercial immunochromatography tests for the detection of panleukopenia virus antigen from feces, blood count and monitoring of cats infected with parvovirus in veterinary offices, as well as necropsy for deceased animals were used.

Key words: Feline panleukopenia virus, epidemiology, hematological results, feline parvovirus, symptoms

Introduction.

Feline infectious panleukopenia, also known as "cat parvovirus", "infectious leukopenia of cats", "infectious gastroenteritis of cats", "typhus of cats", is an infectious-contagious disease, of virotic epidemic nature encountered in felines, clinically characterized by fever syndrome, serious digestive disorders, accompanied by leukopenia, anorexia, depression, prostration and anatomopathologically by catarrhal-hemorgic gastroenteritis lesions. (Perianu Tudor *et al*., 2012).

According to the literature, the incubation period is 2-10 days, with an average of 4-6 days. The disease begins with a fever syndrome, accompanied by gastroenteric manifestations. Depending on how quickly and how seriously it evolves.

The symptomatology of the disease can be classified into four clinical forms: hyperacute, acute, subacute and atypical (Perianu Tudor *et al*., 2012). The infection is widespread in many countries around the world, in Romania being first reported in 1933, when it evolved as a serious epizootic that caused the death of a large number of cats, especially in Bucharest. The importance of the disease lies in contagiousness, high percentage of morbidity and mortality. (Perianu Tudor et al., 2012).

In this study 210 cats were considered that were presented in Veterinary Clinics and Practices in both urban and rural areas from the north-eastern region of România, more specifically, in Iaşi, Botoşani, Vaslui, Suceava counties, between 2021 and 2023 *(Table 1).*

Table 1

Housing conditions and origin of cats with panleukopenia

Housing conditions				Roosting			Origin		Total
One-cat household		Multi-cat household	Animal shelter	Inner	Outside	Mixed	Rural area	Urban area	
No. of cases	38	94	78	49	52	109	161	49	210
%	18.09	44.76	37.14	23.33	24.76	51.90	76.66	23.33	100

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MATERIAL AND METHOD

Material and method. In order to achieve the purpose of the paper, research methods commonly available in veterinary clinics and offices in counties in the north-eastern region of Romania were applied, comprising 210 cases of cats with suspected feline panleukopenia.

In order to clarify the suspicion of feline parvovirus infection, rapid commercial immunochromatographic tests for the detection of panleukopenia virus antigen in faeces were performed.

In order to evaluate the hematological parameters was used the URIT Medical Electronic CO analyzer.

Monitoring studies of clinical and anatomopathological signs were carried out in veterinary offices in Iași, Botoșani, Vaslui and Suceava counties.

The cases covered by the study included cats that had been diagnosed with panleukopenia, or suspected panleukopenia, who received medical care between 2021 and 2023.

RESULTS AND DISCUSSIONS

Results and discussions. Out of the 210 animals diagnosed with feline parvovirus, 76.66% of them come from rural areas, being housed both outdoors and indoors, having a diet based mainly on wet food, cooked in households; 23.33% of them come from urban areas, being housed in apartments, isolated, the diet being based on dry food, alternated with wet food purchased from specialty stores, and 37.14% of cats come from stray animal shelters, which were abandoned beforehand, their health being degraded when they were found.

Out of 37.14% of abandoned felines, 66.66% were found outside the built-up areas, in places where they could not get food, water or shelter, and the other 33.33% were found in urban areas, near common public sanitation bins.

18.09% of cats come from single-cat households in both rural and urban areas.

Looking at the gender distribution, males were the most affected, at 55.23%, compared to females who accounted for 44.77% of all patients *(Table 2)*.

Inconsistent with the literature (Prittie, 2004), 49.53% of cats over 12 months of age developed the disease during the assessed period, followed by cats younger than 6 months 29.53% *(Table 2).*

Table 2

Age and sex categories of patients diagnosed with feline panleukopenia

		No. of cases	%
sex	male	116	55.23
	female	94	44.77
age	< 6 months	62	29.53
	6-12 months	30	14.28
	12-24 months	104	49.53
	>24 months	14	6.66

After performing the anamnesis and corroborating with the clinical signs specific to panleukopenia in the specialized literature, rapid tests for detecting the virus antigen in feces were performed, proved the concordance of the suspicion of disease with the test results, in 90% of cases, with positive results *(Table 2).*

In the remaining 10% of cases, the rapid test for parvovirus antigen was 71.42% negative, and 28.56% of the tests were false negative.

The symptoms of patients whose test result was negative, and the haematological examinations and data collected from the anamnesis, were similar to those described in the literature as specific to feline panlecupenia, were based on the consideration of intermittent elimination of the virus through faeces. For this reason, the diagnosis of Feline Panleukopenia has not been excluded, the animals following the appropriate treatment regimens *(Table 3).*

Table 3

Results of rapid immunochromatographic tests for the detection of panleukopenia virus antigen in faeces

Total no. of samples	No. positive (%)	No. negative (%)	
210	90	10	
		No. negative (%)	No. false negative (%)
		71.42	28.58

From a hematological point of view, it was possible to perform the blood count in 42 of the patients (20% of the total number of analyzed cases), and the results were as follows: in 88% of the analyzed patients, there was a severe leukopenia with monocytopenia, neutropenia and polycythemia, considered aspects constantly encountered in the evolution of parvovirus, known to affect the bone marrow as well.

12% of haematological analyses performed on patients showed no parvovirus-specific changes, which could align on an early stage of the disease *(Table 4).*

Table 4

Results of haematological tests of patients with suspected feline panleukopenia

		%
No. samples	42	100
No. patients with changed blood counts	37	88
No. Patients with unchanged blood counts	5	12

Cats from stray animal shelters that have previously abandoned have very different haematological test values, much lower than the values of cats also diagnosed with feline panleukopenia, but with the owner, although the age is similar.

The differences between the two categories of animals are represented by housing conditions, food administered and stressors to which cats have been exposed *(Table 5).*

Table 5

Age and lowest values of leukocytes revealed by haematological examination in stray cats and cats with owners

With owner		Without owner	
Age	No. Leukocytes /mm3	Age	No. Leukocytes /mm3
18 months	1500	12 months	450
16 months	4300	18 months	500
12 months	4500	18 months	550
24 months	6000	24 months	800
12 months	8000	12 months	1000

In terms of clinical manifestations, 95.23% of cats were confirmed with feline panleukopenia virus and showed symptoms specific to the disease and 4.77% of them showed mild symptoms of the disease, a state of apathy, without digestive symptoms or obvious hematological changes.

The symptoms that patients manifested included the following: apathy (92.85%), hyperthermia (85.71 %), ataxia (85.71%), polydipsia-however, animals refuse liquids, (77.61%); dehydration (80%), vomiting (92.85%), diarrhea (85.71%) (*Figure 1*), tonic muscle contractions (1.9 %), aggression (1.9%) and epileptiform seizures (1.9%) *(Table 6).*

Table 6

The main symptoms present in the studied cats

Symptoms	No. of cats	%
Apathy	195	92.85
Hyperthermia	180	85.71
Ataxia	180	85.71
Polydipsia	163	77.61
Dehydration	168	80
Vomiting	195	92.85
Diarrhea	177	84.28
Tonic muscle contractions	4	1.9
Aggressivity	4	1.9
Epileptiform seizures	4	1.9

Figure 1 **- Clinical aspects - cat with diarrhea with yellowish liquid content**

Regarding the results of the performed necropsies, the macroscopic anatomopathological lesions observed are those specific to dehydration and anemia, with the mention that in rehydrated animals edema and drops can be detected due to hypoproteinemia, it was found at the general examination of corpses that all animals were dehydrated, without bodily lesions, conjunctival mucous membranes, pale mouth, pearl color.

The lesions that drew attention when analyzing the corpses were the characteristic lesions that occur in the ileum and jejunum that are relaxed, cherry-red in color, the mucosa being infiltrated, congested, with fibrinous deposits and ulcers, and these lesions were observable in 4 corpses *(figure 2),*

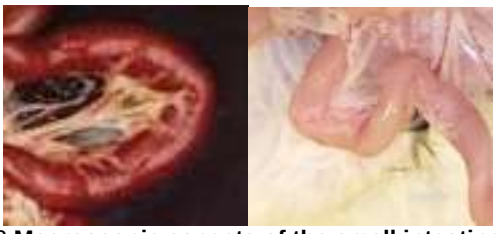

Figure 2 **Macroscopic aspects of the small intestine in cats, Left: Inflamed gut loops, Right: normal appearance**

CONCLUSIONS

The epidemiological, hematological, and serological data recorded are matched by those extracted from the literature, so in all the years studied, males were most frequently diagnosed with panleukopenia, compared to females, but, regarding the age at which the disease usually evolves, it was found that most clinical forms were observed in cats aged 12-24 months. This may be due to the origin of cats, most of which come from stray animal shelters after being abandoned in various out-of-town areas.

Poor weather conditions, homelessness and poor feeding conditions have led to decreased immunity of these felines, making them more susceptible to various pathogens, including feline panleukopenia virus.

A definitive diagnosis of feline panleukopenia should be based on the corroboration of all epidemiological, haematological, serological, clinical and anatomopathological aspects, not on the confirmation of a single aspect mentioned above, specific to this disease or a single test.

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ANALIZE OF THE RIDER POSITION ON A HORSE SIMULATOR A CASE STUDY

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Abstract

The experimental study regarding the influence of the rider position on the horse simulator was conducted on the Racewood simulator. The evaluated results were compared on three different types of saddles. The experiment showed that is a very unique way to determine the impact of the designed equipment in horses, the position of the rider according to the center of gravity and also to measure objectively the outcome of the experience and reactions of the rider.

Key words: horse riding simulator, position of the rider, saddle

Introduction

In the world of equestrian sports every detail counts for the success or failure in competitions. Apart from genetics and environmental factors that contribute to the horse development, the performance it is also influenced by the training and rider. The rider could have a major impact on the result of performance given the psychological part of the rider-horse relation. It is known that the horse can perform different under different riders, so this relation it is also very important. A good example could be the difference that comes with the experience of the rider (amateur or professional riders). Mechanical horse-riding simulators consist of a device that mimics the movement of a real horse, generating between 50 and 100 three-dimensional physical movements (forward and back, left and right, up and down) (J.G. Dominguez-Romero *et al*, 2020). Mechanical horse-riding simulator (HRS) is a type of intervention based on hippotherapy, consisting of a robotic device with a dynamic saddle that imitates the movement of a horse. (Sung Y.H. *et al*,2013)

Based on the hippotherapy research literature, during riding, the rider's pelvis moves in a soft, rhythmic, and repetitive pattern, being a movement similar to that performed by our pelvis during normal human walking. (J.G. Dominguez-Romero *et al*, 2020) Repetitive movements improve postural coordination and rhythm and allow reciprocal movement, in addition to facilitating postural control through stimulation of balance reactions (Sung Y.H. *et al*, 2013) and adaptive behaviors and movement strategies, due to the changing environment in which the session takes place (Yoo J.H. *et al*, 2014). Maintaining the center of gravity within the support base while the animal is walking means that the human rider has to anticipate and compensate for postural adjustments by reducing the center of gravity in order to remain safe on a moving surface such as the rump of the horse, stimulating multiple sensory inputs and efferent outputs (Temcharoensuk P *et al*, 2015), providing continuous motor, visual, somatosensory, and vestibular inputs to the rider (Kim S. *et al* 2013).

The following study tries to reveal the importance of the rider experience on the horse performance. Of course, the saddle and the position of the rider on the horse will have an important impact.

The study was performed by the same rider, with different saddles, on the same simulator that can record the correctness of the riding and to spot the faults that the rider is doing and be able to identify the differences according to the different saddles.

MATERIAL AND METHOD

The study was realized as a experimental case study, on the Racewood horse simulator which analyzed the parameters of the rider position on three different types of saddles.

Racewood simulators

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Racewood products appeared in the 1990s, first created to follow a renowned jockey, with the aim of continuing to improve in his discipline. Gradually, other models were developed for all skill levels, from beginners to professional riders. The development team has worked closely with instructors and professional riders to ensure that the products meet the real needs of the field.

On the Racewood simulator are three sensors to detect the movements and pressure of the rider's legs on the simulator. Again, for the analysis of the results obtained, we used a code (0, +, ++, +++, ++++) according to the pressure exerted on the various sensors, thus:

- 0 : no pressure detected
- + : light pressure
- ++ : medium pressure
- +++ : significant pressure
- ++++ : very strong pressure

Different models are possible, for flat races only, or for horse riding in general. For the latter, there are different modes, for dressage, jumping, outdoor, cross etc. In our case, it was the riding model, as presented below (Figure 1).

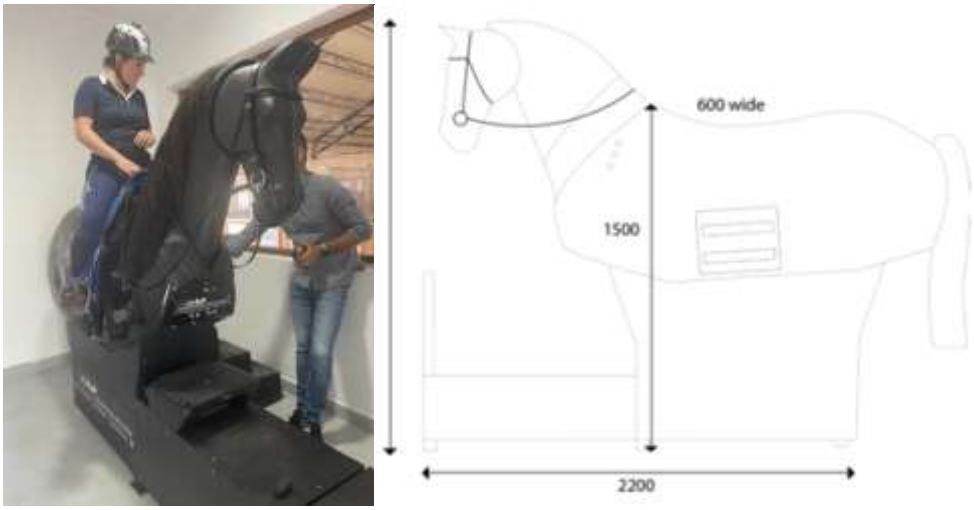

Figure 1 **Racewood simulator**

Multiple sensors are present to establish an idea of the most reliable position possible. There are sensors at the bridle to assess the impact of hand strength, sensors under the saddle and others for the legs. These are three in number, located behind the strap passage, one after the other. The latter are also used to request the start at a gallop during training on the simulator. Option for position analysis on the Racewood simulator

On the simulator, it is possible to train in different environments and on different activities, but there is also a position analysis mode. The analysis time is 2 minutes. It includes walking, trotting, and cantering with both hands. In front of the rider there is a screen, where you can see the evolution of the exercise and the live position.

We tested the position of one rider on the simulator under on 3 different saddles (Figure 2).

Each saddle has been tested twice, once for seated cantering, and a second time for balanced cantering. In Figure 3, we can observe different parameters for each gait: in A, we observe the distribution of the rider's load on the right or left on the saddle. In B, we observe the vertical movements of the rider on the saddle, forward / backward (F for "front" and B for "back"). In C', it is the center of gravity of the rider on the saddle and in C, it represents the movements of the rider, represented in the form of points of different colors according to the gait. In D we see the representation of the use of both hands. And to finish in E, the use of the legs can be observed, when the latter come into contact with the sensors 1, 2, or 3 (the 1 is close to the strap and the 3, the farthest).

To find a reliable way to get the datas, we created a positive and negative number code (figure 4). The evaluation of the rider's center of gravity (CG) is done along two axes: the vertical axis, i.e. the forward/back movements (Front/Rear), and the horizontal axis, i.e. the left/right movements (L/R). The displacements to the left of the CG with respect to the center of the saddle are presented by the values (in centimeters) negative, and positive if the CG is displaced to the right. Similarly, forward movements correspond to positive values and backward movements correspond to negative values.

1 **2** **3**

Figure 2 **Saddles used on the mannequin (original) 1 a podium endurance saddle - Champion Desert seat in leather ; 2 Bua saddle; 3 Kieffer dressage saddle - Ulla Salzberger**

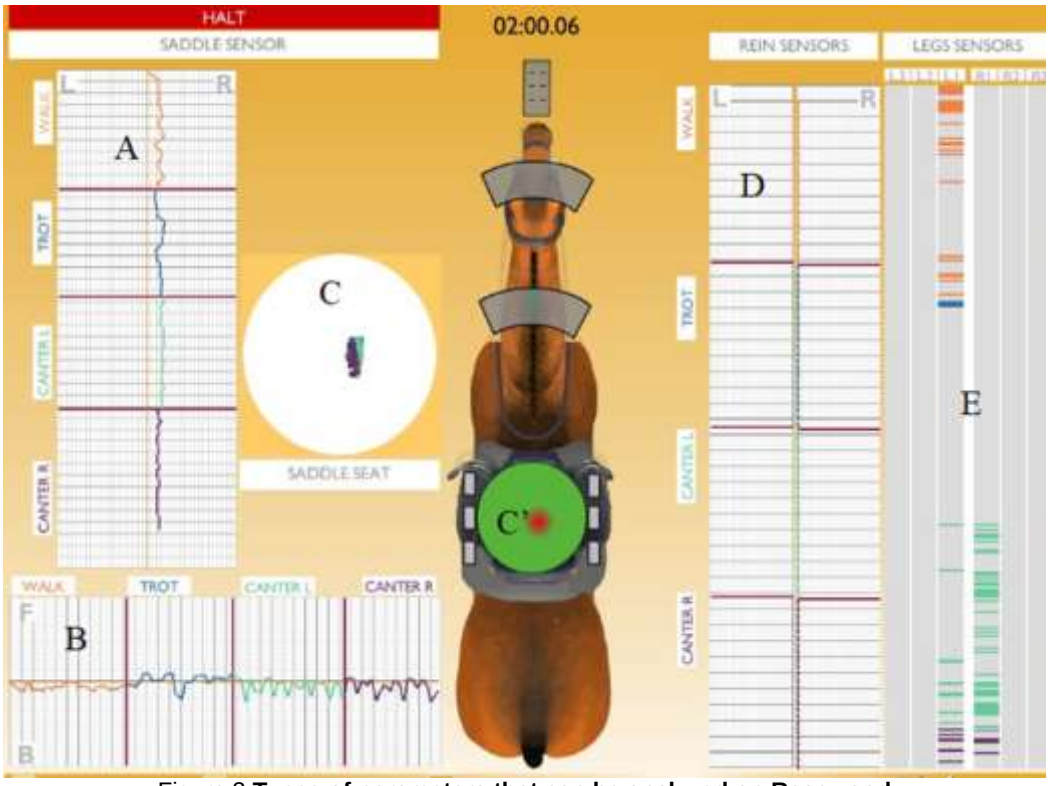

Figure 3 **Types of parameters that can be analyzed on Racewood**

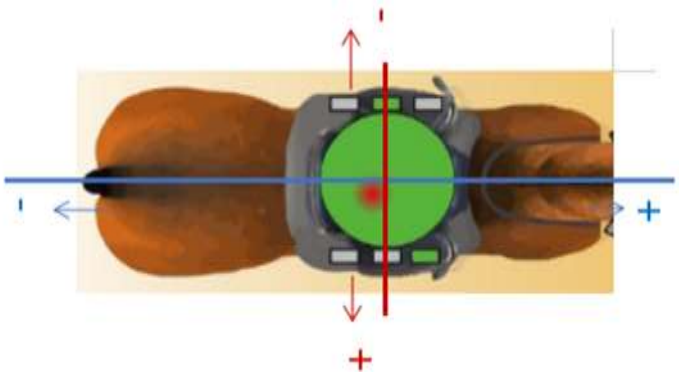

Figure 4: **Scoring method of the center of gravity value**

RESULTS AND DISCUSSION

The data obtained with the RaceWood simulator are presented in the same form as figure 11. No raw data in numerical value could be recovered. The results for the center of gravity were read using the grid scale. We note that the sensors under the saddle show position variations of 5 centimeters around its central point (1 square ≈ 0.25cm). The reading therefore remains very approximate because it is a visual evaluation of the curve. The results should therefore be taken with caution.

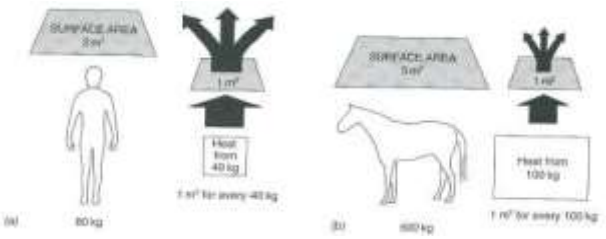

Figure 5 **Body surface area to weight ratio (Tribout P., 2013)**

In table 1, the results are noted at the top of the table and are accompanied by the position applied only to the gallop (measurements are in centimeters). After the collection of the data, we can see that, overall, regardless of the saddle used, the rider has the Center of Gravity shifted on average by 0.89cm to the right, which is therefore intrinsic to the rider.

On the other hand, we note a great variability Front/Rear of the Center of Gravity according to the saddle used at the walk: for the dressage saddle, the Center of Gravity is at point 0, it is therefore centered on the saddle, but if we compare it to the Bua saddle, the Center of Gravity is moved back 2 cm. The saddle therefore has a noticeable impact on the rider's Center of Gravity.

Table 1

Results for Center of Gravity (CG) according to the saddles types

	Podium seated	Podium balance	Bua seated	Bua balance	Dressage seated	Dressage balance
Center of Gravity Left/Right general galloping	0,75	0,75	0,75	1	1	1
Center of Gravity Front/Rear at walk	-0,25 0		-2 -1,75		0,25 0	
Center of Gravity Front/Rear galloping Left	0,25/-0,75	2,5/3	-0,75/-3	2,75/3,5	0,25/-0,75	2,5/3,25
Movement of the center of gravity Front/Rear galloping to Left	1	0.5	2.25	0.75	1	0.75
Center of Gravity Front/Rear galloping Right	0,25/-1	2,25/2,75	-0,75/-3	2,75/3,75	0/-0,75	2/2,75
Movement of the center of gravity Front/Rear galloping to Right	1.25	0.25	2.25	1	0.75	0.75

Logically, we find a Center of Gravity that is always more advanced when galloping in balance compared with galloping seated (Figure 6)

It can be seen that whatever the saddle used, the rider's center of gravity is always more stable when galloping in balance than when galloping seated. We were able to determine this by the ever-decreasing difference between the front/rear values when galloping in balance compared to when seated. For example, for the Bua saddle in seated gallop, the center of gravity moves over 2.75cm, against only 1cm in balanced gallop.

The changes observed between the right hand and the left hand (according to the different saddles and positions) are small and therefore cannot be interpreted in our study. We therefore averaged the median results for right and left hands, for all saddles and for both positions (Figure 7).

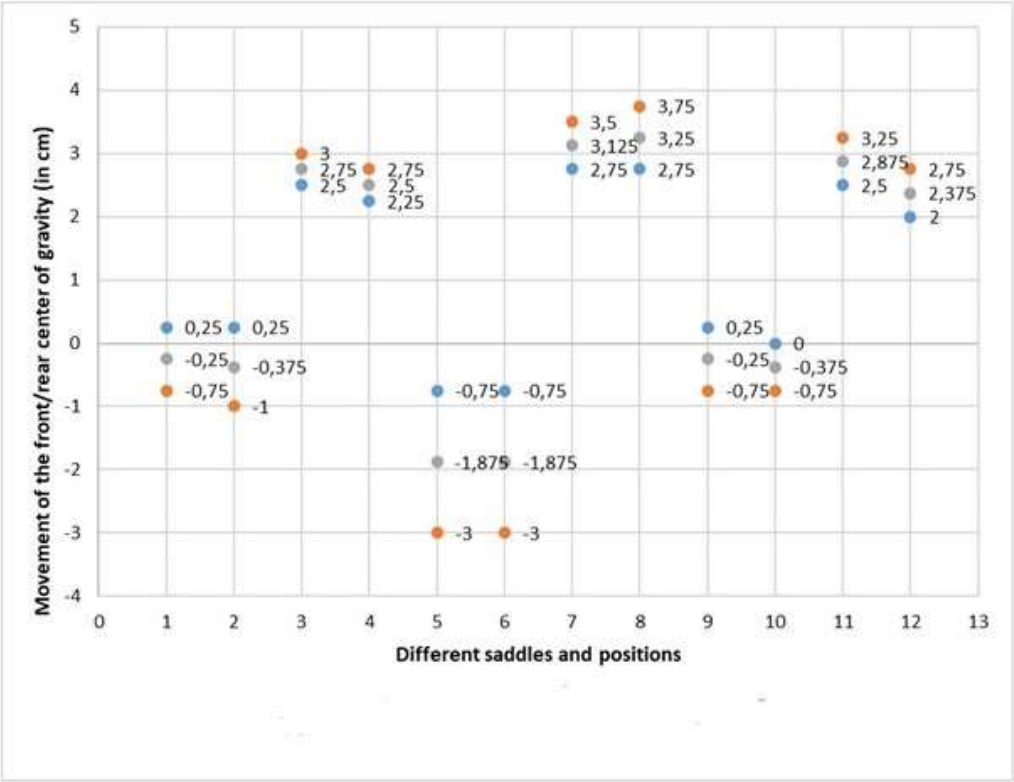

Figure 6 **Graphic representation of the front/rear Center of Gravity of the rider according to the saddle and according to the position**;

Legend:

- Average values of the most prominent positions
- Average values of the furthest back positions
- Averages of both

1: Left hand seated podium saddle, 2: Right hand seated podium saddle, 3: Left hand balanced podium saddle, 4: Right hand balanced podium saddle, 5: Left hand seated Bua saddle, 6: Right hand Seated Bua saddle, 7: Left hand balance Bua saddle, 8: Right hand balance Bua saddle, 9: Left hand seated Dressage saddle, 10: Right hand seated Dressage saddle, 11: Left hand balanced Dressage saddle, 12 Right hand balanced Dressage saddle.

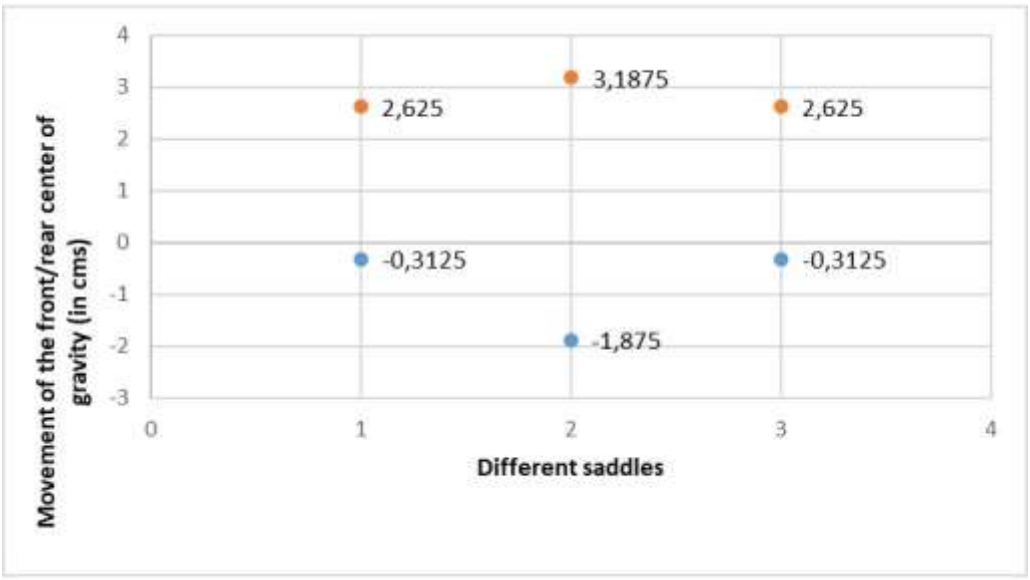

Figure 7 **Graphic representation of the rider's average Front/Rear Center of Gravity according to saddle and position**

Legend

- Seated averages
- Average equilimbrium

1: Podium saddle, 2: Bua saddle, 3: Dressage saddle

It is observed that the Center of Gravity averages are identical for the Podium saddle and the Dressage saddle. We therefore probably have the same range of motion in the saddles: seated, the Center of Gravity is slightly moved back from the center of the saddle (0.31cm), and forward of the center for the balanced position (2.63cm). When the rider moves from a seated position to a balanced position, the Center of Gravity is moved forward (2.94cm). For the Bua saddle, its Center of Gravity is shifted 5.06cm forward when passing into balance.

The results that we obtained are presented in the tables 2 and 3 according to each saddle used.

For the gallop seated on the left hand, the left leg is at the strap, and only slightly touches L1; the right leg is slightly more in contact with R1. This corresponds to a classic position for galloping (left leg at the strap and right leg moved back).

For the gallop seated on the right hand, the same situation is found, but reversed (right leg at the strap and left leg moved back).

For the balanced gallop, the legs are further back because they come into contact with the Sensors 2 and 3. As for the seated gallop, we find a slight gap between the two legs (legs with the strap/leg back). In addition, we find that the pressure exerted on the sensors is greater when galloping in balance compared to galloping seated.

Table 2

Results of the pressure exerted on different sensors by the rider for the Podium saddle

	PODIUM SEATED		PODIUM BALANCE	
	Gallop Left	Gallop Right	Gallop Left	Gallop Right
L1 (Sensor 1 Left)	+	++	+	0
R1 (Sensor 1 Right)	++	+	0	+
L2 (Sensor 2 Left)	0	0	++++	+++
R2 (Sensor 2 Right)	0	0	+++	+++
L3 (Sensor 3 Left)	0	0	0	0
R3 (Sensor 3 Right)	0	0	+++	+

Table 3

Results of the pressure exerted on different sensors by the rider for the Bua saddle

	PODIUM SEATED		PODIUM BALANCE	
	Gallop Left	Gallop Right	Gallop Left	Gallop Right
L1 (Sensor 1 Left)	++++	+	0	No results registred
R1 (Sensor 1 Right)	+++	++++	0	
L2 (Sensor 2 Left)	0	+++	++++	
R2 (Sensor 2 Right)	+	0	++	
L3 (Sensor 3 Left)	0	0	+	
R3 (Sensor 3 Right)	0	0	+++	

For the left-hand seated gallop, the left leg is behind the strap, it touches the L1 sensor with significant pressure. The right leg is slightly further back and touches R1-R2, (left leg at the strap and right leg back).

For the seated gallop with the right hand, the same situation as with the left hand is observed but reversed (right leg just behind the girth and left leg further back).

For the balanced gallop, the legs are further back because they come into contact with sensors 2 and 3. As for the seated gallop, we find a slight gap between the two legs (legs with the strap/leg back). We have no data recorded for canter to the right in balance.

For the Keiffer saddle, whatever the position and the hand, the legs do not touch the sensors, they remain at the strap and are fixed, without perceptible movements , so that the sensors on the simulator were not activated and thus they dint registered any results.

CONCLUSION

In scientific literature, there are few reports for the applications of the horse riding simulators, so our study is a case study that is original and needs further development to apply this methodology at a large scale.

Through this study, we can conclude that the rider s center of gravity is offset to the right regardless of the saddle used. In this study, it

differs according to the saddle used, thus clearly showing an impact of the saddle.

The balanced position moves the Center of Gravity forward compared to the sitting position, and this difference in Center of Gravity depends, among other things, on the saddle.

The balanced position seems to give a more stable Center of Gravity in the movement than if the rider is seated: the difference between the extreme values is reduced.

The movement of the legs depends on the saddle used. The dressage saddle induces a good position of the legs of the rider: they are fixed to the strap. For the other two, the legs are further back compared with the dressage saddle. In conclusion, for these last two saddles, the position of the legs in balance is further back than in the seated gallop.

After analyzing the results of evaluation for the three saddles for the Center of Gravity and the movement of the legs, we can conclude that the most suitable saddle for the rider is the dressage saddle.

The Racewood system it is an important tool for training of the riders, professionals or amateurs, because it can help preparing the riders before mounting a real horse, thus without having a possible negative impact on the real horse. Also, it is an important way to evaluate accurately the rider's performance and can help him progress to a superior level. Another important fact is that it helps the riders understand the processes and the mechanics involved in equestrian sports. For the research, we consider that is a unique way to determine the impact of the designed equipment in horses, the position of the rider according to the center of gravity and also to measure objectively the outcome of the experience and reactions of the rider.

ACKNOWLEGMENTS

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*** www.racewood.com

VETERINARY CARE NEEDS IN KENNELS OF BRACHYCEPHALIC AND NON-BRACHYCEPHALIC DOGS - PILOT STUDY

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Abstract

This is a descriptive, observational pilot study, based on the results obtained by applying an original questionnaire addressed to purebred dog breeders, speakers of the French and Romanian languages, regarding the perceived need for medical-veterinary assistance in canine reproduction. 44 answers were obtained (24 in French, 20 in Romanian) about 167 (100%) bitches from 33 brachycephalic and non-brachycephalic dog breeds, aged between 2 and 7 years, of which 75 (44.91 %) declared pregnant. The need for estrus monitoring by a veterinarian varied by group and breed type (68.62% of non-brachycephalic females, 41.66% brachycephalic), "small non-brachycephalic" breeds were monitored more intensively (90.90 %), artificial insemination was necessary in 49.33% of the gestation obtained (50.98% in non-brachycephalic breeds; 45.83% in brachycephalic breeds). All the breeders declared ultrasound confirmation of pregnancy, in brachycephalic breeds caesarean section was necessary in 45.8% of cases, post-partum veterinary control was requested only in 22.7% of cases, more frequently (37.5% of cases) to "large and medium brachycephalic" breeds (76.9% of answers). The puppies from the "giant brachycephalic" breeds were tested by a veterinarian, an aspect declared by 43.8% of the Romanian breeders and only 38.1% of the French respondents. Conclusion: Due to the type of research chosen, the results obtained in the present study cannot be extrapolated to the entire population of dog breeders, but it is a potential means of evaluating the needs felt and expressed by veterinary medical assistance in dog breeding.

Key words: brachycephaly dog breeds, nonbrachycephalic dog breeds, canine breeders

Introduction

Dogs from canine breeds (over 300 breeds) were, and remain, among the most frequent patients of the veterinarian. Purebred dog breeders turn to this professional for advice and preventive, curative and recuperative interventions, both for physiological conditions (gestation) and for various pathologies.

The different breeds of dogs do not require the presence of the veterinarian in all the stages of the life cycle and the individual variability contributes a lot to the diversity of the needs for specialized medical-veterinary intervention. In reproduction, due to the genetic predispositions of certain breeds of dogs towards specific health and/or reproductive problems, the veterinary specialist is requested more frequently for certain breeds of dogs.

A dog is considered brachycephalic based on the morphology of its skull:

"Brachycephalians have a short and broad head, with a rounded skull, without an external sagittal crest but with an absent or very weak nuchal crest. This particularity allows them to be differentiated from two other groups of dogs: mesocephalic and dolichocephalic (Homo N., 2008)

The proportions of the skull are defined by two indices:

- "Cephalic index (CI), which is defined as the ratio of head width to head length: (head width / head length) x 100. It varies from 50 in extreme dolichocephaly (Greyhound), 70 in mesocephalic and 90 in extreme brachycephalic (Pug).
- the craniofacial index, defined as the ratio between the distance from the external occipital protuberance to the frontonasal suture, and that from the frontonasal suture to the rostral end of the nasal bone. It ranges from 10/7 dolichocephalic to 10/3 brachycephalic.

These two morphological clues reveal the compact appearance of the head of dogs from brachycephalic breeds. It fits in the dimensions of two squares" (Lignereux Y. *et al*, 1991; Homo N., 2008)

[1] USAMV Cluj

The current research allowed the comparative evaluation of some parameters regarding the reproduction of purebred dogs; with an impact on reproduction management (gestation duration depending on the size of the breed), or those that have a direct economic impact (number of pups born and weaned, frequency of dystocia and cesarean operations, etc.) as well as the stages of the veterinarian's intervention, their frequency, depending on the different brachycephalic dog breeds, prone to dystocia, and non-brachycephalic breeds.

MATERIAL AND METHODS

This is an observational, descriptive study and it was developed based on an original working questionnaire with 17 items, addressed to dog breeders, speakers of French, Romanian, English, made through an online form through the Internet.

The purpose of the research was to evaluate the attitude of dog breeders regarding the perceived need for specialized veterinary intervention in the management of the reproduction of puppies of different breeds.

The objectives of the study consist in knowing the stages of the veterinarian's intervention depending on the size and type of dog breed (small, medium, large or giant breeds and brachycephalic or non-brachycephalic).

The administration of the questionnaire was done online, the data collection was by self-registration.

The selection of participants was made by contacting different discussion/sharing groups through social networks, direct contact of breeders through their website, but also of breeders known from their own veterinary activity, who were ask them to send the questionnaire to other known breeders, collecting the data. The respondents accepted participation in the research and gave their consent to be included in the study by completing and returning the questionnaire.

To facilitate the interpretation of the results, the dog breeds were classified into several groups according to their adult weight and their phenotype, brachycephalic or non-brachycephalic.

Inclusion criteria for brachycephalic breeds:

- giant breeds: breeds for which adults weigh more than 45 kg;
- large breeds: breeds for which adults weigh between 25 and 45 kg
- medium breeds: breeds where adults weigh between 10 and 25 kg
- small breeds: breeds where adults weigh less than 10 kg

Inclusion criteria for non-brachycephalic breeds:

- giant breeds: breeds for which adults weigh more than 45 kg
- large breeds: breeds for which adults weigh between 25 and 45 kg -
- medium breeds: breeds for which adults weigh between 10 and 25 kg.
- small breeds: breeds where adults weigh less than 10 kg

Some breeds present in this study that can be classified into two different groups, based on individual factors, were classified in their upper weight limit (French Bulldogs - in the class of medium brachycephalic dogs, Boxers in the class of large brachycephalic dogs, etc.).

RESULTS AND DISCUSSION

Out of the more than a hundred purebred dog breeders contacted, 44 responded to the questionnaire (24 French/Swiss, 20 Romanian), accepted participation in the research and gave their consent to be included in the study, by completing and returning it.

Following the application of the questionnaire, in the study, data were obtained on 167 (100%) bitches from 33 dog breeds belonging to kennels whose owners' accepted participation in the research and completed the questionnaires. (Figure 1)

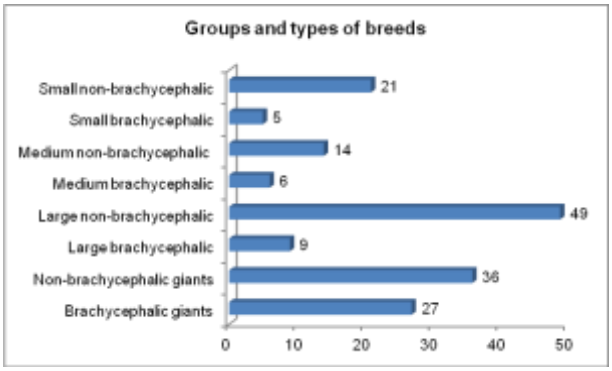

Figure 1 **Distribution of the group and type of breeds in the total panel enrolled**

From all the females studied, 75 (44.91%) were declared pregnant at the time of data collection. It should be noted that the owners of kennels for "large brachycephalic and non-brachycephalic" breeds who completed the questionnaire had a weight of 72.45% of the total panel and 69.33% of the total declared pregnant bitches.

By grouping dogs according to phenotype and without considering their group, we found that data were collected on 47 (100%) brachycephalic bitches, of which 24 (51.06%) were pregnant and 120 (100%) of bitches belonging to non-brachycephalic breeds, of which 51 (42.50%) bitches were pregnant. Without considering the

weight of the bitches, we can see that the number of non-brachycephalic females (120) in the studied group is more than twice as high as the number of brachycephalic females (47) declared by the respondents.

In our study, the age of the bitches declared pregnant at the time of the research shows that 50.66% of the bitches in the panel are between 2 and 3 years old, 33.33% are between 4 and 5 years old, 14.66% are between 6 and 7 years old and that no bitch is over 8 years old.

In dogs, puberty occurs on average between 4 and 18 months, according to Fontbonne *et al* reproduction of bitches from the first heat is not indicated, because at this stage the development of the pelvis is not yet finished and the maternal instinct/behavior of the bitches at this age is not adequate. Mating is recommended in at least the second heat in small dogs (usually around two years) and the third heat in large dogs. After 6-7 years, bitches show a progressive degeneration of the ovules. The bitch may be pregnant but the risks of malformed and small pups with calving difficulties (by non-release or obstructive dystocia due to too large a puppy) increase. It also increases the risk of disease (eclampsia) and reduced breastfeeding capacity. (www.centrale-canine.fr)

Analyzing the answers of the breeders participating in our study regarding an important stage in the reproduction cycle, that of monitoring heat in bitches, most of the respondents declare that they called on the expertise of the veterinarian, thus, for each breed and regardless of the size of the dogs or the category of brachycephaly or non brachycephalic, the number of specialized veterinary interventions exceeded that of the supervision carried out by the breeder. (Figure 2)

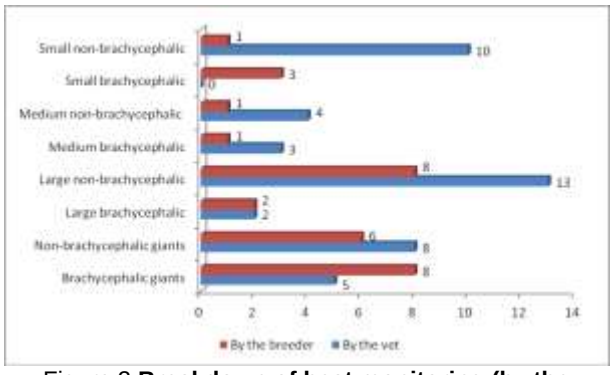

Figure 2 **Breakdown of heat monitoring (by the veterinarian or breeder) according to the group and type of breed**

It should be noted that the answers given by the participants in our study support that the "small non-brachycephalic" breeds were monitored more intensively than the other groups: in fact, 90.90% of the dogs in the research group were thermally monitored by the veterinarian and in all specimens they were given the progesterone test, alone or accompanied by a vaginal smear. In contrast, the so-called "small and brachycephalic" breeds were not watched at all by a veterinarian during heat; it was the breeder who monitored the heats of the females, either by smearing or observing the vulva or behavioral changes.

Monitoring of heat by a veterinarian therefore varies by group and breed type. Of the total number of pregnant females, 60% had, prior to pregnancy, thermal monitoring, vaginal smear, hormonal dosing or follicular stimulation, performed by a veterinarian.

The frequent call to a veterinarian at this stage is explained by the fact that the breeder is thus more certain to correctly detect the most favorable moment to have the maximum fertility rate of the bitches, which increases the chances of pregnancy.

Depending on the type of breed, heat monitoring is variable. We note that 68.62% of the non-brachycephalic females had heat monitoring performed by the veterinarian, compared to only 41.66% of the brachycephalic females.

Analyzing the type of litters made for the 75 pregnant bitches, it can be noted the presence of natural litters (38=50.66%) in weight close to artificial insemination (37 litters=49.33%). (Figure 3)

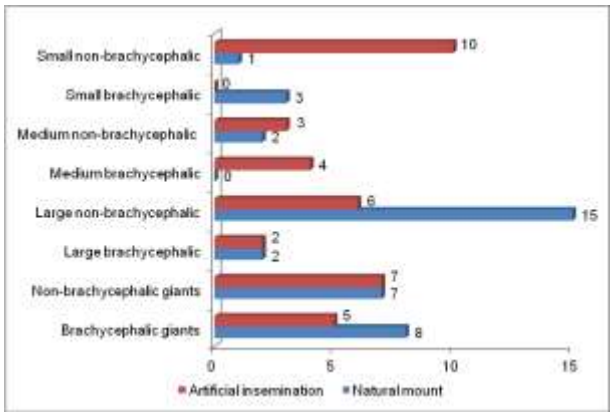

Figure 4 **Distribution of the type of insemination (natural/artificial) according to the group and type of breeds**

Regardless of weight, in total, in the brachycephalic category, 13 natural mounts (54.16% of the total mounts made in this category) and 11 artificial mounts (45.83%) were reported. In the non-brachycephalic breeds, out of the 51 (100%) foals reported, those achieved naturally represented 25 cases (49.01%) and those through artificial insemination were 26 (50.98%). In our study, the "large and non-brachycephalic" breeds had a share of natural reproduction of 71.42%, in contrast to the "small and non-brachycephalic"

breeds in which artificial insemination was used in 90.90% of cases.

It should be noted that in both analyzed categories (brachycephalic and non-brachycephalic) litters made by artificial insemination represented approximately half of the total litters, an important aspect that demonstrates the need for specialized veterinary assistance for their realization.

Our panel being composed of a majority of so-called giant brachycephalic dog breeds, this explains the results obtained as this category does not necessarily need mating assistance, unlike certain "medium and brachycephalic" breeds such as the Bulldog French from our study, where we found that artificial insemination was chosen by all respondents who are breeders of such dogs. (Figure 3)

The choice of the type of mount depends on the breeder, for efficiency or practicality, as well as on the breed of the dog. Indeed, some dog breeds such as English Bulldog, French Bulldog, Pug, require the intervention of a professional for mating (artificial insemination). In our study, the intervention of the veterinarian at this stage was necessary in 49.33% of the herds.

Natural mating remains the most frequently used method of reproduction (Badinand F. *et al*, 1998), (Greco D.S., 2008). This can be explained by the fact that artificial insemination represents an additional cost compared to that of a natural mating and requires the intervention, and therefore the cost, of the veterinarian.

The veterinarian intervened in this stage of canine reproduction by artificially inseminating 50.98% of females from non-brachycephalic breeds and 45.83% of females from brachycephalic breeds.

The ultrasound examination is a safe, precise, fast, early (starting from 25 days after fertilization) and risk-free means, and the least expensive compared to other verification methods (by measuring relaxin or radiography) and is the most used to establish the pregnancy diagnosis. Breeders prefer to call the veterinarian at this stage to be sure of the pregnancy.

In the current study, all the breeders (100%) declared that the confirmation of the state of pregnancy and the establishment of the diagnosis of pregnancy was made by the veterinarian, based on the ultrasound scans and the specialized consultation.

The veterinarian does not necessarily intervene during eutocical parturition. On the other hand, they are required during dystocia. In the literature, the need for caesarean section intervention appears:

- when the female has exceeded the gestation period: the first signs listed above appear and no signs of labor occur for more than 24 hours, or if no birth occurs within 36 hours after the progesterone falls below 2 ng/ml;
- when the female exhibits strong expulsive efforts for more than 20-30 minutes without any fetus emerging;
- when there is green vulval discharge but no fetus is expelled. More generally, when the female has vaginal discharge for more than 2-3 hours without signs of labour;
- when the time between each litter is more than 4 hours. The average expulsion time between each litter should be 20 to 30 minutes;
- when there are stillborn foetuses;
- when the total time of expulsion for all litters is more than 4-8 hours, with possible lengthening in primiparous or high litter bitches;
- when these parameters are not met, veterinary intervention is required.

According to Fontbonne A. *et al*, dystocia is "the inability to expel fetuses without assistance. Dystocia exists when a female has a full-term pregnancy and no signs of parturition appear or when she has started labor but is clearly unable to expel her fetuses alone.

This dystocia are due to uterine contractions that are too weak, unproductive or too prolonged, without fetal expulsion, or to obstructions (disproportion between the size of the fetus(es) and the mother's pelvic symphysis, insufficient dilation of the soft tissues, torsion/rupture of the uterus...).

According to Gravilovic B.B. *et al*, the incidence of dystocia in dogs is on average 5%, but is influenced by the breed and age of the pregnant bitch: an increase in dystocia is observed in bitches over 7 years of age.

According to Bergström A. *et al*, the breeds most at risk are miniature and so-called giant breeds. The incidence can reach up to 100% in certain breeds, especially in brachycephalic dogs (Linde-Forsberg C. *et al*, 1998).

According to Evans K.M. *et al*, 10 breeds are distinguished by a higher number of dystocia: Boston Terrier, Bulldog, French Bulldog, Mastiff, Scottish Terrier, Miniature Bullterrier, German Wirehaired Pointer, Clumber Spaniel, Pekingese, Dandie Dinmont Terrier.

Your vet can intervene in different ways: by injecting substances that dilate the soft tissues, substances that increase contractions (oxytocin), obstetric maneuvers or surgery such as caesarean section when the obstruction is upstream of the vagina or if medical treatment does not work. It is the most commonly used surgery for dystocia. In our group of bitches, veterinary intervention for

parturition, by performing cesarean section, had a low frequency in every brachy or non-brachycephalic group, except for the so-called "medium brachycephalic" breeds, for which the frequency of veterinary intervention in specialized assistance for parturition (cesarean section) was 100% (Figure 4).

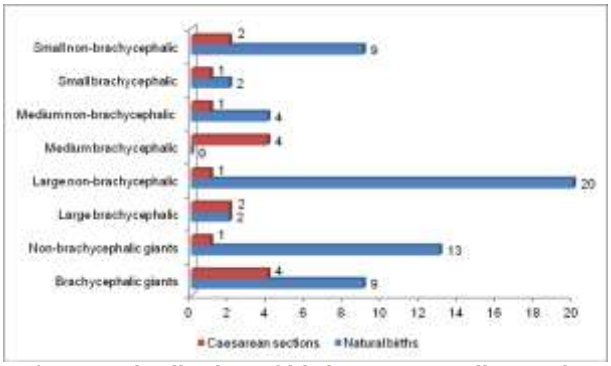

Figure 4 **Distribution of birth type according to the group and type of breed**

For the so-called "large and brachycephalic" breeds, there were 50% natural births and therefore 50% caesarean sections. The group for which the veterinarian intervenes the least is the so-called "Large and Brachycephalic" breeds where 95.20% of births were natural; closely followed by the so-called "Giant Brachycephalic" and "Small Brachycephalic" breeds with 92.90% and 90.2% of natural births respectively.

For non-brachycephalic dog breeds we can see that with a proportion of 90.19% natural births, the veterinarian intervened little at this stage. In this group, only 9.80% of fetuses were completed by caesarean section. As for the brachycephalic breeds, the veterinarian intervened 45.8% in our group of females, which shows a more frequent veterinary intervention at this stage of reproduction in brachycephalic breeds than in non-brachycephalic breeds.

Long-term selection of the phenotype of dogs led to the observation of changes in pelvic geometry. In brachycephalic patients, for example, there is a dorso-ventral flattening of the pelvic canal, which predisposes them to mechanical dystocia. Larger size and skull morphology also favor obstructive dystocia. Finally, bitches of these breeds often have weak abdominal musculature and breathing difficulties that can make expulsion of the fetus difficult and thus may require caesarean section.

Postoperative complications, especially post-caesarean section, are possible. There are risks of endometritis, retained placenta, hemorrhage, uterine prolapse, mastitis, wound infection, peritonitis and postpartum hypocalcaemia. The veterinarian will therefore take all necessary measures to avoid as far as possible all these complications and postoperative follow-up of the bitch is strongly recommended.

The veterinarian can intervene with advice to breeders for advice on the diet of the pregnant and post-pregnancy dog; as well as the bitch, or with expert answers to any other questions the breeder may have.

At the post-partum check-up stage, in our study, the veterinarian was consulted in only more than a fifth of cases (22.70%) by the breeders participating in the research. We note that most requests were for "large brachycephalic" and "medium brachycephalic" breeds. The types of veterinary specialist intervention were for scar control/removal of sutures, feeding advice for the puppies and/or for the lactating bitch.

Overall, 15.70% of non-brachycephalic bitches, had postpartum follow-up, including scar checks, suture removal and/or diet advice. A large majority of breeders, 84.30%, stated that they supervised this stage without the help of a veterinarian.

For brachycephalic bitches, the rate of veterinary intervention at this stage was higher (37.50% of cases). These percentages can be explained by the fact that there were more caesarean operations (45.8%) and that the breeders declared that they preferred to request postpartum follow-up by a veterinarian.

At this stage, veterinary assistance is recommended especially to breeders of females who have completed gestation by caesarean section, in order to follow the good evolution of the plague / scar. This check-up is most often free of charge to encourage breeders to request a follow-up of their dog. In our study there were also situations recorded where some breeders of naturally faected bitches also consulted their veterinarian for advice on feeding their puppies or nursing bitches.

The batches for which puppies were tested the least were the breeds 'large brachycephalic', 'medium brachycephalic' and 'small brachycephalic'.

On the other hand, the breeds for which, affirmatively, puppies were frequently tested, and this in 76.9% of the responses, were the "giant and brachycephalic" breeds, in which tests for dysplasia (of hips and/or elbows), genetic, cardiac or deafness tests were performed. These were carried out according to the breeds and their predisposition to certain diseases

For the other groups, the proportions of puppies tested varied, but in general, the majority of puppies were not tested, according to the breeders participating in this study. It is worth

mentioning that in our study, in large and small brachycephalic puppies, as well as in medium non-brachycephalic puppies, no post-partum testing of puppies was reported.

Affirmatively, the veterinarian intervened at this stage on 50% of the brachycephalic bitch puppies in our group by performing hip/elbow radiographs to check for dysplasia; blood sampling for genetic testing for pathology; heart/kidney ultrasounds to detect possible malformations; tests to detect deafness and exclude puppies from breeding.

For non-brachycephalic breeds, the veterinarian intervened in only 35.3% of cases.

In France, there is a website (www.centrale-canine.fr) that provides for registration for: "Any mention of screening results for genetic defects, whether congenital (autosomal) [...], hereditary only or considered hereditary, but, it is desirable to also register data on hip (HD-) and elbow dysplasia, patellar luxation and retinal ectopia".

These examinations testify not only to a selection specific to the act of breeding, but also to a contribution of the breeder to the traceability of origins for the future, especially if the puppy sold would be used for reproduction, [...]."

These tests for genetic defects are therefore not compulsory and not all breeders do them and thus take the risk of having dogs with genetic defects.

These tests for genetic defects are therefore not compulsory and not all breeders do them and thus take the risk of having dogs with genetic defects.

The analysis of the answers given by the French and Romanian speakers regarding the postpartum testing of puppies revealed that almost half of the Romanian breeders (43.80%) participating in the study declared that they had tested their puppies, the French breeders declaring this activity in only 38.1% of the cases.

CONCLUSIONS

The study assessed the opinion of a group of respondents from France and Romania, breeders of pedigree dogs, on the perceived need for veterinary intervention throughout the reproductive cycle of bitches, according to groups and types of dog breeds.

The study analysed data on 167 bitches, of which 75 were pregnant, belonging to 33 different breeds of dogs.

The results revealed the influence of breed group and breed type on the stages and frequency of veterinary intervention in the reproductive cycle of female dogs, in agreement with data from the literature.

The veterinary interventions, felt as needs for specialist assistance, expressed by the breeders participating in this study were: estrus supervision, choice of artificial insemination, choice of pregnancy diagnosis or caesarean section in medium-sized brachycephalic breeds, testing of puppies, postpartum monitoring of bitches.

Due to the type of research chosen, the results obtained in the present study cannot be extrapolated to the entire population of dog breeders but it is a potential means of assessing the felt and expressed needs of veterinary nurses who are passionate about dogs and involved in dog breeding.

This study is just a first look at the role that the veterinarian plays in the reproductive cycle of the female dog, depending on the breed type. In order to confirm the trend observed in this study, it would be necessary to extend the study to a larger number of breeders as well as to a national representative sample, which would allow the estimation of the real need for veterinary care for this important category of pets.

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MYCOPLASMATIC (ENZOOTIC) PNEUMONIA OF PIGS AS A HEALTH PROBLEM IN FATTENING UNITS

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Abstract

Mycopasmatic or enzootic pneumonia is the most common disease of the respiratory system under in the intensive pig production. It is clinically manifested by coughing, a chronic inflammatory process in the lungs, high morbidity and a relatively low percentage of deaths. The infection can be transmitted horizontally and vertically. *Mycoplasma hyopneumoniae* invades the epithelial cells of the trachea, bronchi, bronchioles and alveoli and disrupts the function of the ciliary body. Evagination of epithelial cells occurs, so that the cleansing of the airway mucosa by the mucociliary apparatus is inhibited. As a result, bacterial complications (*Pasteurella, Bordetella, Klebsiella, Actinobacillus, Hemophilus*) are common.*M.hyopneminia* can play imortant role in PRDC. The development of *Mycoplasma hyopneumoniae* is favoured by large congregations of pigs in small spaces, inadequate environmental conditions (microclimate), parasitic infections and inadequate nutrition. *Mycoplasma hyopneumonia* can be a significant health problem on the fattening farm, exacerbated by the influence of non-specific factors as well as the spread of other bacterial pathogens.

Key words: *Mycoplasma, pneumonia,* fattening, pigs, intensive breeding

After completion of the rearing phase, the further technological process in intensive pig breeding comprises several production lines: fattening, rearing of female breeding material (gilts) to maintain the parity structure and repair the udder, and rearing of boars to obtain semen for artificial insemination. Pig fattening is the finnish phase of meat production. The piglets arrive at the fattening center facilities at the age of 10 weeks and with a body weight of about 25 kg. They remain in the fattening center until they reach their final body mass. (Mrvaljević, 1995).

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Successful fattening depends on numerous factors, such as genetic potential, balanced nutrition, microclimatic or zoohygienic conditions, and general health of the sow. On the quality of external and internal biosecurity measures and health management depends the extent of direct and indirect damage caused by health disorders in fattening farms (Bojkovski et.al. 2011,2022, Prodanović et al. 2021; Prodanov-Radulović et al., 2020a). One of the most common diseases in fattening is mycoplasmatic or enzootic pneumonia. Mycoplasmatic or enzootic pneumonia is a multifactorial disease of the respiratory tract of pigs whose primary pathogen is *Mycoplasma hyopneumoniae* and in which a number of so-called risk factors are involved. The source of infection is sick pigs, which transmit the pathogens to individual pigs by direct contact. Piglets are most commonly infected through contact with an infected sow or contaminated

environment (aerogenic infection). The horizontal route of transmission is particularly pronounced in chronically infected pigs. After infection, an immune reaction occurs and specific antibodies and sensitized lymphocytes are produced. All previous studies have shown that sensitized lymphocytes play an important role in the defense against mycoplasma infection. All the above facts should be taken into account in the program to control enzootic pneumonia. This is especially important since vaccination has been shown to be the most effective control method in many countries. In addition, an effective strategy to control enzootic pneumonia would have to include correction of management, housing conditions, microclimate, all-in/all-out pig manipulation, strategic medication, and, of course, implementation of effective vaccine programe.Each of the above measures should be adapted to the specific conditions, especially the type of farm, the production system, the origin of the infection, the time of its occurrence, and other non-specific factors (Prodanov-Radulović, 2020b). *M. hyopneumoniae* is sensitive to light, high temperatures, and drying. Most disinfectants and detergents quickly inactivate it. Under humid and cold conditions, it can be maintained in an infectious state for 2-3 days. The bacterial species *Mycoplasma hyopneumoniae* has spread worldwide and causes major economic losses in intensive pig farming. Studies have shown that total production losses can be as high as 25%, even in the absence of secondary infections *(*Stevenson, 1998, Bojkovski et.al.2021). Damage results from reduced daily gains, weaker feed conversion, and individual growth retardation, which together extend the time it takes for the animal to reach adequate body weight. Because of the difference in growth rates, animals on infected farms must be sorted multiple times, which increases the cost of fattening. Infection with the *M. hyopneumoniae* species also leads to an increased likelihood of lung infections with other microorganisms that further complicate the inflammatory processes in the lungs and increase mortality.
As mentioned earlier, mycoplasmas can live as commensals on the mucous membranes of organs and cause disease under certain conditions, such as a decrease in the body's defenses. Mycoplasmas are transmitted transovarially by direct contact between animals, cohabitation, coitus, through the secretions and excretions of infected individuals. Whether infection occurs depends on numerous factors that interact, but the most important is certainly the resistance of the animal. Prolonged, direct and indirect contact between animals is sometimes required for infection with mycoplasma to develop. Bronchial secretions, urine, milk, fetal fluids, joint contents may be contaminated with mycoplasma. The possibility of transmission of the pathogen through food is not excluded, but the infection is most often introduced through the purchase and introduction of new animals with unknown health status.

Epizootiology

The source of infection for the youngest categories of pigs are sows and older gilts. Infection is predominantly by droplet infection and is transmitted by airborne or direct contact with nasal discharge. The infection spreads relatively quickly from litter to litter. The youngest categories of pigs are also most susceptible to infection, although in most cases latent infection occurs in the youngest pigs. In piglets, the infection may rarely cause lesions on the teat. When pigs are in pre-fattening, when they are exposed to other micro-organisms due to the mixing of animals from different farms and when non-specific factors are present, bronchopneumonia may occur in a larger number of animals. Since the pathogen is already in the pig's body, the housing conditions and the immune status of the animal play an important role in the development of the disease. Air, frequent temperature changes, mixing or bringing in animals from other areas, unbalanced diet, possible parasite infestation and other unfavourable conditions contribute to the occurrence of the disease (Pavlović, et al., 2007, Došen, 2007)

Pathogenesis

M. hyopneumoniae colonizes the upper part of the respiratory system of swine and adheres to the ciliated epithelium of the bronchi and bronchioles, where it remains without further invasion of the cells or parenchyma. It adheres exclusively to the cilia of the ciliated epithelium, and in some cases structures resembling adhesion pili have been observed to attach the mycoplasma by using cells. When mycoplasmas are taken up by cells, they damage the cell membrane using metabolites (H_2O_2)., In this case the infected cells lose the ability to degrade hydrogen peroxide. The pathogen spreads and colonizes the respiratory system by ingesting the ciliated epithelial cells of the trachea and bronchi in the cranioventral parts of the lung (Sarradell et al., 2003). During evolution, mycoplasmas have lost all genes involved in the biosynthesis of amino acids, fatty acids, and vitamins, so they obtain all of the above substances from the host cell in which they parasitize. Furthermore, *M. hyopneumoniae* disrupts the cellular receptors and transport mechanisms of the cell to which it binds, causing additional damage through toxic metabolites. Because of the aforementioned actions, the activity of the cilia ceases, they become blind, and eventually the affected cells die and slough off. The consequence dysfunctionality the ciliated epithelium is the accumulation of mucus and inflammatory exudate and the obstruction of the airways. In the acute phase of the disease, neutrophils and macrophages accumulate in the airways and surrounding tissues. As the disease progresses, the peribronchial and perivascular areas are densely infiltrated with mononuclear cells (lymphocytes and macrophages), resulting in hyperplasia of the lymphoid tissue associated with the bronchi (BALT - "Bronchi alveolar lymphoid tissue"). Cytokines secreted by macrophages (IL -1, IL -6, IL -8, prostaglandin E2, TNF) stimulate the activation and accumulation of inflammatory cells, but also have a cytotoxic effect on the endothelium of the alveoli and the epithelium of the airways. The accumulation of mucus and inflamed exudate due to the loss of function of the ciliated epithelium, the increased activity of the glandular cells of the mucosa, bronchoconstriction due to the action of chemical mediators of the inflamed cells, and the increased pressure of the lymphoid tissue lead to airway constriction and atelectasis of the surrounding alveoli. Secondary infections with microorganisms that are physiologically present either on the mucosa or in the immediate environment are common. The most common infection is the bacterium *Pasteurella multocida*, which potentiates the pathological process and doubles the lung surface area affected by the changes. In addition to this bacterium, other microorganisms can also act as agents of secondary infections; the most common are *Actinobacillus pleuropneumoni, Haemophilus parasuis, Streptococcus suis*; some viruses (PRRS - "Porcine Reproductive and Respiratory Syndrome", SIV - "Simian immunodeficiency virus") or other species of mycoplasma *(M. hyorhinis*) *(*Ciprian et al. 1988). If the infection with *M. hyopneumoniae* is not complicated by common infections with the already mentioned microbes, the changes on the lungs may remain localized and gradually detach from the healthy tissue, but they remain permanent (scarring changes are most often found on the slaughter line) (Burch, 2004 , Ivetić et al. 2005).

Clinical picture

Enzootic swine pneumonia caused by the bacterium *M. hyopneumoniae* is characterized by high morbidity and low mortality; secondary infections are common and complicate the course, increasing the number of deaths, i.e., mortality. The course of disease can be acute or chronic. The acute form of the disease occurs only when animals first exposed to the *M. hyopneumoniae* species become infected. The incubation period lasts 2-8 weeks (Zimmereman 2012 Šamanc, 2009,). Severe acute pneumonia may occur, with respiratory distress, painful and nonproductive but audible coughs, dehydration, elevated body temperature, apathy, and clumsiness, and mortality is high in all age groups. It is not uncommon for the disease to manifest with only mild pneumonia. The chronic form often occurs in farms where the M. hyopneumoniae species has been present for some time, i.e., it is an enzootic infection of the sputum. Symptoms may occur for several weeks or even months and are more frequent and intense when animals are disturbed (e.g., during morning feeding, rehousing, etc.); in addition to coughing, weaker feed conversion and consequently reduced growth are observed. If the primary infection is not complicated by additional bacterial infections, the sick pigs recover spontaneously. Despite the clinical improvement, this disease, i.e. its causative agent, persists in breeding, so the cure, i.e. eradication of this disease from breeding, is problematic.(Šamanc, 2009 , Fano et.al. 2005,

Zimmereman 2012).It is manifests in from of increased body temperature, fatigue, inappetence, dyspnea, and in the most severe cases, death. Subclinical infections, where the disease progresses without visible clinical signs (carriers), are very common and therefore represent a major problem in intensive pig farming.

Pathological changes

The first changes in the lungs are seen on the 7. to 10. day of infection and peak after four weeks. They consist of clearly demarcated areas of purple to gray consolidated lung tissue, the extent and distribution of which depend on the stage of the disease, the resistance of the individual animal, the virulence of the causative species, and possible secondary infections. The apical and cranial lobes are most commonly affected, and changes in the caudal lobes are found only in severe cases of disease and complicated secondary infections (Prodanov-Radulović et al., 2020a; Vicca, 2005). Macro pathologically, the cross-section shows a large amount of catarrhal exudate from the trachea, bronchi, and bronchioles, and the tissue is edematous and fleshy in consistency. The bronchial and mediastinal lymph nodes are often markedly enlarged. Three to five weeks after infection, the small airways in the affected portions of the lungs become visible as white spots, which is a consequence of the severe peribronchial inflammation. In cases where there is no secondary infection, the changes are resolved after 12 to 14 weeks. Affected areas become gray, have a hard consistency, are atelectatic, and have even greater demarcation. After cessation of the disease, gray scars remain visible, and in milder cases, healing may be complete, with no visible scars on the lung tissue (*Leneveu et al., 2005*). In the initial phase of the disease caused by the *M. hyopneumoniae*, following histological changes: Loss of airway ciliated epithelium, flaking of ciliated epithelium, and accumulation of neutrophils and macrophages in the lumen and around the airways). In cases where the disease or infection progresses,following changes can be seen: catarrhal bronchointerstitial pneumonia of the cranioventral parts of the lung, peribronchial, peribronchial and perivascular infiltration of lymphoid cells, formation of lymphoid follicles, thickening of the alveolar septa and obliteration of the bronchiolar lumen, and atelectasis of the adjacent alveoli. Hyperplasia of airway lymphoid tissue (BALT "bronchus-associated lymphoid tissue") is the most significant histologic change in enzootic pneumonia and consists of macrophages, dendritic cells, T and B lymphocytes, plasma cells, CD4 cells, and some CD8 cells (*Sarradell et al ., 2003*). Histopathologic findings can be further complicated by secondary infections. For example, necrotic pneumonia is found in infection with the bacterium *P. multocida*, fibrinous-hemorrhagic-necrotic pneumonia, pleural adhesions of yellowish color and massive fibrinous infiltration in infection with the species *Actinobacillus pleuropnumoniae*, and secondary infection with the species *M. Hyorhinis* as well as *Haemophilus parasuis* manifests as catarrhal pneumonia, pleurisy, pericarditis, and polyserositis (Prodanov-Radulović et al, 2020a).

Diagnostic

Epizootiologic control and anamnestic approach are the first steps in suspecting *M. hyopneumonia*e infection in a farm. Enzootic pneumoniae usually occurs in previously uninfected breeding animals after the purchase of new animals and after the mixing of fattening piglets of different origins when a new farm is established. The diagnosis of enzootic pneumonia is difficult to make on the basis of the clinical picture, because the signs of the disease are sparse and often without characteristic symptoms. Despite its obvious advantages (ease of performance, low cost, no burden), clinical surveillance without other diagnostic methods is not sufficient to objectively diagnose enzootic pneumonia. This is confirmed by studies that have shown that in 30% of infected farms, the presence of the disease could not be detected by clinical surveillance alone (*Levonen, 2000*). Macropathological and pathohistological findings are characteristic. However, they are not pathognomonic. The cranioventral distribution of pulmonary changes is also seen in *Streptococcus suis* infection, and subacute infection with porcine influenza virus can produce changes similar to those seen in the early phase of enzootic pneumonia (Zimmermann et.al.2012*)*. Lung lesions caused only by the *M. hyopneumoniae* species are resolved by 5-6 weeks, and by 2 months after infection, the changes may no longer be apparent (Morris et al., 1995). Diagnosis of enzootic pneumonia by isolation and culturing of the pathogen is very difficult and time consuming..Clinical material for laboratory examination should be collected and transported as soon as posible. The age of the pathologic process also affects the success of isolation, in the chronic course of the disease, secondary bacterial infections isolation of *M. hyopneumoniae often is* unsuccesful. Special culture media are used for laboratory detection and isolation of *M. hiopneumoniae*, and an atmosphere with elevated

CO_2 concentration is required. Colonies grow after 7-10 days, they are about 5 mm in size; they only exceptionally have a denser center grown into the substrate, and the appearance of "film and spots" is weak. Because of its slow growth, other microoganisms often cover it most commonly by the species *M. hiorhinis* and *M. flocculare*. The species *M. flocculare* is considered part of the physiological microflora of the respiratory system of healthy pigs, while *M. hyorhinis* often settles in the inflamed, altered lung tissue after primary infection with the species *M.hyiopneumoniae*. In many diagnostic laboratories, immunofluorescence with labeled polyclonal antibodies is used as the standard method for detecting *M. hyopneumoniae*. The disadvantage of this method is the frequent cross-substitution with the species *M. flocculare and M. hyorhinis*. The immunohistochemical method on lung specimens fixed in formalin is also available for the detection of the species *M. hyiopneumoniae*, but it is not sensitive enough (Rautiainen, 1998). Of the serological methods, RVK and ELISA are used and are considered the most sensitive, but still not sufficiently specific, methods for the diagnosis of enzootic pneumonia. Special antigen preparation avoids cross-reactions with the bacterial species *M. hyorhinis* and *M. flocculare*. In addition to blood serum, antibodies can also be detected in colostrum. The advantages of using colostrum are that it is easy to collect and the test is very sensitive, since colostrum has a higher concentration of antibodies than sow serum in the first 24 hours after farrowing. The disadvantage of antibody detection methods is that it is not always possible to distinguish diseased from vaccinated animals based on the results, and they cannot be used for early diagnosis of this disease because of the long period required for seroconversion. With advances in molecular biology, new diagnostic methods have emerged, the most important of which is the polymerase chain reaction (PCR), which has been shown to be highly specific, sufficiently sensitive, and rapid. To date, various PCR methods have been developed (single PCR, multiplex PCR, realtime PCR). Currently, the so-called "nested PCR" is used in most laboratories. This involves two consecutive PCR procedures, where the PCR product of the first procedure is immediately used as starting material for the second PCR procedure. This method is suitable when an extremely small amount of mycoplasma is expected in the test material, which cannot be detected by the usual PCR method (*Baumeister et al., 1998*), *Caron et al., 2000*). Material for the detection of *M. hyopneumoniae* can be lung tissue, if it is a dead animal, or swabs of the nasal mucosa, i.e. bronchoalveolar lavage from live animals*(*Otagiri et.al.200*5).*

Therapy

Despite the variety of antibiotics and chemotherapeutic agents used in the treatment of enzootic pneumonia in pigs, it is very difficult to cure the disease completely and to remove *M. hyopneumoniae* from the cultures. Because *M. hyopneumoniae* colonises the surface of the ciliated epithelium without penetrating the tissue, accessing and achieving a therapeutic dose of the drug is difficult. An additional obstacle to antibiotics is the accumulation of infected cells and the constriction of blood vessels in the affected area. An additional problem with on-site treatment is the simultaneous presence of animals in different stages of disease. The drugs used today to control and treat enzootic pneumonia in pigs are: tetracyclines, macrolides (tylosin, tilmicosin), lincosamides (lincomycin, clindamycin), pleuromutilins (tiamulin, valnemulin), fluoroquinolones (enrofloxacin, flumequin, danofloxacin, ciprofloxacin), aminoglycosides (streptomycin, gentamicin, tobramycin) and fluorfenicol (Prodanov-Radulović et al., 2020b; Vicca, 2005). Of the above-mentioned preparations, tetracyclines, macroloid antibiotics and pleuromutilins are most frequentlyused. The combination of chlortetracycline and valnemulin and chlortetracycline and tiamulin proved to be particularly effective. Fluoroquinolones and aminoglycosides are the only ones that have a mycoplasmicidal effect, which is why their use is recommended in programmes to eradicate enzootic swine pneumonia. The susceptibility of field isolates of *M.hyopneumoniae* to various antimicrobial preparations has been studied only rarely and on a small number of isolates, due to the difficult cultivation of this microorganism and the lack of standardised procedures (Burch, 2004). It is particularly important to emphasize that the stand-alone use of antibiotics under in vivo conditions cannot eliminate all microbes, especially when dealing with co-infection with viruses (PRSV and PCV_2), which significantly weaken the pig's immune system. Thus, treatment with antibiotics not only fails to kill the pathogen *(M. hyopneumoniae),* but also allows the emergence of resistant strains that can cause disease of epidemic proportions. The emergence of species resistant to certain antimicrobial preparations to which they were previously sensitive has also been noted; isolates of *M.hyopneumoniae* moderately sensitive to oxytetracycline have been isolated in Japan, and several authors noted reduced sensitivity of

certain species to chlortetracycline. They were the first to demonstrate acquired resistance of certain field isolates to macroloides (tylosin and tilmicosin), lincosamides (lincomycin) and fluoroquinolones (flumequine and enrofloxacin) (Vicca et al. 2005). The mechanism of resistance emergence is based on the high frequency of mutations in the genes encoding the target site to which antibiotics bind, which is a consequence of the low amount of genetic information destined for DNA repair systems. The frequency of occurrence of resistant *M. hyopneumoniae* species under field conditions is probably low, and some of the possible reasons for this are: the inability to transfer this type of resistance between different species, the instability of the gene mutation causing the resistance, and the more difficult spread of resistant species *(Bojkovski, et al. 1997 ., Vicca, 2005*).As enzootic pneumonia is a disease of intensive pig production, animals are usually treated in groups. Parenteral administration of the drug is usually used only in animals with an acute form of the disease for the first three days, after which therapy is continued (usually by one drug if water-soluble preparations are used for group therapy). For the most common, chronic form of the disease, all animals in the breeding are treated. Antibiotics are added to the drinking water. The problem with this application is the weak appetite of the sick animals. To prevent this, feed intake must be carefully monitored. Similar difficulties arise when antibiotics, which is an effective form of group therapy, are administered in the drinking water, as consumption is closely related to temperature, feed composition and many other factors. When selecting an antibiotic and its dose, many other factors must be taken into account (absorption of the antibiotic after oral administration and its distribution in the target tissue, solubility, secondary infections). Therefore, the focus of control of enzootic pneumonia should be on prophylaxis and control measures.

Prophylaxis

Prophylactic measures can be divided into those carried out in areas where infection with *M. hyopneumoniae* has not yet been detected and those carried out when this pathogen is already present in the herd. One of the preventive measures is the purchase and introductioninto the herd new animals that are free from *M.hyopnemoniae* and formation of a new mycoplasma-free herd.. Similarly, it would be good to introduce only animals from single source animals with , tested breeding farm, as mixing animals of different origins and health status reared on different farms increases the possibility of disease outbreaks. Whether farms remain free of the pathogen depends on the location of the farm itself. Thus, if there is an infected farm within a radius of three kilometers, it is only a matter of time before the infection spreads to healthy farms. Therefore, in areas with developed pig farming, it is almost impossible to keep the farm free of infection. By applying external biosecurity measures, the possibility of introducing mycoplasmas can be reduced. Uninfected farms should be kept as closed as possible, genetic material should only be introduced by artificial insemination, and newly arrived pigs must be quarantined for a period of eight weeks. Among the quarantined pigs, some animals from the own breeding should be placed and tested at the beginning of the quarantine and after five weeks. All animals in quarantine should be serologically tested and, in case of death, tissue samples should be submitted for molecular diagnostics. For farms raising only fattening animals, the "all in all" husbandry system should be applied. out ". In farms where *M.hyopnoniae* is already present and in areas where safe husbandry is not possible, the following measures are taken: Compliance with prescribed zootechnical conditions, vaccination and strategic medication. New animals introduced into farms where *M.hyopneumoniae* is already present may be infected with this bacterium, but should be free of other diseases such as swine dysentery or PRRS. If animals are free of the pathogen causing enzootic pneumonia and they are planned to be introduced into farms where this disease is already present, it is recommended to add antibiotics to the feed during the first weeks. Measures to reduce the risk of spread or clinical manifestation of the disease include: 1. avoiding overcrowding of the house, 2. ensuring good ventilation and air circulation, 3. maintaining a high level of hygiene, 4. reducing the amount of biologically active dust, micro-organisms, CO_2 and ammonia in the air, 5. reducing unnecessary manipulation of the animals and other stress factors, 6. Maintaining an optimal air temperature according to the pig categories, 7. Ensuring a balanced and high quality diet, 8. Avoiding dietary changes at sensitive life stages, 9. Monitoring the presence of other respiratory disease agents (PRRSV, SIV). Numerous vaccines have come onto the market in the last decade and are used with varying degrees of success in most countries with developed pig farming. When planning a vaccination programme, each breed should be considered as an individual case. In order for the expected outcome of vaccination to be as successful as

possible, the dynamics of the spread of infection at the level of a particular farm, the presence of competing diseases and the relationship between cost and expected gain should be taken into account. Vaccinating sows in the last stage of pregnancy provides protection for piglets in the first weeks of life. Most of the antibodies are absorbed by the piglets in the first six hours after colostrum intake, and the antibody level in the piglets' serum is the same as that of the sow about 24 hours after farrowing. The half-life of the antibodies is about 15 days, so that in piglets with a high initial titre, significant amounts of antibodies can still be detected 60 days after farrowing. In such piglets, infection is possible at a later stage of rearing and the disease usually occurs at the end of fattening. In contrast, in piglets with a low initial titer, a relatively low antibody level is observed after only 30 days. *(Burch, 2004, Hodgins et.al.2004*). It has been found that piglets with a high maternal antibody titre show a significantly weaker response to vaccination (interference phenomenon). In such cases, it is better to vaccinate 6-8 weeks after farrowing to allow a natural decline of maternal antibodies (passive immunity) and to achieve an adequate immune response in most vaccinated animals (active immune response). Piglets without maternal antibodies or with low titers can be vaccinated as early as one week after farrowing, as the age of the piglets has been shown to have no significant influence on the effect of the vaccination(Thackeret.al. 2000,Siugzdaite et.al.2002, Meyns et.al.2006,Valčić,2007)..

Control of Mycoplasma pneumonia in pigs in the Republic of Serbia

Respiratory diseases have become one of the biggest problems in modern pig production. Within this disease complex, *Mycoplasma pneumonia and Actinobacillus pleuropneumonia* occupy a prominent place. For this reason, the following was carried out: Examination of pig blood serum for the presence of antibodies against *Mycoplasma hyopneumoniae* and *Actinobacillus pleuropneumoniae* by ELISA. Blood samples were collected from sows, gilts, boars and piglets from 5 pig farms and analysed. The tests were performed by the indirect ELISA method using the following diagnostic kits: Herd Chek *M.hyo*: *Mycoplasma hyopneumoniae*, antibody test kit, and Chekit APP –Apx IV*: Actinobacillus pleuropneumo*niae (App) antibody test kit. A total of 1100 pig blood sera were tested, including 458 sera from sows, 434 sera from gilts, 88 sera from boars and 120 sera from piglets. Antibodies against *Mycoplasma hyopneumonia* were found in 176 (38.42 %) sow sera, 217 (50 %) gilts sera, 36 (40.90 %) boars sera and 30 (25 %) piglets sera. The percentage of positive sera varied between farms, ranging from 21-80 % in gilts, 17-65 % in sows, 16.67-100 % (10/10) in boars and 0-60 % in piglets. Antibodies against *Actinobacillus pleuropneumoniae* were found in 320 (69.86 %) sera from sows, 333 (76.72 %) sera from gilts, 43 (48.86 %) sera from boars and 66 (55 %) sera from piglets. The percentage of positive sera between farms ranged from 60.32-79.64% for sows, 64.54-89.58% for gilts, 34.09-72.73% for boars and 51.43-60% for piglets. The test results show that infection with *Mycoplasma hyopneumonia*e and *Actinobacillus pleuropneumoniae* is present in pigs in all 5 farms tested. The intensity of infection differs between farms and also between production and technology categories of animals. We highlight here gilts as a category in which the highest percentage of positive sera was found for *M. hyopneumoniae* with 80% and for *A. pleuropneumoniae* with 89.58%. (Žutić M, 2009, Žutić J.2008).

Successful control of *Mycoplasma pneumoniae and Actinobacillus pleuropneumoniae* depends on effective prevention of transmission of the pathogen both between farms and between certain categories of animals on the same farm. Good results can be achieved by strict application of reliable serological methods. Serological control of gilts is particularly important to detect infected animals before insemination and to remove them as such from the herd, as dams transmit the infection to their offspring after farrowing.

Control of Mycoplasma pneumonia in pigs in North Macedonia

In the studyconducted by Angelovski et.al.2023,antibody against *M.hyopneumoniae* were detecte3d in 58%(145/250) of pigs with highest seroprevalence observedin finishing pigs(86%,43/50). Lowest seroprevalence was detected in youngest pigs at 6 weeks of age(26%,13/50). In the same study ,high precentage (91,2%) of lungas with enzootic pneumonia like3 lesions was observed during slaugheter checks in finishing pigs.(Angjelovski et.al. 2023*).*

CONCLUSION

Enzootic pneumonia is widespread throughout the world and causes major economic losses in intensive pig farming. the pathogen type *M. hyopneumoniae* is found exclusively in pigs and is mainly transmitted by carriers or airborne. In farms where the pathogen is enzootic, the disease is chronic, and the constant occurrence of cramp-like, unproductive coughing on the farm, weaker feed conversion and consequently lower growth are often the only signs of the disease. Additional problems are caused by very frequent secondary infections with bacteria and viruses. The acute course of the disease occurs in pigs first exposed to *M. hyopneumoniae* and the visible signs of the disease are either very mild changes in the respiratory system to severe acute pneumonia associated with high mortality. Clearly demarcated, purple to grey areas of consolidated lung tissue, usually distributed over the apical and cranial lobes, are characteristic pathoanatomical findings for enzootic pneumonia in pigs. ELISA, polymerase chain reaction (PCR) and RT - PCR (real life polymerase chain reaction) have proven to be the most reliable methods for diagnosing enzootic pneumonia Antimicrobials most commonly used today to control and treat this disease are: Oxytetracycline, Chlortetracycline, Florfenicol, Macrolide antibiotics, Pleuromutilins and Fluoroquinolones. After treatment, *M. hyopneumoniae* can still be detected in the lungs and relapses are very common. Disease suppression and eradication measures include compliance with external and internal biosecurity rules, adherence to prescribed zoohygienic conditions, vaccination and, depending on the farm technique, the use of strategic medication .

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FOOD DEFNSE, FOOD SAFETY AND FOOD INDUSTRY

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Abstract

The potential of this study is to investigate issues regarding potential application of Food Defense concepts for Food Industry. According to Larson (2023), consumers face the risk that their food is unsafe because of natural and accidental contamination (traditional food safety problems) or deliberate contamination (food defense problems). Food Safety refers to a potential accidental hazard (physical, chemical, or microbiological) that may occur and Food Defense concerns a hazard that may be intentionally introduced, including by acts of terrorism. The study is based on exploratory research. A qualitative approach based on interviews with the Managers from the Food Industry. Other secondary data were collected through a private certification database.

Key words: Food Defense, Food Safety, Food Industry

According to Bogadi et al., (2016), at present, food business operators are increasingly required to comply with food quality and safety management systems to expand their business at national and international level. The main initiators of food defense implementation in the food supply chain are retail networks, who condition their producers' certifications in accordance with one of the food safety systems' standards.

Food defense is the effort to protect food from causing harm to the consumer, encompassing active steps, protection activities and/or security assurance procedures that deliver product safety regarding intentional acts of adulteration (Manning & Soon, 2016). Intentional adulteration may take several forms, such as acts of terrorism, tampering by discontented employees, consumers, or competitors, as well as economically driven adulteration (Bogadi et al., 2016).

In recent years, thorough measures to improve food safety in the food chain for consumers have become a necessity (Sarno, 2021)

To avoid the risk of food-related health hazards, it is necessary for businesses to promote food protection measures and for consumers at the end of the food chain to adopt the appropriate measures, such as food hygiene measures (Riaz, 2016).

The potential of this study is to investigate issues regarding potential application of Food Defense concepts based on ethics and protected against food fraud.

The approach is based on a study case which implements Food Safety procedures that are more open to ethics principles and then protected against Food Fraud or incorrect labeling, etc.

Food Safety refers to a potential accidental hazard (physical, chemical, or microbiological) that may occur and Food Defense concerns a hazard that may be intentionally introduced, including by acts of terrorism.

With frequent incidents of falsified expiration dates and contamination of food with foreign substances, the interest in food safety has increased considerably. Both situations are criminal incidents that involve employees from Food Industry. These incidents show the reality of "using food to cause health problems" and "contaminating food with foreign substances out of dissatisfaction with the company."

Moreover, they clearly demonstrate that food safety measures that only assume external crimes are insufficient. As a result, the term food defense has been repeatedly used in the media and is now a concept common not only in the food industry but also among consumers. Food defense is a countermeasure against food contamination caused by the intentional introduction of foreign substances (Newkirk, 2011; Kanagawa,2014).

Intentional adulteration incidents have been recorded at every major point along the farm-to-fork continuum: pre-harvest, processing, transportation, retail and at the consumer level (Fredrickson, 2014).

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Food-related companies and businesses must implement not only food hygiene measures but also food defense measures to ensure food safety. Food defense refers to practicing "safety management to protect against attacks on food, such as intentional contamination of food with foreign substances or contaminants" (Jurica, 2019; Xirasagar, 2010).

For implementing a Food Defense Plan, three major conditions must be implemented: Regulation, Food Safety Procedures, and a Contingency Plan.

A Food Defense Plan should be implemented based on Assessment Vulnerability - a process used to identify specific points in the food supply chain where intentional contamination has the greatest potential to cause economic and public health harm or to identify and prioritize the weaknesses (vulnerabilities) in a specific food operation chain.

Food Defense is an improvement for Food Safety Procedures. Food Safety represents one of the most important topics for the Food Industry.

Public health pays an important contribution to protect citizen's health through public health policies, laws and procedures based on risk analysis (Jensen and Sandoe, 2002).

Ethics will have a great contribution to food safety in three levels of risk analysis: first stage - risk assessment with value judgments in the process of risk assessment, the second - risk management, involving the process of weighing policy and technological alternatives to accept, minimize, or reduce assessed risks, to select and implement options by facilitate decision making, and the third stage, risk communication. Other contributions of ethics on risk management includes risk reduction (Sperling, 2010). These principles and values of public health ethics will also help balance various proposals to deal with the scientific food risk and determine the best (or least harmful) solution.

These principles and values include the salience of population health, safety, and welfare; fairness and equity in the distribution of services; and respect for the human rights of individuals and groups (Gostin, 2003).

Ethics of food safety is a dynamic area that continues challenging our perceptions of food consumption, health risks, and public responsibility for food borne illness. Food ethics may involve, for example, genetically modified organisms used in food (GMO), incorrect labeling or food fraud (substances that can change the composition or interfere with the biological states or processes in food).

Table 1

Assessing the implementation of food defense requirements in industrial food processors

Requirement description	Proportion of compliance (number of industries)
Compliance proportion of food defense requirements in industries (n=38) certified by the IFS standard (International Featured Standards, 2014)	
Food defense responsibilities are clearly defined. Those responsible should be key staff/ have access to top management team. Sufficient knowledge in this area should be demonstrated.	27/38 (71%)
A food defense hazard analysis and associated risks assessment must be performed and documented. Based on this assessment and on legal requirements, critical security areas must be identified. Food defense hazard analysis and risk assessment should be conducted annually, or upon changes affecting food integrity. An appropriate alert system must be defined and periodically checked for effectiveness.	0/38 (0%)
If legislation makes registration or on-site inspections necessary, evidence of these must be provided.	38/38 (100%)
Based on a hazards analysis and assessment of associated risks, critical security areas should be adequately protected to prevent unauthorized access. Access points should be controlled.	24/38 (63%)
Procedures must be in place to prevent tampering and/or allow identification of tampering.	28/38 (74%)
Visitor policy must include aspects of food defense plan. Delivery and loading staff in contact with the product must be identified and must comply with company's access rules. Visitors and external services providers must be identified in product storage areas and should be registered upon access. They should be informed about site policies and their access controlled accordingly.	35/38 (92%)
All employees must be trained in food defense on an annual basis or when significant program changes occur. Training sessions must be documented. Employee hiring and termination practices should consider security aspects as permitted by law.	20/38 (53%)
A documented procedure should exist for managing external inspections and regulatory visits. Relevant personnel must be trained to execute the procedure.	36/38 (95%)

Compliance proportion of food defense requirements in certified industries (n=6) by the FSSC 22000 standard (Foundation Food Safety System Certification 22000, 2019).	
Each organization shall assess the potential danger of acts of sabotage, vandalism, or terrorism to their products and should establish protection measures.	5/6 (83%)
The organization shall identify, preferably in the facilities plan, the areas considered more sensitive or susceptible to vandalism, sabotage and terrorism. Access to these places should be denied to unauthorized personnel using locks or electronic keys.	5/6 (83%)

MATERIAL AND METHOD

The study is based on exploratory research. A qualitative approach based on interviews with the Managers from the Food Industry. Other secondary data were collected through a private certification database. Food defense audit of the industrial units and then comparison of food defense vulnerabilities in the audited industries with those of other certified companies.

Interviews with 20 managers was conducted with questions based on requirements of IFS, BRC and FSSC 22000 (British Retail Consortium, 2015; Foundation Food Safety System Certification 22000, 2019; International Featured Standards, 2014). The questions with yes and no like an answer mixed into four groups: 10 external security; 2) internal security; 3) personnel security and 4) general requirements. Each interview was completed by un audit that included: facilities assessment, staff interviews, documents examinations, closing meeting with main findings, assess of probable causes and conclusions.

The selection criteria for the audited food industries included: being a meat-based food producing industry officially approved for food processing and regularly inspected by food authorities and having a food safety management certification system according to standards that included food defense requirements.

To compare the food defense audit results with those of other industries certified according to standards that consider food defense requirements (BRC, IFS and FSSC 22000), a consultation to a national private database was carried out. This database belongs to a private organization which operates globally and is concerned with certification of management systems, services, products, and individuals, providing audit, inspection, and training services.

RESULTS AND DISCUSSIONS

Analyzing the responses of the managers we also find out only 2 cases of incorrect labeling and addition of preservatives where reported, but due applicable procedures where corrected and 8 of food fraud were reported and the products where recall from the markets.

Based on Pilot Project EIR report' Analysis of food integrity in Romania (MADR, 2015), the top 10 of products most at risk of fraud in the Romanian food sector is: Olive oil, Fish, Organic food, Milk, cereals, Honey and maple syrup, Tea and coffee, Spices, Wine, and Certain fruit juice.

Analyzing the Food Fraud Network reports we find out that most incorrect labeling cases are connected with (place of origin; addition of water; dates; health claim; nutrition claim; denomination; ingredients; treatment and/or process; weight and/or volume; others) is the principal cause of the alleged violation in 2014 and 2015, followed by falsified documents, substitution, prohibited substances (additives; growth promoters; pesticides; veterinary medicines; others) and the suspicion of illegal export.

Auditing food defense requirements for the case studies contains: 1) external security assessment- external perimeter, building and structure, shipping, and dispatching; 2) internal security - storage of raw and subsidiary materials; 3) personnel security assessment- employee hiring, visitors or washing uniforms and 4) general requirements – preventive maintenance for premises and equipment, water distribution, mail, pest control, traceability, supplier control and emergency contacts.

To compare the food defense audit results of industries, audit reports on other previously certified food industries (according to at least one standard including food defense requirements: BRC, IFS and/or FSSC 22000) were assessed. Thus, a total of 45 food industries were considered, of which 38 were certified by the IFS standard, 6 by the FSSC 22000 and 1 by the BRC standard. All industries assessed had mature food safety management systems.

Considering the IFS and FSSC 22000 standard, Table 1 displays the proportion of compliance of the food industries certified by that standard.

CONCLUSIONS

If food intentionally contaminated with a foreign substance is sold and delivered to consumers, it is possible that consumers will eat it and experience health problems. Therefore, it is crucial for not only food manufacturers but also food delivery service providers to consider food

defense measures. Additionally, promoting consumer education and awareness regarding food defense can contribute to enhancing food safety throughout the food chain.

Food defense is a relatively unexplored concept. Several reasons seem to explain these observations, namely the novelty of food defense requirements as part of food safety management systems and the familiar character of food businesses. As an initial intervention strategy, food defense training, to get both the staff and managers acquainted with the concept, would be of upmost importance for these industries, pointing out tat the personnel is the most important resource.

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International database for the study includes companies certified between January 2014 and September 2016

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ASSESSMENT OF WIHDRAWAL PERIOD OF OXYTETRACYCLINE POST TREATMENT OF PIGS AND POULTRY IN ROMANIA

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Abstract

The presence of antimicrobial residues in animal products must be lower than maxim residue level (MRL), other way may have adverse effects on consumer health such as allergic reactions and resistance development. Withdrawal periods are used to avoid animas to be slaughter before the concentration of MRL declines with respect for public health's and waste food. The paper investigates the use of oxytetracycline in pigs and poultry with a focus on the differences on the withdrawal periods for different products used in Romania. The original question is whether compliance with the withdrawal period can be used to judge compliance with the MRL and its applicability for pigs and poultry industry.

Key words: Withdrawal period, antimicrobial, risk management, consumer health

The duration of the withdrawal period is listed in the specific product summary of all legal, veterinary medicinal products (VMP) used in production animals. The withdrawal period is set nationally or internationally and is based on the maximum residue limit (MRL), which is the maximum allowed concentration of a given residue in a carcase or a food product due to the treatment of an animal using a certain VMP (European Medicines Agency, 2023).

The MRL is derived from the acceptable daily intake value over a lifetime (ADI), wherein no pharmacological effect is expected in humans due to the residue in food products (European Medicines Agency, 2012). The ADI used in the MRL calculation is generally the lowest of the two ADIs (EU Commission, 2018).

Tetracyclines are the second most used drug class in European livestock production, following penicillins (European Medicines Agency,2021). It is mostly used orally in weaner pigs to treat post-weaning diarrhoea (Moura et al., 2023). Oxytetracycline is a tetracycline that can be used for intramuscular and intravenous injection or oral treatment and is often used to treat respiratory diseases and Mycoplasma- induced lameness in finishers and sows (Papich, 2021).

Official testing of live animals, carcases or meat thereof based on a suspicion of residue of a non-illegal VMP was possible from 1996 (EU Council, 1996). This allowed the competent authority under defined circumstances to test whether the live animal(s) for slaughter or the carcase(s) or the meat thereof, was complying with Indiscriminate use of antibiotics, lack of guidance and failure to notice drug withdrawal period, lack of consumer awareness are some primary reasons for occurrence of antibiotic residues in poultry edible tissue (Singh et al., 2014).the MRL and, therefore, considered as safe for human consumption. This practice was abolished in 2019 (EU Commission, 2019a).

The original question is whether compliance with the withdrawal period can be used to judge compliance with the MRL. Working group 1 (WG1) within the RIBMINS COST Action network investigated this for oxytetracycline for pigs, then we complete the investigation applied for Romania with poultry.

The usage of antimicrobials in poultry has increased due to their incorporation in diet as prophylactics, therapeutics, and growth promoters (Nonga et al. 2010). Despite of its therapeutics properties, oxytetracycline causes toxic effects like aplastic anemia, thrombocytopenic purpura, neutropenia, hemolytic anemia, thrombocytopenia, hypoglycemia, nausea, vomiting, diarrhea, esophageal ulceration, renal failure, hepatotoxicity, photosensitivity, rashes and tooth discoloration (Zaenglein et al., 2016).

Antibiotics are widely used in poultry production not only to treat diseases but also to maintain health, promote growth and enhance feed efficiency (Okerman et al., 2007). However, this practice may lead to deposit drug residue in poultry meat and products that is related with adverse

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health hazard to the consumers (Sarker et al., 2018). These hazards include toxic effects, immune-pathological effects, carcinogenicity, mutagenicity, nephropathy, hepatotoxicity, reproductive disorders, bone marrow toxicity and allergy.

Indiscriminate use of antibiotics, lack of guidance and failure to notice drug withdrawal period, lack of consumer awareness are some primary reasons for occurrence of antibiotic residues in poultry edible tissue (Singh et al., 2014).

Tetracycline, a broad-spectrum antibiotic is commonly used in poultry industry for its antibacterial and growth promotion effects. (Sarker et al., 2018). Oxytetracycline (OTC) a member of tetracycline family is difficult to be metabolized and partly excreted in the environment in the form of parent compounds due to its high solubility in water (Widiastuti et al., 2015). Higher OTC are being used due to its spectrum, availability, relatively cheaper price, and easy oral administration through drinking water or feed (Slana and Dolenc, 2013). Doxycycline (DC), a semi-synthetic, second-generation tetracycline is also used in poultry farm due to its wide coverage of organisms like Rickettsia, Chlamydia, Mycoplasma etc. and bacteriostatic activity (Hsiao et al., 2016; Prats et al., 2016).

Excessive use of these antibiotics in poultry farms causes accumulation of residue in meat that could be transferred to humans through the food chain, so idea of the study is to verify if after withdrawal period the meat is safe to be consumed. An interesting perspective might be consumer perception about this issue.

Non-prudent use of antibiotics in animal and poultry production such as using large doses, consistent use and ignoring withdrawal period could result in deposition of drug residues in different tissues of animal which threaten consumer's health (Sanz et al., 2015).

Surveys about consumer perceptions have shown that European consumers are increasingly concerned about the quality of their food. Three out of 10 Europeans mentioned chemical residues from pesticides (31%), antibiotics (30%) and pollutants like mercury and dioxins (29%) as risk to be "very worried" about - according to a European survey about consumer perception about food safety (TNS, 2010).

Therefore, monitoring of veterinary drug residues in foods of animal origin is important to prevent potential hazard of residues to public health. To prevent the risk of antimicrobial residues in animal products, maximum residue limit (MRL) which is the maximum permissible level of residues in the food with no adverse effect on consumer health was set by international organizations and associations such as WHO, FAO and European Community (Lateefat et at., 2022).

Table 1

Table Number of products with minimum and maximum withdrawal period for oxytetracycline

Pork – oxytetracycline 10 % authorised for intramuscular injection			Poultry – oxytetracycline oral suspension 20 % and 50 %		
No of products	Minimum withdrawal days	Maximum withdrawal days	No of products	Minimum withdrawal days	Maximum withdrawal days
5	7	28	2	3	10
*Long – acting oxytetracycline products are available on Romanian market.					

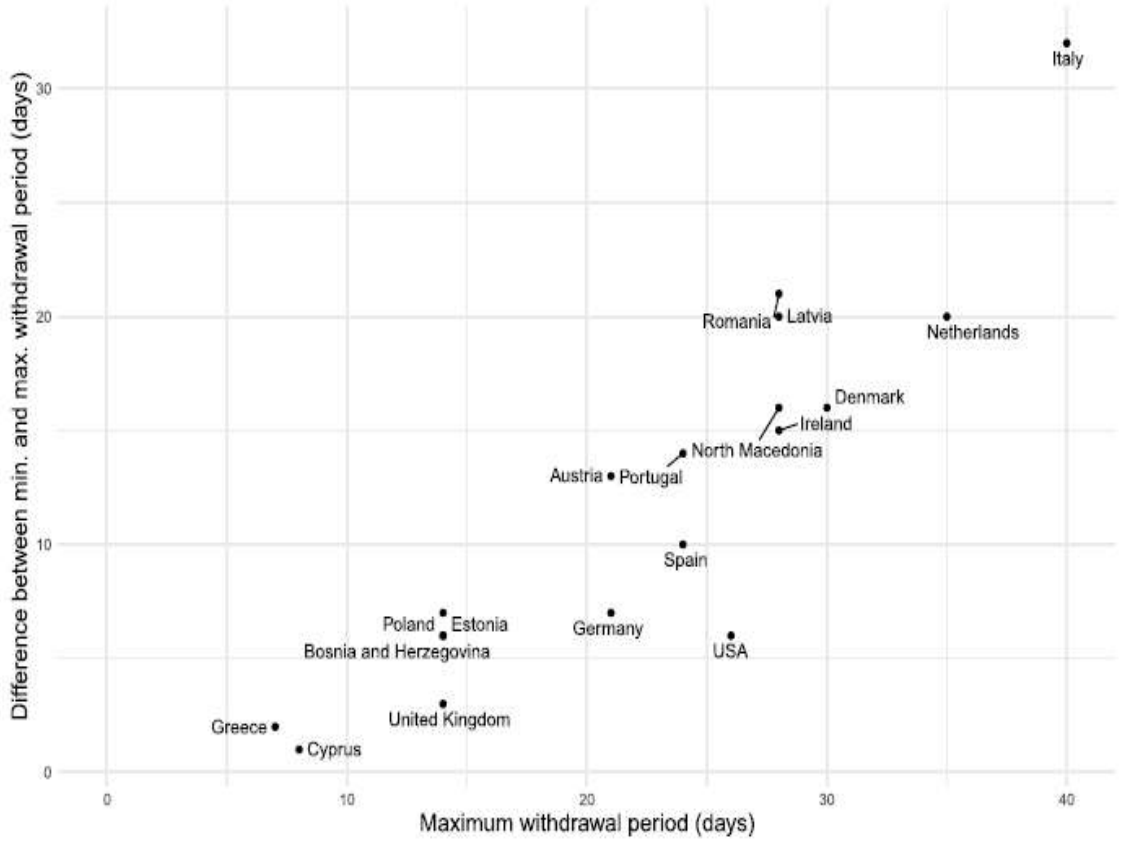

Figure 1. **The maximum withdrawal period plotted against the difference between the minimum and maximum withdrawal period for 18 countries in which multiple products registered (Lund et al., 2023)**

MATERIAL AND METHOD

In the original study, the RIBMINS WG 1 collected information about all oxytetracycline products used in pigs' treatment, fulfilling the inclusion criteria through their network. Then the information's collected for Romania were completed with similar ones from poultry production industry.

As mentioned before, the research project was divided in two contexts: first, assessing pig producers using a questionnaire survey, then secondly, screening of antibiotic residues in pigs and poultry. It is important that animal products be assessed by a method with good detection capability at or below MRL for most of the antimicrobial drugs used in food animal production.

We focus data collection to oxytetracycline products with 10 mg / ml concentration, corresponding with 10 % and authorised for intramuscular injection in pigs and oral suspension 20 % and 50 % for poultry. For the pigs we studied 5 products and for poultry 2 products.

The focus was on the recommended dosage, whether it was long-acting or not, duration of the withdrawal period, marketing name, authorisation holder and year of first authorisation (when available) in the studied country. The oxytetracycline products were found by searching the internet, focusing on major veterinary medicinal products (VMP) databases of specific product summaries in each country. Particularly, the oxytetracycline product had multiple withdrawal periods depending on whether a 24-h or 48-h dosage regime was administered or it s a single dosage regime.

We focus on identifying for the studied country, medium, median, and maximum withdrawal periods as showed in Table 1. Next, the difference between the maximum and minimum was plotted against the maximum withdrawal period in each country.

Some Hypotheses occur from the research:
Hypothesis 1. The year of the first market authorisation is correlated to the length of the withdrawal period, so older products have a longer withdrawal period than newer products.
Hypothesis 2. The duration of the withdrawal period is longer if the product is a long-acting formulation.

RESULTS AND DISCUSSIONS

The original study was based on 68 products from 29 countries in- and outside the EU. The withdrawal period ranged between 5 and 40 days with a median of 14 days. There was a clear correlation between the maximum withdrawal period and the maximum difference between the minimum and maximum withdrawal period in the

same country as presented in Figure 1 (Lund et al., 2023).

Based on questionnaires analysis we made some evaluation of issues Hypothesis.

The first hypothesis evaluated if exists an association of the length of the withdrawal period based on the year of first market authorisation.

The second hypothesis evaluated whether the duration of the withdrawal period between short- and long-acting products differed. The analysis showed that long-acting products had a shorter withdrawal period than short-acting products, verified by a Wilcoxon ranked sum test. The short-acting products had a wider range in withdrawal periods. Thus, the short acting exhibited a greater variation in withdrawal periods, whereas the long acting had a more concentrated distribution partly due to fewer products.

On the screening of antimicrobials for pigs (meat and kidney) or poultry (carcass without skin and liver) no antimicrobials present. If antimicrobial residues present, the carcasses will be condemned and destroyed.

CONCLUSIONS

Our survey identified that a minimum, median, and maximum withdrawal period of 5, 14 and 40 days, respectively, are in force for oxytetracycline 100 mg/ml for intramuscular use in pigs and a period of 3, 7 and 10 days are in force for oxytetracycline oral use for poultry.

The year of the product authorisation have not a significant effect on the duration of the withdrawal period.

Another significant contributing factor was whether the product was long- or short-acting. The finding that long-acting products were associated with a shorter withdrawal period than short-acting products further points to the need for updated and harmonised withdrawal periods. The reason for the observed variation is the prior lack of a harmonised approach to determine the withdrawal periods. According to Lund et al. (2023), until the withdrawal periods are harmonised, we suggest using a risk-based approach to calculate the actual concentration of a given VMP at the time of slaughter when an animal is accidently delivered prematurely. In this way, the concentration can be compared with the MRL. If it is higher than MRL, safe ways of handling can be identified through intended use compared to the ADI.

ACKNOWLEGMENTS

The original work was undertaken by a working group (WG 1) within the European COST Action, RIBMINS CA18105. RIBMINS is an acronym for risk-based meat inspection and integrated meat safety assurance. Please see https://ribmins.com/ for more information. In this paper, the aims were to: collect information about current ways of monitoring the presence of AM residues in pigs and pork and develop best practices depending upon the objective of monitoring and control in the individual country. Later, the research was completed with poultry study case applied in Romanian framework.

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DIAGNOSTIC METHODS USED TO DETECT *TOXOPLASMA GONDII* INFESTATION IN CATS - CASE REPORT

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Abstract

The results regarding the prevalence of toxoplasmosis in humans in the city of Iasi during one year, show a rate of 6,6% of cases detected with positive IgM, and 32.7% of cases detected with positive IgG, compared to the prevalence of toxoplasmosis in cats which shows a rate of 0.7% of positive cases detected with positive IgM; which denotes that toxoplasmosis is underdiagnosed in veterinary medicine. A very important role is played by the diagnostic method used. The article deals with a case study, a 1,8-year-old cat with cerebellar ataxia, dysmetria and hypermetria, with moderate opacification of the entire corneal surface, panuveitis, chorioretinitis and corneal edema. Following the paraclinical investigations, the diagnosis of toxoplasmosis was made, using the Welltest *Toxoplasma* IgG/IgM immunochromatographic test, confirming the acute phase of the disease with positive IgM and negative IgG. Using the molecular detection techniques through qRT PCR, the result was negative, emphasizing the fact that the protozoan *Toxoplasma gondii* uses the blood as a way of spreading in the body, the relatively short phase that can induce a negative result, despite the presence of severe symptoms. The conclusions emphasize the importance of using a correct diagnostic method, molecular techniques, despite their high sensitivity, are not always recommended. In toxoplasmosis, the recommended diagnostic method is the serological one to detect IgG/IgM antibodies.

Key words: *Toxoplasma gondii*, qRT PCR, IgG/IgM antibodies

Introduction. Toxoplasmosis is a very important zoonosis caused by the intracellular protozoan *Toxoplasma gondii*, having as its intermediate host almost all warm-blooded animals, including humans, in which transplacental transmission can be life-threatening to the fetus causing death or severe neurological damage, inflammation and retinochoroiditis (Molaei S. *et al*., 2022; Torrey, E.F.; Yolken, R.H. 2013). Immunocompromised patients are associated with severe central nervous system damage, lethal encephalitis and myocarditis (Galvan-Ramirez M., 2013).

The reproductive sexual cycle of *Toxoplasma gondii* occurs only in definitive hosts, represented by domestic and wild felids, as they may also be intermediate hosts. First, tachyzoites develop an active multiplication in tissues, associated with a rapid invasion that produces harmful effects. They have a tropism especially for the central nervous system and striated muscles, where they remain dormant as bradyzoites, leading to a long-term chronic infection until a definitive host ingests the tissue. After 16-21 days of infection, cats excrete oocysts in feces, contaminating soil and water (Calero-Bernal, R. & Gennari, S. 2019; Dubey, J. 2004; Dubey, J. & Jones, J. 2008; Weiss, L. M. & Dubey, J. P. 2009; Silva, J. C. R. *et al*., 2001; Dubey, J. *et al*., 2020).

Toxoplasma infection in cats is in most cases asymptomatic, complicating the diagnosis of the disease, increasing the risk of infection in humans and animals.

This parasitosis has repercussions on public health, producing a wide range of symptoms in humans, as well as repeated abortions, significant economic losses in animals (Hatam-Nahavandi K. *et al.*, 2021; Dubey J.P. *et al*., 2020). Clinical signs are not conclusive to distinguish toxoplasmosis from other infections (Molaei S. *et al.*, 2022). Serological methods are the most commonly used in the diagnosis of toxoplasmosis (Shieh M. *et al*., 2017; Dard C. *et al.*, 2016). New techniques with higher selectivity and accuracy are needed for direct determination of biomarkers for *Toxoplasma gondii*.

Toxoplasma gondii infection has an acute phase of manifestation, which in immunocompetent patients is often asymptomatic,

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and a chronic phase (Pittman K.J. *et al*., 2014). In recent years biosensors have been defined as ideal for the diagnosis of toxoplasmosis due to their sensitivity and selectivity compared to formal procedures (Anik Ü. *et al*., 2017).

In terms of the clinical picture, there is a variety of symptoms, correlating with the different categories of toxoplasmosis, including the acquired form in immunocompetent patients, during pregnancy and congenitally, a reactive form in immunocompromised patients, as well as ocular infections. But these symptoms are not specific and vary from asymptomatic forms in immunocompetent patients to ocular forms, congenital neurotoxoplasmosis being often fatal. Thus, the use of several different diagnostic methods is necessary for a diagnosis of certainty (Ybanez R.H.D. *et al*., 2020; Strharsky J. *et al*., 2009).

Diagnosis is crucial for surveillance, prevention and control of toxoplasmosis. Laboratory diagnostic approaches include immunological, molecular and immunohistochemical methods. Thus, diagnosis is divided into indirect methods by immunological tests and direct methods of parasite detection by microscopy and molecular methods (Müller de Barros R.A. *et al*., 2022). Thus, we have the Sabin-Feldman dye test (DT) enzyme-linked immunosorbent assay (ELISA), immunosorbent agglutination assay (ISAGA), indirect hemagglutination test (IHA), indirect fluorescence antibody test (IFAT), modified agglutination test (MAT), latex agglutination test (LAT) and Western blot (WB) (Liu *et al.*, 2015; Sun *et al.*, 2015; *Dard et al*., 2016).

In human medicine, the detection of specific anti-toxoplasma antibodies (IgM, IgE, IgG and IgG progress) in serum samples of affected patients is a priority (Montoya J.G. *et al.*, 2002; Rostami A. *et al*., 2018; Wassef R. *et al*., 2019). Direct molecular methods are commonly used for the diagnosis of toxoplasmosis, especially in immunocompromised patients with serum antibody deficiency or prenatal and congenital transmission. Thus, various molecular methods, including conventional PCR, RAPD-PCR, RT-PCR, high-resolution melting and microsatellite analysis, are used to improve diagnostic methods (Teixeira L.E. *et al.*, 2013; G. Saadatnia *et al*., 2012; K. Khanaliha *et al*., 2021; Witter R. *et al.*, 2020).

The sensitivity and specificity of PCR techniques depend on a number of factors such as the gradients of the amplification reaction, the primers used and the method of DNA extraction from biological samples such as whole, pleural and peritoneal blood (Montoya J.G. *et al.*, 2002; Molaei S. *et al*., 2022). Sensitivity and specificity between PCR techniques, were between 70-95% and 85-100%, respectively, in several studies performed on different samples (Khanaliha K. *et al*., 2021; Ferreira Ade M. *et al.*, 2004; Soltani Tehrani B. *et al*., 2020). In humans the PCR technique, allowed the detection of *Toxoplasma gondi* DNA in clinical samples such as amniotic fluid, aqueous humor, cerebrospinal fluid, bone marrow and blood (Edvinsson B. *et al*., 2008; Mattos C.C. *et al*., 2011; Camilo L.M. *et al*., 2017), the technique being recommended especially in AIDS patients, who have a poor immunological status.

MATERIAL AND METHOD

The study aimed to establish a diagnostic protocol for toxoplasmosis in veterinary medicine, given that this disease is under-diagnosed in animals as compared to the high number of positive cases in humans. Thus, we consulted the data on toxoplasmosis cases diagnosed in Iasi County in humans, in the Praxis laboratory, in order to demonstrate that the incidence of this disease in cats in Iasi County is not real and the need for caution in diagnosis is required.

Case description

A case was presented in the Faculty of Veterinary Medicine Iasi: cat, common breed, 1 year and 8 months with apathy, inappetence, cerebellar ataxia, dysmetria and hypermetria.

Ocular ultrasound showed a reduction in the size of the eyeball, of the anterior and posterior chamber; areas of increased hyperechogenicity in the uvea, and of the projection area of the optic nerve papilla with an increase in size of these structures. In both eyes there is thickening of the posterior structures with increased echogenicity (choroid and retina). The diagnosis of panuveitis, chorioretinitis and corneal oedema was established. Nervous signs and ocular damage led to the suspicion of toxoplasmosis. The acute stage fades in a few days to months, leading to the latent stage. Latent infection is normally asymptomatic; however, in immunocompromised patients (such as those infected with HIV or transplant recipients on immunosuppressive therapy), toxoplasmosis may develop. The most notable manifestation of toxoplasmosis in immunocompromised patients is toxoplasmic encephalitis, which can be fatal. If infection with *T. gondii* first occurs during pregnancy, the parasite can cross the placenta, possibly leading to hydrocephalus, intracranial calcification and chorioretinitis, with the possibility of miscarriage or intrauterine death. It has also been tested for FIV and FELV, with negative results.

The *Toxoplasma* IgG/IgM Antibody (TOXO Ab) rapid test, using the double layer sandwich lateral flow immunochromatographic method, was used for the diagnosis. The test aims at the qualitative detection of *Toxoplasma* IgG and IgM antibodies in animal blood samples, serum or plasma.

The rapid test for veterinary use - Well Test Toxoplasma gondii Ag- a rapid test using the double-layer, sandwich, lateral flow immunochromatographic method was also used for the qualitative detection of *Toxoplasma gondii* antigens in faecal, serum or plasma samples.

Microscopic examination of blood smears and diagnosis by RT-PCR were used as direct methods. Real-time PCR is the fastest and most reliable method to achieve accurate detection of *T. gondii.* The DNA extraction, was made using BioMagPure 12 Plus (Biosan, Latvia). Concisely, genomic DNA was extracted from 200 µl whole blood using Blood DNA Extraction Kit 200, according to the manufacturer's protocol.

For RT-PCR diagnosis, Nzytech's *Toxoplasma gondii* quantitative qPCR kit was used, which is a highly specific product designed for real-time PCR (qPCR) applications. The qPCR method serves as the gold standard in molecular diagnostics due to its exceptional accuracy, specificity and sensitivity. A *T. gondii* specific primer and probe mix is provided and can be detected through the FAM channel in a Real-time PCR experiment.

The primer and probe mix provided exploits the so-called TaqMan® principle. During PCR amplification, forward and reverse primers hybridize to the T. gondii DNA. A fluorogenic probe, which consists of a DNA sequence labelled with a 5`-dye and a 3`-quencher, is included in the same reaction mixture to hybridize specifically in the DNA target region between the two primers. During PCR amplification, the probe is cleaved and the reporter dye and quencher are separated. The kit includes a positive control template that allows controlling the PCR set-up and is also useful for copy number determination. This can be used to generate a standard curve of T. gondii copy number / quantitation Cycle (Cq) value.

BioRad's CFX96 equipment was used, using the thermal cycling conditions recommended by the kit (*table 1*).

Tabel 1

RT-PCR program used

Cycles	Temperature	Time	Notes
1	95°C	2min	Polymerase activation
40	95°C	5 s	Denaturation
	60°C	30 s	Annealing/Extension

RESULTS AND DISCUSSIONS

Prevalence of toxoplasmosis in Iasi County

Diagnosis in the Praxis laboratory is performed on request, using immunochromatographic tests for qualitative determination of IgG and IgM antibodies. But the diagnosis of toxoplasmosis is much more complex and must combine both direct and indirect methods for a certainty result.

The results of epidemiological investigations carried out at the Praxis laboratory, Iasi County in the period July 2022-July 2023 showed 226 requests for anti-*Toxoplasma gondii* IgM antibody screening (ELISA), of which 208 in women and 18 in men, with 15 positive cases in women and no positive cases in men (*figure 1*).

Figure1 **Distribution of toxoplasmosis by sex in humans**

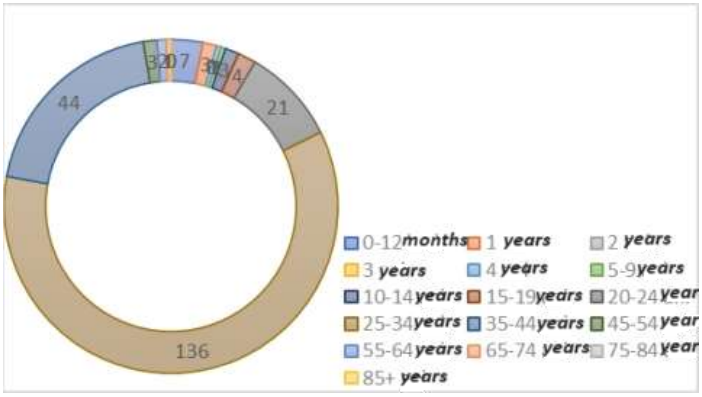

Figure 2 **Distribution of toxoplasmosis by age groups for the presence of IgM**

Out of a total of 226 tests performed for women and men, 60.17% (136) were performed in the age category 25-34 years, 19.46% (44) were performed in the age category 35-44 years, 9.29% (21) of the tests were performed in the age category 20-24 years, 3.09% (7) of the tests were performed in the age category 0-12 months, and the rest of the tests were performed in the other age categories (*figure 2*). The high proportion of testing in the 25-34 age group is strictly related to the number of pregnant women in this age group.

Out of a total of 220 positive tests, 134 (60.90%) were performed in the age category 25-34 years, 37 (16.81%) were performed in the age category 35-44 years, 20 (9.09%) in the age category 20-24 years, and the remaining 29 (13.18%) in other age categories (*figure 3*). The presence of the majority of IgG-positive cases in the age category 25-34 years is also directly proportional to the high number of cases tested in this category, closely related to the testing during pregnancy.

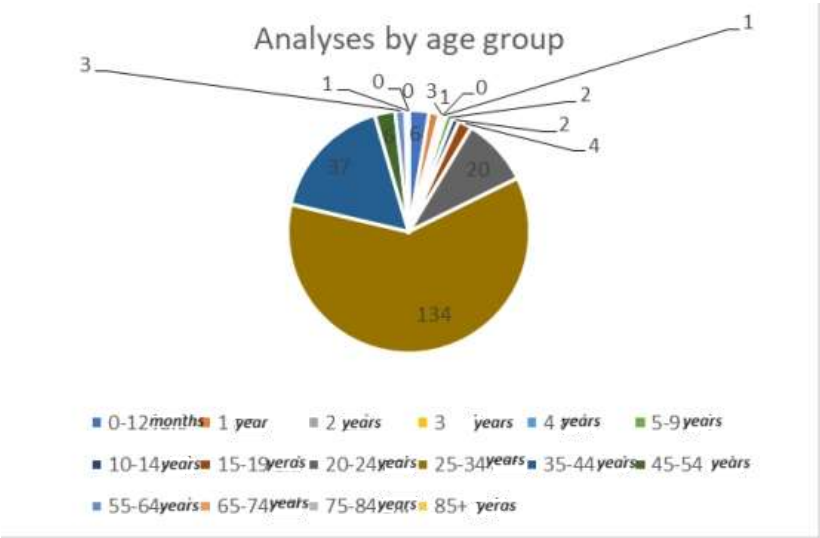

Figure 3 **Distribution of toxoplasmosis by age groups for the presence of IgG**

Studies show that IgM antibodies peak at 2 months post-infection, making the sensitivity and specificity of serological tests dependent on the timing of post-infection testing.

The case presented at the Faculty of Veterinary Medicine underlines the need to combine diagnostic methods in toxoplasmosis.

The *Toxoplasma* IgG/IgM Antibody (TOXO Ab) rapid test was weakly positive for IgM and negative for IgG. The WELLTEST *Toxoplasma gondii* Antigen test was negative (*figure 4*).

Cytological examination - Smear exam (MGG/DQ stain):

A. Schizocytes, erythrocyte agglutination, anisocytosis (reduced degree);
B. Nf reactive, Nf total 88.6% (35.90x 10^3/µl), Nf young (1-2 lobes) 62.4%, Nf (3 lobes) 29.4%, Nf (4-5 lobes) 8.2, %, Eo 0% (x10^3/µl), Mo 4.1% (1.66x 10^3/µl), Lf 7.3% (2.96x10^3/µl).
C. Giant platelets.

Cytological and haematological diagnosis (reference values The Merck Veterinary Manual,

ed 8): anaemia, leukocytosis, neutrophils, eosinopenia, lymphopenia.

Figure 4 **Results of the WELLTEST *Toxoplasma gondii* Antigen *and Toxoplasma* IgG/IgM Antibody (TOXO Ab) rapid test respectively**

Diagnosis by RT-PCR was negative, which underlines the importance for diagnosis of using an appropriate biological sample and of collecting it at the right time in the course of the disease. Although RT-PCR is considered the gold standard in diagnosis, in toxoplasmosis it is only useful in the dissemination phase in the body, when blood is used as a biological sample.

The detection of oocysts in the faeces is not a reliable method of diagnosis because they look similar to those of some other parasites. Additionally, cats can also shed oocysts for only a short period of time and often are not shedding oocysts when they are showing signs of disease. Clinically manifested toxoplasmosis occurs during dissemination and intracellular replication of tachyzoites. It usually occurs as a reactivation of a latent infection, and more rarely after a newly acquired infection. If a carrier cat is immunosuppressed, bradyzoites in tissue cysts rapidly replicate and disseminate again as tachyzoites.

CONCLUSIONS

Following a 2020 study by Mahbobeh Montazeri et al. on the basis of official reports it is estimated that more than one billion people would be infected with *T. gondii*, mainly through consumption of food: water, vegetables and fruit contaminated with sporulated oocysts shed by cats and through consumption of raw or undercooked contaminated meat. CDC (Center for Disease Control and Prevention) reports toxoplasmosis as the second most common cause of death due to food-borne diseases - approximately 327 deaths and the fourth leading cause of hospitalizations attributed to food-borne diseases (approximately 4428 hospitalizations) in the US in the mid to late 2000s.

Regarding the definitive host, a global survey conducted between 1967 and 2017 estimated the seroprevalence of *T. gondii* at 35% in domestic cats and 59% in feral cats. It is most widespread in Australia and Africa, where the seroprevalence of *Toxoplasma gondii* in domestic cats reaches 52% and 51% respectively. Asia ranks last with a seropositivity of 27% in domestic cats. As for *Toxoplasma gondii* seroprevalence values in feral cats, it is estimated at 74% in Africa, 67% in Asia, 67% in Europe and 66% in South America.

The article points out that this disease is under-diagnosed in both human and veterinary medicine. Although the number of cases is much higher in human medicine, it is found that the requests are correlated with the period of pregnancy, when this toxoplasmosis screening test is mandatory, and is not suspected or monitored in the human population.

Also, toxoplasmosis in cats is often asymptomatic, with few requests for diagnosis, except in cases of severe symptoms.

The importance of using the correct diagnostic method in toxoplasmosis according to the stage of the disease has been highlighted in this case study. Thus, molecular methods using blood as a biological sample are not recommended, as they may be false negatives.

The protozoan is rarely found in blood, and occasionally in CSF, fine needle aspirates of organs (e.g., lymph nodes) and transtracheal or bronchoalveolar lavages and are common in peritoneal fluids of animals developing ascites, but collection of these biological samples is invasive and poses risks to the patient. Detection of tachyzoites confirms the diagnosis.

We conclude a need for the use of combined methods in the diagnosis of toxoplasmosis, recommending the use of serological methods, but still considering the development of IgM antibodies, differently depending on the immune status of the host, considering that it is necessary to use both direct and indirect methods.

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DIAGNOSIS AND TREATMENT OF *AELUROSTRONGYLUS ABSTRUSUS* INFESTATION IN CATS - CASE REPORT

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Abstract

Studies on *Aelurostrongylus abstrusus* infestation in domestic cats are limited, both for Europe and globally. Diagnosis is quite laborious and often the infestation is not included in the differential diagnosis of respiratory diseases, a fact which leads to a late diagnosis that endangers the life of the animal, even causing its death. In case of massive infestation, respiratory symptoms are complemented by a diarrhoeal syndrome and anorexia. In June 2023 at the Faculty of Veterinary Medicine in Iasi, Romania, a cat (common breed, male, aged 1 year and 4 months) came with respiratory symptoms, chronic cough, shortness of breath, mucopurulent nasal discharge, accelerated breathing and loss of appetite. The cat had previously been treated for chronic bronchitis. Radiological interpretation was of an intensified interstitial lung pattern with nodular and bronchial appearance, compatible with chronic micro-bronchitis. The first diagnosis was pneumonia, but following symptomatic treatment the results were not satisfactory. Following coproparasitological examination the result was infestation with *Aelurostrongylus abstrusus* and antiparasitic treatment was instituted.

Key words: *Aelurostrongylus abstrusus*, laborious diagnosis, coproparasitological examination

The metastrongyloid nematode *Aelurostrongylus abstrusus* is a worldwide occurring feline lungworm found in the lower respiratory tract, particularly in the bronchioles and alveoli (lung parenchyma) of felines. Female nematodes are oviparous, and from the eggs laid the first larval stage hatches in the alveoli and alveolar canals. L1 larvae pass from the respiratory tract into the gastrointestinal tract and are released into the external environment with the faeces. The infesting L3 larval stage develops inside snails, which are the intermediate host in the life cycle of the parasite. Cats become contaminated by carnivorism, consuming small mammals, birds, reptiles or amphibians, which feed on gastropods, representing paratenic hosts. In cats, larvae enter the upper gastrointestinal tract on the first day of infection and reach the lungs shortly afterwards. After two more clutches, females begin laying eggs in the fourth week after infection (Anderson, 2000; Bowman *et al*., 2002; Grewal *et al*., 2003).

Since the original description of the parasite *A. abstrusus* isolated from cats in 1890, the global distribution of this parasite in cats has been documented through numerous case reports and epidemiological surveillance (Bowman *et al.*, 2002). The clinical picture in cats ranges from mild symptoms (e.g., nasal discharge or cough) to severe respiratory failure. Although most cases are asymptomatic, a number of respiratory problems associated with *Aelurostrongylus* infestation in cats have been reported (Grandi *et al*., 2005; Payo-Puente *et al*., 2005; Iannino *et al*., 2013), and pathological changes of varying degrees have been shown necropsically (Dennler *et al.*, 2013). Thus, a misdiagnosis or delay in treatment may result in death of the host.

A study in Switzerland showed that altitude and temperature are limiting factors for *Aelurostrongylus* infestation in cats, being more common in regions with average temperatures above -2°C and in regions below 700m above sea level; serological testing can help to improve the identification of infected animals by assessing risk factors at population level and for better management at individual level, overcoming the challenges posed by faecal examination which is not available to everyone (Gueldner *et al.*, 2018).

Cats that have access to the free-ranging environment are at risk of contamination with a series of potentially life-threatening parasites. Some parasitic infestations are underdiagnosed and

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can cause life-threatening pulmonary and cardiovascular disorders. Thus, the mistakes made in establishing the diagnosis or delaying treatment can lead to the death of the host (Studzinska *et al*., 2017).

Carnivores are parasitized in the lungs by numerous nematode species that localize in the trachea, bronchi, lung tissue but also in the right ventricle and pulmonary artery, causing the development and progression of distinct morbid conditions. We can name oslerosis, crenosemiasis, aelurostrongylosis, angiostrongylosis and pulmonary filariasis. Metastrongyloid parasite infestation of dogs and cats is poorly studied in Romania.

Recent European studies indicate that these parasites are spreading in Europe (Lange *et al.*, 2018). A study on lungworm infestation of wild cats in Europe, conducted on 16 carcasses, from 16 European feral cats (*Felis silvestris silvestris*), revealed the presence of *Aelurostrongylus abstrusus*, the most common, followed by *Troglostrongylus brevior*. Three specimens of *Angiostrongylus chabaudi* found in the pulmonary arteries of a feral cat were also reported. Histologically, the most common lesions were mild to severe chronic catarrhal bronchitis and chronic interstitial pneumonia with smooth muscle hypertrophy associated with *T. brevior* and *A. abstrusus,* respectively. These results demonstrate that European feral cats may harbour several species of lungworms that can affect their health and well-being. Also, *F. s. silvestris* represents a potential reservoir for respiratory nematodes in domestic cats (Lange *et al.*, 2016).

A study conducted in Italy on 250 cats showed the presence of antibodies against *A. abstrusus* in forty-five (21.4%, 95% CI: 16.1-27.6%) samples. This study confirms the occurrence of *A. abstrusus* in endemic areas of Italy and indicates that one-fifth of randomly selected cats have or had lungworm infection with antibody production (Di Cesare *et al.*, 2018).

MATERIAL AND METHOD

In January 2023 at the Faculty of Veterinary Medicine in Iasi- Romania, a cat (common breed, male, aged 1 year and 4 months) appeared with respiratory symptoms, chronic cough, shortness of breath, mucopurulent nasal discharge, accelerated breathing and loss of appetite. The cat had previously been treated for interstitial pneumonia.

In view of previous unsuccessful attempts to treat respiratory complaints, a coproparasitological examination was recommended to rule out suspected infestation with *Aelurostrongylus sp.*

The coproparasitological examination was performed using the Willis flotation technique and also a settling method and a Baermann larvoscopic method.

Control examination of faecal samples was performed using the Baermann method at 21 days, 1.5 months and 3 months after therapy.

RESULTS AND DISCUSSIONS

The result of the coproparasitological examination using the Willis flotation method was negative, and one larva of *Aelurostrongylus abstrusus* was found through the settling method (*figure 1*). The Baermann larvoscopic method revealed a massive infestation with *Aelurostrongylus abstrusus.*

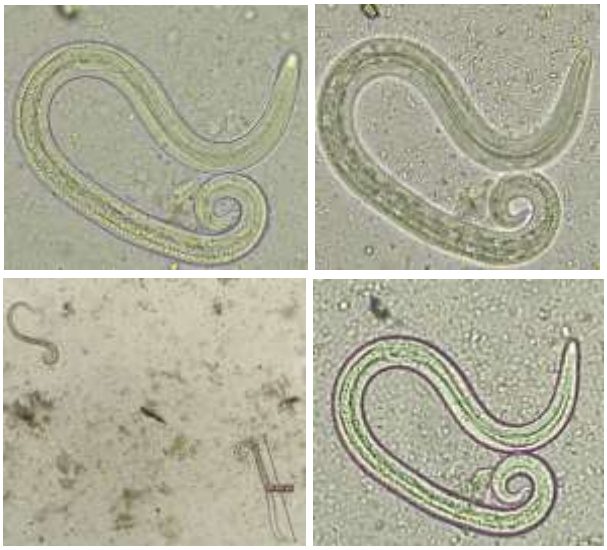

Figure 1 **L1 larva of Aelurostrongylus abstrusus**

Radiological examination shows a thickened pleural line with irregular appearance. Diffuse interstitial-bronchial mixed lung pattern in the lung parenchyma with fluid-specific radiopacity in the left diaphragmatic lobe. Slightly radiologically enlarged right heart silhouette, trachea with normal calibre and trajectory. Radiological appearance compatible with parasitic pathology/ bronchopneumonia (*figure 2*).

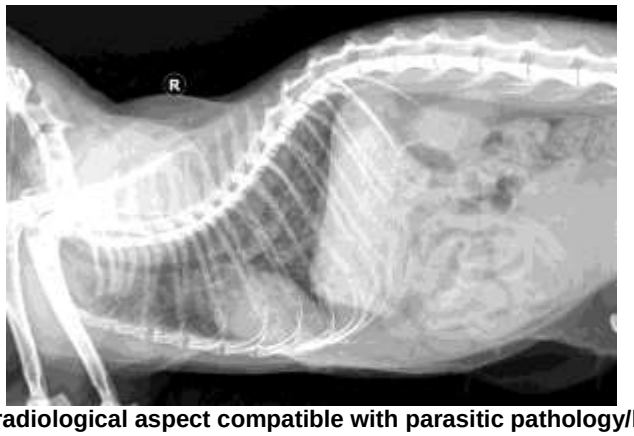

Figure2 **X-ray examination – radiological aspect compatible with parasitic pathology/bronchopneumonia**

The treatment consisted in the administration of Panacur paste 4.8g, containing fenbendazole 187.5 mg/g, the dose administered was 50 mg fenbendazole/kg body weight administered for 3 consecutive days. As adjuvant treatment probiotics were administered Viyo Recuperation Cat, 1 sachet/day for 7 days and RX Hepato Support 1 capsule/day for 30 days.

After 3 weeks the coproparasitological examination was repeated (flotation method), and the result was positive for *Aelurostrongylus abstrusus* infestation. It was recommended to continue treatment with Nexgard Combo (Esafoxolaner 3.60 mg, Eprinomectin 1.20 mg, Praziquantel 24.90 mg) 1 administration/month for 3 months. The coproparasitological examination at 1.5 months and at 3 months confirmed negative diagnosis of *Aelurostrongylus abstrusus* infestation, as the cat remained under its owner observation.

CONCLUSIONS

Some parasitic infestations are underdiagnosed and can cause life-threatening pulmonary and cardiovascular disorders. Thus, a mistake in diagnosis or delay in treatment can lead to the death of the host. In *Aelurostrongylus abstrusus* infestation, diagnosis is quite laborious, requiring coproparasitological diagnostic methods performed and interpreted by a specialist parasitologist. The article underlines the fact that aelurostrongylosis is an underdiagnosed parasitosis in veterinary medicine, often diagnosed and treated as bronchopneumonia, making it necessary to monitor this parasitosis by specialist coproparasitological examinations. The difficulty of effective treatment is also emphasized, as regular specialist rechecks are necessary to monitor the efficacy of antiparasitic therapy.

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THE USE OF SOME EXOMETABOLITES FROM MICROMYCETES FOR THE FORTIFICATION OF RESISTANCE INDICES IN BEE

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Abstract

Abstract. The goal of the proposed research was focused on the use of exometabolites of micromycetes to increase the physiological resistance of bee families after the winter period, as well as to stimulate their productive indices. From the 21 strains of micromycetes taken from the National Collection of Nonpathogenic Microorganisms of the Institute of Microbiology and Biotechnology, TUM, were selected 3 strains (Ps.sp.11, Ps.sp.19 and Ps.sp.62) which showed more productive indices of the development on culture media, as well as more pronounced bactericid properties. Exometabolites were prepared from the mentioned strains and administered to 3 experimental grups of bee familes in doses of 10, 25 and 50 ml per kg of wheat flour cakes. The productive indices of the bee families were examined over 12, 24 and 36 days after the administration of the biomass of exometabolites. As a result, it was established that the highest index - 47.1 squares of hatched brood, was registered at 24 days after the administration in the 1st experimental group of bees which was fed with a dose of 25ml/kg of wheat flour cakes. The difference between this group and the control group was 19.4 squares of hatched brood. At the same time, the honey collection per beech was 3.4 kg in the 1st experimental group of bees, representing 0.8 kg more compared to the control group and the prolificacy index was 34.5% higher compared to the control group.

Key words: exometabolites, micromycetes, bees, honey, culture media, prolificacy

Introduction

All bee species are extremely important to balancing different ecosystems. Due to bees, many plant species are pollinated in forests, agricultural plants, fruit trees and other various ecosystems; resulting in the production of fruits, vegetables and cereals, which serve as food for humans and animals. It is known that worldwide more than 300 species of cultivated plants are totally or partially dependent on pollination, and the production of 75% of the crops that provide products traded on the world market depends on pollination. In some agricultural crops (over 90), bees increase production by at least 30% (cotton, medicinal plants, agricultural crops, animal feed), and about 10% of entomophilous agricultural crops depend entirely on bee pollination [4,7].

The bees are among the most evolved social insects, which means that they jointly carry out a whole series of activities necessary for the survival of the species: raising offspring, gathering, and processing food, etc. behaving in this way like an organism. Bees are eusocial insects with close interaction with their environment. For this reason, the health of bees is impacted by the effects produced during the collection of nectar and pollen. Nonetheless, a poor nutrition, especially at the end of winter or early spring (lack of microelements, carbohydrates, protein substances, vitamins) and inadequate food sources (pollen soaked with agrochemical and biocides products) of bee colonies, can cause a microbiological dysbiosis; therefore, leading to a decreased ability of the colonies to respond to the environment factors. Bees have a lower diversity of detoxification genes than the genome of other insects. For this reason, to degrade potentially toxic molecules, bees can also rely on other components, which shape their physiology, such as the intestinal microbiome [1,5,9].

It is known that the environment plays a major role in shaping the bee microbiome. Agricultural lands which are being treated with different chemical substances (pesticides, insecticides), contribute to the disruption of the bacterial status in the bees' gut, increasing the vulnerability of bee colonies to different infectious germs (viruses, bacteria, fungal) or to different specific parasites. Therefore, to protect the bee colonies, it is recommended to be placed in less humid or shaded locations the apiaries with more sensitive bee colonies [8, 10]. The composition of the microflora of bee colonies also varies

depending on the place of collection and type of the collected pollen. In pollen, there are various symbiotic communities of microbes that provide a variety of benefits to bees. Microbes associated with pollen supplies are promontory of bee health, but can also represent a major food resource for developing bee larvae. At the same time, the diversity and composition of the intestinal microbiota in bees, differs depending on the ecosystem in which they operate [6,9].

According to scientific data, the composition of the microbiome in bees and bee products consists mainly of lactic bacteria from the genera Lactobacillus and Bifidobacterium, which form a favorable symbiotic environment [2]. The composition of species and the number of bacteria in the intestinal micromyoma of bees depends on several factors: the season, the environment, the source, the quantity and quality of the nectar, the state of the bee, the presence of microorganisms in the nectar [3,10].

The bees and the lactic acid microflora mutually evolved from each other: the bacteria receive a niche with available nutrients, and the bees receive protection from harmful microorganisms. In order to maintain a balanced microbial status and a satisfactory physiological resistance in the bee colonies, as well as to reduce the risk of apathy of some infectious diseases,

it is necessary to systematically monitor the bee colonies. This can be achieved by taking samples for examination of the microbial status, as well as strengthening the physiological status of the bees' body through the additional feeding of biologically active preparations.

In this context, the goal of this proposed research was focused on the use of exometabolites of micromycetes to increase the physiological resistance of bee colonies after the winter period, as well as to stimulate bees productive indices [5,7].

MATERIAL AND METHODS

The researches were carried out in the microbiology laboratory of the Department of Food Safety and Public Health, of the Faculty of Veterinary Medicine of TUM, in the laboratory of the National Collection of the Institute of Microbiology and Biotechnologies. As a material for investigations served the bee families from the experimental apiary of the Institute of Microbiology and Biotechnology, UTM. In orer to obtain the exometabolites from micromycetes from the National Collection of Nonpathogenic Microorganisms, were selected 21 strains of micromycete which were isolated from the soil of the central area of the Republic of Moldova. As nutrient culture mediums for the isolation and the study of morphologycal properties of the strains of micromycetes were used Malt-agar and Czapek mediums. To preserve the micromycetes in the collection of microorganisms, were used the malt-agar medium.

The cultivation of isolated micromycete strains was carried out in a thermostat at a temperature of 28oC for 14 days. The cultures were examined visually according to the morpho-cultural characters, as well as microscopically. The antimicrobial properties of the micromycete isolates were studied according to the diffusometric method by using agar blocks. The method was based on the diffusion capacity of the metabolites produced by the studied microorganisms in the depth of the agar and the action of the active substance in the diffusion zone on the test cultures. The morphological properties of the micromycete strains were studied over 4, 7 and 14 days of cultivation. From the 21 strains of micromycetes used for the investigation of the bee families were selected 3 strains (Ps.sp.11; Ps.sp.19; Ps.sp.62) which demonstated the best development parameters on culture media, as well as more pronounced antibacterial and antifungal actions.

The biomass of exometabolites was prepared from the mentioned strains of micromycetes, and was administered with cakes from wheat flour which were thoroughly homogenized and placed in the hives on the honeycombs to be consumed by the bees.

RESULTS AND DISCUSSION

In order to increase the resistance of the bees' body after the winter period and to stimulate the productive parameters; additional food consisting of cakes of wheat flour with exometabolite of micromycetes biomase were given to bee families. The results of these investigations are presented in table no. 1. where can be seen that were formed 3 experimental groups whith 9 hives in each and one control group. To the bee families from the first group were administered exometabolites of the micromycete strain Ps.sp.11 on 3 dilutions, respectively 10ml, 25ml and 50ml of exometabolites per 1kg of wheat cakes. To the bees from the second group were administered exometabolites of the micromycete strain Ps.sp.19 with delutions 10 ml, 25 ml and 50 ml of exometabolites per 1 kg of wheat cake, and to the families of bees from the third group were administered exometabolites of the micromycete

strain Ps.sp. 62 with delutions of 10 ml, 25 ml and 50 ml of exometabolites per 1 kg of wheat cakes.

At 12, 24 and 36 days after the administration of exometabolites of micromycetes were examined the number of brood squares of the bees in the experimental groups, compared to the bees from the control group.

Analyzing the data presented in table 1, it was determined that the highest index of the number of plots with brood was in the first experimental group of bees, to which was administered the biomass of exometabolites with the strain of micromycetes Ps. sp.11. This index was at 24 days after the administration of the food supplement in a dose of 25ml/kg/cake mass constituted 40.4 squares of brood of bee, compared with 24.7 squares of brood of bee in the control group.

The highest index of - 47.1 squares of brood of bee was established in the first experimental group at 24 days after the administration, representing a diference of 19.4 squares of brood of bee when compared to the control group.

If making a global comparison with the strains of micromycetes Ps. sp. 19 and Ps.sp.62 at 24th day after the administration of exometabolites with indications in the dilution of 25ml/kg/cake mass, this represents an increase of 7.97 and 11.17 squares of brood of bee since they had indices of 35.67 and respectively 38.87 squares of brood of bee.

Table 1

The results of the action of exometabolites of micromycetes to the number of squares of brood of bee

Mycr. strain	Ps. sp.11			Ps.sp.19			Ps.sp.62			Control group		
Dilution / Days	10ml/l	25ml/l	50ml/l	10ml/l	25ml/l	50ml/l	10ml/l	25ml/l	50ml/l	I	II	III
12 days	39,50	40,40	40,25	27,87	28,16	28,33	35,50	36,50	27,60	24,75	28,75	29,00
24 days	41,07	47,10	44,25	35,37	35,67	36,33	34,87	38,87	35,30	27,7	29,25	30,25
36 days	39,93	43,10	42,37	27,62	28,67	31,00	34,50	32,63	28,80	26,25	27,62	27,38

Another indicator that was monitored in the bee families that were additionally fed with the exometabolite biomass was the amount of honey collected from the bee of experimental hives, compared to the control group.

The results of this study are presented in table no. 2. After performing the study, it was determined that the highest collection of honey in the bee families of the first experimental group where the biomass of exometabolites of the strain Ps.sp.11 was administered as additional feed in the dose of 25ml/kg/ wheat cakes was recorded at 24 days after feeding and the collection per beehive constituted 3.4 kg of honey.

Table 2

The amount of honey collected on frames from the beehives of the additional feeding of bee families with the biomase of micromycete exometabolites.

Mycr. Str.	Ps.sp.11			Ps.sp.19			Ps.sp.62			Control group		
Dilu-tion Days	10ml/l%/kg	25ml/l%/kg	50ml/l%/kg	10ml/l%/kg	25ml/l %/kg	50ml/l%/kg	10ml/l%/kg	25ml/l%/kg	50ml/l%/kg	I %/kg	II %/kg	III %/kg
12 days	104 2,6	112 2,8	108 2,7	108 2,7	112 2,8	108 2,7	88 2,2	92 2,3	100 2,5	92 2,3	108 2,7	100 2,5
24 days	109,8 3,0	124,5 3,4	113,5 3,1	106,2 2,9	113,5 3,1	113,5 3,1	98,9 2,7	100 2,5	112 2,8	95,2 2,6	106,2 2,9	98,9 2,7
36 days	121,2 3,6	131,3 3,9	127,9 3,8	104,3 3,1	111,1 3,3	128,2 3,5	109,8 3,0	106,2 2,9	104,3 3,1	97,6 2,9	104,3 3,1	97,6 2,9

In the bee families that were fed with exometabolites biomass from the micromycete strain Ps. sp.19 the honey collection was 3.10kg per beech. At the same time, in the third experimental group of bees which was feed with biomass of exometabolites of strain Ps.sp.62 the honey collection constituted 2.5kg per beehive. In the bees families of the control group, the amount of honey collected from one beehive was 2.6 kg. In conclusion, the difference of amount of honey per beehive in the bee families from the first experimental group was with 0.8kg more per beehive, compared to the bee families from the control group.

Another index that was monitored in the bee colonies after the administration of exometabolites from micromycetes consisted in establishing the degree of prolificacy in dynamic. The results of this study are presented in table 3.

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Table 3

Indices of the prolificacy of the bee families under the action of exometabolites of steptomycetes.

Mycr. strain	Ps. sp.11			Ps.sp.19			Ps.sp.62			Con-trol group
Dilution Days	10ml/l, %	25ml/l, %	50ml/l,%	10ml/l,%	25ml/l,%	50ml/l,%	10ml/l,%	25ml/l,%	50ml/l,%	--, %
12 days	75,67	77,39	77,11	53,40	53,95	54,27	68,01	69,90	52,87	52,68
24 days	78,69	90,23	84,77	67,77	68,33	69,60	66,81	74,47	67,62	55,71
36 days	76,49	82,57	81,18	52,92	54,92	59,38	66,09	62,51	55,17	51,98

The data in the table 3 shows that the highest prolificacy index - 90.23%, was established in the first experimental group of bees, at 24 days after the administration of the biomass of exometabolites of the micromycete with strain Ps.sp.11 in the dilution of 25ml/kg of wheat flour cakes. At the same time, in the second and third experimental groups, to which were administered the biomass of the stains of Streptomyces's Ps. sp.19 and Ps.sp.62, at 24 days after administration, the prolificacy index was 68.33% and 74.47%, respectively. If comparing the experimental groups, the difference in prolificacy between first and second experimental groups was 21.13% and 15.76%, respectively. However, if comparing the first experimental group with the control group, the index of prolificacy was 34.52% higher.

CONCLUSION

1. In order to increase the resistance of the bees' body after the winter period and to stimulate the productive parameters, it is recommended to administer the exometabolites of micromycetes as additional food in the mixture with wheat flour cakes, placed inside the hives on the honeycombs.
2. The use of exometabolites of micromycetes of the strain Ps.sp 11 in a dose of 25ml/kg/ wheat cakes, stimulated the formation of seedlings representing an increase of 24.7 squares of brood of bee if compared with the control group.
3. Feeding the bee families with exometabolites of micromycetes supplements increased the prolificacy index by 21.13%, and the honey collection by 0.8kg per beehive, compared to the bee families from the control group.

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INVESTIGATING NEUTROPHIL SUBPOPULATION DYNAMICS IN A MOUSE MODEL OF SARS-COV-2 INFECTION

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Abstract

The project largely involved studying neutrophil dynamics or kinetics in a mouse model of SARS-CoV-2 infection. The study is carried out in a mouse model using transgenic mice expressing the human ACE-2 receptor, which allows the virus to enter cells.

Keywords: virus, mouse model, neutrophil population

INTRODUCTION

Since the first reports of an outbreak of a severe acute respiratory syndrome (ARDS) caused by coronavirus 2 (SARS-CoV-2) in China in December 2019 (*1*, *2*), the coronavirus disease 2019 (COVID- 19) have grown to be a global public health emergency. SARS-CoV-2 infection is characterized by a range of symptoms including fever, cough, fatigue and myalgia in the majority of cases and occasional headache and diarrhea (*1*, *3*). Among reported cases, approximatively 80% present mild conditions, 13% serious, and 6% developing critical case requiring intensive care associated, with fatality rate of 2-8% of reported cases (*4*). More severe cases of COVID-19 show development of ARDS and acute lung injury, leading to mortality caused by damage to the alveolar lumen. A high number of patients with ARDS secondary to COVID-19 developed life-threatening thrombotic complications (*5*). Coronavirus infections in the past have been characterized by the onset of a virus-induced inflammation associated with a cytokine storm that begins at the infection site and spreads throughout the body via the systemic circulation (*6*). It is therefore reasonable to postulate that the inflammatory response measured both at cellular and molecular levels would represent a main prognostic signature for the disease. Molecular assays have been the gold standard to directly detect for the presence of the virus as well to respond to the demand of clinicians to characterize the infection onset, notably cytokine storm, an uncontrolled inflammatory response, resulting in viral sepsis, ARDS, respiratory failure, shock, organ failure, and death (*7*, *8*). However, there is a lack of prognostic markers on complications onset in severe cases.

MATERIAL AND METHODS

A retrospective cohort of 201 patients with confirmed COVID-19 pneumonia revealed that older age, neutrophilia, and organ and coagulation dysfunction were the major risk factors associated with the development of ARDS and progression to death (*9*). ARDS and sepsis are among the most frequently observed common complications in deceased patients (*3*). In severe cases, bilateral lung involvement with ground-glass opacity is the most common chest computed tomography (CT) finding but more surprisingly, abnormal CT scans were also observed on asymptomatic COVID-19 patients (*10*). Immune transcriptome profiling from broncho-alveolar lavage fluid of COVID-19 patients showed hypercytokinemia (*6*). In addition, serum concentrations of both proinflammatory cytokines and anti- inflammatory cytokines, including IL-6, TNF-α, and IL-10 increased in the majority of severe cases and were markedly higher than those in moderate cases, suggesting cytokine storms might be associated with disease severity, providing insights into immune therapeutics (*3*, *11*). The cytokine storm has been associated with

massive influx of innate immune cells, namely neutrophils and monocytes, which may aggravate lung injury. However, little is known about the innate immune features and the molecular mechanisms involved in COVID-19 severity. Increasing clinical data indicated that the neutrophil-to-lymphocyte ratio (NLR) is a powerful predictive and prognostic indicator for severe COVID-19 (*12–14*). Lymphopenia, neutrophilia, and high NLR are commonly presented and associated with more severe viral infection (*12*, *15*). However, there are very few treatments (if none) specifically tackling neutrophil functions that could alleviate inflammation and facilitate infection resolution.

RESULTS AND DISCUSSION

The first comprehensive evaluation of whole blood circulating neutrophils in septic (*16*) and COVID-19 patients was made (16). High dimensional mass cytometry revealed a specific neutrophil signature of sepsis severity that does not overlap with other inflammatory biomarkers, and that distinguishes patients with sepsis from those with non-infectious inflammatory syndrome (*16*). Unsupervised analysis of 40-dimesional mass cytometry data characterized previously unappreciated heterogeneity within the CD64+ immature neutrophils and revealed two new subsets distinguished by CD123 and PD-L1 expression. These immature neutrophils exhibited diminished activation and phagocytosis functions. Critically, the proportion of CD123-expressing neutrophils correlated with clinical severity. To test the hypothesis of a virally-driven neutrophil profile that could be a good COVID patients' disease-state indicator, the multi-parametric neutrophil profiling strategy was applied. This strategy was based on known neutrophil markers to distinguish COVID-induced phenotypes in critical (in intensive care unit) compared to severe symptomatic patients (in infectious departments). After this strategy, two new CD10-CD64+ immature neutrophil subsets expressing either LOX-1 or CD123 that were specific to COVID-19 were identified. In addition, previous work showed that LOX-1 is important mediator of inflammation and neutrophils dysfunction in sepsis and cancers (*17, 18*).

CONCLUSIONS

Despite these very interesting results the functional characteristics of these neutrophil subsets and their role in COVID-19 pathogenesis is still unknown.

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CARDIOVASCULAR CONSEQUENCES AND COVID-19 INFECTION: ESTABLISH THE MODEL

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Abstract

Recent studies have highlighted that the risks for developing cardiovascular alterations are significantly increased in patients who previously suffered from Covid-19. This study aims at determining the functional and structural long-term effects of Covid-19 disease on the cardiovascular system using a specific and original humanized mouse model recapitulating the endogenous cardiovascular expression of the SARS-CoV-2 main receptor ACE2 (Angiotensin Converting Enzyme 2). We will focus on studying the systemic and pulmonary vessels and the cardiac tissue to understand how SARS-CoV-2 infection leads to cardiac and vascular tissue remodelling and function alteration.

Keywords: virus, cardiovascular, SARS-CoV-2 infection

INTRODUCTION

Covid-19 is a multifaceted disease which often combines a pneumonia with anomalies in the function of other organs including the cardiovascular system. In patients with a severe form, the infection causes microangiopathy (microvascular thrombosis) and endothelial inflammation at the pulmonary, cardiac, hepatic and cerebral levels in patients. Recent studies have highlighted important long-term cardiovascular consequences after SARS-CoV-2 infection even without hospitalization with increased risk of myocardial ischemia, stroke, myopericarditis, arrhythmias, atrial fibrillation or pulmonary fibrotic lesions (1). This risk depends on the severity of the disease but it remains significant in patients with a less severe form. At the pulmonary level, clinical reports observe that, following infection, pulmonary vascular wall is thickened and that several patients developed pulmonary hypertension. Systemic vessels are also modified after Covid-19 as patients show persistent endothelial dysfunction and increased arterial stiffness several months after infection (14). Hence some studies suggest that infection could increase the risk of developing hypertension.

Different mechanisms were proposed to explain the cardiovascular effects of the virus. First, the infection-induced massive immune response (cytokine storm) can activate endothelial cells leading to endothelial dysfunction, permeability and thrombi formation (2). SARS-CoV-2 could also directly infect cardiovascular cells through ACE2 binding. This was observed in vitro (3) but is difficult to observe in patients (4,5). At the vascular level, recent publications and our own preliminary observations suggest a very low endothelial expression of ACE2 and a high expression by smooth muscle cells (SMC) and pericytes (6,7). At the cardiac level, ACE2 is expressed by some cardiomyocytes (8) and we also observed expression at the epicardial level in mice. The consequences of virus infection of vascular and cardiac cells are still poorly understood. It was recently suggested that it could induce myofibrogenic transition of pericytes (9) and reduce expression of contractile proteins in cardiomyocytes (10,11). ACE2 hydrolyses angiotensin (Ang) I and II leading to Ang-(1-7) formation. Interestingly, virus binding could drive ACE2 internalization (12) leading to decreased vascular ACE2 activity, increased Ang II levels (pro-hypertensive and pro-inflammatory) and decreased Ang-(1-7) levels (anti-hypertensive and anti-inflammatory), thereby promoting local inflammation, vasoconstriction and vessel wall pressure increase. Most observations of SARS-

CoV-2 infection-induced tissue effects have been made on patients post-mortem specimens, which does not allow to understand the mechanisms involved as well as the temporality of the lesions and their fate over time.

MATERIAL AND METHODS

In this study we used hACE2-KI transgenic mice, and epithelial cells-expressing hACE2-K18 mice in order to differentiate the effects due to the immune and inflammatory reaction (K18 mice) from the effects related to the infection of cardiovascular cells (KI mice). We used higher virus dose (20000 pfu/mouse) leads to a more severe disease in hACE2-KI mice to determine the sub-lethal dose of virus to be used for the project.

Mice (hACE2-KI, 2 to 5 months of age) infected with a sub-lethal virus dose (administered intranasally under anaesthesia) will be studied early after at 5 and 10 days in order to determine if the virus is present in the different organs studied (lung, heart, aorta, small resistance mesenteric vessels) and to identify infected cells. The study was performed by RT-PCR and immunofluorescence assays. We used markers of the different cell types (endothelial vWF, SMC α-SMA, cardiomyocytes α-actinin, epicardial progenitors WT1 (13), fibroblasts, and vascular progenitors PDGFRα (14)) to detect co-labeling with SARS-CoV-2 Spike protein. To assess infected mice susceptibility to pulmonary hypertension, hACE2-KI mice infected with a sub-lethal virus dose was followed for 1 and 3 months. Infection was confirmed by RT-PCR detection of viral RNA in the mice stool 2, 5 and 8 days after infection. Cardiac function was studied by echo-Doppler and ECG-TUNNEL for the analysis of the electrical signal, in particular the duration of P waves, and for the detection of rhythm abnormalities and the quantification of extrasystoles. Systemic arterial pressure (tail cuff and carotid catheter) and right ventricular pressure (RVSP, reflecting pulmonary arterial pressure) was measured to detect the presence of systemic or pulmonary hypertension. Heart will be either processed for immunohistological analysis to assess tissue cardiac remodeling or will be separated to weigh the 2 ventricles and assess right ventricular hypertrophy.

hACE2-KI mice was infected or not with a sub-lethal dose and kept for 1 and 3 months (estimated from the results obtained in the previous experiments) to determine their susceptibility to pulmonary hypertension by subjecting the mice to chronic hypoxia for 21 days. The mice was subjected to echocardiography and measurement of RVSP and right ventricular hypertrophy.

In all groups, we assessed the remodelling of tissues of interest: mainly lung, systemic vessels, ventricles, and right atrium. Using immunofluorescence, histological staining, RT-PCR, we measureed fibrosis, cardiomyocyte size, pulmonary arterial muscularization, systemic and pulmonary vascular wall thickness, inflammation, proliferation and apoptosis of the different cell types (cardiomyocytes, endothelial cells, SMC, pericytes, fibroblasts/interstitial cells, progenitors (13, 14).

RESULTS AND DISCUSSION

SARS-CoV-2 does not bind to the murine receptor mACE2. We have set up and compared the effects of SARS-CoV-2 infection in two mouse models expressing the human receptor hACE2: the widely used K18-hACE2 transgenic mouse model, expressing hACE2 in keratin 18 (K18)-expressing epithelial cells, and the novel hACE2-KI (knock-in) model (from Cyagen) where the hACE2 expression is controlled by the endogenous mACE2 promoter and completely comparable to the endogenous mACE2 (our observations). hACE-K18 mice infected with a low virus dose (5 pfu/mouse) show a high mortality rate (60%) with severe pneumonia and weight loss. In comparison, hACE2-KI mice response to higher virus doses (50 to 5000 pfu/mouse) is very attenuated, with small weight loss and 100% survival. Our main novel finding is that, although hACE2-KI mice develop only mild pneumonia following SARS-CoV-2 infection, they display after 3 weeks a significant late vascular remodeling with neomuscularization of small pulmonary vessels. Importantly, such a vascular remodeling was absent in K18-hACE2 mice lungs, despite a more severe pulmonary disease with major inflammation, suggesting that it depends on ACE2 expression in pulmonary vascular cells (e.g. pericytes or SMC).

CONCLUSIONS

Our hypothesis is that infection of ACEE2-expressing vascular cells could lead to long-term vascular remodeling, muscularization of the pulmonary vessels and pulmonary fibrosis and could increase the susceptibility to the development of pulmonary hypertension.

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ANIMAL MODELS FOR CCHFV AND BSL-2, BSL-3 SURROGATE MODELS

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Abstract

Crimean-Congo hemorrhagic fever virus (CCHFV) is an important tick-borne human pathogen endemic throughout Asia, Africa and Europe. The pathogenic mechanisms of CCHF are poorly understood, largely due to the dearth of animal models. However, several important animal models have been recently described, including novel murine models and a non-human primate model. This review, we examine the current knowledge of CCHF-mediated pathogenesis and describe how animal models are helping elucidate the molecular and cellular determinants of disease. This information should serve as a reference for those interested in CCHFV animal models and their utility for evaluation of medical countermeasures and in the study of pathogenesis.

Keywords: virus, zoonotic, surogate models

INTRODUCTION

In 1973, Crimean-Congo hemorrhagic fever virus (CCHFV) was identified as the singular causative agent of two separate illnesses, Congo fever (identified in 1956) and Crimean fever (identified in 1944). CCHFV is a member of the *Nairoviridae* family in the order *Bunyavirales*, a group of enveloped tri-segmented negative stranded RNA viruses. Despite having been originally identified in West Central Africa and the Crimea, today the virus is endemic throughout a wide geographical area that includes Africa, Asia and Europe. The presence of the virus in these regions is directly correlated with the presence of the main arthropod vector of CCHFV, *Hyalomma spp* ticks. While CCHFV is endemic in many areas, the expansion of the host-range of the ticks is allowing the virus to emerge in new areas [4].

CCHFV has a dichotomous relationship with animals and humans. While CCHFV infects a large number of wild and domesticated mammalian species, including bovines and ovines, and some avian species such as ostriches, the virus does not cause severe disease in these species. Instead, infections in these animals are predominantly asymptomatic, often resulting in a viremia that can last >5 days which helps maintain CCHFV in nature. In marked contrast, CCHFV infection in humans can lead to a severe, even life-threating, disease with key features that include coagulopathy, hepatic injury and neurological disorders. An in-depth understanding of CCHFV-mediated pathogenesis has been hampered by the lack of animal models. However, several murine and non-human primate models have recently been developed which will provide a means to investigate CCHFV pathogenesis, in addition to providing a platform to bridge medical countermeasure (MCM) development to humans.
One of the major problems to study this virus is the lack of BSL4 facilities.

Small Animal Models

CCHFV does not cause disease in immunocompetent adult rodents, including mice, rats, guinea pigs and hamsters. Until 2010, the only available models were neonatal mice and neonatal rats which were first used in 1967 by Chumakov and colleagues. However, Bereczky, S. et al. discovered that strain 129 mice lacking the type I interferon receptor A ($IFNAR^{-/-}$) were susceptible to CCHFV and produced a lethal/severe disease model. Subsequently, these

studies were repeated in C57BL/6 mice also lacking the type I interferon (IFN-I) receptor. Additionally, CCHFV produces severe disease in STAT-1$^{-/-}$ mice and mice lacking both the IFN-I receptor and IFN-gamma receptor (IFNAGR$^{-/-}$). These animals have deficiencies in both IFN-I and type II interferon (IFN-γ) signaling. We recently developed a novel murine system by exploiting an antibody against IFN-I receptor A (MAR1-5A3) that was previously shown to produce severe disease models with other unrelated viruses. This antibody produces a transient IFN-I blockade in mice and results in consistent lethal/severe CCHFV infection. The advantage to this model is it creates the same phenotype as an IFN-I receptor knockout animal in virtually any wild-type or transgenic mouse without the need for cross-breeding

The disease produced in the antibody-mediated IFN-I blockade model is essentially identical to the disease observed in genetic KO animals with similar mean times to death. In addition to conventional mouse systems, Spengler et al. developed a novel humanized mouse model by transferring human CD34^{+} stem cells into NOD-SCID-gamma (NSG)-SGM3 mice, which are extremely immunodeficient mice lacking mature T-cells, B-cells, and natural killer (NK) cells and have defects in cytokine signaling due to lack of the common gamma chain. Infection of these mice with CCHFV produces neurological disease. Below we describe how these murine systems are being used to evaluated CCHF pathogenic processes in addition to MCM development.

Non-Human Primate Models

The development of an NHP model that recapitulates human CCHF disease has been a challenging area of research. Earlier studies of CCHFV infection of African green monkeys, baboons, and patas monkeys were unsuccessful. Recently, a cynomolgus macaque severe disease model was described that establishes the first immunocompetent animal model for CCHF. NHPs were infected with the European human clinical isolate of CCHFV, strain Kosova Hoti, using an intravenous (IV) or combined IV and subcutaneous (SC) high dose (5 $\log_{10}$ $TCID_{50}$) exposure. The animals became viremic and developed a severe and sometimes fatal disease characterized by inflammatory immune responses, elevated liver enzymes, increased clotting times, thrombocytopenia, leukopenia and fever, which are all representative of human cases of CCHF. Histopathology demonstrated that CCHFV mainly targeted the liver and spleen where in situ hybridization identified viral RNA in the hepatocytes, Kupffer cells, and endothelial cells.
The development of the cynomolgus macaque model represents an important advancement in the field where an immunocompetent CCHF animal model is now available to study pathogenic disease mechanisms and evaluate candidate medical countermeasures. Adding further value is the ability to use two genetically unrelated strains, Hoti and Afg09-2990, which both produce disease in the NHP. This model should be further refined to determine reproducibility by evaluating variables such as virus strain/stock, dose, and genetic background of NHPs. Furthermore, the mechanism and impact of viral RNA persistence on the development of long-term sequela is an important area of future research in the NHP model.

Surrogate Models

Because CCHFV research requires BSL4 containment and many researchers do not have access to such facilities, several groups have developed surrogate nairovirus murine models. Hazara virus (HAZV) is a nairovirus isolated from the *Ixodes redikorzevi* tick and is a member of the CCHFV serogroup. Evidence to date indicates that HAZV is non-pathogenic in humans and can be manipulated in BSL2 environments. Dowall, et al. demonstrated that similar to CCHFV, HAZV is pathogenic in IFNAR$^{-/-}$ mice. HAZV infection in IFNAR$^{-/-}$ mice led to severe disease with a MTD of ~5 days depending on viral dose. Histopathological changes in the liver and spleen were detected and are analogous to that of CCHFV infection of mice. Recently a novel nairovirus called Tolfa virus (TFLV) was isolated from *Haemaphysalis flava* ticks and *Haemaphysalis fomsensis* ticks in Japan. TFLV is also in the CCHFV serogroup. Shimada, et al. found that this virus, though considered non-pathogenic in humans, produced severe disease in IFNAR$^{-/-}$ (A129 background) mice. Infection in these mice resulted in pathological effects in the intestinal tract and was lethal with a MTD of ~4–5 days. Liver involvement in TFLV murine infection was not specified in the published reports.

In addition to HAZV and TFLV, another nairovirus termed Dugbe virus (DUGV) has shown promise as a CCHFV surrogate. DUGV is a member of the Nairobi sheep disease virus serogroup.

Infection of mice either immunocompromised by cyclophosphamide treatment (within 48 h) or IFNAR$^{-/-}$ mice results in a lethal disease which included respiratory tract

involvement (lung edema) and also a neurological disease. Contrary to HAZV and TFLV, DUGV has been reported to occasionally cause human disease, particularly in children [101]. For this reason, study of DUGV requires BSL3 containment. Interestingly, one report suggested that a human isolate of DUGV (IbH11480), contrary to tick-isolates, could produce disease in immunocompetent mice. Despite DUGV not being in the same serogroup as HAZV and TFLV, the possibility that tick and human isolates have differing phenotypes in immunocompetent mice may allow for important insight into viral genetic factors influencing nairovirus pathogenesis. Overall, the use of BSL2 and BSL3 surrogates for CCHFV is promising and suggest that these viruses, in particular HAZV, should continue to be investigated as surrogate models for CCHFV pathogenesis.

CONCLUSIONS

There is an urgent need for not only rapid diagnostics to identify CCHF cases, but also MCMs that can mitigate disease, particularly in a post-exposure setting. The advent of new models for studying disease in rodents and NHPs lays the foundation for important advancements for CCHFV research. These systems will be critical in elucidating the complex host-pathogen dynamics leading to CCHFV-induced organ injury and severe disease.

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Animal models: important tools for studying SARS-Cov-2 infection

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Abstract

Ever since the appearance of COVID-19, the pathophysiology of SARS-CoV2 infection, the identification of treatments and the development of vaccines have been priorities. This search for preventive and therapeutic strategies has been carried out using animal models adapted to the problem under study.

Keywords: Animal research, models, SARS-CoV-2

SARS-COV-2 INFECTIVITY

SARS-CoV2 (Severe Acute Respiratory Syndrome CoronaVirus type 2), the cause of COVID-19, is a single-stranded RNA virus belonging to the Coronaviridae family, a name derived from the presence of a halo of "spike" viral protein trimers forming a crown of spicules. This family comprises 7 viruses that infect humans, of which SARS-CoV2 is the third after SARS-CoV (or SARS-CoV1) and MERS-CoV (Middle East Respiratory Syndrome-CoV) to cause a fatal epidemic. The genome sequence of SARS-CoV2 is 79.4% similar to that of SARS-CoV1 and 50% similar to that of MERS-CoV. The SARS-CoV2 genome contains 11 genes enabling the production of 29 to 33 viral proteins. Of these, 3 proteins are present in the virus envelope: the membrane protein (M), the envelope protein (E) and the spicule protein, S. The S protein is the viral protein responsible for SARS-CoV2 infectivity in humans. It consists of 2 subunits, S1 and S2. S1 contains an angiotensin-converting enzyme 2 (ACE2)-binding domain. ACE2 is an enzymatically active protein of the renin-angiotensin system, considered to be the most widely recognized SARS-CoV2 receptor by the scientific community. Since 2003, ACE2 has been recognized as a receptor for SARS-CoV1. S2 contains the sequence that enables fusion of the viral envelope with the cell membrane, a fusion that leads to endocytosis of the virus and thus entry of the viral genome into the cell. The pathogenicity of SARS-CoV2 is greater than that of SARS-CoV1, due to its greater affinity for ACE2.

Animals models and their specificity

Mouse. Rodents are the most widely used laboratory animals today, and among them the mouse is the animal of choice in biology. Wild mice are not naturally susceptible to SARS-CoV2, which does not bind effectively to their Ace2 protein. Genetically modified mouse models stably expressing the human ACE2 protein (hACE2) have therefore proved essential. Most of these models were generated during the SARS-CoV1 epidemic in 2003 [2; 3]. These models express hACE2 under the control of different promoters that exhibit tissue- and cell-specific expression [3]. The most widely used model is one in which hACE2 expression is under the control of the cytokeratin K18 promoter (K18-hACE2). In this model, hACE2 mRNA and hACE2 protein were detected in the lungs, encephalon, trachea, digestive tract organs, kidneys and testes, with highest expression in the lungs and encephalon [4]. The encephalic presence of hACE2 is greater in the K18-hACE2 model than in humans. The lungs and brain of these mice are severely affected. K18-hACE2 mice show very high mortality, peaking 6 to 7 days after infection at high infection rates (2x10-3/2x10-4 PFU) and around 10 days after infection at lower

rates [3]. As in humans, SARS-CoV2 infection leads to systemic and local (pulmonary and encephalic) cytokine shock; in the K18-hACE2 model, the pulmonary cytokine response precedes encephalic cytokine shock, the latter being synchronous with the encephalic viral peak and peak mortality, suggesting that encephalic involvement is responsible for the severity of the phenotype developed by these mice [3; 5; 6]. In this model, infection induces a hypoxic environment favoring the expression of HIF1α (Hypoxic Induced Factor), which increases ACE2 addressing to the neuronal membrane, making cells more susceptible to SARS-CoV2 binding. This model, characterized by severe nervous tissue damage (very high neuroinflammation) and a very high mortality rate, is therefore relevant for studying the pathophysiological mechanisms observed in humans developing severe COVID-19. However, this model is not very suitable for studying the long-term effects of infection, or for assessing the value of certain treatments, at least until future studies have determined the infection conditions (viral dose) that will allow better survival of the animals for longer-term study.

The ferret. The respiratory tract of the ferret (Mustela putorius furo), and in particular its long trachea which allows easy compartmentalization of the upper and lower respiratory tracts as in humans, make it a relevant animal model for studying the virulence and spread of respiratory viruses. The ferret is susceptible to infection by SARS-CoV2 (its ACE2 is close to human ACE2), but clinical manifestations are mild (mild respiratory symptoms and fever), with none of the severe symptoms seen in humans and no significant mortality. The ferret coughs and sneezes, and can be infected by indirect contact with conspecifics over a distance of more than one meter; this model is therefore used to simulate the transmission of SARS-CoV2 in humans [1; 7]. On the other hand, the ferret's immune responses to SARS-CoV2 infection are similar to those of humans (similar inflammatory cytokine profile in the airways), which led to its use in the development of antiviral and vaccine treatments prior to the launch of clinical trials [7].

The hamster. Two hamster lines were studied: the golden hamster (Mesocricetus auratus) and the Roborovski hamster (Phodopus roborovskii). These hamsters are naturally susceptible to SARS-CoV2; their ACE2 protein interacts with the virus' spike protein. Hamsters can be infected by both intranasal and ocular routes. Following intranasal infection, hamsters rapidly display many of the symptoms described in humans, with the exception of fever and thermal chills, which are only seen in Roborovski hamsters, where infection is 100% fatal, whereas it is never fatal in golden hamsters [8]. In golden hamsters, the onset of symptoms and their regression are identical in males and females: they present a peak of respiratory distress, a decrease in body mass, lethargy and reduced mobility between 2 and 4 days after infection. Symptoms are less pronounced after ocular infection [9]. An improvement in general condition is observed from 7 days post-infection, and recovery generally occurs 14 days after infection. Virus titration or the presence of viral particles has been demonstrated in the lungs, accompanied by pronounced inflammation leading to severe pneumonia. The presence of viral particles or inflammation in other tissues remains controversial. Golden hamsters were therefore used to study the immunity conferred by a primary infection with SARS CoV2, and also for transmission from one congener to another. This work has shown that a second exposure to SARS-CoV2 does not lead to visible symptoms, indicating that immunity is established after a primary infection. The golden hamster is therefore being used in vaccine trials. Viral transmission from one congener to another is possible, and the course and severity of symptoms are similar to those observed in intranasal infection. Hamsters have also been used to study the anosmia and agueusia often described in patients with COVID-19 [10]. This work demonstrated that infected hamsters showed anosmia and agueusia that persisted as long as the virus was present in the olfactory epithelium.

Macaques. Among non-human primates, rhesus macaques (Macaca mulatta) have been the most widely used [11]. Their infection with SARS-CoV2 causes predominantly respiratory symptoms, with the development of pneumonia present from the day after infection and lasting at least the 1st week post-infection, which then subsides. These respiratory symptoms are accompanied by a lack of appetite, but fever is rarely reported. Several routes of infection have been tested: intratracheal, intranasal and aerosol; the mode of infection has little influence on the course of the disease. In addition to the lungs and respiratory tract, the virus is also found in the central nervous system, liver, heart, kidneys, spleen and intestines. With regard to central nervous system involvement, despite the absence of neurological signs, astrocytic activation has been described in the cortex two weeks after infection, and neuroinflammation accompanied by microglial activation and the presence of alpha-

synuclein aggregates in Lewy body-like structures in the ventral region of the midbrain has been demonstrated 5-6 weeks after infection [12]. These findings suggest that the rhesus macaque is a relevant model for the study of neurological disorders associated with COVID-19, and in particular for the study of phenomena associated with "long COVID". Furthermore, secondary infection with the same variant as the primary infection indicates the development of immunity. Rhesus macaques were therefore used for vaccine testing

CONCLUSIONS

THE K18-hACE2 mice are the most suitable model for studying the neurological changes associated with SARS-CoV2 infection. It should be noted, however, that since the infection conditions required for significant animal survival have not yet been established, this model is not suitable for studying long-term effects.

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IDENTIFICATION OF ARTHROPODS BY THE MALDI TOF TEHNIQUE

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Abstract

MALDI-TOF mass spectrometry is a relatively new diagnostic technique that has revolutionised clinical microbiology by accurately identifying species of bacteria, filamentous fungi and yeasts. Recently, new applications to identify parasites and arthropods of medical interest have been developed, but few have developed MALDI-TOF analysis protocols for characterizing arthropod species. Thus, there is a need for a standardization in terms of the anatomical part(s) to be used according to arthropod families (head, thorax, legs) and the steps for protein extraction and homogenization. In this study we created a bank of 47 specimens including the following species: *Aedes caspius, Anopheles hyrcanus, Anopheles maculipennis, Coquillettidia richiardii, Culiseta annulata, Culex pipiens.* Thus, the aim of this study was to compare the quality of spectra and of results in the identification between different anatomical parts of mosquitoes, head, thorax (with wings) and legs, in order to optimize the use of the MALDI-TOF spectrometry tool. We evaluated the bank using the "bank versus bank" test (database provided by the laboratory of parasitology and mycology of Paris, Sorbonne University), each specimen had 4 deposits of protein extracts and the identification threshold log(score) was set to 1.7. Identifications were confirmed by morphological identification keys. There were differences in the protein profiles between each anatomical part. Leg spectra had the lowest number of high intensity peaks compared to those of the head or thorax.

Key words: Maldi Tof mass spectrometry; database; arthropod;

MALDI-TOF (*Matrix assisted laser desorption and ionisation-Time of flight*) mass spectrometry is a method that has revolutionised clinical microbiology by identifying species of yeast, bacteria and filamentous fungi against a bank of reference spectra (Sanguinetti and Posteraro, 2017; Angeletti, 2017; Wolk and Clark, 2018). More recent applications have also been developed, such as the identification of parasites (Murugaiyan and Roesler, 2017) and more recently arthropods of medical interest (Yssouf *et al*, 2016; Laroche *et al*., 2017; Murugaiyan and Roesler, 2017).

The mass spectrometry method is based on the detection of protein fingerprints (characteristics of a species, strain or physiological state) constituted by mass spectra and/or protein profiling in search of biomarkers.

This method is widely used in northern countries becoming of interest and increasingly accessible in countries endemic for infectious and tropical diseases. The process is automated, simple and fast to use, providing highly accurate results with a minimum of technical expertise. Thus, for entomological field studies, the use of the MALDI-TOF spectrometry instrument is being considered. Indeed, studies have shown that it is possible to identify by mass spectrometry species of mosquitoes (*Diptera: Culicidae*), Culicidae (*Diptera*: *Ceratopogonidae*), ticks (*Acari: Ixodidae, Argasidae*), but also phlebotominae (*Diptera: Psychodidae: Phlebotominae*), fleas (*Siphonaptera*), tsetse flies (*Diptera: Glossinidae*) (Yssouf *et al*., 2016), and bedbugs or "triatomines" (*Hemiptera: Reduvidae*) (Laroche *et al*., 2018).

The method has been validated for the identification of all adult, egg and larval stages, representing a great advantage, especially for the imago stages that are more difficult to identify. Protein profiling studies have also shown biomarkers of infection in arthropods, such as infection with *Rickettsia spp.* or *Borrelia spp.* in ticks (Diarra *et al*., 2017; Fotso Fotso *et al*., 2014; Yssouf *et al*., 2015), with *Bartonella spp.* in fleas (El Hamzaoui, Laroche, & Parola, 2018) and with *Plasmodium spp.* in *Anopheles* (Laroche *et al*.,

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2017). Other studies have shown the presence of biomarkers that make it possible to identify the host from which the blood mass was taken (Niare *et al*., 2016) or to identify closely related species of *Anopheles* (Müller *et al*., 2013). However, few have developed MALDI-TOF test protocols for characterizing arthropod species. Thus, there is a need for standardization in terms of the anatomical part(s) to be used according to arthropod families (head, thorax, legs) and of protein extraction and homogenization steps.

Overall, MALDI-TOF is a promising tool for characterizing mosquito vectors. If used for mosquito monitoring, this proteomic method will avoid molecular test, with a gain in terms of the speed and cost of the test. Like DNA sequence databases, accessibility via online applications is essential for the widespread use of MALDI-TOF MS databases. Such online platforms have already been proposed for fungi (Diarra *et al.*, 2017) and *Leishmania* species (Laroche *et al.*, 2017) and are currently being set up for mosquito species identification (El Hamzaoui *et al*., 2018).

MATERIAL AND METHOD

During the period 03-09.2022 mosquitoes were caught in the Danube Delta and Iasi City, using CDC Light Traps, by using dry ice as attractant. The captured mosquito specimens were stored at -20°C, in dry condition, being the preservation method with the best results. (Halada, Hlavackova, Dvorak, *et al.*, 2018) For the identification of mosquitoes by the MaldiTof technique, legs, head, thorax and wings were used. Recent studies have shown a significant sensitivity of legs, as the results may be influenced by capture, transport and storage conditions (Diarra *et al*., 2019; Loaiza *et al*., 2019; Rakotonirina *et al*., 2020). Also, the cephalothorax should be cut with great care, as there is a high risk of blood contamination (Müller *et al*., 2013).

Thus, the aim of this study was to compare the quality of spectra and identification results between the different anatomical parts of mosquitoes, head, thorax (with wings) and legs, in order to optimize the use of the MALDI-TOF spectrometry instrument. The automated MALDI-TOF mass spectrometry system, Microflex LT Bruker Daltonics®, was used for the analysis.

Figure 1 **Automated MALDI-TOF mass spectrometry system, Microflex LT Bruker Daltonics®**

RESULTS AND DISCUSSIONS

To carry out this study, we created a bank of 47 specimens including the following species: *Aedes caspius, Anopheles hyrcanus, Anopheles maculipennis, Coquillettidia richiardii, Culiseta annulata, Culex pipiens.*

We evaluated the bank by the "bank versus bank" test (database provided by the laboratory of parasitology and mycology of Paris, Sorbonne University), each specimen had 4 protein extract deposits and the log (score) identification threshold was set to 1.7. Identifications were confirmed by morphological identification keys. Between each anatomical part, there were differences in terms of the protein profiles. Leg spectra had the lowest number of high intensity peaks as compared to those of the head or thorax. The reproducibility of the spectra varied between anatomical parts. Leg spectra were the least reproducible and head spectra showed the best reproducibility, regardless of any experimental conditions. The distribution of

log (scores) varied significantly between anatomical parts, but the head part provided the highest log (scores).

We obtained 98% and 96% correct identification for the head and legs respectively. For the thorax, correct identification rates were lower (81%), which could be partly explained by blood contamination from the abdomen after dissection of frozen specimens.

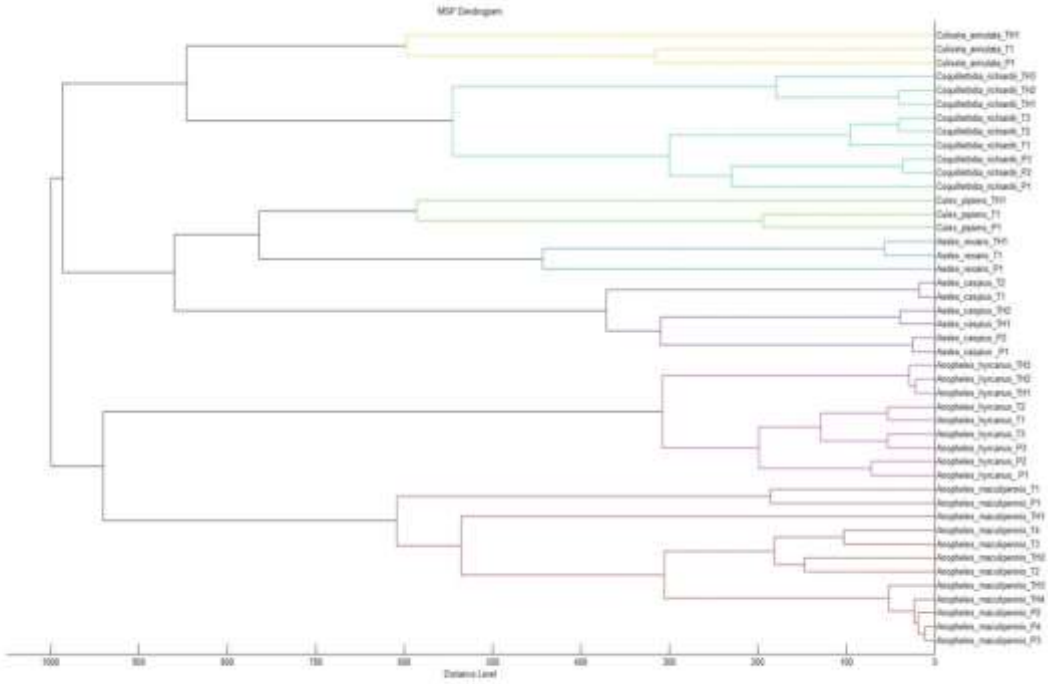

Figure 2 **Dendrogram of matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectra constructed with the 47-mass spectral library (MSL) specimens**

The dendrogram was calculated using Maldi Biotyper v4.1 software and the distance units correspond to the relative similarity of the mass spectra. Cluster analysis of the dendrogram (*figure 2*) showed that specimens belonged to 5 different species, either male or female (*Aedes caspius*, *Anopheles hyrcanus*, *Anopheles maculipennis*, *Coquillettidia richiardii*, *Culiseta annulata*, *Culex pipiens*).

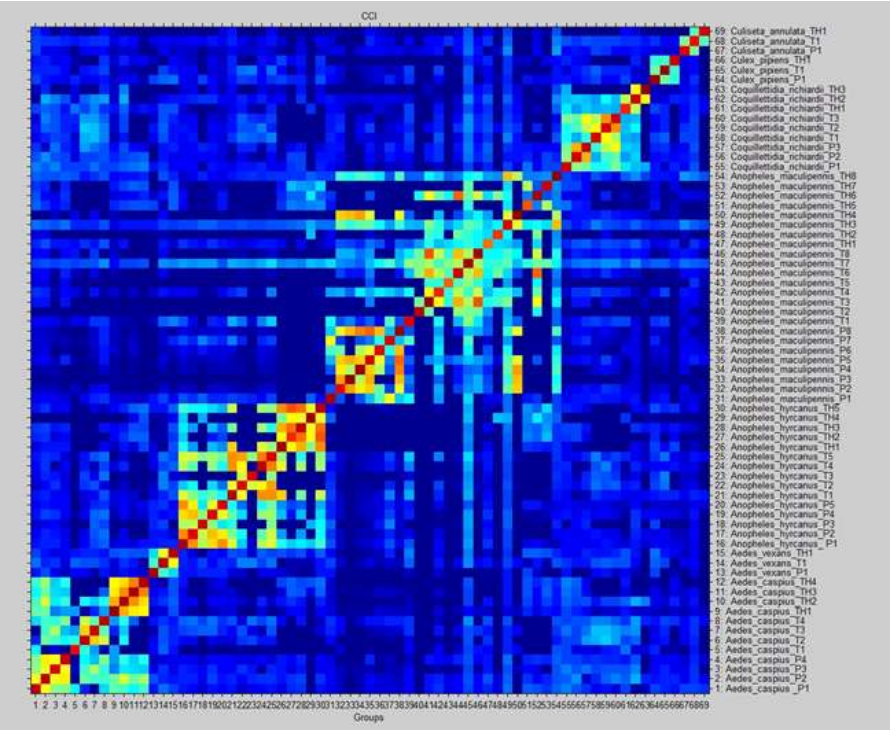

Figure 3 **Heatmap grid of the composite correlation index (CCI) of mass spectra-protein profiles**

Species are indicated on the right side of the heat map. The reproducibility levels of the mass spectra are indicated in red and blue, revealing the relationship and respectively the incongruence between the spectra. The CCI matrix was calculated using Maldi Biotyper v4.1 software with default settings (*figure 3*).

The best results were obtained for specimens stored in the dry state at -20°C.

To increase the reproducibility of the spectra and the speed of testing, the homogenization of protein extracts was done with glass beads. The identification scores obtained were satisfactory (LS≥1.8) and therefore the method was considered suitable.

The extraction was carried out in a mixture of 70% formic acid and 100% acetonitrile. Transport of mosquitoes was done at room temperature in tubes containing silica gel then frozen at -20°C.

CONCLUSIONS

The entomological characterisation of vector mosquitoes is in some cases very complicated, especially when we talk about morphologically twin species, and is an expanding field of research. MALDI-TOF spectrometry is a proteomic tool that requires technical expertise but offers a speed of analysis compatible with its use for entomo-epidemiological monitoring. In this study we proved the speed of this spectrophotometric technique, the accuracy of species identification, and it is a method of the future in medical entomology. Bioinformatics tools coupled with MALDI-TOF spectrometry have paved the way for promising new applications in medical entomology, such as differentiation of similar species, age determination, pathogen infection or blood meal history. Future applications remain to be explored, such as identifying the host from which the blood meal was taken or detecting insecticide resistance.

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THE INFLUENCE OF CLIMATIC FACTORS IN THE TRANSMISSION OF VECTOR BORNE DISEASES

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Abstract

The prevalence of mosquito-borne diseases differs geographically, and transmission times may change in response to the interaction between pathogens, vectors, hosts and the environment. In the context of global warming there is a need to monitor the risk of emergence and re-emergence of vector-borne diseases in Romania. The forecast made in this study shows an increase in temperature until 2050 by 0.78°C, which demonstrated the possibility of extending the transmission period of Plasmodium protozoa until November, of West Nile virus until October and of Dengue fever from June to the first half of September. The results underline the need to introduce vectors and vector-borne disease monitoring and control programmes in Romania in the context of global warming.

Key words: *West Nile, Dengue* fever, *Plasmodium*

External factors, as well as anthropogenic actions, can have a very large impact on global climate (Torres-Vélez and Brown, 2011). Vector-borne diseases are the most sensitive to climate change, as their distribution and evolution is directly controlled by the distribution of vectors, which are influenced by climate change. The effects of global warming and its influence on vector-borne diseases, such as malaria and dengue fever, are a topic of interest to scientists. There is a direct link between climate and the incidence of vector-borne diseases and many studies report a number of patterns explaining the spread and transmission of vector-borne diseases under the influence of temperature (Sutherst, 2004). Mosquito-borne pathogens can affect both animals and humans and are even more difficult to manage once established in a given territory, especially if the reservoir in the wild is represented by wild animals (Tolle, 2009).

The prevalence of mosquito-borne diseases differs geographically, and transmission times can change in response to the interaction between pathogens, vectors, hosts and the environment (Gangosoa *et al*., 2020). The emergence or re-emergence and spread of mosquito-borne diseases are associated with a number of changes in the distribution of their main vectors, either as a result of their accidental introduction or changes in environmental conditions (Ebi, 2013). In Europe, outbreaks of West Nile in humans have occurred mostly in years when temperature anomalies, namely temperature extremes, have occurred (Tran *et al*., 2014; Tabachnick, 2016). Climatic factors, associated with other factors such as human population growth, livestock numbers, intercontinental travel, global trade, urbanisation and land use change, greatly increase the risk of introducing new vector-borne diseases into Europe (Watts *et al*., 2021).

In recent decades, Europe has seen increasing health risks, especially with the intensification of vector-borne diseases such as chikungunya, West Nile virus, Crimean-Congo haemorrhagic fever and Dengue fever (Hotez, 2016; Olesen, 2017). Thus, out of approximately 593 viral diseases that have been identified in animals, on average 29% are vector-borne (Johnson *et al*., 2015). The epidemiology of these vector-borne diseases is influenced by climate and climate change, which play an important role in altering the disease cycle (Randolph, 2009). Temperature increases between different climatic components have direct effects on human and animal health, primarily due to changes in the frequency and intensity of heat waves (Gaughan *et al.*, 2010). The presence of competent vectors in the territory, of favourable climatic factors and evidence of climate change can lead to the re-emergence of diseases, such as malaria, in

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countries where it was previously eradicated. Thus, indigenous cases of malaria have been reported in Germany (Kruger *et al*., 2001), Spain (Santa-Olalla Peralta *et al*., 2010), the Netherlands (Sankatsing *et al*., 2013), Italy (Baldari *et al*., 1998), the UK (ECDC, 2019), France (Armengaud *et al*., 2006) and Greece (Danis *et al*., 2011). The IPCC has stated that human activities have already led to a warming of about 1.0 °C since pre-industrial times, which is likely to reach 1.5 °C between 2030 and 2052 if it continues to increase at the current rate. The 2018 IPCC report highlights that global warming is having a major impact on organisms and ecosystems, as well as it further increases the risk for transmission of vector-borne diseases such as malaria. Global warming determines the increase of the vector capacity of mosquitoes to transmit malaria by shortening the extrinsic incubation period of the parasite, as well as prolongs the mosquito breeding period and increases mosquito density (Kuhn *et al*., 2002; Jetten *et al*., 1996).

The most prevalent mosquito-borne virus is estimated to be dengue virus, which infects 390 million people per year (Bhatt *et al*., 2013) and malaria, despite intensified intervention efforts, causes over 400,000 deaths per year (WHO, 2016).

MATERIAL AND METHOD

In order to follow the evolution of temperatures in Romania and their effects on vector-borne diseases, meteorological data from the National Meteorological Administration from 1961 to the present were processed. Data for each meteorological station were provided, obtaining an average monthly temperature in five geographical regions: Iași, Arad, Bucharest, Sibiu and Tulcea.

This calculated average was used to provide a forecast of temperatures in Romania until 2050. This statistical procedure was obtained through a function within the Excel package called Forcast. This tool predicts a value based on historical data along a linear trend over a time horizon. The Forcast function calculates predictions of future values using linear regression. Choosing this analysis model results in a graphical representation of the forecasted data and a table with the historical and forecasted data used. Thus, in this study we aimed to calculate the climate warming in Romania until 2050, as well as the risk of transmission of some vector-borne diseases: malaria, Dengue fever and West Nile virus.

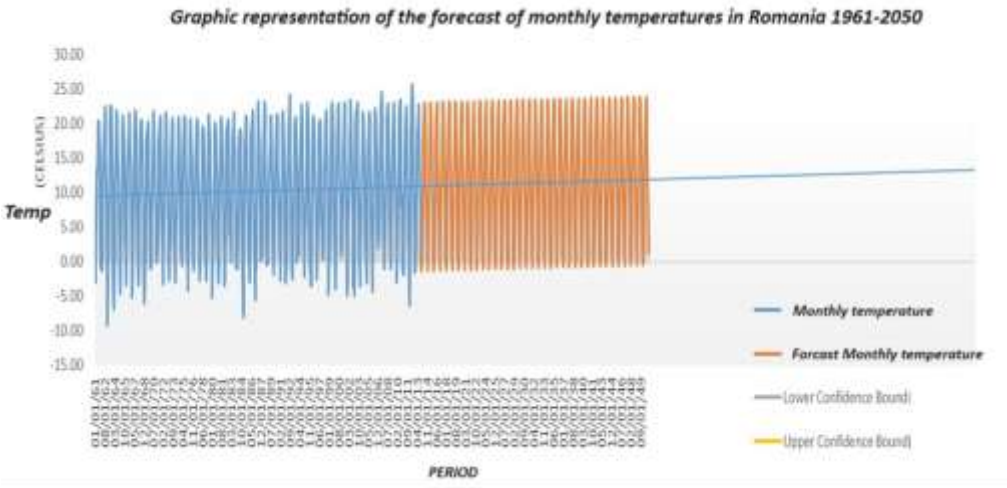


Figure 1 **Graphical representation of monthly temperature forecast in Romania from 1961-2050**

RESULTS AND DISCUSSIONS

The mean temperature obtained was used to indicate the evolution of temperatures over a 28-year horizon compared to the present.

The graph in *Figure 1* shows the evolution of mean annual temperatures recorded in Romania from 1961 to 2013 and is indicated by the blue colour. Temperatures projected up to 2050 are shown in red. The appearance of the graph is specific to seasonality, given by the repetition of the four seasons throughout the year. This shows an increasing trend in temperatures, with a rising trend represented by the line crossing the middle of the graph. In order to better highlight the temperature evolution during the years under study, an indicator called moving average (MA) has been introduced. The role of this indicator is to reduce short-term fluctuations and to highlight long-term trends or cycles in order to eliminate seasonality. With the help of this indicator, we have established the value of the moving average every three years in order to graphically show the evolution of temperatures. This graph gives a result

with an estimation accuracy of 95%, with the remaining 5% representing the range in which unexpected events are taken into account, and this can be seen on the graph in *Figure 2*, by means of the Upper Confidence Bond.

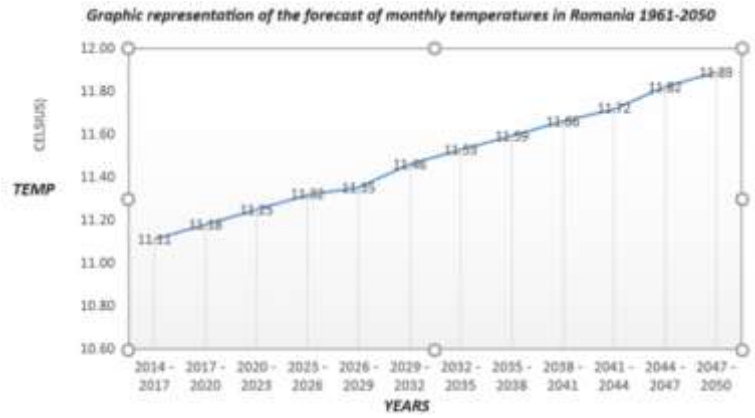


Figure 2 **Average temperatures every three years in Romania from 2014 to 2050**

As can be seen in Figure 2, applying the moving average method, a graph with which the removal of seasonality can be seen was obtained, in order to clearly illustrate the dynamics of temperatures.

The graph suggests that from 2014 to 2050, the temperature increase was estimated to be of almost 1 ºC, i.e., 0.78 ºC.

Malaria forecast 2013-2025 in Romania

Numerous studies have shown that climate change can affect the introduction and spread of vector-borne diseases, affecting the reproduction and development of vector mosquito populations, as well as their vector capacity. Temperature influences the development of the pathogen within the vector, playing a crucial role in the transmission of some vector-borne diseases. In zoonoses, climatic variables cause variations in the distribution and abundance of vertebrate hosts acting as reservoirs of infection.

To forecast the dynamics of malaria cases, a table including three sets of data was developed. The first series is the monthly mean temperature forecast until 2025. The second series is the upper confidence limit. And the third series includes the maximum (mean) temperature, being the average of the warmest days of the month for each station (Iași, Bucharest, Arad, Sibiu and Tulcea).

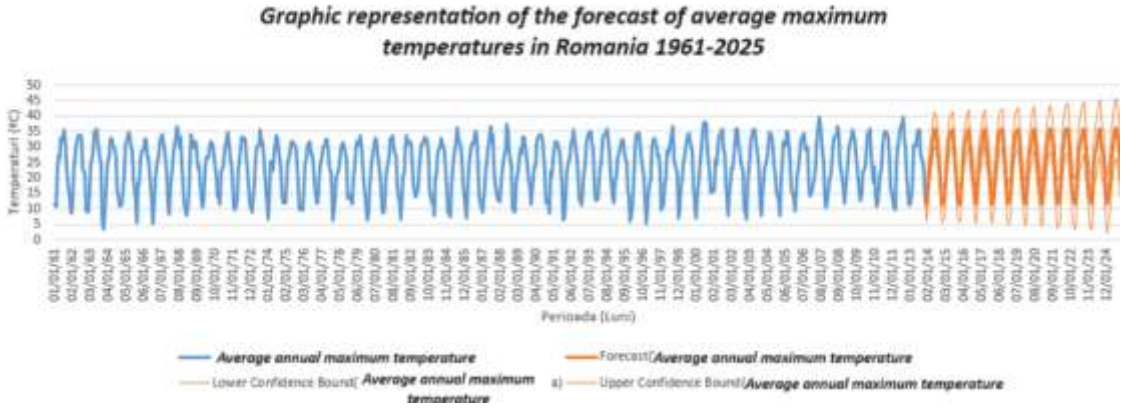


Figure 3 **Graphical representation of the forecast of average maximum temperatures from 1961 to 2025**

Figure 3 shows the average maximum temperature analysis. The meteorological data obtained were recorded for each region. Thus, it was necessary to calculate the average of the warmest days of the month for each station in order to obtain a national analysis. Maximum temperatures for the years 1961-2013 are shown in blue. The projected period to 2025 shows an increasing trend in temperature. The extensions of the forecast period, called Upper Confidence Bounds, illustrate the maximum limit which the

mean temperatures can reach, taking into account the forecast error coefficient value of 5%.

Data on the number of days required for parasite development inside the mosquito at 20°C, 25°C and 28°C were also included for this analysis.

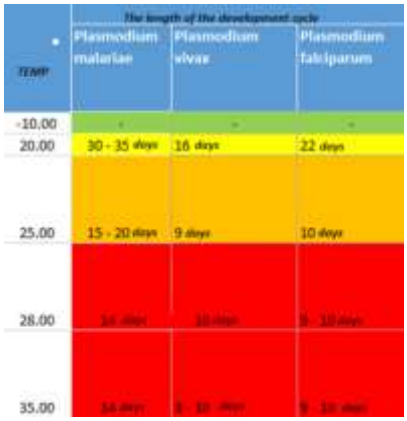


Figure 4 **Developmental cycle time for *Plasmodium* species**

For a more efficient representation of the influence of temperatures on the development of *Plasmodium* species, a colour specific to the temperature required for the development cycle has been included. The yellow colour indicates a longer range of days required for Plasmodium protozoa development at 20°C. The orange colour is attributed to a medium range at 25°C and the red colour indicates the shortest range of *Plasmodium* species development at 28°C (*figure 4*). The interpretation comes down to the inverse proportionality between the temperature evolution and the duration of the development cycle. Therefore, as the temperature increases, a decrease in the development time of the protozoan can be observed, which means a prolongation of the transmission period throughout the year. The result of the analysis is illustrated in tabular form marking the most favourable period for the development of the parasite inside the *Anopheles* female in the year 2023 and the forecast made for the year 2025 shows a shortening of the development period of the protozoan inside the vector.

Table 1

Range of development period of *Plasmodium* species inside the vector for the year 2023

Forecast data				The upper limit of the average monthly temperature			Maximum monthly temperature (average)			Average monthly temperature		
YEAR	Average monthly temperature	The upper limit of the average monthly temperature	Maximum temperature of the month (average)	Plasmodium malariae	Plasmodium vivax	Plasmodium falciparum	Plasmodium malariae	Plasmodium vivax	Plasmodium falciparum	Plasmodium malariae	Plasmodium vivax	Plasmodium falciparum
01/01/23	-1.12	4.84	11.68	-	-	-	-	-	-	-	-	-
02/01/23	-0.18	5.80	13.62	-	-	-	-	-	-	-	-	-
03/01/23	5.19	11.18	21.14	-	-	-	30 - 35 days	16 days	22 days	-	-	-
04/01/23	12.08	18.10	26.84	-	-	-	15 - 20 days	9 days	10 days	-	-	-
05/01/23	17.38	23.41	30.37	30 - 35 days	16 days	22 days	14 days	8 - 10 days	10 days	-	-	-
06/01/23	21.09	27.14	33.99	15 - 20 days	9 days	10 days	14 days	8 - 10 days	10 days	30 - 35 days	16 days	22 days
07/01/23	23.19	29.25	35.74	14 days	8 days	9 - 10 days	14 days	8 - 10 days	10 days	30 - 35 days	16 days	22 days
08/01/23	22.65	28.73	36.00	14 days	8 - 10 days	9 - 10 days	14 days	8 - 10 days	10 days	30 - 35 days	16 days	22 days
09/01/23	17.05	23.15	30.33	30 - 35 days	16 days	22 days	14 days	8 - 10 days	10 days	-	-	-
10/01/23	11.06	17.18	26.15	-	-	-	15 - 20 days	9 days	10 days	-	-	-
11/01/23	6.66	12.79	20.39	-	-	-	30 - 35 days	16 days	22 days	-	-	-
12/01/23	0.51	6.66	13.57	-	-	-	-	-	-	-	-	-

Table 2

Range of development period of Plasmodium species within the vector for the year 2025

Forecast data				The upper limit of the average monthly temperature			Maximum monthly temperature (average)			Average monthly temperature		
YEAR	Average monthly temperature	The upper limit of the average monthly temperature	Maximum temperature of the month (average)	Plasmodium malariae	Plasmodium vivax	Plasmodium falciparum	Plasmodium malariae	Plasmodium vivax	Plasmodium falciparum	Plasmodium malariae	Plasmodium vivax	Plasmodium falciparum
01/01/25	-1.08	5.64	11.76	-	-	-	-	-	-	-	-	-
02/01/25	-0.14	6.60	13.69	-	-	-	-	-	-	-	-	-
03/01/25	5.23	11.99	21.21	-	-	-	30 - 35 days	16 days	22 days	-	-	-
04/01/25	12.13	18.91	26.92	-	-	-	15 - 20 days	9 days	10 days	-	-	-
05/01/25	17.42	24.22	30.44	30 - 35 days	16 days	22 days	14 days	8 - 10days	9 - 10days	-	-	-
06/01/25	21.14	27.96	34.06	15 - 20 days	9 days	10 days	14 days	8 - 10days	9 - 10days	30 - 35 days	16 days	22 days
07/01/25	23.24	30.08	35.81	14 days	8 - 10days	9 - 10days	14 days	8 - 10days	9 - 10days	30 - 35 days	16 days	22 days
08/01/25	22.70	29.57	36.08	14 days	8 - 10days	9 - 10days	14 days	8 - 10days	9 - 10days	30 - 35 days	16 days	22 days
09/01/25	17.10	23.99	30.40	30 - 35 days	16 days	22 days	14 days	8 - 10days	9 - 10days	-	-	-
10/01/25	11.11	18.02	26.22	-	-	-	15 - 20days	9 days	10 days	-	-	-
11/01/25	6.70	13.64	20.47	-	-	-	30 - 35days	16 days	22 days	-	-	-
12/01/25	0.55	7.51	13.64	-	-	-	-	-	-	-	-	-

In the range 2021-2025, the upper temperature limit marks the reaching of a new threshold marked by the red colour, of the frequency of periods favourable to the etiological agent of malaria, in July and August. While at the beginning of 2014-2019, the suitable period for Plasmodium was during the summer months, as time goes on, an increase in this period is observed.

For each species, the most suitable period for transmission was identified according to the temperature and the length of the parasite's development cycle inside the mosquito. Thus, it can be observed that the summer months with the extension of the last month of spring and the first month of autumn are the most suitable for malaria transmission (*table 1 and table 2*).

Forecast of West Nile cases in Romania between 2023 and 2025

The West Nile virus has been present in Romania since 1950. The clinical picture and the spread of the virus have led to the need to monitor and control the disease. The importance of West Nile virus infection lies in its impact, sometimes fatal among equines and through transmission to humans.

In the table below, we have illustrated the range of temperature values, organised according to their growth. For reference, the literature says that West Nile virus is transmitted conditionally at the temperature threshold of 23.7 °C, represented by the orange colour. This is the temperature range at which the virus develops, attaching the upper and lower limits of the optimal temperature. As can be seen, a temperature in the lower limit is below the optimum value of 23.7°C, being represented by the absence of a development cycle. But the upper limit is marked by the red colour, exceeding the optimal threshold (*figure 5*).

Tables 3 and *4* show an extension of the West Nile virus transmission period from April to October inclusively, underlining the need to monitor mosquito populations throughout the year, as well as the bird and horse populations that represent the natural reservoir of the virus.

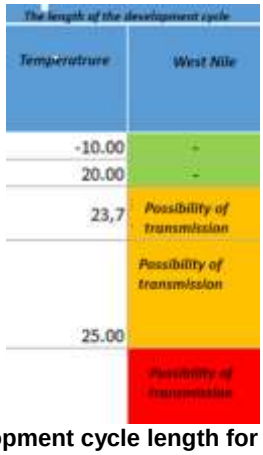
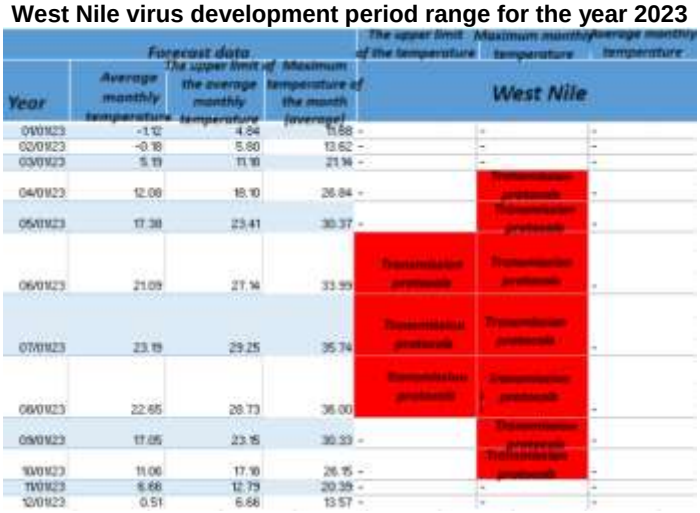


Figure 5 **Development cycle length for West Nile virus**

Table 3

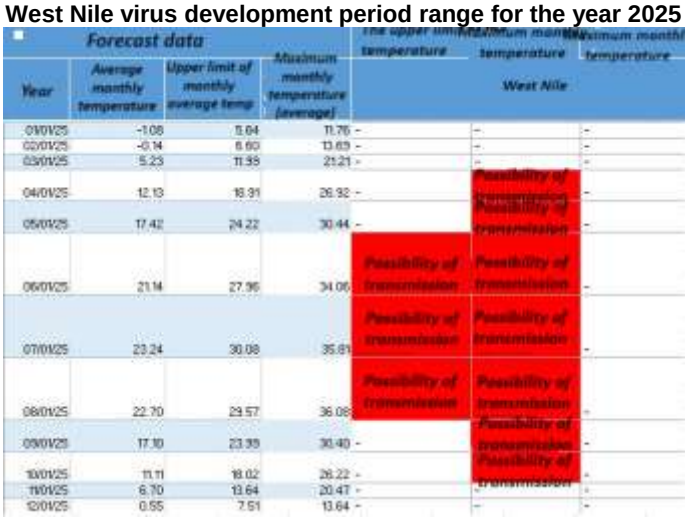
West Nile virus development period range for the year 2023



Forecast data				West Nile		
Year	Average monthly temperature	The upper limit of the average monthly temperature	Maximum temperature of the month (average)	The upper limit of the temperature	Maximum monthly temperature	Average monthly temperature
01/01/23	-1.12	4.84	11.88	-	-	-
02/01/23	-0.18	5.80	13.62	-	-	-
03/01/23	5.19	11.16	21.14	-	-	-
04/01/23	12.08	18.10	26.84	-	[illegible]	-
05/01/23	17.38	23.41	30.37	-	[illegible]	-
06/01/23	21.09	27.14	33.99	[illegible]	[illegible]	-
07/01/23	23.19	29.25	35.74	[illegible]	[illegible]	-
08/01/23	22.65	28.73	36.00	[illegible]	[illegible]	-
09/01/23	17.05	23.15	30.33	-	[illegible]	-
10/01/23	11.06	17.16	26.15	-	[illegible]	-
11/01/23	6.66	12.79	20.39	-	-	-
12/01/23	0.51	6.66	13.57	-	-	-

Table 4

West Nile virus development period range for the year 2025



Forecast data				West Nile		
Year	Average monthly temperature	Upper limit of monthly average temp	Maximum monthly temperature (average)	The upper limit of the temperature	Maximum monthly temperature	Maximum monthly temperature
01/01/25	-1.08	5.04	11.76	-	-	-
02/01/25	-0.14	6.00	13.63	-	-	-
03/01/25	5.23	11.39	21.21	-	-	-
04/01/25	12.13	18.31	26.92	-	[illegible]	-
05/01/25	17.42	24.22	30.44	-	[illegible]	-
06/01/25	21.14	27.96	34.06	[illegible]	[illegible]	-
07/01/25	23.24	30.08	35.81	[illegible]	[illegible]	-
08/01/25	22.70	29.57	36.08	[illegible]	[illegible]	-
09/01/25	17.10	23.35	30.40	-	[illegible]	-
10/01/25	11.11	18.02	26.22	-	[illegible]	-
11/01/25	6.70	13.64	20.47	-	-	-
12/01/25	0.55	7.51	13.64	-	-	-

Dengue cases forecast in Romania between 2023 and 2025

A comparison with West Nile virus shows that the optimal threshold of 23.7°C required for West Nile virus is increased by another 3°C required for Dengue virus. This explains why Dengue fever is specific to tropical areas, but also raises the alarm about its presence in an area characterised by a temperate continental climate such as Romania. In the case of this virus, only temperatures above 27°C are conducive to its development. This is marked only by the red colour, signalling the strict conditions of a high temperature (*figure 6*).

The length of the development cycle	
Temperature	Dengue fever
-10.00	-
20.00	-
25.00	-
27.00	[illegible]
37.00	[illegible]

Figure 6 **Duration of the Dengue virus development cycle**

After the 2018-2022 interval, a further period of increase in the maximum limit is expected, visible in the years 2023, 2024 and 2025 with the addition of September (*table 5*).

Comparing with 2014, over an 11-year time horizon there has been an increase in the upper limit series, which means that there is an increase in temperature and thus more favourable conditions for the spread of Dengue virus in the summer months, extending into September (*table 6*).

Table 5

Dengue virus development period range for the year 2023

Forecast data				Dengue		
	Average monthly temp	The upper limit of the average monthly temperature	Maximum temperature of the month (average)	The upper limit of the average temperature	Maximum monthly temperature	Average monthly temp
01/01/23	-1.12	4.84	11.68	-	-	-
02/01/23	-0.18	5.80	13.62	-	-	-
03/01/23	5.19	11.16	21.14	-	-	-
04/01/23	12.08	18.10	26.84	-	-	-
05/01/23	17.38	23.41	30.37	-	possibility of transmission	-
06/01/23	21.09	27.14	33.99	possibility of transmission	possibility of transmission	-
07/01/23	23.19	29.25	35.74	possibility of transmission	possibility of transmission	-
08/01/23	22.65	28.73	36.00	possibility of transmission	possibility of transmission	-
09/01/23	17.05	23.15	30.33	-	possibility of transmission	-
10/01/23	11.06	17.18	26.15	-	-	-
11/01/23	6.66	12.79	20.39	-	-	-
12/01/23	0.51	6.66	13.57	-	-	-

Table 6

Dengue virus development period range for the year 2025

Forecast data				Dengue		
Year	Average monthly temp	Upper limit of monthly average temp	Maximum temperature of the month (average)	The upper limit of the average temperature	Maximum monthly temperature	Average monthly temp
01/01/25	-1.09	5.64	11.76	-	-	-
02/01/25	-0.14	6.60	13.69	-	-	-
03/01/25	5.23	11.99	21.21	-	-	-
04/01/25	12.13	18.91	26.92	-	-	-
05/01/25	17.42	24.22	30.44	-	Possibility of transmission	-
06/01/25	21.14	27.96	34.06	Possibility of transmission	Possibility of transmission	-
07/01/25	23.24	30.08	35.81	Possibility of transmission	Possibility of transmission	-
08/01/25	22.70	29.57	36.08	Possibility of transmission	Possibility of transmission	-
09/01/25	17.10	23.98	30.40	-	Possibility of transmission	-
10/01/25	11.11	18.02	26.22	-	-	-
11/01/25	6.70	13.64	20.47	-	-	-
12/01/25	0.55	7.51	13.64	-	-	-

CONCLUSIONS

Projections made up to 2050 show a temperature increase by 0.78 ºC, which can influence up to 100% the increase in mosquito populations and also prolong vector-borne disease transmission periods. The forecast for 2025 shows an extension of the transmission period of *Plasmodium vivax* and *Plasmodium falciparum* species until November when a single complete cycle of the protozoan may develop during the lifetime of the *Anopheles* vector.

For West Nile virus the forecast shows an extension of the transmission period up to and including October, with the risk of the virus surviving inside the mosquito over winter, with the transmission cycle continuing into spring from March.

As regards the risk of emergence of Dengue fever in Romania, the data show that in the summer months of June-August, extending into the first part of September, the extrinsic development of the virus is possible, with the risk of epidemics in the context of imported cases. Thus, we

conclude the need to introduce monitoring and control programmes for vectors and vector-borne diseases in Romania, in the context of global warming.

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ASSESSMENT OF THE CONTRIBUTION OF WILDLIFE AND DOMESTIC PIGS IN HEPATITIS E VIRUS TRANSMISSION AND ZOONOTIC POTENTIAL IN EASTERN ROMANIA

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Abstract

Hepatitis E virus (HEV) has been confirmed within the landscape of the European food industry, representing a significant factor in the dissemination of HEV among European citizens. Food-borne transmission of HEV appears to be a major route in Europe, with pigs and wild boars being the main source. The results of this study highlight an overall HEV seroprevalence of 12.8% (95%CI: 7.95-17.75) in wild boars and the detection of HEV RNA in all three fresh pig liver batches sampled from a slaughterhouse in Iași County. Given the prevalent dietary preferences in Romania, pork stands out as a highly favored food choice among the populace. However, the popularity of pork also raises concerns, as there exists the occasional risk of contamination with HEV, presenting a potential threat to consumer health. Ongoing surveillance, regulatory measures, and public awareness initiatives collectively may represent a comprehensive strategy to protect the consumers and ensure the safety of pork products in the market.

Keywords: hepatitis E virus, swine, wild boar

INTRODUCTION

Hepatitis E virus stands out as a prominent global contributor to acute hepatitis, holding the leading position among viral causes. The family *Hepeviridae*, *Orthohepevirinae* subfamily comprises single-stranded RNA viruses organized into four distinct genera. Among these, only strains within the *Paslahepevirus* (HEV) and *Rocahepevirus* (RHEV) genera have exhibited zoonotic potential, posing a significant health threat (Purdy et al, 2022). Within the Paslahepevirus genus, two species, *P. balayani* and *P. alci*, exist. The *P. balayani* species further delineates into eight genotypes (HEV A1-8) based on the host species it infects. Genotypes 3 and 4 are identified in pigs; 3, 4, 5, 6 in wild boars; 3 in rabbits, mongooses, and deer; 4 in yaks; and 7 and 8 in camels (Ahmed et al, 2023). The expanded host range indicates the high variability of these HEV strains and their zoonotic potential.

Five HEV genotypes (HEV1-4 and HEV-7) are recognized to induce hepatitis in humans. In low-income countries, during epidemics, the fecal contamination of water reservoirs serves as the source of infection for genotypes 1 and 2.

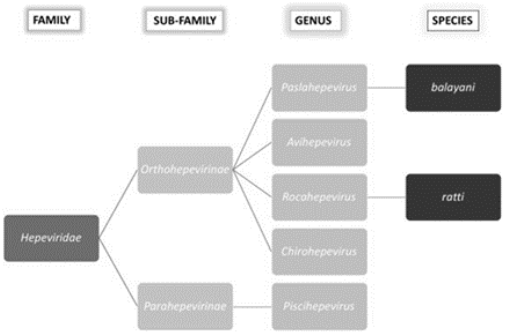

Fig. 1 Taxonomy and structure of *Hepeviridae* family

Autochthonous hepatitis E in the developed world primarily results from HEV-3 transmission occurring through the consumption of undercooked pork or cervid meat, especially the liver of these animals, and also through contact with contaminated animals. More recently, it has been demonstrated that the environment can be contaminated by zoonotic sources. Other foods such as salads, field raspberries, or shellfish (mussels, oysters) are potential sources of contamination. The frequency of human clinical forms associated with HEV infection is not

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known, but it does not appear to depend on the route of contamination (contact or food-borne) (Geng et al, 2023). There are clustered cases of exposure to HEV through food, but not all infected individuals (showing seroconversion) have reported acute hepatitis.

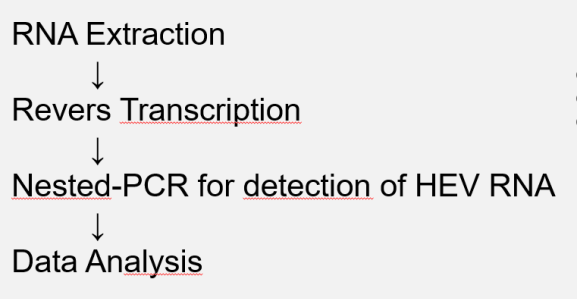
The present study was designed to provide data from a selected group of hunting founds and a slaughter house from Iași County, aiming to highlight the presence of the hepatitis E virus in the pig reservoir. The investigation framework encompasses a seroprevalence survey, delving into the prevalence of antibodies against HEV in wild boars and a molecular investigation focusing on the characteristics of the present viral strains in offal's of domestic swine. Through these multifaceted analyses, the study seeks to contribute valuable insights into the dynamics and potential pathways of zoonotic transmission of hepatitis E infection.

MATERIAL AND METHOD

The investigations war carried out between 2021 and 2022, involving the sampling of 179 wild boar sera from various hunting founds in Iași County. Additionally, 16 liver tissue samples were collected from two distinct slaughtering batches of pigs aged 5-6 months. The domestic pigs weighed approximately 100 kg at the moment of slaughtering.

The PrioCHECK® VHE Ab porcine kit (Applied Biosystems) was employed for the detection of HEV antibodies in serum samples from wild boars. The protocol followed a four-step protocol, including sample preparation, sample incubation, conjugate incubation, and detection. The ELISA plate is coated with recombinant hepatitis E virus (HEV) antigens from the ORF 2 and 3 of genotypes 1 and 3, being used for the purposes of monitoring and surveillance of potential zoonotic infection in swine.

For fresh liver samples, the protocol consisted first of RNA extraction and reverse transcription. Nested PCR was employed to identify HEV RNA in pig liver samples, utilizing a modified PCR technique aimed at enhancing the sensitivity and specificity of the assay. The method incorporates two sets of primers and two consecutive PCR reactions. Following the initial PCR reaction, the resulting amplicon served as a template for a second set of primers and an additional amplification step. The targeted region corresponds to a fragment of the ORF2 from the HEV genome. The specific primers employed in the reaction are detailed in figure 3.



3156N	AATTATGCYCAGTAYCGRGTTG	ORF2
3157N	CCCTTRTCYTGCTGMGCATTCTC	
3158N	GTWATGCTYTGCATWCATGGCT	
3159N	AGCCGACGAAATCAATTCTGTC	

Figure 2. Steps of the molecular diagnosis protocol and the primers used for HEV detection

RESULTS AND DISCUSSIONS

Pigs and wild boars are recognized as reservoirs for infections in humans in European countries. The transmission of these infections to humans occurs through various means, including direct contact, the consumption of raw or undercooked pork meat, and the ingestion of products derived from wild boars, such as sausages (Renou et al, 2014). Notably, several field studies have underscored the prevalence of HEV, revealing that between 2% and 11% of pork livers available in supermarkets across Japan, Europe, and the United States have tested positive for HEV RNA. Moreover, the infectivity of HEV has been corroborated through experiments using animal models.

In addition to direct transmission, there exists the potential for indirect transmission through contact with the manure of pigs infected with HEV (De Schryver et al, 2015). Interestingly, HEV has demonstrated its adaptability beyond traditional sources. It has been detected in unexpected places, such as strawberries cultivated in Canada, frozen raspberries, and salad vegetables in Europe. Furthermore, the versatility of HEV extends to seafood, where genotypes 3 and 4 of the virus are frequently identified. Oysters, flat oysters, mussels, and clams, among other seafood, have been found to harbor HEV. This occurrence is attributed to the bioaccumulation of the virus from water, resulting in its concentration within the digestive tissues of these marine organisms (Takuissu et al, 2022).

Hepatitis E has become the leading cause of acute viral hepatitis in Europe. Recent studies

on blood donor populations, conducted using the same serological test with validated analytical performance in terms of sensitivity and specificity, reveal anti-HEV IgG seroprevalence rates exceeding 20% in countries such as France (22%) (Gallian et al, 2014), Germany (29%), and the Netherlands (27%) (van Gageldonk-Lafeber et al, 2017). The seroprevalence is 16% in the United Kingdom (Hewitt et al, 2014). The rise in seroprevalence with age reflects cumulative exposure to the virus. The prevalence of asymptomatic forms contributes to its relatively low awareness. At the other end of the pathological spectrum, one can observe deadly fulminant hepatitis and chronic forms, especially in transplant recipients (Kamar et al, 2012).

The natural infection kinetics of pigs in farming settings reveal a predominant susceptibility among the animals during their early stages, specifically at the juncture of weaning and the decline in maternal immunity transfer. The transmission pathway primarily involves oro-fecal transmission, typically manifesting around the 10th week of age. Subsequently, the virus undergoes active replication within the liver, with substantial excretion observed in feces between the ages of 12 and 18 weeks before eventual elimination.

2021

Hunting Found	No. of samples
1. Poieni	8
2. Cătălina	1
3. Sinești	1
4. Crivești	6
5. Grajduri	45
6. Schitu-Duca	7
7. Pietrosu	22

2022

Hunting Found	No.of samples	Hunting Found	No.of samples
1. Bivolari	1	17. Cornești	1
2. Horlești	5	18. Prisecani	2
3. Țibănești	8	19. Gropnița	1
4. Stolniceni-Prăjescu	4	20. Valea Lupului	1
5. Stroiești	1	21. Larga-Jijia	1
6. Victoria	5	22. Gorban	2
7.Țuțora	1	23. Moțca	2
8. Popești	1	24. Pocreaca	2
9. Brădicești	4	25. Fermă de suine (Gorban)	13
10. Mogoșești	3	26. Bunești	1
11. Hârmănești	3	27. Bârnova	1
12. Gheorghițoaia	10	28. Strunga	1
13. Turia Perieni	4	29. TătărușI	1
14. Dagâța	3	30. Minoslovești	2
15. Crivești	2	31. Brăiești	1
16. Grajduri	1	32. Poieni	1

Fig. 3. Details of wild boar samples tested for HEV antibodies

It's noteworthy that during the viremic phase, the virus may extend its presence to other organs, including muscles such as the longissimus, biceps femoris, and iliopsoas (Bouwknegt et al, 2009). In certain instances, infections may occur later in the animals' development, leading to the arrival of individuals in the active phase of viral multiplication in the liver and viremic animals at the abattoir. This scenario poses a pronounced risk of exposure to Hepatitis E Virus (HEV) through the consumption of meat products. Turning attention to wildlife, wild boars and deer are identified as carriers of the virus, although the specifics of HEV dissemination within these reservoirs are not yet fully elucidated. Nonetheless, these species remain crucial reservoirs, with HEV prevalence reaching 40% in wild boars and 34% in deer within specific European regions (Pavio Net al, 2010). The evidence of the Hepatitis E Virus (HEV) in food products originating from pork, particularly pork liver, has been substantiated through comprehensive investigations across diverse regions. Five distinct studies conducted in Japan, the Netherlands, India, South Korea, and the United States have collectively shed light on the prevalence of HEV in these consumables. Notably, these studies disclosed that a range of 1% to 11% of commercially available pork livers tested positive for HEV (Di Cola et al, 2021). It's essential to highlight that certain liver-based preparations, such as semi-dry liver sausages, pose a potential risk when consumed in their raw state. These products, as recently underscored in a report from France (Colson et al, 2010) may harbor the virus and could be implicated in human cases. This emphasizes the need for continued vigilance and research to comprehend and

mitigate the risks associated with HEV transmission through pork-derived food items.

In the present study, HEV circulation in suid populations in Iasi County was asses through the detection of HEV antibodies in wild boars and evaluation of the presence of HEV RNA in commercial pig fresh liver.

Following a meticulous analysis of the results derived from serological testing aimed at detecting antibodies against the hepatitis E virus in samples collected from wild boars throughout the year 2021, 19 seropositive animals were identified, with a seropositivity rate of 21.11% (95% CI: 12.68-29.54%). The wild boars that tested positive for HEV hailed from four distinct hunting founds: Poieni, Crivești, Grajduri, and Pietrosu. The scrutiny of results stemming from serological testing for HEV antibodies in samples collected from wild boars throughout the year 2022 highlighted four seropositive animals out of 90 wild boars tested. The seroprevalence for the samples examined in the year 2022 stands at 4.4% (95% CI: 0.9 – 8.70%). Noteworthy is the fact that the wild boars positive for HEV antibodies originated from four distinct hunting founds: Poieni, Stolniceni-Prăjescu, Gheorghițoaia, and Mogoșești (Figure 4).

2021

Hunting Found	No. of positive samples	%	CI 95%
Poieni	4	50%	15.35-84.65
Crivești	1	16.7%	-13.15-46.49
Grajduri	2	1.44%	-1.58-10.47
Pietrosu	12	54.5%	33.74-75.35
Total	**19**	**21.11%**	12,68-29,54

2022

Hunting Found	No. of positive samples	%	CI 95%
Stolniceni-Prăjescu	1	25%	-17.44 - 67.44
Gheorghițoaia	1	9%	-8.59 – 28.59
Mogoșești	1	33.3%	-20 – 86.68
Poieni	1	100%	100
Total	**4**	**4.4.%**	0.9 – 8.70

Fig.4 Results of the detection of HEV antibodies in wild boar population

Similar results have been reported in two previous studies conducted in the Eastern region of Romania, highlighting the HEV seroprevalence at values of 10.29% and 9.61% in wild boar populations (Porea et al, 2018). Two studies from France and the Netherlands have also shown that HEV is endemic in these territories, with HEV seroprevalence in wild boars reaching values of 14% and 12%, respectively (Carpentier et al, 2012; Rutjes et al, 2010). A recent study conducted in Spain reported a 23% prevalence of HEV RNA and suggested that the dynamics of hepatitis E virus in wild boars may be seasonal, peaking at the beginning of the hunting season in late October and November (Rivero-Juarez et al., 2018). Furthermore, individuals with professional exposure, such as hunters and forestry workers, show an increased risk of the presence of specific anti-HEV antibodies, indicating a heightened risk of hepatitis E virus infection among them (Dremsek et al, 2012).

In the study involving domestic pigs, the results of the molecular analysis of the 16 liver samples, highlighted that all samples were

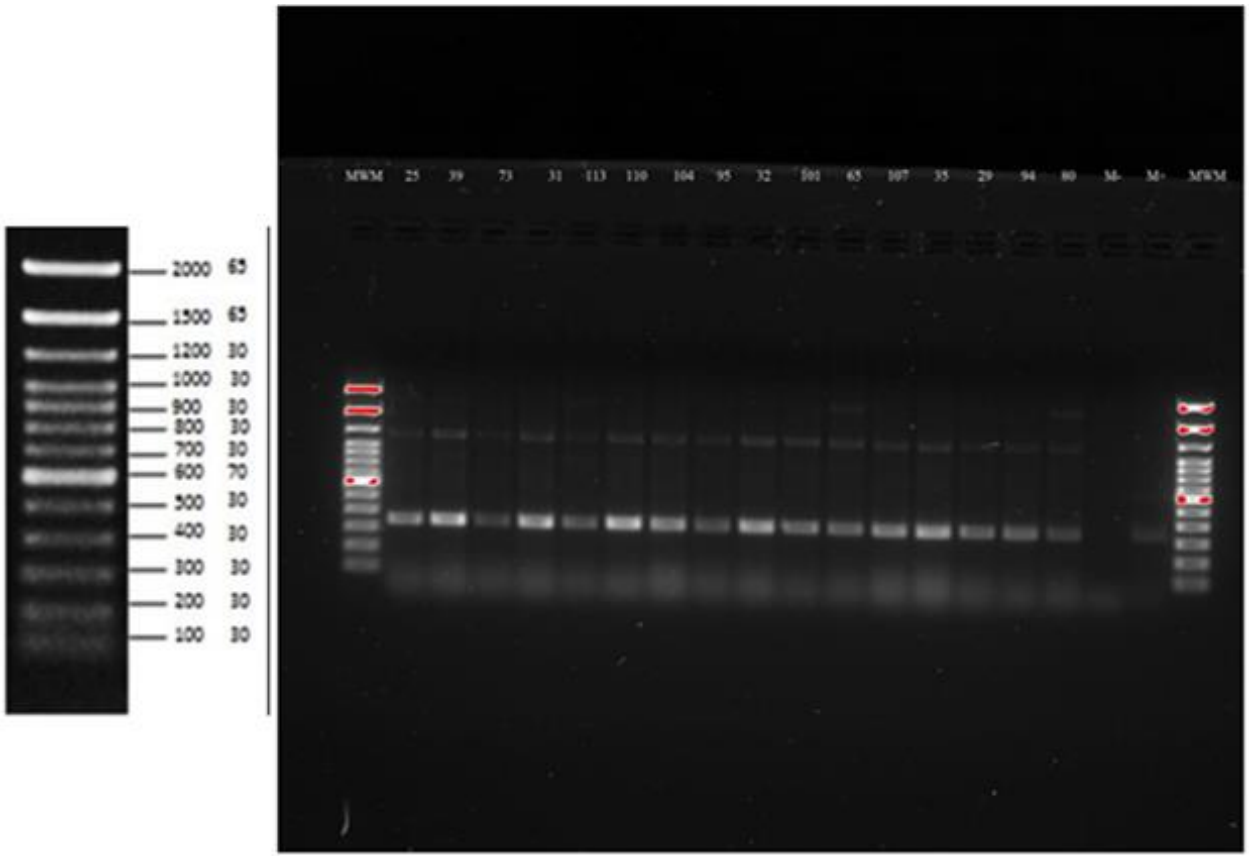


Figure 5. The results of the nested-PCR for detection of HEV RNA in pig liver samples. The expected positive PCR product length: 347 nt

positive for HEV RNA (Figure 5). This noteworthy finding underscores a uniform positivity across all three slaughtering batches, irrespective of the particular sample under examination. This consistent positivity across different batches strongly reinforces the assertion that HEV is a zoonotic disease, capable of transmission through improperly cooked pig meat.

In this context, the farm environment has been identified as a potential source of HEV in pigs. However, understanding the specific risk factors associated with on-farm and between-farm transmission of HEV remains elusive for interpretation. The last research studies highlighted the fact that HEV infections are not uncommon among pigs, the virus being detected in pig liver around four months old (Widen, 2016). Pork and liver undergo diverse industrial food-processing techniques to enhance their safety, flavor, and shelf life. These methods encompass a spectrum of approaches, including fermentation, acidification, high-pressure processing steps, and thermal treatments. Despite the application of thermal processing to pork meat and products, there remains a noteworthy concern regarding the stability of the hepatitis E virus. Published literature indicates that certain temperatures commonly employed in cooking may not effectively inactivate the virus, even when subjected to thermal treatment (EFSA Panel on Biological Hazards, 2017).

CONCLUSIONS

Presently, the substantial prevalence of the hepatitis E virus in animal reservoirs, particularly in pigs, underlines its status as a significant source of contamination for humans. To effectively mitigate the risk of zoonotic human infection, a comprehensive approach is essential. This involves not only the continued monitoring of pigs but also the active surveillance of all animal reservoirs. Recognizing the presence of HEV in animal offal's emphasizes the critical need for advancing diagnostic methodologies within food products. Implementing rigorous processing procedures in the food industry is imperative to curtail the potential for contamination. Furthermore, practical cooking methods should be emphasized to inactivate the virus, thereby enhancing overall food safety and

minimizing the risk of transmission to humans during consumption.

These multifaceted measures, encompassing surveillance, diagnostics, and processing protocols, collectively contribute to a comprehensive strategy aimed at reducing the incidence of zoonotic transmission of HEV to humans through the food chain.

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