ABSTRACT

Animal shelters play an important role in human and animal health, facilitating the spread of parasites to newly admitted animals and shelter staff if there is overcrowding and frequent exposure to a contaminated environment, with dogs thus becoming a source of infection for humans or other animals.

Parasitic diseases are usually underestimated by the population and society in general, but they are of particular importance and similar to infectious diseases.

Due to the fact that the incidence of vector-borne diseases at the European level is on a continuous rise due to intensive globalization and major climate changes, this study was necessary to establish the epidemiology of parasitic diseases in shelter dogs.

Globally, ticks are surpassed only by mosquitoes in terms of the number of diseases they can transmit, being responsible for vectoring a wide variety of pathogenic microorganisms: protozoa, rickettsiae, spirochetes, viruses. Therefore, ticks are important vectors that equally affect animals and humans.

Considering the fact that the number of stray dogs is very high in Romania, this represents a significant risk factor for public health from an epidemiological standpoint, due to the massive contamination of the environment with the feces of stray dogs that do not receive antiparasitic treatments. There is thus a high probability that they are carriers of various pathogens that can easily be transmitted to humans, representing a source of contamination in public areas and thus contributing to the maintenance of the zoonotic chain.

The doctoral thesis titled "**Research on parasitoses in stray animals from shelters**" is structured in accordance with the current regulations, in two main parts: the first part titled "**Current State of Knowledge**," which contains 30 pages, and the second part, "**Personal Contributions**," which presents the results obtained during the study period, comprising 82 pages. In addition to these two parts, the thesis contains a table of contents, acknowledgments, an introduction, a summary, a list of bibliographic sources, a list of abbreviations, a list of figures, and a list of tables. The present work contains 52 figures, 36 tables and 451 bibliographic sources. Part I corresponds to 25% of the thesis content, while Part II comprises 75% of the content, meeting current requirements.

The first part, "**Current State of Knowledge**," is structured into four chapters and presents information regarding the main parasitic diseases encountered in shelter dogs and the main vector-borne diseases transmitted by ticks and mosquitoes.

The first chapter, titled "**Main digestive parasitic infections found in shelter dogs, general aspects and their importance,**" is divided into two subchapters, "Protozooses" and "Helminthoses," and within these subchapters are described the main parasitic infections found in shelter dog. Thus, in the subchapter "**Protozooses**," Giardiasis and Coccidiosis are described, while in the subchapter "**Helminthoses**," Ancylostomiasis and Uncinariasis, Toxocariasis, Toxascaris, and Trichuriasis are described.

The second chapter, "**Main respiratory and circulatory parasitic diseases found in shelter dogs: general aspects and their importance**," consists of three subchapters that focus on describing the main respiratory and circulatory parasitic diseases encountered in shelter dogs. Thus, in this chapter, dirofilariasis, angiostrongyliasis, and capillariosis are described, presenting data from specialized literature regarding etiology, pathogenesis, symptomatology, and diagnostic methods.

The third chapter, "**Vector-borne arthropod diseases**," is divided into two subchapters and provides information on the main tick species reported in Romania and Europe, as well as their importance for dogs and public health.

The fourth chapter, "**Main vector-borne diseases found in shelter dogs**," consists of three subchapters in which the main parasitic and bacterial diseases transmitted by ticks are described: babesiosis, anaplasmosis, ehrlichiosis, and borreliosis. This chapter presents general data taken from the literature regarding etiology, pathogenesis, symptomatology, and diagnostic methods.

The second part of the thesis, "**Personal Contributions**," consists of five chapters, each chapter presenting in detail the obtained results.

Chapter 5 presents the location of the experiments, the purpose, and the objectives of the research. The purpose of the doctoral thesis "**Research on parasitoses in stray animals from shelters**" is to identify existing pathogens and possible co-infections in shelter dogs in the South-East region of Romania, determine the degree of contamination based on the type of shelter they are housed in, and estimate the zoonotic risk of parasitic infestations from stray carnivores.

The **first objective** of the thesis was to identify digestive parasitoses in shelter dogs and to evaluate the degree of infestation depending on the type of shelter in which they are housed. To achieve this objective, we conducted two studies. The first study aimed to determine the prevalence of intestinal parasitoses in shelter dogs in Constanța County using classical coproparasitological methods, while the second study involved determining the prevalence of canine giardiasis in Constanța County using the SNAP® *Giardia* test kit, which is a rapid immunoenzymatic test for detecting *Giardia* antigen in canine feces to assess the zoonotic risk.

The **second objective** aimed to determine the prevalence of respiratory and circulatory parasitoses in shelter dogs in Constanța County using classical coproparasitological methods of concentration, ovoscopic and larvoscopic methods, the concentration method for isolating microfilariae from blood, and molecular biology techniques.

The **third objective** aimed to determine the prevalence of tick infestations in shelter dogs to assess the risk of vector-borne disease transmission.

The **fourth objective** was to establish the seroprevalence and molecular detection of the main vector-borne pathogens found in shelter dogs in Constanța County, in SouthEastern Romania. Achieving this objective aimed at applying molecular biology techniques to identify vector-borne pathogens and using the SNAP 4Dx Plus Test from IDEXX Laboratories for the

simultaneous detection of antibodies against *Anaplasma phagocytophilum*, *Anaplasma platys*, *Ehrlichia canis*, *Ehrlichia ewingii*, *Borrelia burgdorferi* and the antigen for *Dirofilaria immitis*.

By addressing the four main objectives, a wide spectrum of parasitic diseases could be covered in order to establish an overview of the existing parasitic pressure in shelter dogs in Constanța County.

Although molecular studies of tick-borne pathogens in dogs are limited and bring new elements to the veterinary community, the main novelty lies in the overall approach of the doctoral thesis, which aimed to outline a comprehensive picture of all the pathogens encountered in dog shelters. This unique approach led to the completion of the thesis, which brings new elements to the veterinary community and public health.

The originality of this doctoral thesis lies in:

- the first approach regarding the real parasitic pressure present in dog shelters based on the gradual analysis of all biological systems working together to keep the organism alive, leading to a very high parasitic prevalence that is deeply concerning;

- the first reporting of the prevalence of digestive parasitoses obtained in improvised shelters, which represent a very common type of shelter in our country, but to which no one has had access for sampling purposes.

Chapter 6 of the thesis, titled "**Identification of digestive parasites in shelter dogs**", is divided into two subchapters. The **first subchapter** aims to determine **the prevalence of intestinal parasites in shelter dogs in Constanța County using classical coproparasitological methods**, while the **second subchapter** focuses on determining the **prevalence of canine giardiasis in Constanța County using the SNAP® *Giardia* testing kit**, which is a rapid immunoenzymatic test for detecting *Giardia* antigen in canine feces to assess the zoonotic risk.

The first subchapter is titled "**The prevalence of intestinal parasites in dogs from shelters in Constanța County-Romania.**" In this study, 611 stray dogs from 3 public shelters, 2 private shelters, and 4 improvised shelters in Constanța County were included. Fecal samples were collected and examined through direct observation to detect mobile trophozoites or protozoan cysts, using the flotation method with saturated sodium chloride for observing lighter helminth eggs and coccidia oocysts or sporocysts, and the sedimentation method for recovering heavier helminth eggs.

The main coproparasitological methods used in the study were **the flotation method – Willis**, the free sedimentation method or the **successive washings method**, and the **McMaster** method, which is based on the principle of the flotation of lighter parasitic elements to the surface of a superconcentrated solution and is used for counting helminth eggs in fecal samples.

The results showed that more than half of the dogs (**456/611**) were infected with at least one species of gastrointestinal parasites, and the overall prevalence of gastrointestinal parasites was **74.63%.** *Ancylostoma spp./Uncinaria spp.* eggs were the most frequently observed in fecal samples (55.97%), followed by ***Cystoisospora spp.*** (31.91%), ***T. vulpis*** (27.33%), ***Toxocara canis*** (21.27%), ***Capillaria spp.*** (2.78%), ***Toxascaris leonina*** (1.96%), and ***Demodex*** (0.49%).

Mixed infestations with two or more species of parasites were observed in 239 samples, representing 39.11% of the total samples analyzed, while cases of singular infestation were found in 217 (35.51%) of the analyzed dogs. The most frequently detected co-infections were: *T. vulpis/Ancylostoma spp.* (8.83%) and *T. vulpis/Cystoisospora spp./Ancylostoma spp.* (5.4%).

Comparing the prevalence of parasitism in the different types of shelters, it was observed that the prevalence obtained for *Ancylostoma spp./Uncinaria spp.* in improvised shelters is 85.08%, which is much higher than that in public shelters, where it was 55.62%. For *T. vulpis*, if in improvised shelters the obtained prevalence was 53.59%, in public shelters it was half as much (20.71%). In contrast, *Toxocara canis* recorded a prevalence of 19.88% in improvised shelters, while in public shelters the prevalence was 27.8%, which is higher. If for *Isospora canis* the prevalence obtained in improvised shelters (18.23%) was still lower than that recorded in public shelters (47.63%), *Toxascaris leonina* was identified only in improvised shelters, and the obtained prevalence was 6.62%.

Additionally, **the type of flooring** in the shelter has proven to be **relevant for the epidemiology of species**, especially for protozoan infections. A higher prevalence of protozoan infestation was observed in dogs kept without a concrete floor and drainage (56%) compared to dogs kept on a concrete floor (35%).

Five specimens were examined anatomopathologically, and adult parasitic species were observed in the intestinal tract. Thus, *Ancylostoma caninum* were collected from the small intestine, and *Trichuris vulpis* from the cecum and colon.

The quantitative coproparasitological examination was also performed using the **McMaster Method** for 60 samples from improvised shelters, with 20 samples from each shelter. The average OPG obtained for the 20 dogs from the **Ovidiu shelter was 600 eggs/gram of feces**, for the 20 dogs from the **Nicolae Bălcescu shelter OPG=750 eggs/gram** of feces, and for the 20 samples collected from the **Runcu shelter, OPG=1150 eggs/gram** of feces. In public shelters, 20 samples were also processed using the quantitative method, resulting in **OPG=350 eggs/gram** for the public shelter in **Năvodari, OPG=250 eggs/gram** for the public shelter in **Constanța**, and **OPG=600 eggs/gram** for the public shelter in **Hârșova**.

The second subsection aimed to determine **the prevalence of canine giardiasis in Constanța County using the SNAP® *Giardia* testing kit**, which is a rapid immunoenzymatic test for detecting *Giardia* antigen in canine feces to assess the zoonotic risk. This subsection is structured identically to the previous subsection. The study was conducted by analyzing fecal samples from 508 dogs before they were introduced into a private shelter and testing them using the SNAP® *Giardia* kit, a rapid enzyme immunoassay for detecting *Giardia antigen*, to determine the prevalence of *Giardia* in dogs.

The results showed that the overall prevalence of ***Giardia spp.*** was **48.42%,** with 246 samples being positive out of a total of 508 samples analyzed, the highest incidence being recorded in dogs under 1 year old, 30.7% (156/508).

The spread of infestation in younger dogs was significantly higher (156/194 dogs, prevalence 80.4%) than in animals over 12 months old (90/314 dogs, prevalence 28.7%).

Most cases of *Giardia* infection were found in dogs under one year old (30.7%), followed by dogs in the age groups 1-3 years (9.44%), 3-6 years (4.13%), 6-12 years (3.93%), and >12 years (0.2%).

Even though slightly more females were affected - 141/508 (27.75%) than males - 105/508 (20.66%), there was no significant difference between the two groups. Correlating the number of positive tests with the number of dogs entering the shelter monthly, we observe that the month with the highest prevalence of Giardiasis is April (21/27), with a prevalence of 77.8%, while the month with the lowest prevalence is June (18/37) with a prevalence of 20.5%.

Chapter 7, titled "**The Prevalence of Respiratory and Circulatory Parasitoses in Stray Dogs in Shelters in Constanța County**," begins with an introduction, followed by a description of the materials and methods used to conduct the study. In this study, 283 stray dogs from 4 shelters, including 2 public and 2 improvised shelters in Constanța County, were included. Fecal samples were collected from 283 dogs, and at the time of collection, the dogs were already housed in the shelters.

The fecal samples were examined through direct observation, using the **flotation method with saturated sodium chloride**, and through the **Baermann Method** for isolating and identifying certain types of nematode larvae.

Also within the study, 72 blood samples were collected from dogs housed in Public Shelters for the purpose of identifying circulating microfilariae, using the **Knott Method**.

The prevalence obtained for ***Capillaria spp***. was **2.47%,** with 7 positive samples out of a total of 283 analyzed. All positive samples for *Capillaria spp*. came from the public shelter in Hârșova, thus out of the total samples analyzed from the public shelter in Hârșova (25), 7 were positive, resulting in a prevalence of 28% at the shelter level.

Following the use of the Barmann Method for identifying L1 larvae of pulmonary nematodes, no positive samples were obtained.

The 72 blood samples collected from dogs originating from Public Shelters were processed using the Knott Method to identify circulating microfilariae. The total prevalence obtained was **13.89%,** with 10 out of 72 samples being positive for microfilariae. Out of the 10 positive samples obtained using the Knott test, 6/10 samples were positive for *Dirofilaria immitis* microfilariae and 4/10 for *Dirofilaria immitis* and *Dirofilaria repens*.

The highest prevalence was detected in the 4 - 7 years age group, followed by the 1-3 years (young) and 8-12 years group. Out of the 46 females tested, 39 were negative and 8 were positive (17.39%). Out of the 8 samples, 5 were positive for *Dirofilaria immitis* and 3 for co-infection with *Dirofilaria immitis* and *Dirofilaria repens*. Out of the 26 males tested, 24 were negative and 2 were positive (7.69%), with one sample being positive for *Dirofilaria immitis* and one for co-infection with *D. immitis* and *D. repens*.

Following the testing of the 72 blood samples using the real-time PCR technique (qPCR) for the identification of the *Angiostrongylus vasorum* genome, all samples were negative.

Chapter 8, titled "**Prevalence of tick infestation in shelter dogs**," aims to determine the tick infestations in dogs from public shelters. To achieve this objective, 200 canine specimens from public shelters in Constanța County were examined during the period 2023 – 2024. Thus, from the Public Shelter Constanța, 120 dogs were examined, 10 per month, and from the Public Shelter Hârșova, 80 dogs were examined.

At the Public Shelter in Constanța, following the general clinical examination, 219 ticks (46.66%) were collected from 56 dogs (out of 120).

The dogs were infested with ticks almost throughout the year, with the highest prevalence rates (100%) recorded during the months of July and August 2024 and the lowest (0%) in November 2023, January, and February 2024.

An increase in the average intensity of adult tick infestations was recorded during the period from June to August 2024, with the highest value in July 2024. In the same way, the average intensity of the infestation with immatures began to increase in June, peaked in July and August. The average monthly temperature varied from 3.7 °C (January 2024) to over 26 °C (July 2024), and the increase in the average intensity of tick infestation (whether adults or immatures) followed the trend of the average monthly temperatures recorded during the study period. The average intensity of adult and immature stages remained low from October 2023 to February 2024 (the autumn-winter period), dropping to 0 in the months of November, January, and February.

Following the examination of the dogs, the body areas with the highest presence of arthropods were the ears (57%), interdigital areas (18%), and armpits (10%). A total of 219 ticks (126 nymphs, 93 adults) were collected at the end of the study period. All the specimens belonged to the genus *Rhipicephalus (R. sanguineus*).

At the Hârșova Public Shelter, 80 canine specimens were examined. On this occasion, the ticks found attached to the animals' bodies in areas with thin skin were collected.

From a morphological standpoint, three genera of ticks were identified, represented by the following species: ***Rhipicephalus sanguineus*** (84.25%; 107/127), ***Ixodes ricinus*** (10.23%; 13/127), and ***Dermacentor reticulatus*** (5.51%; 7/127).

According to the obtained results, *Rhipicephalus sanguineus* was the dominant species, with a peak of activity during the third examination in August (87 specimens: 23 males; 29 females; and 35 immature stages).

The collected ticks were predominantly adults (67.71%), with different feeding states.

This study identified a high prevalence of the species *Rhipicephalus sanguineus*, at **84.5%** of the total ticks collected from dogs, as follows: 66 adults (37 females and 29 males) and 41 immature stages.

Following the clinical examination of the dogs at the Hârșova Public Shelter, we found that 37.5% of the dogs were not infested with any ticks (30/80), 21.25% (17/80) were infested with one tick, 15% (12/80) were infested with two ticks, and 21.25% (17/80) were infested with three ticks. Additionally, 5% (4/80) of dogs were identified with major infestations, with more than 8 ticks.

Chapter 9, titled "**Seroprevalence and molecular detection of major vector-borne pathogens in shelter dogs in Constanța County, SouthEast Romania**," is structured into three subsections. In the first subsection, the "**Seroprevalence of vector-borne pathogens in stray dogs from a private shelter in Constanța County, in SouthEastern Romania**" was investigated. For this purpose, blood samples were collected from January 2022 to December 2022 from 508 dogs of various breeds and ages ranging from 3 months to 13 years, before entering a private shelter in Constanța County, as a preventive measure.

The samples from the private shelter were examined using the SNAP® 4Dx® rapid test. The overall seroprevalence obtained was **17.71%,** with 90 out of 508 dogs being serologically positive for one or more of the tested pathogens.

Out of the 508 stray dogs, 56 (11.02%) tested positive for anti- *Ehrlichia canis* and *Ehrlichia ewingii* antibodies, 26 (5.11%) for anti- *Anaplasma phagocytophilum* and *Anaplasma platys* antibodies, 33 (6.49%) for *Dirofilaria immitis* antigen, and 0 for *Borrelia burgdorferi* antibodies. To determine which seropositive dogs had an active infection, PCR tests were performed, which showed a molecular prevalence of 1.78% for *Ehrlichia spp.* and 7.69% for *Anaplasma spp.*

66 out of the 508 dogs (12.99%) were infected with at least one pathogen, while 24 out of the 508 dogs (4.72%) were infected with multiple pathogens.

Among the 24 dogs that tested positive for multiple pathogens, the main co-infections encountered were: *Anaplasma spp.* and *Ehrlichia spp.* in 15/508 (2.95%), *Dirofilaria immitis* and *Anaplasma spp.* in 1/508 (0.19%), *Dirofilaria immitis* and *Ehrlichia spp.* in 7/508 (1.37%), and *Anaplasma spp.*, *Ehrlichia spp.*, and *Dirofilaria immitis* in 1/508 (0.19%).

In the **second subchapter**, "**Molecular detection of *Babesia spp.* in asymptomatic stray dogs from a private shelter in Constanța County, Romania**" was conducted, and for this purpose, venous blood samples were collected from 244 dogs. The sampled dogs were apparently clinically healthy and did not show clinical signs of babesiosis. The blood samples were transported to the HistoVet Laboratory for DNA extraction and molecular screening.

The results showed that out of a total of 244 samples, only 6 (2.46%) were positive, identifying the *Babesia spp.* genome through qPCR and melting curve analysis.

Out of the 6 positive samples obtained, 4 came from males and 2 from females, and 4 out of the 6 dogs tested positive were under 3 years old.

This study documented the highest frequency of infections at 11.11% in September and the lowest at 0% in January, February, March, April, June, November, and December.

The third subchapter is titled "**Molecular detection of vector-borne pathogens in stray dogs from public shelters in Constanța County, SouthEast Romania**." This study was conducted by analyzing the whole blood of 102 stray dogs from public shelters to assess the prevalence of vector-borne pathogens in dogs, by detecting the presence of genomic DNA of *Anaplasma spp./Ehrlichia spp.*, *Dirofilaria immitis, Dirofilaria repens*, and *Borrelia spp.* using PCR protocols.

Out of the total of 102 stray dogs included in the study, **60 (58.82%)** were infected with at least one pathogen, while 21 out of 102 dogs (**20.58%)** were infected with **more than one pathogen**. 55 dogs (**53.92%**) tested positive for ***Anaplasma spp./Ehrlichia spp***., 8 (7.84%) for *Borrelia spp*., and 20 for *Dirofilaria spp*. (19.6%). Of the 20 dogs positive for *Dirofilaria spp*., 14 were positive for *Dirofilaria immitis*, 2 for *Dirofilaria repens*, and 4 were co-infected with *Dirofilaria immitis* and *Dirofilaria repens*.

For the ***Ehrlichia spp./Anaplasma spp***. complex, the highest prevalence was found in the Public Shelter of **Constanța (63.2%)**, followed by the Public Shelter of Hârșova with a prevalence of 52%, and the lowest prevalence was obtained in the Public Shelter of Năvodari (30%). For *Borrelia spp*., the highest prevalence was found in the Public Shelter of Hârșova, with 7 positive samples out of 25, resulting in a prevalence of 28%, followed by the Public Shelter of Constanța with a prevalence of 1.75%. In the Năvodari Public Shelter, all samples were negative for *Borrelia spp*.

Regarding dirofilariasis, the highest prevalence was obtained for ***Dirofilaria immitis*** in dogs from the **Hârșova Public Shelter (28%),** followed by the Constanța Public Shelter (15.8%) and the Năvodari Public Shelter (10%). The prevalence for *Dirofilaria repens* was 16% for the Hârșova Public Shelter, 10% for the Năvodari Public Shelter, and 0% for the Constanța Public Shelter.

Out of the 21 dogs infected with more than one pathogen, 10 dogs (9.80%) were co-infected with *Dirofilaria immitis* and *Anaplasma spp./Ehrlichia spp*., 1 dog (0.98%) was co-infected with *Dirofilaria repens* and *Anaplasma spp./Ehrlichia spp*., 3 (2.94%) were co-infected with *D. immitis, D. repens*, and *Anaplasma spp./Ehrlichia spp*., 5 (4.9%) dogs were co-infected with *Anaplasma spp./Ehrlichia spp.* and *Borrelia spp*., 1 dog (0.98%) was co-infected with *Anaplasma spp./Ehrlichia spp*., *D. immitis*, and *Borrelia spp*., and 1 dog (0.98%) was co-infected with *Anaplasma spp./Ehrlichia spp*., *D. immitis, D. repens*, and *Borrelia spp*.

In the final chapter, **chapter 10 – "Final Conclusions"** – The final conclusions present the findings of our research, highlighting new and original results regarding the parasitic pressure present in dog shelters in Constanța County. The analysis of the obtained results contributes to the potential establishment (and monitoring) of objectives aimed to minimize the impact of parasitic diseases on stray dogs, in order to prevent repercussions on human health and to break the zoonotic chain, which invisibly subjugates us alarmingly under its vectorial weight.