

ABSTRACT

Most of the ticks that infest companion animals are species that feed on three different vertebrate hosts, making them such important vectors in the transmission of diseases.

The growing interest of researchers in studying vectors and vector-borne diseases is due to several factors, mainly climate change, urbanization, but also some socio-economic factors.

Vector-borne diseases are considered real challenges for veterinarians, as the clinical signs may be diffuse or overlapping with other pathologies. Also, co-infestation with ticks but also co-transmission of pathogens are relevant for both public and animal health.

The PhD thesis entitled "**Research regarding the bioecology of the ixodid ticks in Eastern Romania and the epidemiology of transmitted diseases**" is structured accordingly to the general rules, in two main parts: a first part entitled "**Current state of knowledge**", which contains a number of 25 pages and a second part, "**Personal contributions**" detailing the results obtained during the studies, with a number of 51 pages. In addition to these two parts, the thesis contains a table of contents, acknowledgements, introduction, abstract, bibliography, list of abbreviations, list of figures and a list of tables. This paper contains 13 figures and 20 tables and 317 bibliographic sources have been consulted.

The first part, "**Current state of knowledge**", is structured in three chapters and presents information on the importance of ticks involved in the transmission of pathogens, the main tick-borne diseases and the clinical implications in dogs with babesiosis and co-infected with other tick-borne diseases.

The first chapter, entitled "**The importance of ticks involved in the transmission of vector-borne diseases**" is divided into three subchapters and begins by describing some aspects of the ecology of ixodid ticks, represented by the appropriate climate and their seasonal activity, the role of global warming and the dynamics of ixodid tick populations. It also provides information on the main species of ticks reported in Romania and Europe, as well as their importance in pets and in human health.

The second chapter, "**Canine tick-borne diseases**" consists of four subchapters focusing on the description of the main parasitic and bacterial diseases transmitted by ticks: babesiosis, hepatozoonosis, anaplasmosis, erlichiosis and borreliosis. This chapter presents general data taken from the literature regarding the etiology, pathogenesis, symptomatology, diagnostic methods and treatment used.

The third chapter, "**Clinical implications in dogs with babesiosis and co-infected with other diseases transmitted by ticks**" briefly describes data identified in the literature regarding the multitude of pathogens that can co-infest animals, these data being relevant also for public health. Co-infections can also change the associated clinical manifestations making it difficult to diagnose and they can change the prognosis of patients.

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

Chapter 4 presents the place of the experiments, aim and objectives of the research. The **aim** of this PhD thesis was to identify existing pathogens and possible co-infections in dogs and ticks collected from them in the eastern Romania.

The first objective of the thesis consisted in **the morphological and molecular identification of the main parasitic and bacterial pathogens found in the blood of dogs infested with ticks**, presented at the Parasitic Diseases Clinic with specific symptoms to infestation with *Babesia* spp.

The second objective was to **collect and morphologically identify the ticks attached to the dogs' bodies, as well as the molecular identification of the main parasitic and bacterial pathogens** in order to evaluate the microbial load and possible co-infections to which dogs were exposed.

The third objective was to **detect co-infections found in dogs in Eastern Romania**.

Little molecular studies of tick-borne pathogens in dogs and possible co-infections have led to this thesis, which brings new elements to the veterinary community and public health.

The originality of this PhD thesis consists in:

- Ⓢ the first identification of *B. rossi* in two symptomatic dogs in Romania, for which we consider that additional studies are needed to confirm the presence of this pathogen and its vector in Europe and in Romania.
- Ⓢ the first report of the symbiote *Candidatus Midichloria mitochondria* in the blood of dogs and ticks collected from them, requiring further investigation to better understand the role and possible implications for animals and public health.

Chapter 5 of the thesis, entitled "**Molecular identification of Babesia species and co-infection with Hepatozoon canis in dogs and ticks in eastern Romania**" begins with an introduction, description of materials and methods used to conduct the study. This study included 90 dogs which were presented to the Clinics with signs compatible with canine babesiosis (hyperthermia, hemolytic anemia, thrombocytopenia, jaundice and hemoglobinuria) (Irwin, 2009), whole blood samples (EDTA) were collected from them in order to be able to analyze microscopically the thin blood smears, stained by the Romanowsky (Diff-Quick) method. The remaining blood samples were stored at -20°C until DNA was extracted for molecular detection of *Babesia* spp. and *H. canis*.

Each dog was submitted to a clinical examination, and all detectable ticks were collected for species identification by standard taxonomic keys (Estrada-Pena et al., 2004; Dantes-Torres et al., 2013). Collected ticks were stored in 70% ethanol and separated by individual animal for morphological detection. **All identified ticks (No.=182) were divided into No. = 100 sub-samples** (nine composed by pools of three ticks, 64 by pools of two ticks,

and 27 sub-samples individually analysed) comprised of specimens collected from the same dog, and homogeneous species that were screened for *Babesia* species and *H. canis* using molecular analyses.

Thirty-five dogs (38.9%; 95% CI = 29.0–49.8) showed mild clinical signs and 55 (61.1%; 95% CI = 50.2–71.0) expressed the acute form, attributable to **babesiosis**. All 90 of the sampled dogs that tested positive for the babesial parasites using a stained thin blood smear were positive for the general piroplasmid PCR. Three *Babesia* species were found in dogs: *B. canis* (85/90 = 94.4%; 95% CI = 86.9–97.9) (99.1–100% identity with GenBank Accession numbers: MK571831), *B. vogeli* (3/90 = 3.3%; 95% CI = 0.9–10.1) (100% identity with GenBank Accession number: KY290979), and *B. rossi* (2/90 = 2.2%; 95% CI = 0.4–8.6) (100% identity with GenBank Accession Number: MT740273). All the dogs resulted **negative for *H. canis***.

Additionally, the dogs that expressed acute clinical signs were positive for *B. canis* with the prevalence of 58.9% (95% CI = 48.0–69.0) and *B. rossi* with the prevalence of 2.2% (95% CI = 0.4–8.6). The dogs that showed mild clinical signs were positive for *B. canis* with the prevalence of 35.6% (95% CI = 25.9–46.4) and *B. vogeli* with the prevalence of 3.3% (95% CI = 0.9–10.1).

Moreover, five dogs have died (5.6%, four infected with *B. canis* and one with *B. rossi*) and for three of them the follow-up remains unknown. Overall, 91.1% of the dogs have recovered.

Dogs that presented with ticks during the routine visit were 33.6% of the total (38/113; 95% CI = 25.1–43.2). Briefly, 71.1% (27/38; 95% CI = 53.9–84.0) of dogs showed low infestation, 21.1% (8/38; 95% CI = 10.1–37.8) showed moderate infestation and 7.9% (3/38; 95% CI = 2.1–22.5) showed high infestation. **A total of 182 ticks were collected from dogs**; of these, 58 ticks were found on 15/90 (16.7%; 95% CI = 9.9–26.3) symptomatic dogs and 124 ticks on another 23 dogs previously analysed for piroplasmosis (as above described). Specifically, 179 adults (113 engorged females and 66 males), 2 nymphs, and 1 larva were found.

One hundred tick sub-samples were prepared and identified as follows: *Ixodes ricinus* (64%; 95% CI = 53.7–73.2), *Dermacentor reticulatus* (33%; 95% CI = 24.1–43.2), and the *Rhipicephalus sanguineus* group (3%; 95% CI = 0.8–9.1). *Babesia canis* (MIR = 81%; 95% CI = 71.7–87.9) (99.8–100% identity with GenBank Accession numbers: MK571831, accessed on 1 March 2021), *B. vogeli* (MIR = 3%; 95% CI = 0.78–9.6) (100% identity with GenBank Accession number: KY290979, accessed on 1 March 2021) and *B. microti-like piroplasm* (MIR = 1%; 95% CI = 0.1–6.2) (100% identity with GenBank Accession number: MN355504,) **was found in ticks**. In addition, **15 ticks were positive for *H. canis*** (MIR = 15%; 95% CI = 8.9–23.9), **six were co-infected with *B. canis*, and one with *B. microti-like piroplasm***.

We herein report the first comprehensive molecular survey of *Babesia* spp. and *H. canis* in owned dogs and in their ticks from the eastern part of Romania. Over 90% of dogs and over 80% of ticks collected from symptomatic dogs were positive for *B. canis*.

Chapter 6, entitled "**Detection of the co-infections with *Borrelia*, *Anaplasma* and *Ehrlichia* species in dogs with babesiosis in north-eastern Romania**" is structured identically to the previous chapter, this time trying to identify possible co-infections with bacterial pathogens. The study was performed by analyzing the whole blood of 66 dogs that were confirmed with babesiosis in the previous chapter, while the attached ticks detected during the general clinical examination of the dogs, were removed and placed in separate tubes with 70% ethanol.

Both blood and tick samples were tested for possible co-infections with bacterial pathogens carried by ticks: *Borrelia* spp., *Anaplasma* spp. and *Ehrlichia* spp.

The analyzed blood samples (n = 66) were collected from dogs of different breeds and aged between 3 months and 13 years, from Iași and the surrounding areas. **15/66 (22.7%) dogs were positive for *Borrelia burgdorferi* s.l., 0/66 for *Anaplasma* spp./*Ehrlichia* spp.** according to the results obtained from the PCR test. To date, the symbiote *Candidatus Midichloria mitochondria* has been identified only in ticks, this being the first report of molecular identification of the bacterium in the blood of dogs. In the present study, ***Candidatus Midichloria mitochondria* was identified in 2/66 (3%) of dogs** included, positive also with *Borrelia burgdorferi* s.l. *Candidatus Midichloria mitochondria* DNA was amplified by primers used to amplify *Anaplasma/Ehrlichia* species, and was identified after sequencing.

A total of 99 adult ixodid ticks were collected from dogs during the clinical examination. The ticks were identified as *Ixodes ricinus* - 48.5% (48/99 - 32 females and 16 males) and *Dermacentor reticulatus* - 51.5% (51/99 - 34 females and 17 males). The most commonly detected pathogenic species in ticks was *Candidatus Midichloria mitochondrii* (25.8%) without clinical significance, followed by *Borrelia burgdorferi* s.l. (12.9%) and **co-infection with *Borrelia burgdorferi* s.l. and *Candidatus Midichloria mitochondria* (6.5%)**.

Even if the *Anaplasma* and *Ehrlichia* species were not detected in the analyzed blood and tick samples, we consider that their pathogenicity is expressed due to the report by other authors (Matei et al., 2017b; Hamel et al., 2012; Morar et al., 2015).

Chapter 7, entitled "**Correlation regarding clinical signs, diagnosis and treatment in canine babesiosis**" presents the materials and methods used to perform this study, which consisted of collecting two samples of peripheral blood (EDTA and without anticoagulant) from each patient suspected of babesiosis, for the following investigations: microscopic examination of the blood smear, hematology examination, blood biochemistry examination and two serological tests: SNAP 4DX Plus (IDEXX), respectively *Babesia gibsoni* antibody test (WellTest).

The results of this study showed that **the most affected dogs among those analyzed were those of common or mixed breed - 40.48% (17/42)**, followed by Pekingese - 11.9%

(5/42) and Husky, German Shepherd and Shepherd Caucasian - 7.14% (3/42). Regarding the gender of dogs, **the number of infestations in males was higher (66.66%), compared to infestations in females (33.34%)**. According to the data obtained from the anamnesis, we found that **76.19% (32/42) of the study participating dogs had not acaricidal treatment**, according to the veterinarians recommendations. **The most common clinical signs** found in the 42 dogs studied were: **fever - 37/42 (88.1%), pale mucous membranes - 31/42 (73.8%)** and **hemoglobinuria - 31/42 (73.8%)**, followed by dehydration (11/90%) and jaundice (4.76%).

For all blood samples collected, 3-5 smears were performed and at least 50 microscopic fields were examined using the x1000 objective. **All blood samples were positive for *Babesia canis***. Hematological changes found - **moderate to severe anemia** (decreased erythrocyte count, hematocrit and hemoglobin) and **thrombocytopenia - 40/42 canine patients**, as well as **changes in renal and hepatic parameters**, resulting from biochemistry tests of the blood.

We also identified ***Babesia gibsoni* antibodies in 2/42 blood samples** positive for canine babesiosis, which showed a moderate form of infestation with *Babesia canis* and responded to treatment with Imidocarb dipropionate.

Chapter 8, entitled "**Prevalence of tick infestation in dogs**" aims to establish the preponderance of tick infestations in the canine patients included in the study and investigate possible changes in the activity periods of the species identified.

The results of this study showed that most of the ticks collected from dogs were adults (99.54%), with different feeding status, morphologically identifying three species of ticks: ***Dermacentor reticulatus* (57.7%; 251/435)**, ***Ixodes ricinus* (41.6%; 181/435)**, and ***Rhipicephalus sanguineus* complex (0.69%; 3/435)**.

The species ***Dermacentor reticulatus* was dominant, with two peaks of activity in spring**, in March (52 specimens: 21 males; 31 females), April (138 specimens: 53 males; 85 females) and **autumn** - September (19 specimens: 10 males; 9 females), October (9 specimens: 6 males; 3 females). We also found **2 specimens of *D. reticulatus* male, in January**, outside the normal period of activity.

The second species identified ***Ixodes ricinus*, shows three peaks of activity, with a decreasing trend in the colder months**, having the most intense period of activity in the **spring** months (March, April), followed by a decrease in May. The second peak is found in **summer**, in June, and the third in **autumn**, in September and October.

The prevalence of *Rhipicephalus sanguineus* complex is low, of only **0.69%** of the total ticks collected from dogs, being active in April and May.

Also, at the clinical examination of the patients, we found that most canine patients - **41.2% were infested with a single tick**, in 23.3% we noticed two ticks, and in 20.6% three ticks. Also, **in the canine patients from which we collected more than two ticks (58.7%)**, we found: **either both species (*Ixodes ricinus* + *Dermacentor reticulatus*) in 20.7%**

(23/111) dogs, or **only one species: *Ixodes ricinus*** - 32/111 (**28.8%**) and ***Dermacentor reticulatus*** - 56/111 (**50.5%**).

The effects of tick parasitism on companion animals and their owners can be limited by the regular application of various methods of protection recommended by veterinarians.

The last chapter, Chapter 9 - "**Final Conclusions**" summarizes the conclusions of our research, creating an overview of the results of the PhD thesis. The results of this study provide new data on the identification of a new species of pathogen in dogs, as well as the detection of a little studied symbiont in this region.