ABSTRACT

The title of this doctoral thesis is **"The epidemiology of vector-borne parasitic diseases of dogs from Romania"** and is composed of several studies meant to help understand the phenomenon that leads to changes in the prevalence and distribution of vector-born parasitic diseases of dogs. These changes have not only been observed in Romania but also all over the world.

This thesis is structured in two parts, according to the required standards: the first part is named **"Current state of knowledge"** and the second part is named **"Personal contributions"** and describes all scientific results obtained during the doctoral thesis.

The first part of the thesis, called **"Current state of knowledge"**, provides up-to-date, general knowledge about each parasitic disease studied in this thesis. Each chapter of the first part presents information that highlights the impact the parasitic diseases studied in this thesis have on the health of dogs but also on that of humans.

The first chapter, called **"The importance of canine leishmaniosis"** is split in five subchapters. In the first subchapter we present data that shows the impact that *Leishmania* spp. infections have on the health of animals and humans. In the second subchapter we describe the biology and ecology of sandflies. These arthropods are vectors for *Leishmania infantum* and represent the main mode of transmission for this parasite. In subchapter three we present the main methods of prevention of canine leishmaniosis. The effectiveness of each method is also discussed and examples for the application of each method are provided. Subchapter four describes the clinical and paraclinical manifestations of *L. infantum* infections in dogs and the last subchapter describes the epidemiological situation of canine *L. infantum* infections in Europe and in Romania.

Chapter II is named **"The importance of canine babesiosis"**, it is divided in four subchapters and presents general and current knowledge that highlights the importance of canine babesiosis for the health of dogs. The first subchapter presents a short history that describes the first reports of *Babesia* spp. infections. Subchapter two describes the life cycle of *Babesia* spp. in the vertebrate and arthropod host.

Subchapter three describes the clinical and paraclinical signs that are specific to *Babesia* species that infect dogs from Europe: *Babesia canis*, *Babesia vogeli*, and *Babesia gibsoni*. The last subchapter explains the epidemiological situation of canine babesiosis in Europe.

Chapter III is called **"The importance of canine hepatozoonosis"** and is structured similarly to chapter II. It is subdivided in four subchapters. The first subchapter is an introduction and the second describes the life cycle of *Hepatozoon canis*. The third subchapter describes the clinical and paraclinical signs of canine *H. canis* infections and the last subchapter describes the epidemiological situation of this infection in Europe

The last chapter of the first part is called **"The importance of canine dirofilariasis"** and is divided in six subchapters. Like in previous chapters, the first subchapter provides an introduction. We describe the first recorded reports of *Dirofilaria immitis* and *Dirofilaria repens* infections, the localisation of the parasite in the case of canine, feline or human infections and also a description of transmission of this parasite. In subchapter two, we present the particularities of *D. immitis* and *D. repens* infection in humans and in subchapter three we discuss about why infections in wild animals play a role in maintaining these infections in a region. Subchapter four and five describe the life cycle of *D. immitis* and *D. repens* in vertebrat host, but also in the arthropod, mosquito host. The final subchapter describes the epidemiology of *D. immitis* and *D. repens* in Europe and particularly in Romania.

The second part of the thesis, named **"Personal contributions"**, is divided in five chapters. In this part we present and discuss the results obtained during the experimental part of the thesis.

Chapter V is the first chapter of the second part and is named **"The epidemiology of canine leishmaniosis in the south of Romania"**. The south of Romania is the region where most canine and human *Leishmania infantum* infections have been reported. In this region there have also been the most reports of vector-competent sandflies. After a period of approximately 50 years since the last autochthonous *L. infantum* infections have been eliminated from Romania, there have been new reports of autochthonous canine infections, but also one infection in a jackal and one human infection.

In this study we present an epidemiological study of canine *L. infantum* infections in the south of Romania. This study is meant to determine if canine leishmaniosis is re-emerging in Romania.

We collected samples of serum, blood and eye swabs from 300 dogs, from the counties of Mehedinți, Dolj, Teleorman and Ialomița. The blood and eye

swabs samples from each dog were used to extract DNA. A qPCR assay was used to detect *L. infantum* specific DNA in the nucleic acid samples. The serum samples were used in an ELISA assay in order to detect anti-*L. infantum* antibodies.

Both qPCR and ELISA tests were negative for all samples but in spite of these results there is still the risk that canine leishmaniosis will become endemic in Romania again.

Chapter VI is entitled **"Seroepidemiological study of canine leishmaniosis in the south-east of Romania"**. As the previous chapter, this study is focused on canine *L. infantum* infections, but this is a serological study, focused on the south-east of Romania. As mentioned in the previous chapter most reports of *L. infantum* infections are from the south of the country, but there is no information about the epidemiology of this infection from the south east of Romania. The role of this study is to determine in dogs from the south-east of Romania are exposed to *L. infantum* infections.

Serum samples were collected from 110 dogs from the counties of Călărași and Galați. All samples were tested using an ELISA assay and 5/110 samples were positive for anti-*L. infantum* antibodies. All five positive samples were from Galați county and the total prevalence was 4.54%. This study shows that dogs from the south-east of Romania are exposed to *L. infantum* infections.

Chapter VII is entitled **"The distribution and prevalence of babesiosis and hepatozoonosis in dogs from the south of Romania"** and is an epidemiological study of canine hepatozoonosis and babesiosis that in the south of Romania. The studied region was the same as that of chapter V. There have been several studies in Romania about *Babesia* spp. infections but there is still a need for a comprehensive epidemiological study on this subject. Canine babesiosis is considered by most veterinarians to be a particularly important parasitic disease.

Hepatozoon canis infections are less pathogenic than *Babesia* spp. infections but there is very little known about the epidemiology of canine hepatozoonosis in Romania. This lack of epidemiological data is the main reason we chose to study this infection.

Blood samples were collected from 300 dogs, as mentioned in chapter V. DNA was extracted from all blood samples. All DNA samples were tested using an aspecific PCR assay meant to amplify DNA fragments of bot *Babesia* spp. and *Hepatozoon* spp. All samples positive to this first assay were tested again using a PCR assay specific for *Babesia* spp. and one for *H. canis.* Samples that were

positive for the specific PCR assays were sequenced in order to confirm the diagnosis.

In this study we report a prevalence of 47.6% for *H. canis* and 9.6% for *Babesia* spp. (*B. canis* 7% and *B. vogeli* 2.6%). We reported statistically significant differences in the prevalence of these infections between counties. The highest prevalence of *H. canis* and *B. vogeli* infections were reported in the most western counties and the highest prevalence of *B. canis* infections was reported in the most eastern counties. The phylogenetic study showed that sequences from this study are similar to sequences previously reported in Romania or in nearby countries, such as Hungary, Turkey or Slovacia.

This study reports a surprisingly high prevalence of *H. canis* in the south of Romania and infections with *B canis* and *B. vogeli* in the counties from the south of Romania. We believe that the information presented in this study is of use to veterinarians working in this region.

Chapter VIII is entitled **"Epidemiology of canine dirofilariasis in shelter dogs from the south of the Romania"**. This is the last chapter of the second part that describes an epidemiological study. This epidemiological study was carried out in the same region as the studies from chapters V and VII. Like all the other diseases presented in this thesis, canine dirofilariosis is also a vector-borne disease that is of importance for veterinarians from Romania.

In Romania there have been published several studies about *D. repens* and *D. immitis* infections in dogs. The majority of these studies are based on morphological identification of microfilaria or the detection of anti-*D. immitis* antibodies. In our study, we take advantage of the sensibility and specificity of qPCR in order to determine the prevalence of filarial infections in dogs from the south of Romania.

Blood samples were collected from 300 dogs and DNA was extracted from all blood samples. All DNA samples were first tested using a qPCR in order to take advantage of the great sensibility of this method. All positive samples to this first screening were tested again using a classical PCR assay that produces a longer amplicon, suitable for sequencing. Coinfected samples confirmed with a third classical PCR assay using primers specific for *D. immitis* and *D. repens*. The amplicons of positive samples were purified and sequenced.

We report a prevalence of 11.66% (35/300) for *D. repens*, 4.66% (14/300) for *D. immitis* and 1.33% (4/300) for *A. reconditum*. There were no statistically significant differences of prevalence between counties.

This study achieved the objective of providing accurate data about the prevalence of *Dirofilaria* spp. infections in stray dogs from the south of Romania.

We believe this information is of use to veterinarians but considering the zoonotic potential of these infections, we consider this study to be of interest to human health professionals as well.

Chapter IX is named **"The use of MALDI-TOF mass spectrometry for the identification of sandflies"**. This is the last chapter of the thesis and it is focused on the use of the MALDI-TOF mass spectrometry technique in order to identify sandflies.

The MALDI-TOF technique has been used for a long time in the field of microbiology as a cheap, fast and reliable alternative method to identify microorganism such as bacteria or fungi. In time, new applications have been discovered for this technique. The use of this technique in the field entomology, to identify important arthropods, is particularly relevant to our study.

Sandflies are arthropods important for the spread of canine leishmaniosis. The bite of female sandflies is the main mode of transmission of *Leishmania infantum*. Not all sandfly species are vectors for canine leishmaniosis and because of this it is very important to be able to identify accurately the species of sandfly. For many years, sandflies have been identified morphologically, but this method is time-consuming and requires specialised, experienced personnel. In recent years, many researchers have chosen to use molecular biology techniques in order to identify sandflies but this can be a very costly alternative. The MALDI-TOF technique has been proven to be an efficient, cheap and fast alternative to reliably identify sandflies.

CDC light traps are indispensable tools for studying hematophagous arthropods with positive phototropism but these instruments come at a significant cost. There are few producers worldwide and because of this, there is often a significant shipping cost added to the total cost of these traps. To circumvent this cost issue, we built our own CDC style light traps using available materials. The light traps were used to collect sandflies form 21 collection points, from all four regions from the north of Greece. We identified morphologically a total of 110 sandflies (44 female and 66 male) of which 48 were identified as *Phlebotomus tobbi*, 21 *Sergentomya minuta*, 25 *Phlebotomus neglectus*, 13 *Phlebotomus perfiliewi*, 1 *Phlebotomus papatasi* and 2 *Phlebotomus simici*.

Sample homogenisation is an important step for the MALDI-TOF analysis but manual homogenisation is time-consuming and very often leads to differences in the quality of the spectra between operators. To avoid this type of issues we developed an automatic homogenisation protocol for sandflies.

Some of the spectra obtained after the MALDI-TOF analysis were used for the creation of a database necessary for identification. We created a database

for the identification of *Phlebotomus tobbi*, *Sergentomya minuta*, *Phlebotomus neglectus*, and *Phlebotomus perfiliewi*. It was not possible to create a database for *Phlebotomus papatasi* and *Phlebotomus simici* because of the small number of samples. These last two species were only identified morphologically.

This last study has completed the objective of facilitating the study of sandflies by improving the methods of capture and identification of sandflies by MALDI-TOF.

The objective of this thesis is to complete and update our knowledge about the epidemiology of canine leishmaniosis, babesiosis, hepatozoonosis and dirofilariosis in the context of the world-wide changes of prevalence and distribution of vector-born diseases.