ABSTRACT

Ticks, after mosquitoes, spread the greatest range of pathogens affecting human and animal health worldwide, carrying a microbial complex of coexisting symbionts, commensals, and pathogens.

Lyme disease, tick-borne encephalitis, Crimean-Congo hemorrhagic fever virus, and rickettsiosis in humans, or, anaplasmosis and babesiosis in livestock, are all transmitted by *Ixodes ricinus*, the most frequent and widespread tick species in Europe.

I. ricinus is the dominant species in Romania (86.9%), followed by *Dermacentor marginatus* (9.5%), *Haemaphysalis punctata* (2.6%), and *Dermacentor reticulatus* (0.02%).

Research on bacterial and viral diversity in ticks has increased significantly in recent years as a result of the development of high-throughput sequencing methods, revealing unexpected microbial diversity. So, in addition to already known pathogens, new viral pathogens have been described and some of these pathogens belong to viral families that already contain arboviruses (for example, *Nairoviridae, Flaviviridae, Phenuiviridae* and *Orthomyxoviridae*).

The spread and abundance of ticks and associated pathogens is influenced by a variety of factors such as environmental, landscape and anthropogenic factors. Climate change has been cited as the primary cause of the expansion of ticks and the increase in tick-borne infections and many studies have demonstrated the importance of temperatures on questing tick densities. In addition, the importation of animals from other areas and migratory birds enlarge the distribution of ticks, leading to exposure of new populations in remote areas.

Romania is one of the most biogeographically diverse countries in Europe, with favourable conditions for outbreaks of tick-borne diseases, especially in the Southern part of the country, which includes the Danube Delta. The Danube Delta Biosphere Reserve (DDBR) is the second largest wetland in Europe, located mainly in Eastern Romania. The DDBR presents a high level of biodiversity and functions. It represents a major center for bird migration from Africa and Asia to Europe, leading to a high risk of introduction of animal pathogens, including both zoonotic and vector agents.

The biological richness of this region is composed by 30 types of ecosystems comprising 2994 species of flora and 4286 species of fauna (amphibians, fish and reptiles), as well as terrestrial mammals and domestic animals (such as carnivores, rodents, insectivores, bats, artiodactyls and lagomorphs). In such an environment, ticks can represent the bridge that may lead to interspecies transmission of pathogens, including to humans.

The aim of the PhD thesis entitled „**Research on the carrying and the zoonotic risk of infectious agents transmitted by ticks”** was to obtain novel information concerning tick-borne pathogens from different ticks species and to assess the risk of tick-borne infections in animals from understudied areas from South-Eastern Romania.

The objectives of this thesis were:

- To assess the seroprevalence of the main bacterial vector-borne infections in animals in South-Eastern Romania
- To survey the circulation of Crimean-Congo haemorrhagic fever virus in Romanian ticks and in small ruminants using serological and molecular biology techniques
- To identify novel arboviruses transmitted by ticks using Next-Generation Sequencing (NGS) techniques followed by the search for antibodies against their viral proteins in exposed animals using high-throughput serological technique (LIPS).

The originality of this thesis consists in:

- The use of state-of-the-art technologies such as NGS to uncover the virome of ticks collected from understudied areas of Romania
- The identification of new viral pathogens in ticks, described for the first time in Romania, including novel viruses with potential relevance for public health
- The assessment of seropositivity in exposed animals using high-throughput serological technique (LIPS).

The thesis is organized according to the general rules into two principal parts: the first one describes the current state of knowledge regarding ticks and their associated pathogens. The last chapter of the first part describes different state-of-the-art sequencing techniques that can be applied to identify pathogens in ticks. The second part presents the personal contributions to the field, summed up in five chapters.

Besides these main parts, the thesis also contains acknowledgments, list of abbreviations, introduction, abstract, table of contents, list of figures, bibliography, and annexes. **The current thesis contains 29 figures and 14 tables, having 205 bibliographic sources consulted**.

The first part, which concentrates on the current knowledge from the literature, comprises four chapters and associated subchapters.

The **first chapter** presents the species of ticks and the geographical distribution of the main species in Romania. **The second chapter** describes the main bacterial diseases transmitted by ticks, including etiology, epidemiology, clinical signs, and the state of knowledge in Romania. The **third chapter** details the major viral tick-borne pathogens, including recently identified tick-borne flaviviruses, following the same structure as in Chapter II. The **fourth chapter** describes the main existing NGS sequencing platforms.

The second part of the thesis, Personal contributions to the field of study, is structured into five Chapters (Chapters V to IX). **Chapters V and VI** describe the organizational and institutional framework in which the research was developed and the purpose and objectives of the current research. The next three describe the studies carried out, including a brief description of material and methods, the results, and the subsequent discussions for each study and the last chapter (Chapter IX) relates the general conclusions of the paper, in which all the remarks are gathered and analyzed in a final form.

Chapter VII, entitled **„***Seroepidemiological and molecular investigation regarding the main pathogens transmitted by ticks to animals***"** is divided in two sections: **the first section** presents two serological surveys regarding Lyme disease, ehrlichiosis, and anaplasmosis in domestic and hunting dogs. The aim of the first study „Serological survey of Lyme disease, anaplasmosis and ehrlichiosis in dogs in two counties in South-Eastern Romania", was to evaluate the presence of IgG antibodies against *B. burgdorferi*, *A.phagocytophilum* and *E.canis* in dogs in Eastern Romania. Canine vector-borne diseases (CVBD) have become a subject of interest in the past few years. In dogs, bacteria such as *Anaplasma phagocytophilum, Borrelia burgdorferi* and *Ehrlichia canis* are among the principal CVBDs, which are an emerging problem in veterinary medicine. These surveys also provide a valuable perspective in the identification of possible risk areas for humans. Dog sera were screened using commercial enzyme-linked immunosorbent assays for specific IgG antibodies against *Borrelia* VIsE antigen, *Anaplasma phagocytophilum* and *Ehrlichia canis* respectively. As a result of ELISA testing, 14 out of 92 tested dogs were detected to be seropositive for specific IgG antibodies, among which six for *Borrelia* spp. (6.5%), five for *Anaplasma phagocytophilum* (5.4%) and three for *Ehrlichia canis* (3.2%). Co-detection of IgG antibodies against two pathogens was observed in four dogs. The second serological survey titled „Seroepidemiological survey of anaplasmosis in domestic and hunting dogs" aimed to evaluate the seroprevalence of *IgG A. phagocytophilum* antibodies in dogs from three different counties from Romania between 2015 and 2019. *Anaplasma phagocytophilum* is an emergent pathogen with a seroprevalence that varies from region to region. The population analyzed consisted of 92 domestic and hunting dogs from Sibiu, Tulcea, and Iasi counties. Dog serums were tested using commercial enzyme-linked immunosorbent assays with the purpose to detect IgG anti-*Anaplasma phagocytophilum* antibodies. This study showed an overall prevalence of 14.% (13 out of 92 tested dogs were identified as seropositive). The highest seroprevalence was recorded in hunting dogs from Sibiu county (58.8%). These results suggest that dogs living outdoors and receiving no treatments are prone to infection with *A. phagocytophilum* and highlight the importance of appropriate antiparasitic treatments to protect against ticks.

The second section presents two serological and molecular surveys regarding viral tick-borne diseases. Firstly, tick vectors were analyzed using molecular techniques, in order to assess the prevalence of Crimean-Congo hemorrhagic fever virus (CCHFV) in *Dermacentor* and *Rhipicephalus* sp. ticks collected from Southern Romania, where previous studies indicated the presence of CCHFV IgG antibodies in small ruminants. The main vector is *Hyalomma marginatum* but CCHFV has been detected in more than 30 tick species, including *Dermacentor* and *Rhipicephalus* sp. For this research, a total of 127 ticks were collected in 2019, from five sites across Tulcea County. Detection of CCHFV in ticks was performed by Real-Time RT-PCR. In all collection sites, the samples tested scored negative for the six CCHFV genotypes. Even if these tick species can transmit CCHFV, the fact that the main vector has not been tested can explain the negative results.

This work was completed with a serological investigation of the vertebrate hosts. Sera from 250 sheep and goats were collected from the same region as in the previous study. The detection of antibodies against CCHFV nucleoprotein in animal sera was performed using ID Screen CCHF Double Antigen Multispecies (IDvet, Grabels, France). Ticks and serum samples were also analyzed by Real Time RT-qPCR targeting CCHFV. The global CCHFV antibody seroprevalence rate for tested animals was 37.7% (CI 95% 31.7-43.7). The estimated seroprevalence in sheep was 29.8% (CI 95% 23.2-36.5), (54/181) and 57.7% (CI95% 46.3-69.2) in goats (41/71). No CCHFV RNA was detected from the tick pools and small ruminant's sera tested by Real Time RT-PCR. These results indicate the circulation of CCHFV or another close nairovirus among small ruminants in Southern Romania.

Chapter VIII entitled „**Next generation sequencing- Uncovering the Romanian tick virome"** is the most detailed part of the thesis. This chapter presents original results about Romanian ticks virome obtained using Next-Generation Sequencing (NGS).

In the first part of the subchapter named **"Identification of novel viruses with potential relevance for public health in Romanian ticks"** are described the results after sequencing by NGS more than 500 adult ticks belonging to three different genera collected from Eastern Romania.

The purpose of this study was to describe the diversity of *Rhipicephalus, Dermacentor* and *Haemaphysalis* sp. ticks collected from relatively unexplored areas of Romania, in order to increase the knowledge of virus diversity in Eastern Europe, comprising novel ones that may have relevance for human and animal health. This study begins by presenting the classification of identified viruses by host spectrum, followed by a detailed analysis of the most important detected viral families. The RNA virome of Romanian ticks was composed of 29 families, with some families restricted to a given tick species (unclassified *Mononegavirales*), while others were shared by all (unclassified *Riboviria*, unclassified *Picornavirales,* unclassified ssRNA+ and *Flaviviridae*) or specific to questing ticks (e.g., *Luteoviridae, Tombusviridae* and *Marnaviridae*).

Among the viral communities infecting Romanian ticks, viruses belonging to the *Flaviviridae, Phenuiviridae* and *Nairoviridae* families were detected and full genomes have been derived. In the *Flaviviridae* family, a virus named Jingmen tick virus (JMTV) was identified in engorged ticks from sheep and goats, suggesting that ruminants may play a role in the life cycle of Jingmenviruses in Romania. Phylogenetic analyses placed Romanian Tacheng tick virus 2 (TaTV2) (*Phenuiviridae* family) in a clade with ticks and human isolates first identified in China, indicating that this virus may be important for human and animal health. The pathogenicity of this virus for humans and animals was later confirmed, TaTV2 being detected in tick bitten patients and in several vertebrate species and also in *Hyalomma asiaticum* ticks. In addition of these findings, other viruses not yet known to be pathogenic (Bole tick virus 4, Nayun tick nairovirus) have been identified and further studies are needed to determine their importance for human and animal health.

The second subchapter entitled „**Identification of a novel *Quaranjavirus* and other viral families in *Ixodidae* ticks from Danube Delta"** describes a novel *Orthomyxovirus* and other viruses. The aim of this study was to identify viruses associated with *Rhipicephalus* ticks collected from small ruminants from Tulcea County, using metagenomics. *Orthomyxoviridae* family are known as argasid tick-borne viruses but recent studies have reported novel quaranjaviruses-like in hard ticks and little is known about their pathogenicity. Three viral families were detected: *Phenuiviridae* (41%), *Chuviridae* (36%) and *Orthomyxoviridae* (22%).

Within the *Orthomyxoviridae* family, sequences of a novel quaranjavirus, tentatively named Cataloi tick quaranjavirus (CTQV) was identified. CTQV is phylogenetically situated in a clade apparently restricted to *Ixodidae* and distinct from the argas-related quaranjaviruses. This presumably high diversity of vertebrate hosts (iguanas, small ruminants, and seabirds) indicates that CTQV and other *Ixodidae*-associated quaranjaviruses probably are tick-specific, independent of the hosts on which ticks feed. However, the ability of ixodid-associated quaranjaviruses to replicate in their vertebrate hosts remains to be elucidated. Future studies are necessary to determine the pathogenic potential of CTQV and other viruses for animals and humans health.

The aim of the third subchapter was to determine if CTQV could constitute a novel tick-borne arbovirus. For this purpose, sera from small ruminants exposed to tick bites from Danube Delta region were screened using LIPS assay to evaluate the range of antibodies responses against CTQV. A significant increase of the signal-to-noise ratio was observed for sheep sera collected in Slava Cercheza or Somova in 2019 and in goat sera collected either in Cataloi in 2019 or in Slava Rusa in 2020. To determine if these sera could be considered positive, we defined a positivity threshold value as the mean of signal-to-noise ratio of non-exposed French sera + 3 standard deviations. Only 8 sheep sera collected in Slava Cercheza in 2019 slightly exceeded this threshold. To determine if these sera could be considered as positive, more-specific serological tests such as seroneutralization are needed to confirm this result, but they require the isolation of the virus.

The last chapter (**Chapter IX**), summarizes all the conclusions of our research and creates a general view of the thesis. This research confirmed the presence of known bacterial and viral pathogens in the study area. In addition to these, new pathogens described for the first time in Romania, with a risk for public health, were identified.