ABSTRACT

Key words: *questing ticks, pathogens, molecular detection, microbial populations*

In Europe, ticks are the most important vectors for pathogens that cause both human and animal diseases. *Ixodes ricinus* ticks are the most common ticks in Europe, and are broadly distributed across the entire continent. They are capable of transmitting a wide variety of tick-borne zoonotic pathogens, such as viruses, bacteria, and protozoa. *I. ricinus* is also the most common tick species in Romania, representing 86.9% of ticks, followed by *Dermacentor marginatus* (9.5%), *Haemaphysalis punctata* (2.6%), and *Dermacentor reticulatus* (0.02%). *Rhipicephalus* spp. are also present in the southeastern regions at lower levels, and two such species, *Rhipicephalus sanguineus* and *Rhipicephalus rossicus*, are reported to parasite dogs and occasionally humans in Romania.

Factors that contributed at the expansion of TBPs are complex abiotic and biotic factors that influence the distribution and abundance of predominantly ixodid ticks, and also have great impact on the transmission dynamics of tick-borne pathogens.

Climate change has been invoked as a primary cause for the expansion of ticks distribution and intensity of tick-borne infections; for instance, the emergence of tick-borne encephalitis, human babesiosis and granulocytic anaplasmosis in North America and Europe occurred simultaneously with warming trends. However these increases in incidence and distribution must be connected with other potential causes such as, the increased awareness of healthcare providers, anthropogenic changes namely habitat fragmentation, urbanization, de- and reforestation, land use changes that were not under a clear impact of climate change.

Romania has a unique particularity in comparison with other European countries that being the separation of territory in five ecoregions represented by a high biodiversity in terms of habitats and variety of mammal species present. In terms of type of habitats found in Romania, there are described 357 types that are categorized in seven classes and 24 subclasses according to the Palaearctic Habitats classification system. Both abiotic and biotic components inside habitats influence the distribution and abundance of tick populations. Because vegetation type influences the presence and movement of host species, it is also likely to influence the presence, density, or persistence of tick species and the probability of successful host

acquisition. Certain ticks associate with a specific microclimate and habitat conditions that are characterized by a serie of variables such as humidity, temperature, and day light hour.

Also, the geopolitical position of Romania at the Eastern border of the EU is strategically important from an epidemiological point of view. It represents a continual risk for emerging disease, not only in Romania, but also as a gateway into Europe.

The doctoral thesis ***„Epidemiological and etiological research regarding emerging vector-borne infections in Eastern Romania"*** is structured in accordance with current legal criteria into two parts: a first part entitled **„Current state of knowledge"** that comprises 44 pages and a second part **„Personal contributions"** containing 102 pages, that details the results obtained during PhD studies. To these two main parts, thesis also contains a table of content, introduction, abstract and references.

The first part **„Current state of knowledge"** is structured in four chapters that present literature data regarding main components in the field of tick-borne diseases and their status in Romania: epidemio-climatic characteristics, information on tick species, bacterial and viral agents vectorised by ticks. Chapter IV describes different next generation sequencing techniques that can be used in screening of pathogens in ticks.

The first chapter entitled **"Ixodid ticks involved in transmitting pathogens to animals and humans in Romania"** starts by describing the epidemio-climatic conditions in Romania that can influence the distribution of ticks. In this chapter it is also given information on the tick species diversity found in Romania. Contributing factors at the expansion of tick-borne pathogens are complex abiotic and biotic factors that influence the distribution and abundance of predominantly ixodid ticks, and also have great impact on the transmission dynamics of tick-borne pathogens.

Habitat variety and the host diversity result for relatively high tick species diversity in Romania, in comparison with neighboring countries. Distribution maps and host spectrum showed that the most widespread ticks in Romania are *Ixodes ricinus* (86.9%), *Dermacentor marginatus* (9.5%) and *Haemaphysalis punctata* (2.6%). However, in southern Romania, thermophilic Palearctic species like *Rhipicephalus bursa* and *Hyalomma marginatum* are also common. *I. ricinus* is the most important zoonoses vector tick in Romania; this argument is supported by its abundance and wide host specificity, high frequency in humans and significant vectorial capacity.

In terms of pathogens occurrence, chapter II „**Bacterial tick-borne pathogens"** describes the main bacterial agents that are already detected in ticks from Romania. *Borrelia*

burgdorferi sensu lato raised the most interest of the tick-borne pathogens due to its great implications in medical health. Studies revealed a mean prevalence of *B. burgdorferi* s.l in *I. ricinus* ticks at a lower level comparing with the European average. Other pathogens such as *Rickettsia* spp., *Bartonella* spp., *Anaplasma* spp. and *Candidatus* Neoehrlichia mikurensis were also detected in ticks and animal hosts from Romania.

In chapter III, "T**ick-borne viruses"**, two viral agents are described due to their occurrence in Romania. Also, other arboviruses are detailed taking into account the low number of studies that focused on the detection of viruses in ticks.

The chapter IV entitled "**Next generation sequencing – exploring the microbiome of ticks"**, describes the sequencing techniques used for the description of microbial populations in different organisms. There are also presented the applications of Next generation sequencing. As the current situation of tick-borne pathogens in Romania is still being evaluated, next generation techniques could be applied for the description of tick microbiome.

The second part of the thesis „**Personal contributions**", contains **five chapters**, each chapter presents in detail, by following a specific structure, the results obtained for the proposed objectives. This part ends with a final chapter in which the final conclusions are shown.

The current epidemiological status of tick-borne pathogens in ticks and/or wildlife in Eastern Romania is still being evaluated. Therefore an in-depth surveillance program is needed, since such pathogens can easily be carried into and throughout Europe.

The goal of this thesis was to obtain new information regarding the epidemiological situation of tick-borne pathogens in Eastern Romania. The study had an integrated approach in accordance with the *One Health* concept and aimed to evaluate the risk of infections transmitted by ticks to animals and humans. In order to accomplish this desideratum, the study had **three main objectives**:

- **serological investigation of main tick-borne infections in Eastern Romania**
- **Identification by molecular biology techniques of tick-borne pathogens in questing ticks**
- **characterization of ticks microbial communities by means of Next Generation Sequencing**

In chapter VI „Seroepidemiological surveys of main tick-borne infections in animals and humans", are shown results obtained after performing seroepidemiological surveys of two main tick-borne infections in animals and humans - Lyme disease in dogs and Crimean-Congo haemorrhagic fever in small ruminants (sheep and goats).

The first serosurvey focused on the detection of **Crimean-Congo hemorrhagic fever virus (CCHF) antibodies** in small ruminants. CCHF is a widespread zoonotic disease caused by a tick-borne virus (Nairovirus) of the Bunyaviridae family. Small ruminants with CCHF undergo a mild infection, sometimes with transient viraemia. Serum samples to test for CCHFV antibodies were collected from 90 small ruminants in various sites in Tulcea and Constanța Counties. All serum samples were tested for the presence of specific IgG antibodies against CCHFV by a commercial ELISA kit (Vectorbest, Novosibirsk, Russia), slightly modified. The serological examination of serum samples for anti - CCHFV infection evidenced **CCHF IgG antibodies in the blood of 16 out of 29 goats (55%) and in 51 out of 61 (85%) sheep**. These results suggest that the CCHF virus may be widespread in traditionally reared small ruminant livestock in Tulcea and Constanța Counties. CCHF infected animals also represent a potential threat to abattoir workers and to public health.

Sheep and goats included in the study were parasitized by ticks belonging to ***Rhipicephalus* spp**. These ticks were collected and tested by rRT-PCR for the identification of CCHF virus; all ticks turned out to be **negative** for this viral agent.

Second subchapter presents the results obtained after surveying dog sera for **Lyme disease** antibodies. Lyme disease is a vector-borne infection caused by spirochetes from *Borrelia burgdorferi* complex transmitted by ticks. In Europe, the most common genospecies isolated from animals and humans are: *Borrelia afzelii*, *Borrelia garinii* and *Borrelia burgdorferi* sensu stricto. Pathogens are transmitted by various species of genus *Ixodes*. In Europe, *Ixodes ricinus* is the most important vector. Dogs are susceptible to tick infestation due to their interactions with the specific habitats of arthropods and so the risk of Lyme disease transmission is high. Many authors have proposed dogs to fulfill the role of "sentinels" making it possible the identification of risk areas for Lyme disease.

The purpose of this study was to determine the seroprevalence of antibodies against *Borrelia burgdorferi* s.l. in dog sera collected from four counties of Romania. Dog sera were analyzed using a commercial enzyme-linked immunosorbent assay (IgG Canine Lyme Borrelia - ELISA, NOVATEC, Germany). A total of 90 serum samples were tested. Following the immunoassay, one sample (1.11%) was positive for *Borrelia burgdorferi* IgG antibodies from the 90 tested samples. The seroprevalence registered in this study was lower than in previous studies conducted in Romania. The fact that most of the samples were collected from dogs kept in animal shelters may be an explanation for the low seroprevalence.

In chapter VII "Tick-borne pathogens detection in questing ticks", the goal was to identify most important pathogens involved in causing human infections and to determine the coinfection rates in questing ticks collected from one urban region dedicated for recreational activities and two forested areas in Eastern Romania.

In Europe, ticks are the most important vectors of pathogens that cause both human and animal diseases. So far, around 60 bacteria, 30 parasites and 100 viruses are registered as potential tick-borne pathogens and the list continues to expand. Numerous studies have indicated the role of ticks in the transmission of pathogens that have implications in human health, and their medical importance has long since been acknowledged. Also, several reports indicated ticks capacity to harbor two or more pathogens and transmit them simultaneously causing multiple infections, events that are not rare in humans. In Romania however, most surveys of ticks have concentrated on the identification of a limited number of bacterial and parasitic tick-transmitted pathogens with low concern for the identification of coinfections and detection of viruses in questing ticks.

The objective of our study was to identify the most important tick-borne pathogens in questing ticks collected from forested and urban areas and evaluate their coinfection rates in Eastern Romania as a gateway into Europe. We analyzed 557 questing adult or nymph hard ticks belonging to three species (*Ixodes ricinus, Dermacentor reticulatus* and *Haemaphysalis punctata*).

Briefly, DNA samples were assessed by PCR and quantitative PCR to determine the infection rates of *Borrelia* spp., *Rickettsia* spp., *Anaplasma phagocytophilum, Bartonella* spp. and *Candidatus* Neoehrlichia mikurensis. We also screened RNA samples by RT-qPCR to identify the infection with tick-borne encephalitis virus and Eyach virus. The global prevalence of ticks infected with one or more pathogens was 45.9%.

Tick-borne pathogens detected in Ixodes ricinus

***Ixodes ricinus* ticks represented 95.9%** (534/557) from the collected ticks with 77 adult ticks and 457 nymphs. The overall prevalence of infection with one or more of the tested pathogens registered a value of 47.9% (256/534).

***Borrelia* spp. DNA was identified in 164 (30.7%) *I. ricinus* ticks**. Overall, *Borrelia* spp. infection rates were not significantly different in adult (32.4%; 25/77) and nymphal (30.6%; 139/457) *I. ricinus* ticks. Nested PCR performed on *Borrelia* spp. positive samples, targeting *IGS 16S-23S* gene, revealed 20 positive samples. The remaining 144 *Borrelia* spp.-positive *samples* that tested negative by nested PCR were included in a real time PCR reaction,

where 113 *Borrelia* spp.-positive *I. ricinus* ticks had specific sequences for one or more *Borrelia* species. The mean infection rate, over all ticks tested, was 14.7% for *B. garinii* (14.2% of the adults and 14.8% of the nymphs), 8.8% for *B. afzelii* (6.4% of the adults and 9.1% of the nymphs), 4.8% for *B. valaisiana* (3.8% of the adults and 5% of the nymphs), 4.8% for *B. lusitaniae* (3.8% % of the adults and 5% of the nymphs), 1.1% for *B. spielmanii* (1.2% of the adults and 1% of the nymphs), 0.9% for *B. miyamotoi* (1.2% of the adults and 0.8% of the nymphs), 0.3% for *B. burgdorferi sensu stricto* (0.4% of the nymphs) and 0.1% for *B. bissetti* (0.2% of the nymphs).

***Anaplasma phagocytophilum* was detected in 7/534 ticks (1.3%) *I. ricinus* nymphs**, no adult tick testing positive. To date, no reported cases of human *A. phagocytophilum* infection in Romania exist but there are many studies indicating circulation of *A. phagocytophilum* in Romanian territory.

Tick-borne rickettsioses are recognized emerging diseases present in Europe and consequently in Romania, that cause a wide range of clinical manifestations. Previous research in Romania focused on the identification of *Rickettsia* spp. in ticks collected from different hosts and no study reported prevalence of spotted fever group (SFG) *Rickettsia* in questing ticks. ***Rickettsia* spp. In this study, DNA was observed in 78/534 (14.6%) *I. ricinus* ticks**. There were no differences in the prevalence of infection between adults and nymphs. DNA was detected in 12/77 adults (15.5%) and in 66/457 nymphs (14.4%).

***Bartonella* spp. DNA was detected in 22 *I. ricinus* adults or nymphs (22/534, 4.1%)**. *Bartonella spp.* infected 7.7% (6/77) adult and 3.5% (16/457) nymphal *I. ricinus* ticks. One positive sample was successfully sequenced and matched *B. henselae*, a pathogen responsible for cat-scratch disease, one of the most common zoonosis acquired from domestic animals in industrialized countries. Competence of *I. ricinus* for this bacterium has been demonstrated under laboratory conditions, and human infections with *B. henselae* following tick bites have also been reported.

***Candidatus* N. *mikurensis* DNA was found in 29/534 (5.4%) ticks**, all of which were *I. ricinus* nymphs, resulting in a prevalence of 6.3% (29/457. Global prevalence was similar to other European studies with values between 3.5% and 7%. *C.* N. mikurensis was recently identified in Romania, in a tick collected from a patient. Another recent study has established *C.* N. mikurensis prevalence in unfed *I. ricinus* ticks from center and north part of the country. The infection rate reported in our study is in accordance with this previous study.

TBEv was detected in 10 RNA samples via specific rRT-PCR, but could not be confirmed by nested PCR. Given the fact that there are limited data on TBEV infection on ticks, it is necessary to evaluate the patterns and distribution of TBEV infection in Ixodid ticks.

There are several published **cases of humans being infected with multiple tick-borne pathogens**; this is somewhat expected, as many ticks harboring two or more infectious agents can transmit them simultaneously. This phenomenon may occur frequently in *I. ricinus* ticks, as they feed on a large variety of host species which act as reservoirs for multiple pathogens.

Co-infections **were identified only in *I. ricinus* ticks** (96/534; 17.9%), which accounted for 37.5% (96/256) of infected ticks. Co-infection prevalence was 15.5% (12/77) in adults and 18.3% (84/457) in nymphs. **Regarding associations between the *Borrelia burgdorferi* s.l. group** (comprising *B. burgdorferi* s.s., *B. garinii*, *B. afzelii*, *B. valaisiana*, *B. lusitaniae*, *B. spielmanii*, *B. bissetti*), 57 *I. ricinus* ticks were positive for association with two species and 8 ticks were infected by three different *Borrelia* species. Most frequent dual *Borrelia* specie association was between *B. garinii* and *B. afzelii* (30/57; 52.6% *I. ricinus* ticks) followed by *B. garinii/B. lusitaniae* (18/57; 31.5% ticks). Association of three *Borrelia* species was found in eight *I. ricinus* ticks, half of them being infected with *B. garinii*, *B. afzelii* and *B. valaisiana*.

Co-infections of *Borrelia* species with other tick-borne pathogens or between the other surveyed agents occurred in 8.2% *I. ricinus* ticks (44/534; 6.4% of adult stages and 8.5% of nymphs) distributed in ticks from all three sampling areas. Dual co-infection between a *Borrelia* specie and other pathogen occurred in 22 *I. ricinus* ticks (22/534; 4.1%); nine ticks had co-infections between *Borrelia* spp. and *Rickettsia* spp. and other seven ticks tested positive for *Borrelia* spp. and *C.*N. mikurensis. We detected co-infection of two pathogens without *Borrelia* species in five ticks (5/534; 0.9%).

These results raise further questions about whether these agents can be co-transmitted to humans (or animals), their incidence, the effect of co-infection on symptom severity, the efficacy of treatments against multiple infection, and the development of new diagnostic tests better adapted to screening for multiple tick-borne diseases.

Our findings confirm that ticks are important pathogen vectors in both forested and urbanized areas. We have generated detailed data on the occurrence of zoonotic pathogens, representing solid foundation for further studies examining the risk of human tick-borne diseases.

Tick-borne pathogens detected in Dermacentor reticulatus

Dermacentor reticulatus is a tick specie considered to be the second most reported after

I. ricinus in central Europe. In Romania however, questing *D. reticulatus* ticks prevalence was estimated at 0.02%. *D. reticulatus* ticks were found only in sampling area 2, four adult females (4/557; 0.7%) being obtained after dragging. Molecular testing for all tick-borne pathogens included in this study, revealed one positive *D. reticulatus* tick for *Rickettsia* spp. The *gltA* sequence obtained from the *Rickettsia*-positive *D. reticulatus* female shared 99% similarity to ***Rickettsia raoultii*** (GenBank accession no. KJ663737.1).

Tick-borne pathogens detected in H. punctata

Haemaphysalis punctata ticks represented 3.4% from the total ticks collected, with a total of 19 ticks (three adults and 16 nymphs). There is limited information regarding identification of tick-borne pathogens in *H. punctata* tick species from Romania, no pathogen being so far successfully identified. After testing for the selected pathogens, three nymphs were infected with a single different tick-borne pathogen (3/19; 15.7%). *Borrelia* spp. DNA was identified in one *H. punctata* nymph (1/19; 0.6%). The positive *Borrelia* spp. PCR product was successfully sequenced and showed 94% identity to ***B. miyamotoi*** sequences (GenBank accession no. CP010308.1). *Rickettsia* spp. DNA was detected in one *H. punctata* nymph (1/19; 0.6%). Valid sequence was unable to be generated for the single *H. punctata* PCR product.

Bartonella spp. DNA was observed in a single *H. punctata* nymph (1/19, 0.6%) 2. Among the 19 *H. punctata* ticks analyzed, there were no positive samples for more than one pathogen.

Chapter VIII entitled **"High-throughput detection of tick-borne pathogens"**, includes a study that analyzed 144 *Borrelia* spp.-positive samples by high-throughput real-time PCR using **BioMark real-time PCR system (Fluidigm, USA)**. Also, 34 RNA pools obtained by mixing 557 RNA samples were tested for 24 viral agents vectorized by ixodid ticks.

After testing the 144 samples for 39 bacteria and parasites, 15 bacterial agents (*Borrelia miyamotoi, Anaplasma marginale, A. platys, A. ovis, A. centrale, A. bovis, Ehrlichia chaffeensis, E. ruminantium, E. canis, Rickettsia conorii, R. slovaca, R. masilliae, R. aeschlimannii, Bartonella quintana, Coxiella burnetii*) and 9 parasites (*Babesia divergens, B. canis, B. bovis, B. caballi, B. bigemina, B. major, B. ovis, Theileria equi, T. annulata*) were not identified. **There were identified seven genospecies from *Borrelia burgdorferi* s.l. group**, *B. garinii* being the most abundant specie (79/144; 54.8%) followed by *B. afzelii* (46/144; 31.9%). *B. valaisiana* and *B. lusitaniae* were detected in 26 tick samples (26/144; 18%).

DNA samples also tested positive for the infection with ***Anaplasma phagocytophilum* and *C.* N. mikurensis** at a low infection rate (1.3%; 2/144 and 2.7%; 4/144 respectively). For

Rickettsia spp., 60 samples tested positive (60/144; 41.6%), BioMark real-time PCR system being able to identify only one specie - ***Rickettsia helvetica*** **with a prevalence of 11.8%** (17/144). Ticks showed specific sequences for parasitic agents as well. There were detected **three species from *Babesia* genus**: *B. microti, B. vogeli and B. EU1*; these species had a prevalence of 0.6% (1/144), 1.3% (2/144) and 2% (3/144) respectively.

Multiple pathogen infections were identified in 90 samples (62.5%) being noticed **43 different associations of pathogens**; 23 (23/144; 15.9%) ticks had association of two *Borrelia burgdorferi* s.l genospecies, most frequent being the one between *B. garinii and B. afzelii* (11/144; 7.6%) followed by *B. garinii* and *B. lusitaniae* in nine ticks.

After testing the 34 pools for **24 viral agents**, **one pool was positive for the infection with West Nile virus and another pool had infection with Bourbon virus** (*Orthomyxoviridae*). Further detection methods performed for the confirmation of Bourbon virus infection (rRT-PCR and nested-PCR) did not show positive samples.

The BioMark real-time PCR system as a surveillance method represents a major improvement in epidemiological studies, able to facilitate comprehensive testing of tick-borne pathogens, and which can also be customized to monitor emerging diseases.

Chapter IX **„Characterization of tick microbial communities using Next-Generation Sequencing”**, shows the potential of Next-Generation Sequencing as a tool for metagenomic studies that aim to describe ticks microbiome**.**

In the first part of this chapter are described the results after screening three pools of tick sample for the presence of **viruses** by Next Generation Sequencing (NGS) on the Illumina NextSeq platform. Worldwide, ticks are responsible for transmitting the highest number of pathogens compared to any other arthropod. Identification of bacterial tick-transmitted pathogens was the spotlight for most surveys carried out in Romania, with less concern for the viral agents vectorized by ticks.

The objective of the study was to characterize the virome of questing ticks from Eastern Romania as a gateway into Europe, by using high-throughput sequencing (HTS). The study included 557 questing adult or nymph ticks (534 *Ixodes ricinus*, 4 *Dermacentor reticulatus* and 19 *Haemaphysalis punctata*) randomly distributed in three pools, collected from three distinct areas in Eastern Romania.

Analysis of HTS data uncovered sequences with similarity to viruses that associate to vertebrates, arthropods and plants. The number of total viral reads obtained after the assembly step was 21 368 211, 32 337 393 and 29 676 963 respectively for each pool. Subsequently,

assembled viral contigs generated from each pool totalized 436 956, 1 052 524 and 272 492 contigs. Taxonomic assignation of contigs considered as distant to known vertebrate viruses, **revealed homology to viral agents of *Bunyaviridae* family**, each pool recording a number of 58, 243 and 11 contigs. Among them, contigs mapped to recently described viruses from *Nairovirus* genus- **South Bay virus**, and from *Phlebovirus* genus namely **blacklegged tick phlebovirus-1** and **blacklegged tick phlebovirus-2**. Prevalence of viral sequences in ticks was screened by PCR from cDNA of individual ticks, using specific primers for the sequences found by HTS. South Bay virus-like cDNA was detected in three *Ixodes ricinus* (one nymph, a female and a male) with a global prevalence of 0.5%, while no ticks were positive for *Phlebovirus*-like sequences. Two sequences South Bay virus-like were validated and proved to be highly homogeneous (99% identity), originating from ticks collected at the same site (male and female). After establishing the prevalence and distribution of South Bay virus-like in ticks, the following step will be the attempt to isolate this new viral agent by intracerebral inoculation of type I interferon receptor knock-out mice.

In the second part of this chapter it is described the **metagenomic profile of bacterial communities** associated with questing ticks in Romania revealed after applying Next Generation Sequencing on the Illumina NextSeq platform.

Total number of reads for bacterial agents was 606.368 for pool-1, 480.101 for pool -2 and 1.216.785 for pool -3; reads were assembled to contigs that matched to a high number of bacterial families, some with species known to be transmitted by ticks. **There were detected potential pathogenic bacterial genera that have a wide distribution in Europe**: *Borrelia, Anaplasma, Rickettsia, Bartonella, Coxiella, Francisella, Ehrlichia* **and also recently identified pathogenic agents** - *Candidatus* Neoehrlichia mikurensis and *Orientia* sp. ***Rickettsia* genus** had the highest number of reads in all three pools (72.333 in pool-1, 47.541 in pool -2 and 354.511 in pool -3) followed by ***Francisella* and *Borrelia***.

High-throughput sequencing technologies provide a complete picture of the bacterial communities present within questing ticks under natural conditions. This study demonstrates a novel detection strategy for the microbiomes of arthropod vectors in the context of epidemiological and ecological studies. Although further research is required to investigate the functional and the ecological implications of the bacterial communities associated with ticks, this work represents an initial effort in data acquisition and an innovative analysis towards the characterization of the tick microbiome including disease-causing bacteria under natural condition.