

PhD THESIS

Identification of vectors and potential natural reservoir species for *Rickettsia* genus

(SUMMARY OF THE PhD THESIS)

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INTRODUCTION

Ticks represent a significant group of hematophagous ectoparasites, considered to be important vectors of infectious diseases in Europe. Ticks are vectors of several parasitic, bacterial or viral pathogens, with a strong impact on human and animal life. The 20th century witnesses an increase in vector diseases incidences and diversity (BOULANGER ET AL., 2019). Tick-borne bacterial pathogens have been described worldwide as being a potential risk for both animal and human health. The tick's population and community structure as well as their abundance are related to the geographical origin, being influenced by a large variety of factors mainly related to the climatic features, including the degree of vegetation cover (DANTAS-TORRES ET AL., 2012; TAGLIAPIETRA ET AL., 2011) or hosts availability (HOFMEESTER ET AL., 2017). The climate conditions to which these species have adapted include the subarctic climate of Northern Europe (PEEL ET AL., 2007), as well as warmer and wetter climate areas in the South (DANTAS-TORRES AND OTRANTO, 2012). *Ixodes ricinus*, one of the most widespread tick species in Europe over the last 30 years, possesses a great capacity to cross different landforms, having an increased adaptability (CICCULLI ET AL., 2019), characterized as ecological plasticity (ROSÀ ET AL., 2018).

Some of vector-borne pathogens are agents of important zoonoses: *Borrelia burgdorferi*, the etiological agent of Lyme disease, a vector borne disease with a wide distribution including Romania (KALMÁR ET AL., 2013), which was described for the first time in 1977 by rheumatologist Allen Steere (BUSH ET AL., 2018); *Anaplasma phagocytophilum*, the etiological agent of granulocytic anaplasmosis, also widely distributed in Europe and in Romania (MATEI ET AL., 2016), and *Babesia microti*, *Babesia divergens*, etiological agents of babesiosis (GORENFLOT ET AL., 1998).

The *Rickettsia* species are transmitted mainly through arthropod vectors, with an important number of them being transmitted by ticks. Among tick-borne diseases spotted fever group (SFG), *Rickettsiae* genus, may cause non-specific symptoms which make clinical diagnosis difficult. *Rickettsia* spp. are gram-negative, intracellular bacteria, belonging to the Class α -proteobacteria, Order Rickettsiales, Family *Rickettsiaceae* (NCBI TAXONOMY, 2022).

Rickettsia species of medical concern transmitted by ticks, described so far in Europe are: *R. rickettsii* the etiological agent of Rocky Mountain spotted fever (RMSF) (WILSON AND CHOWNING, 1904), *R. conorii* the etiological agent of Mediterranean spotted fever (MSF) (BRUMPT, 1932), *R. sibirica mongolitimonae* because the main

clinical manifestations include lymphangitis, the acronym LAR (lymphangitis-associated rickettsiosis) has been proposed (OTEO AND PORTILLO, 2012), *R. slovacica* the etiological agent of scalp eschar and neck lymphadenopathy after tick bite (SENLAT) (LAKOS, 1999), *R. monacensis* the etiological agent of MSF-like (OTEO AND PORTILLO, 2012), *R. helvetica* (OTEO AND PORTILLO, 2012), *R. aeschlimannii* (TOSONI ET AL., 2016) and *R. massiliae* (OTEO AND PORTILLO, 2012). In *I. ricinus*, one of the most widespread tick species in Europe (OTRANTO ET AL., 2018), the reported *Rickettsia* spp. are: *R. helvetica* (BORAWSKI ET AL., 2019), *R. monacensis*, *R. raoultii*, (KLITGAARD ET AL., 2019, WALLMÉNIUS ET AL., 2012), *R. slovacica*, *R. sibirica* and *R. sibirica mongolotimonae* (WALLMÉNIUS ET AL., 2012). In addition, species with unknown pathogenicity such as Candidatus "*R. mendelii*" (HAJDUSKOVA ET AL., 2016), *R. bellii* (STAŃCZAK ET AL., 2018) were reported in this tick species in countries such as Czechia (HAJDUSKOVA ET AL., 2016), Germany (PAROLA ET AL., 2013), Poland, (STAŃCZAK ET AL., 2018) and Sweden (WALLMÉNIUS ET AL., 2012).

Romania possesses a high biodiversity in its 21 ecoregions of the five biogeographical regions (ANPM, 2022), including a high diversity of tick species and their hosts (MIHALCA ET AL., 2012A, 2012B).

Up to date, in Romania several SFG *Rickettsia* were detected in ticks collected from the environment or in different hosts and less in tissue samples. A review of *Rickettsia* species diversity, hosts association and distribution in Romania showed presence of the following *Rickettsia* species: *R. helvetica*, *R. raoultii* (ANDERSSON ET AL., 2018B; RĂILEANU ET AL., 2018; ZAHARIA ET AL., 2016), *R. conorii* (BORŞAN ET AL., 2021; IONITĂ ET AL., 2016; PIŢIGOI ET AL., 2013), *R. monacensis* (ANDERSON ET AL., 2018B; MĂRCUŢAN ET AL., 2016; BORŞAN ET AL., 2021; MATEI ET AL., 2021), *R. slovacica* (IONITĂ ET AL., 2016, ANDERSSON ET AL., 2018B; ZAHARIA ET AL., 2016), *R. aeschlimannii* (ANDERSSON ET AL., 2018B), *R. felis* (BORŞAN ET AL., 2021), *R. massiliae* (MĂRCUŢAN ET AL., 2016; ZAHARIA ET AL., 2016). The detection was made either in quested ticks such as: *D. marginatus* and *D. reticulatus* (BORŞAN ET AL., 2021; MATEI ET AL., 2018), *I. ricinus*, *H. punctata* (BORŞAN ET AL., 2021), *D. reticulatus* (RĂILEANU ET AL., 2018), *H. punctata* (BORŞAN ET AL., 2021) or fed ticks and even in tissue samples in birds or small mammals (MĂRCUŢAN ET AL., 2016; BORŞAN ET AL., 2021) or in human hosts (ZAHARIA ET AL., 2016; PIŢIGOI ET AL., 2013; ŞERBAN ET AL., 2009).

AIM AND OBJECTIVES OF THE RESEARCH

Although Romania is ranked in top 15 in Europe, based on the surface area, a characteristic to which a high population of ticks and the presence of a considerable diversity of hosts are added, there are relatively few data related to the bacterial load of ticks regarding the genus *Rickettsia* spp. Knowing the vectors of the large group of ticks with a potential of natural reservoir for the genus *rickettsia* will allow the development of a database concerning their distribution and their capacity to be a natural reservoir and vectors of transmission.

The aim of this research was to identify the diversity of *Rickettsia* species in Romania and the associations between ticks and *Rickettsia* species and between *Rickettsia* species and vertebrate hosts, all in order to assess the capacity of these arthropod and the vertebrate species to be considered potential natural reservoir hosts and/or vectors of species of the genus *Rickettsia*.

For this aim, the following general and specific objectives were established:

General objectives:

1. Establishing associations between tick species-*Rickettsia* species;
2. Establishing associations between vertebrate species-*Rickettsia* species;
3. Establishing the diversity of *Rickettsia* species in Romania;

Specific objectives:

1. Choosing the samples to be included in the studies and databases creation;
2. Amplification of specific DNA by PCR and visualization through electrophoresis;
3. Purification and sequencing of the positive samples;
4. Making associations between ticks and pathogens with zoonotic potential of the *Rickettsia* genus in Romania;
5. Finding the involvement of ticks with vector potential in rickettsiosis epidemiology;
6. Detection of *Rickettsia* species in small mammals and bats tissues samples from our country;
7. Finding the role of small mammals and bats in *Rickettsia* spp. epidemiology;
8. Assessing the presence of rickettsial agents in domestic and wild carnivores in Romania
9. Outlining the reservoir population in rickettsiosis epidemiology;
10. Statistical analysis of data obtained to provide scientific arguments for research and development of scientific articles.

STRUCTURE OF THE DOCTORAL THESIS

The thesis entitled “Vectors and potential natural reservoir species for *Rickettsia* genus identification” consist in 153 pages written according to the current editing norms at academic and national level. The doctoral thesis is structured in two parts consisting of 10 chapters and it contains 28 figures, 18 tables and 388 bibliographic references.

The first part of the doctoral thesis entitled “Current state of knowledge” is structured in 3 chapters and comprises 30 pages. This part synthesizes information regarding the taxonomy, morphological characteristics, pathogenicity, epidemiology, vectors and reservoirs of *Rickettsia* and associations between *Rickettsia*, ticks and their geographical distribution in Europe (**Chapter 1**). It also includes the presentation of most important zoonotic or with zoonotic potential rickettsia species in Europe, such as: *R. rickettsii*, *R. conorii*, *R. sibirica mongolitimonae*, *R. slovacae*, *R. helvetica*, *R. monacensis*, *R. aeschlimannii* and *R. massiliae* (**Chapter 2**). The last chapter of this part (**Chapter 3**) includes data related to diagnostic tools for *Rickettsia* spp. infection, based on serological or molecular methods or even *Rickettsia* cultivation.

The second part of the doctoral thesis is allocated to original research. This is structured in 7 chapters and comprises 100 pages.

Chapter 4 presents the research motivation, the aims and goals of the research. We aimed to evaluate and review the presence of rickettsial DNA in all types of biological samples collected during previous research, for understanding the diversity of SFG *Rickettsia* species in Romania.

Chapter 5 of this part sintetize the materials and methods used. All studies were conducted in Romania, using questing or engorged ticks, tissue samples collected from carcasses of insectivorous bats, small mammals and wild carnivores accidentally killed, mainly by collisions, and blood collected from domestic carnivores. Ticks were tested for the presence of *Rickettsia* DNA by PCR amplifying *Rickettsia* spp. *gltA* and 17-D genes fragments, tissue samples from small mammals, insectivorous bats and wild carnivores as well as blood collected from domestic carnivores were tested using PCR detection amplifying a 381 bp fragment of the *gltA* gene. For amplifying *Rickettsia* spp. 17-D gene fragment a nested PCR protocol was used.

Chapter 6 contains the **Study I: SFG *Rickettsia* spp. diversity in ticks population and the first report of *Rickettsia hoogstraalii* in Romania**, and it aimed to evaluate the diversity of SFG *Rickettsiae* in ticks belonging to five species. All *Ixodes ricinus*, *Dermacentor marginatus*, *D. reticulatus*, *Haemaphysalis punctata* ticks included in this study were questing ticks collected by flagging. Considering the lack of data on SFG *Rickettsia* in *Rhipicephalus rossicus*, a tick present in the steppe region of southeastern

Romania, *R. rossicus* collected from owned dogs (*Canis familiaris*) were also included in the study. A total of 2028 questing and engorged ticks were tested by PCR amplification of *Rickettsia* spp. gltA and 17-D gene fragments.

Chapter 7 includes **Study II: SFG *Rickettsia* screening in small mammals' population and insectivorous bats species**. Motivated by the high diversity and ubiquity of small mammals and bats in Romania, as well as by the fact that they are known reservoir hosts for important zoonotic viruses and because of their potential to harbor SFG rickettsiae, the aim of this study was to detect *Rickettsia* species in tissue samples. All 20 bats species and 31 small mammals' species were identified using morphological keys. The samples used from bats (n = 322) was represented by heart tissue and by splenic tissue for small mammals (n = 791). All animals used for the purpose of this study were accidentally killed, mainly by collisions.

Chapter 8 contains the **Study III: Domestic and wild carnivores' involvement in *Rickettsia* spp. ecoepidemiology**. This study aimed to evaluate the presence of rickettsial agents in domestic and wild carnivores in Romania, considering their potential role as sentinel species for tick-borne diseases. The study included 357 blood samples from domestic carnivores and more than 1200 samples originating from wild carnivores' carcasses.

In the **Chapter 9** are presented the conclusions and recommendations driven from the results of the present research. In the **Chapter 10**, the originality and innovative contributions of the thesis are presented.

RESULTS OF THE ORIGINAL RESEARCH

Chapter 6. The aim of the current study was to review the diversity of SFG *Rickettsiae* in ticks collected from all 41 romanian counties. A total of 2028 questing and engorged ticks collected in Romania belonged to five species were evaluate by PCR tehniques of *Rickettsia* spp. gltA and 17-D gene fragments: *I. ricinus* (n = 1128), *D. marginatus* (n = 507), *D. reticulatus* (n = 165), *Rhipicephalus rossicus* (n = 128) and *Haemaphysalis punctata* (n = 100). *Ixodes ricinus* and *H. punctata* assessed all developmental stages, as for *Dermacentor* spp. and *R. rossicus* only adults were assessed in the case.

The overall SFG *Rickettsia* spp. infection prevalence was 5.82% (118/2028; 95% CI: 4.88–6.92). Although the prevalence varied considerably (between 7 and 24.22%) between tick species, with the highest value for *R. rossicus* ticks. According to BLAST analysis, the most prevalent species was *R. monacensis*, with a prevalence of 43.22%

(51/118; 95% CI: 34.13–52.66%) of the total positive ticks. The sequences were detected in *I. ricinus* (7 females, 11 males, and 12 nymphs), *R. rossicus* (12 females and 2 males), and *Hae punctata* (1 male, 2 nymphs, and 1 larva) ticks. The second most prevalent species detected was *R. helvetica*, representing 38.14% (45/118, 95% CI: 29.35–47.53) of the positive ticks. It was isolated from *I. ricinus* (12 females, 13 males, and 10 nymphs) and *R. rossicus* ticks (6 females and 4 males). *Rickettsia raoultii* was identified in 15.25% of the positive ticks (18/118, 95% CI: 9.3– 23.03%), *Rickettsia raoultii* was detected in *D. marginatus* (4 females and 4 males), *I. ricinus* (2 males and 3 nymphs), *R. rossicus* (2 females and 1 male), and *Hae. punctata* ticks (1 male, nymph, and larva). *Rickettsia slovacica* had a 1.69% prevalence (2/118, 95% CI: 0.21–5.99%). It was detected only in *D. marginatus* ticks (1 female and 1 male). *Rickettsia hoogstraalii* was detected in a sequence isolated from one *R. rossicus* collected from a dog representing 0.85% prevalence.

Infection with *Rickettsia* spp. was detected in 22 of the 41 Romanian counties (53.66%; 95% CI: 37.42–69.34), with a variable prevalence ranging between 1.35% and 50%.

Chapter 7. Based on the high diversity and ubiquity of small mammals and bats, moreover the raportation of seven out of eight SFG rickettsiae species with zoonotic potential in Romania, the aim of this study was to detect *Rickettsia* species in tissue samples in small mammals and bats. In previous studies, a number of 791 small mammals from 31 species were collected from a variety of habitats in Romania (14 counties). At the same time, 322 samples of bats belonging to 20 species coming from 13 locations and caves were examined. Splenic tissue sample was collected from each small mammal. The reference organ for bats was represented by the heart.

All 791 small mammals tested, showed negative results for the presence of rickettsial DNA. SFG rickettsiae DNA was detected in 14.6% (n=47/322, 95%CI: 11.02–19.04) of samples. Positive samples were detected in *Nyctalus noctula* (22 out of 188 tested), *Pipistrellus pipistrellus* (n=13/55), *Myotis alcathoe* (n=5/12), *P. nathusii* (n=4/25), *Plecotus auritus* (n=1/6), *Vespertilio murinus* (n=1/4) and *P. pygmaeus* (n=1/2). The overall prevalence of *Rickettsia* spp. was 23.64% (95%CI: 13.23–37.02) in *P. pipistrellus* and 11.7% (95%CI: 7.48–17.18) in *N. noctula*. Among the 47 samples, only 17 were suitable for sequencing (>30 ng/μl DNA concentration). The sequences analysis showed 99.7% to 100% identity with *R. monacensis*. Four obtained sequences of gltA fragments (MT741493– MT741496) were deposited in the GenBank database. The overall *R. monacensis* prevalence was 5.28% (n=17, 95%CI: 3.2–8.84): 9.09% (n=5, 95%CI: 3.02–19.95) in *P. pipistrellus* and 6.38% (n=12, 95%CI: 3.34–10.88) in *N. noctula*. All the confirmed positive samples originated from two distinct locations *N. noctula* in Cheile Bicazului and *P. pipistrellus* from “Huda lui Papara” Cave.

Chapter 8. This study aimed to evaluate the presence of rickettsial agents in domestic and wild carnivores from Romania, considering their potential role as sentinel species for tick-borne diseases. In addition, motivated by the inconsistency in the detection of vector-borne pathogens and *Rickettsia* spp detection in tissue samples, the study also aims to compare the presence of rickettsial DNA in different types of samples.

Blood samples from 357 dogs were collected over 2 years in other previous projects. The chosen subjects came from areas within four altitude ranges: coastal 0 to 5 m, plain 6-100 m, hilly 200 to 400 m and low mountains >500 m. 1215 samples were collected from 550 wild carnivores. Samples taken consisted in blood clots, heart tissue, liver, lung, spleen, kidney, lymph nodes and bone marrow. Resulting 833 samples from 95 animals. For the rest of the animals only the spleen was tested.

DNA amplification from blood, heart tissue, liver tissue, lung tissue, spleen tissue, kidney tissue, lymph nodes and bone marrow samples collected from domestic and wild carnivores resulted in negative results for the detection of *Rickettsia* spp. in all the samples tested.

GENERAL CONCLUSIONS

In the **Chapter 9** of the thesis the general conclusions formulated based on the results obtained are presents as follows:

The average prevalence of *Rickettsia* spp. in collected ticks from Romania showed a moderate value (~5%), within European limits.

The diversity of rickettsia species, belonging to the spotted fever group according to the results obtained by screening both environmental and fed ticks, was represented by: *R. helvetica*, *R. hoogstraalii*, *R. monacensis*, *R. raoultii* and *R. slovacica*.

This study represents both the first detection of *R. hoogstraalii* in Romania and the first detection in *R. rossicus* tick collected from a dog.

The reporting of *R. hoogstraalii* in Romania completethe known diversity in our country, represented by: *R. asemonensis*, *R. conorii*, *R. helvetica*, *R. massiliae*, *R. monacensis*, *R. raoultii* and *R. slovacica*.

The importance of detection of *R. helvetica*, *R. monacensis*, *R. raoultii* and *R. slovacica*, in significant percentages (over 98% of all positive samples) is due to the zoonotic nature of these species, thus representing a major risk to public health.

The most prevalent species in our country, according to the studies presented, seems to be *R. monacensis*, in both cases of ticks and vertebrate hosts.

Rickettsia monacensis and *R. raoultii* detection in *Hae. punctata* not fed larvae suggests the possible transovarian transmission of the two *Rickettsia* species in the

Hae. punctata tick species. These observations came in addition to the detection of *R. monacensis* and *R. helvetica*, in *I. ricinus* larvae, reported in previous studies.

The detection of several rickettsia species as: *R. helvetica*, *R. monacensis*, *R. hoogstraalii* and *R. raoulti* in *Rh. rossicus*, collected from the dog, suggest either the involvement of the *Rh. rossicus* species in the epidemiology of these rickettsia, or the involvement of dogs in their epidemiology.

The detection of *R. monacensis* in biological tissue samples from insectivorous bats (*P. pipistrellus* and *N. Noctula*) with a relatively high prevalence, suggests the possible involvement of bats in the epidemiology of this rickettsia.

Failure of rickettsia DNA detection in tissue samples collected from micromammals, despite the presence of positive ticks collected from the same individuals, reported in a previous study, suggests the existence of a short-lived rickettsemia and the lack of involvement of micromammals as reservoirs in the epidemiology of rickettsia.

The lack of rickettsia DNA detection in tissues collected from domestic and wild carnivores in Romania suggests either the non-involvement or the minor role of these species in the epidemiology of rickettsiae, as competent hosts and/or reservoir.

RECOMMENDATIONS

Following the previous conclusions, zoonotic rickettsia species monitorisation is recommended in Romania. We also recommend increasing awareness of the risks to public health represented by the presence of zoonotic species on the territory of our country.

It is necessary to raise awareness by popularizing these results, among medical practitioners, for an appropriate differential diagnosis of tick-borne diseases in dogs.

Considering the detection of *R. hoogstraalii* on the territory of our country and the lack of data regarding the pathogenic potential for humans and animals of this species, as well as the largely unknown epidemiology of this species, further studies to clarify these aspects are recommended.

Considering the detection of *R. monacensis*, a zoonotic species being the agent of Mediterranean fever-like, in tissue samples from bats, it is necessary to evaluate these animal species, in the epidemiology of *R. monacensis* and the possible risk to human health.

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