IAȘI UNIVERSITY OF LIFE SCIENCES
FACULTY OF VETERINARY MEDICINE
DOCTORAL SCHOOL OF VETERINARY MEDICINE
DOCTORAL FIELD: VETERINARY MEDICINE
SPECIALIZATION: INFECTIOUS DISEASES

DOCTORAL THESIS

PhD Coordinator,
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BRĂTULEANU Bianca Elena (BAISAN)

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TEZĂ DE DOCTORAT

Conducător de doctorat,
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IAȘI
2022

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RESEARCH ON THE CARRYING AND THE ZOONOTIC RISK OF INFECTIOUS AGENTS TRANSMITTED BY TICKS

PhD Coordinator,
Prof. PhD. Gheorghe SAVUȚA,

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IAŞI
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CERCETĂRI PRIVIND PORTAJUL ȘI RISCUL ZOONOTIC AL UNOR AGENȚI INFECȚIOȘI TRANSMIȘI DE CĂTRE CĂPUȘE

PhD Coordinator,
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BRĂTULEANU Bianca Elena (BAISAN)

IAȘI
2022

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ACKNOWLEDGEMENTS

On the occasion of accomplishing my PhD thesis, I'd like to express my gratitude to everyone who helped and encouraged me along the process.

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Firstly, I would like to express my sincere gratitude to my PhD advisor, Prof. Gheorghe Savuta, for accepting to supervise my PhD, for guiding me, and for all the support shown during the doctoral period.

****

Special thanks to prof. Marc Eloit, who closely coordinated my thesis, for his patience, motivation, and immense knowledge, but also for giving me the opportunity to conduct my research in the „Pathogen Discovery" laboratory, Institute Pasteur, Paris, France. Without his precious support, it would not be possible to conduct this research.

****

I am particularly thankful to a special person-Sarah Temmam, because she's been a real help the whole time, starting with research ideas, giving me good advice to improve and interpret my work and who learned me to trust myself, no matter what. I could not have imagined having a better mentor.

****

I would like to thank every member of the „Pathogen Discovery" laboratory for giving me the opportunity to work in a pleasant environment. Among them, I would like to mention Delphine Chretien, who always has the patience to answer all my questions and who helped me from the beginning in my research activites.

****

I would like to address my sincere thanks to my colleagues from FMV Iasi, for the help offered in samples collection.

****

Also, I thank the direction of the „Ion Ionescu de la Brad" Iasi University of Life Sciences, for the opportunities offered during my doctoral studies.

****

Last but not the least, I want to express my gratitude to my family and friends, whose unconditional support meant a lot to me. I am grateful to my husband who always encouraged and supported me through every difficult situation. My sincere thanks also goes to my parents, without you none of this would indeed be possible.

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.

REZUMAT

La nivel mondial, căpușele sunt depășite doar de țânțari în ceea ce privește numărul de patogeni pe care îi pot transmite animalelor și oamenilor, adăpostind o mare varietate de agenți simbionți, comensali și patogeni.

Ixodes ricinus este cea mai răspândită specie din Europa, fiind vectorul mai multor boli de importanță medicală și veterinară, precum boala Lyme, encefalita transmisă de căpușe, febra hemoragică Crimeea-Congo și rickettsioza la om sau anaplasmoza și babesioza la animale. De asemenea, *I. ricinus* este specia dominantă și în România (86,9%), urmată de *Dermacentor marginatus* (9,5%), *Haemaphysalis punctata* (2,6%) și *Dermacentor reticulatus* (0,02%).

Datorită modernizării tehnologiilor de secvențiere de ultimă generație, cercetările privind varietatea bacteriană și virală a căpușelor au crescut considerabil în ultimii ani, dezvăluind o diversitate microbiană neașteptată. Astfel, pe lângă agenții patogeni deja cunoscuți ca fiind vectorizați de căpușe, au fost descrise noi virusuri, aparținând familiilor *Nairoviridae, Flaviviridae, Phenuiviridae* și *Orthomyxoviridae*. Factorii de mediu, peisagistici și antropogeni, influențează distribuția și abundența căpușelor, având un impact major și asupra dinamicii de transmitere a agenților patogeni. Schimbarile climatice au fost citate ca fiind cauza principală a expansiunii căpușelor și a creșterii numărului de infecții transmise iar numeroase studii au demonstrat importanța temperaturilor asupra densității căpușelor din mediu. La această expansiune, contribuie deopotrivă importul de animale din alte zone, dar și păsările migratoare.

România este una dintre cele mai diverse țări din punct de vedere biogeografic din Europa, fapt care influențează dinamica de răspândire a populațiilor de căpușe, mai ales în partea de sud a țării, ce cuprinde Delta Dunării. Delta Dunării reprezintă principala escală a păsărilor în rutele migratorii spre Africa și înapoi spre Europa, ceea ce conduce la un risc ridicat de introducere de noi agenți patogeni în zonă. În acest areal există aproximativ 30 de tipuri de ecosisteme, care cuprind 2994 specii diferite de floră și 4286 de specii de faună (amfibieni, pești, și reptile), precum și mamifere domestice și sălbatice. Într-un astfel de mediu, căpușele reprezentă „puntea" care duce la transmiterea interspecifică a agenților patogeni, inclusiv omului.

Scopul lucrării „**Cercetări privind portajul și riscul zoonotic al unor agenți infecțioși transmiși de către căpușe**" a fost acela de a obține informații privind noi agenți patogeni transmiși de către diferite specii de căpușe din Sud-Estul României și de a evalua potențialul lor zoonotic pentru sănătatea animală și umană.

Pentru a îndeplini scopul propus, au fost stabilite următoarele **obiective generale**:

- Evaluarea seroprevalenței principalelor infecții bacteriene transmise de către căpușe la animale în Sud-Estul României
- Evaluarea circulației virusului febrei hemoragice Crimeea-Congo prin tehnici serologice și de biologie moleculară la căpușe și rumegătoare mici
- Identificarea de noi arbovirusuri transmise de căpușe prin tehnici de secvențiere de ultimă generație (NGS) și detectarea anticorpilor împotriva proteinelor virale la animalele expuse, prin tehnici serologice de ultimă generație (LIPS).

Originalitatea acestei teze constă în:

- Utilizarea secvențierii de ultimă generație (NGS), pentru a descrie viromul căpușelor colectate din Sud-Estul României
- Detectarea de noi agenți patogeni virali în căpușe, identificați pentru prima dată în România, inclusiv virusuri noi relevante pentru sănătatea publică
- Evaluarea seropozitivității la animalele expuse folosind tehnica de imunoprecipitare a luciferazei (LIPS)

Prezenta lucrare de doctorat este organizată în conformitate cu normele în vigoare, în două părți principale: **prima parte** descrie „Stadiul actual al cunoașterii", ce condensează informații actualizate din literatura de specialitate privind principalele specii de căpușe din Romania și agenții patogeni vectorizați de acestea. De asemenea, sunt descrise metodele de secvențiere utilizate în studii de metagenomică. **A doua parte** prezintă contribuțiile personale, în care sunt descrise metodele de lucru și rezultatele obținute ca urmare a cercetărilor realizate pe parcursul perioadei de studii doctorale, rezumate în 5 capitole.

Pe lângă aceste două părți principale, lucrarea actuală mai conține mulțumiri, lista abrevierilor, introducere, rezumat, cuprins, lista figurilor, bibliografie și anexe. **Teza conține 29 figuri și 14 tabele, având și 205 surse bibliografice consultate.**

Prima parte se concentrează pe cunoștințele actuale din literatura de specialitate și cuprinde 4 capitole și subcapitolele aferente. **Primul capitol** prezintă principalele specii de căpușe și distribuția lor geografică în România. **Al doilea capitol** descrie cele mai importante boli bacteriene transmise de căpușe precum boala Lyme, anaplasmoza canină și ehrlichioza, prezentând etiologia, epidemiologia, semnele clinice și stadiul actual al cunoșterii în România. Cel de-**al treilea capitol** prezintă agenți patogeni virali ca febra hemoragică Crimeea-Congo, encefalita transmisă de căpușe, dar și flavivirusuri recent identificate, vectorizate de căpușe, urmând aceeași structură ca și în capitolul II. Cel de-**al patrulea capitol** descrie platformele de secvențiere (NGS) existente.

Cea de-a doua parte a tezei, **Contribuții personale**, este structurată în 5 capitole (capitolele V-IX). **Capitolele V** și **VI** descriu cadrul organizatoric și instituțional în care au fost efectuate cercetările, precum și scopul și obiectivele acesteia. Următoarele 3 capitole prezintă studiile realizate, incluzând descrierea materialului și metodelor utilizate, rezultatele și discuțiile ulterioare, pentru fiecare studiu în parte. Capitolul IX (*Concluzii finale*), concentrează concluziile generale ale lucrării, desprinse în urma supravegherilor serologice și analizei meta-transcriptomice efectuate.

Capitolul VII, intitulat "*Cercetări seroepidemiologice și moleculare privind principalii agenți patogeni transmiși de căpușe la animale*", este împărțit în două secțiuni: **prima secțiune** prezintă două studii serologice privind boala Lyme, ehrlichioza și anaplasmoza la câinii domestici și de vânătoare. Scopul studiului "*Investigații serologice privind borrelioza Lyme, anaplasmoza și ehrlichioza la câinii din două județe din sud-estul României*" a fost de a determina seroprevalența anticorpilor IgG împotriva *B. burgdorferi, A.phagocytophilum* și *E. canis* la câinii domestici din Sud-Estul țării. *B. burgdorferi, A.phagocytophilum* și *E. canis* se numără printre principalele bacterii care afectează populația canină, fiind un subiect de interes în ultimii ani. Ținând cont de similitudinile dintre factorii de risc ai bolii Lyme la câini și oameni, sistemul de supraveghere serologică a câinilor poate fi o metodă eficientă de stabilire a riscului și a răspândirii bolii. În

studiul de față au fost testați 92 de câini pentru detecția anticorpilor anti-*Borrelia* VIsE, *A. phagocytophilum* și respectiv, *E. canis*. În urma testului ELISA, 14 din cei 92 de câini testați au fost detectați ca fiind seropozitivi, dintre care 6 au prezentat anticorpi anti-*Borrelia spp*. (6,52%), 5 anti-*A. phagocytophilum* (5,43%) și 3 anti-*E. canis* (3,26%). La 4 câini a fost observată co-infecția cu doi agenți patogeni diferiți.

Cel de-al doilea studiu serologic intitulat "*Cercetări seroepidemiologice asupra anaplasmozei la câinii domestici și de vânătoare*" a avut ca scop evaluarea seroprevalenței anticorpilor IgG anti-*A. phagocytophilum* la câini selectați din trei județe: Iași, Sibiu și Tulcea, în perioada 2015-2019. *A. phagocytophilum* este un agent patogen emergent, cu o seroprevalență care variază de la o regiune la alta. Populația analizată a fost alcătuită din 92 de câini domestici și de vânătoare.

Acest studiu a prezentat o prevalență globală de 14,13% (13 din 92 de câini testați au fost identificați ca fiind seropozitivi). Cea mai mare seroprevalență a fost înregistrată la câinii de vânătoare din județul Sibiu (58,8%), care deseori nu primesc niciun tratament antiparazitar împotriva căpușelor.

A doua secțiune prezintă două cercetări bazate pe tehnici serologice și moleculare asupra virusului febrei hemoragice Crimeea-Congo (FHCC). Primul studiu s-a bazat pe detectarea FHCC în căpușele *Dermacentor* și *Rhipicephalus* sp., colectate din județul Tulcea, regiune unde studii anterioare au indicat prezența anticorpilor IgG împotriva FHCC la rumegătoarele mici. Principalul vector al CCHFV este *Hyalomma marginatum*, dar virusul a fost detectat în peste 30 de specii de căpușe, inclusiv *Dermacentor* și *Rhipicephalus* sp. Aproximativ 127 de căpușe au fost colectate în anul 2019, din cinci locații din județul Tulcea. Căpușele au fost testate prin Real-Time RT-PCR pentru detectarea ARN-ului viral pentru cele șase genotipuri ale virusului FHCC. În niciunul dintre cele 5 situri de colectare nu s-a înregistrat rezultate pozitive. Chiar dacă speciile de căpușe analizate în acest studiu pot transmite FHCC, faptul că vectorul principal (*H. marginatum*) nu a fost testat, poate explica rezultatele negative.

FHCC circulă în natură prin intermediul unui ciclu căpușă - gazda vertebrată – căpușă, astfel că pentru a avea o imagine de ansamblu asupra circulației FHCC în România a fost determinată seroprevalența la gazdele vertebrate, fiind colectate seruri de la 250 de ovine și caprine din aceeași regiune, ca și în studiul anterior.

Detectarea anticorpilor anti-FHCC a fost realizată prin tehnica ELISA, utilizând kitul ID Screen CCHF Double Antigen Multispecies (IDvet, Grabels, Franța). Seroprevalența globală la animalele testate a fost de 37,7%. Seroprevalența obținută la ovine a fost de 29.8% și de 57.7% la caprine. Căpușele și probele de ser au fost, de asemenea, analizate prin RT-qPCR vizând detectarea virusului FHCC, toate probele fiind însă negative. În România nu au fost înregistrate cazuri FHCC la om, până în momentul actual. O primă ipoteză pentru seropozitivitatea obținută în rândul rumegătoarelor ar fi faptul că în regiune circulă o tulpină FHCC nepatogenă pentru om, din moment ce animalele sunt în mod obișnuit asimptomatice. De asemenea, trebuie luată în considerare existența unui alt virus apropiat de FHCC, care poate genera reacții serologice de încrucișare.

Capitolul VIII, "*Caracterizarea viromului căpușelor prin tehnica secvențierii de ultimă generație*", este partea cea mai detaliată și originală a tezei. Acest capitol prezintă potențialul

secvențierii de ultimă generație pentru studiile metagenomice care au ca scop descrierea microbiomului unor organisme diverse.

În prima parte a subcapitolului intitulat "*Noi virusuri relevante pentru sănătatea publică identificate în căpușele din România*" sunt descrise rezultatele în urma secvențierii utilizând platforma Illumina NextSeq500, a peste 500 de căpușe adulte aparținând unor specii diferite, colectate din Estul României. În țara noastră, majoritatea studiilor s-au axat pe identificarea agenților bacterieni transmiși de căpușe, cu o mică preocupare pentru agenții virali.

Scopul acestei cercetări a fost acela de a descrie viromul căpușelor *Rhipicephalus, Dermacentor* și *Haemaphysalis* sp., colectate din zone relativ neexplorate din România, pentru a spori cunoștințele privind diversitatea virusurilor în Europa de Est, incluzând virusuri noi, care pot avea relevanță pentru sănătatea umană și animală. Studiul de față cuprinde o descriere generală a compoziției virusurilor ARN și ADN, ce diferă în funcție de specia de căpușe, urmată de o analiză detaliată a celor mai importante familii virale detectate.

Virusurile ARN au fost reprezentate de 29 de familii virale, unele familii virale fiind limitate la o singură specie de căpușă (*Mononegavirales*), în timp ce altele au fost identificate în toate cele 3 specii analizate (spre exemplu, *Riboviria, Picornavirales* , ssRNA+ și *Flaviviridae*), sau detectate doar în căpușele nehrănite (*Luteoviridae, Tombusviridae* și *Marnaviridae*).

Printre comunitățile virale identificate, au fost detectate virusuri ce aparțin familiilor *Flaviviridae, Phenuiviridae* și *Nairoviridae*. Spre exemplu, Jingmen tick virus (JMTV) aparține familiei *Flaviviridae,* fiind identificat în căpușele colectate de pe ovine și caprine din județul Tulcea, indicând faptul că rumegătoarele joacă un rol major în ciclul acestui virus.

Un alt virus identificat a fost Tacheng tick virus 2 (TaTV2) (familia *Phenuiviridae*), plasat din punct de vedere filogenetic intr-un grup cu alte izolate provenite de la căpușe și de la oameni din China, astfel că TaTV2 prezintă o importanță deosebită pentru sănătatea umană și animală. Patogenitatea acestui virus pentru oameni și animale a fost confirmată ulterior, TaTV2 fiind detectat la pacienții mușcați de căpușe și la mai multe specii de vertebrate, precum și în căpușele *H. asiaticum*. Mai mult decât atât, au fost identificate și alte virusuri a căror patogenitate nu se cunoaște încă (Bole tick tick virus 4, Nayun tick nairovirus), fiind necesare studii suplimentare pentru a determina infectivitatea lor pentru vertebrate.

Scopul celui de-al doilea subcapitol intitulat "*Identificarea unui nou Quaranjavirus și a altor familii virale în căpușele Ixodidae din Delta Dunării*", a fost acela de a identifica virusuri asociate căpușelor *Rhipicephalus sanguineus* colectate de pe rumegătoare mici din județul Tulcea, prin tehnica secvențierii de ultimă generație. *Quaranjavirusurile* sunt transmise în majoritatea lor de căpușele argaside, dar studii recente au identificat noi secvențe aparținând acestui gen viral și în căpușele dure-ixodide, cunoscându-se puține date despre patogenitatea acestora.

În prezenta cercetare au fost detectate trei familii virale: *Phenuiviridae* (41%), *Chuviridae* (36%) și *Orthomyxoviridae* (22%). În familia *Orthomyxoviridae,* au fost detectate secvențe virale ce aparțin unui nou quaranjavirus, denumit Cataloi tick quaranjavirus (CTQV). Acest virus a fost situat din punct de vedere filogenetic, într-un grup aparent limitat la căpușele ixodide, diferit de quaranjavirusurile transmise de căpușele argaside. Capacitatea de replicare a quaranjavirusurilor asociate căpușelor ixodide, rămâne de elucidat.

Scopul celui de-al treilea subcapitol a fost de a determina dacă CTQV ar putea constitui un nou arbovirus transmis de căpușe. În acest sens, serurile provenite de la rumegătoare mici

expuse la mușcături de căpușe din regiunea Deltei Dunării au fost analizate cu ajutorul testului LIPS pentru a evalua prezența anticorpilor împotriva CTQV. S-a observat o creștere semnificativă a raportului semnal/zgomot pentru serurile de ovine colectate în Slava Cercheză sau Somova în 2019 și pentru serurile de caprine colectate fie în Cataloi în 2019, fie în Slava Rusă în 2020. Pentru a determina dacă aceste seruri pot fi considerate pozitive, am definit o valoare de prag de pozitivitate ca fiind media raportului semnal-zgomot al serurilor franceze neexpuse + 3 deviații standard. Doar 8 seruri de ovine colectate în Slava Cercheză în 2019 au depășit acest prag. Pentru a confirma dacă aceste probe sunt pozitive, sunt necesare teste serologice mai specifice, cum ar fi seroneutralizarea, dar acestea necesită izolarea virusului.

Ultimul capitol (**capitolul IX**), sintetizează toate concluziile cercetării și creează o viziune generală asupra tezei. Cercetarile efectuate au confirmat persistența unor agenti patogeni bacterieni și virali cunoscuți în zona studiată. Pe langă aceștia, au fost identificați noi agenți patogeni descriși pentru prima dată în România, cu risc pentru sănătatea publică.

INTRODUCTION

Ticks harbor a wide variety of microorganisms, such as nematodes, fungi, protozoa, bacteria and viruses, and are implicated in the transmission of several pathogens. So far, around 160 arboviruses have been identified in ticks, 25% of them associated with human and animal diseases.

The health consequences of tick-borne diseases in Europe and elsewhere have only recently started to be appreciated. Anthropogenic factors, variation in vertebrate faunal composition, social-recreational changes, and climatic trends have contributed to changing tick distribution and risk patterns for tick bites in humans. This is coupled with the increasing awareness of new tick-borne pathogens and growing recognition of the importance of established tick-borne pathogens for human and animal health.

The recent development of viral metagenomics and the characterization of the viral diversity from an organism or an environment using state-of-the-art technologies such as Next Generation Sequencing (NGS) is encouraging for the surveillance of diseases and can be accomplished by analyzing the viromes of selected animals and arthropods that are closely in contact with humans.

During the last decade, many new viruses have been discovered in arthropods, including ticks, and their identification has provided new insights on the diversity and evolution of RNA viruses. Nevertheless, little is known about their ability to infect vertebrates despite the growing number of studies.

Romania's geopolitical position on the Eastern border of the EU is strategically important from an epidemiological perspective. Romania is one of the most biogeographically diverse countries in Europe, with suitable conditions for the establishment of tick-borne virus foci, particularly in the Southern half of the country, via importation of animals from other areas and migratory birds. The epidemiological situation of tick-borne pathogens in Romania is still under investigation and data is limited. A detailed surveillance program is necessary, therefore, as these pathogens can be easily transported into and throughout Europe.

The aim of this thesis was to obtain novel information concerning tick-borne pathogens from different tick species and to assess the risk of tick-borne infections in animals from understudied areas in South-Eastern Romania.

The thesis had three main objectives:

- 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 NGS techniques followed by the search for antibodies against viral proteins in exposed animals using high-throughput serological technique (LIPS)

PART I
CURRENT STATE OF KNOWLEDGE

CHAPTER I
MAIN TICK SPECIES INVOLVED IN THE PATHOGENS TRANSMISSION TO HUMANS AND ANIMALS IN ROMANIA

Ticks are blood-feeding ectoparasites that belong to the class *Arachnida*. *Ixodidae* (hard ticks), *Argasidae* (soft ticks), and *Nuttalliellidae* are the three primary families in this class (Anderson and Magnarelli, 2008). The *Ixodidae* family is the largest, with four genera and over 700 tick species found all over the world (Brites-Neto et al., 2015).

In Romania, 27 tick species have been identified, with 25 belonging to the *Ixodidae* family (Table 1.1) and two to the *Argasidae* family (*Argas persicus, Argas reflexus*) (Coipan and Vladimirescu, 2011). The predominant tick species is *Ixodes ricinus* (86.9%) followed by *Dermacentor marginatus* (9.5%) and *Haemaphysalis punctata* (2.6%) (Mihalca et al., 2012a).

Table 1.1

The main *Ixodidae* tick species present in Romania (After Coipan et al., 2011)

Dermacentor sp.	***Haemaphysalis sp.***	***Hyalomma sp.***	***Ixodes sp.***	***Rhipicephalus sp.***
D. marginatus	*H. concinna*	*H. aegyptium*	*I. apronophorus*	*R. annulatus*
D. reticulatus	*H. inermis*	*H. detritum*	*I. arboricola*	*R. bursa*
	H.parva	*H. scupense*	*I. crenulatus*	*R. rossicus*
	H. punctata	*H. marginatum*	*I. hexagonus*	*R. sanguineus*
	H. sulcata		*I. laguri*	*R. turanicus*
	H. erinacei		*I. redikorzevi*	
			I. ricinus	
			I. rugicollis	
			I. simplex	
			I. trianguliceps	
			I. vespertilionis	
			I.canisuga	
			I. lividus	

Ixodes ricinus (Figure 1.1.A) is the most abundant species on the European continent (Reis et al., 2011) and is also the most common species in wooded areas in Romania (Mihalca et al., 2012b). Tick-borne encephalitis virus, Lyme disease, anaplasmosis and ehrlichiosis (Rizzoli et al., 2014) are among the more than 25 infections that *I. ricinus* is known to transmit to humans and domestic animals (Coipan et al., 2013).

After *I.ricinus*, ***Dermacentor reticulatus*** (Figure 1.1.B) is the second most common hard tick species in Europe (Paulauskas et al., 2018). *D. reticulatus*' distribution area has lately expanded to higher latitudes and elevations over Central Europe (Germany, Poland, Hungary, Slovakia, and Romania) (Mihalca et al., 2012a; Foldvari et al., 2016). Domestic animals such as sheep, goats, cattle, horses, cats, dogs, and pigs are the most common vertebrate hosts reported (Siroky et al., 2011).

D. reticulatus is a vector for a variety of infections (Rudolf et al., 2016) and is a carrier of a variety of bacteria (Foldvari et al., 2009; Foldvari et al., 2016) and viruses including tick-borne encephalitis virus and Omsk haemorrhagic fever virus (Ruzek et al., 2010).

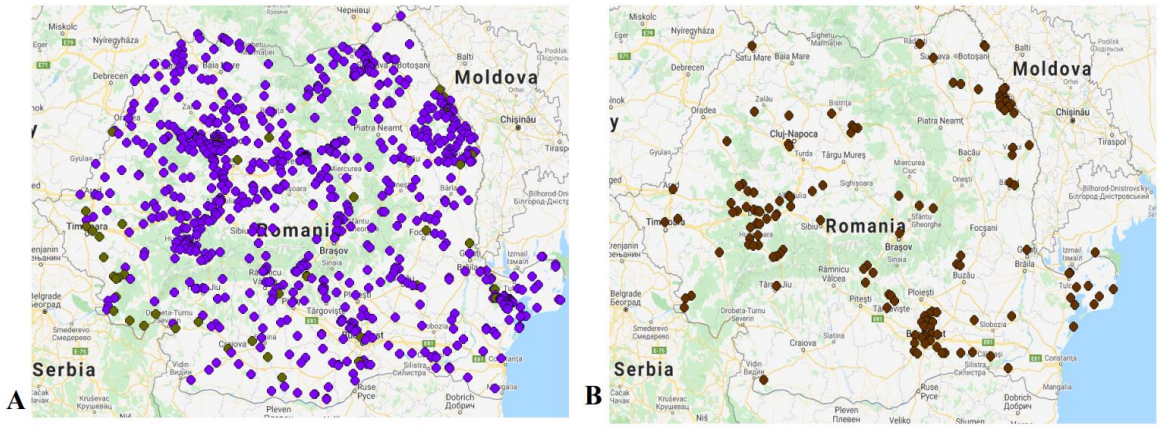


Figure 1.1- Geographical distribution of *Ixodes ricinus* (A) and *Dermacentor reticulatus* (B) questing ticks in Romania (VectExcel)

Despite occasional reports from the Central and Northwestern areas of Romania, ***Rhipicephalus bursa*** (Figure 1.2.C) seems to be restricted to the Southern lowland region of Romania (Mihalca et al., 2012b). ***R. bursa*** feeds on ruminants, other domestic animals, wildlife, and humans, and is known to transmit a number of significant livestock infections (Masala et al., 2012; Aktas, 2014; Erster et al., 2016; Ferrolho et al., 2016).

Rhipicephalus sanguineus (Figure 1.2.D) also known as the "brown dog tick" feeds mostly on dogs, but can also infest a variety of domestic and wild hosts, including cats, rodents, birds, and humans (Iori et al., 1996; Estrada-Pena and Jongejan, 1999; Dantas-Torres et al., 2006). Many infections are transmitted to dogs by *R. sanguineus* (Ewing et al., 2002; Dantas-Torres, 2008) and recent research have indicated that *R. sanguineus* exposed to high temperatures is more likely to attack humans (Parola et al., 2008).

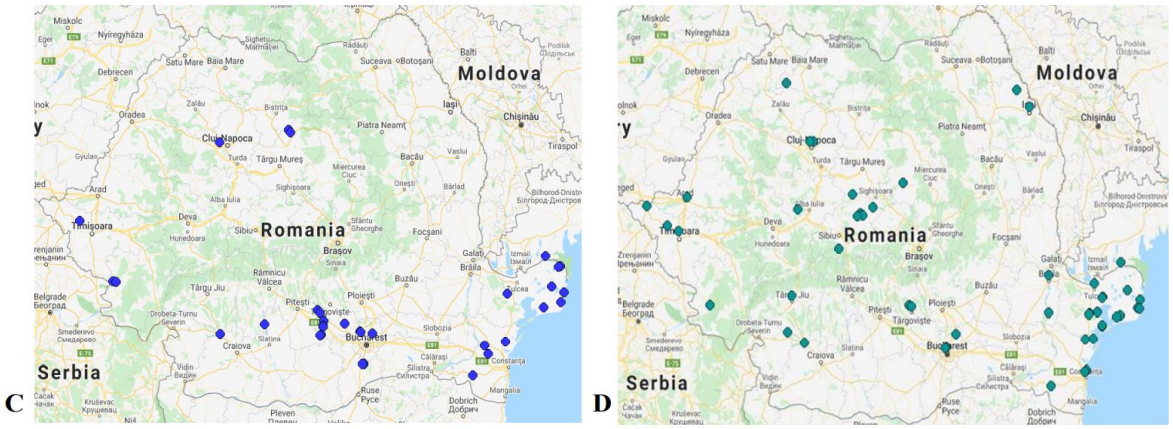


Figure 1.2- Geographical distribution of *Rhipicephalus bursa* (C) and *sanguineus* (D) questing ticks in Romania (VectExcel)

Haemaphysalis punctata (Figure 1.3.F) is generally assumed to be a species with a Mediterranean distribution (Estrada-Pena et al., 2013) whose main hosts are carnivores, wild and domestic ruminants, hedgehogs, birds and rodents (Nosek, 1971). *H. punctata* is the most common species in Romania (Mihalca et al., 2012b) and it serves as a vector for significant viruses like Crimean-Congo hemorrhagic fever virus and tick-borne encephalitis virus (Estrada-Pena and Jongejan, 1999).

The Mediterranean ***Hyalomma*** tick, often known as the Crimean–Congo hemorrhagic fever tick, is one of the most important vectors of the disease. ***H. marginatum*** (Figure 1.3.E) is typically found in Romania's southern regions (Mihalca et al., 2012a).

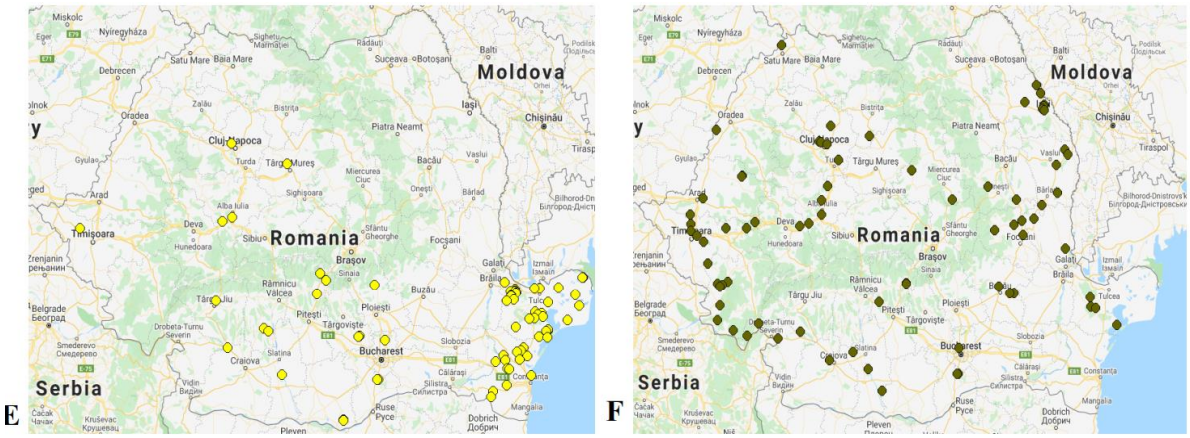


Figure 1.3- Geographical distribution of *Hyalomma marginatum* (E) and *Haemaphysalis punctata* (F) questing ticks in Romania
(VectExcel)

CHAPTER II
THE MAIN BACTERIAL TICK-BORNE PATHOGENS

In Europe, ticks are the most common vectors, infecting both humans and animals. The major species in Europe is *I. ricinus*, which is found all over the continent. This species of tick can transmit a wide range of infections, including viruses, bacteria and protozoa. *I. ricinus* is also the dominant species in Romania (86%), followed by *D. marginatus* (9.5%), *H. punctata* (2.6%) and *D. reticulatus* (0.02%).

Numerous investigations on the prevalence of diseases in ticks collected from vegetation and animal hosts have been performed in Romania. Most of this research focused on detecting a single or limited number of pathogens. Ticks and wildlife are the major reservoirs of tick-borne pathogens (TBP), which cause a wide range of tick-borne diseases in humans and animals, including anaplasmosis, babesiosis, borreliosis, ehrlichiosis, and rickettsioses. TBP circulation, particularly zoonotic TBP, should be investigated in a multidisciplinary manner in accordance with "One Health" concepts.

The implementation of such an approach requires collaboration among key stakeholders (such as veterinarians, entomologists and researchers), to identify infection and exposure risks and implement effective prevention strategies.

In the category of tick-borne diseases, Lyme disease has the highest number of infections in Europe. *Borrelia burgdorferi* sensu lato is responsible for around 80.000 cases per year. However, it is commonly acknowledged that precisely evaluating disease incidence, distribution, and trend over time remains a difficulty for surveillance across European countries.

2.1. Lyme disease

Etiology

More than 18 genospecies comprise the *B. burgdorferi* sl complex (Stanek and Reiter, 2011). The most frequent circulating genospecies in Europe are *B. afzelii* and *B. garinii*, followed by *B. burgdorferi* ss and *B. valaisiana* (Table 2.1.1). All pathogenic genospecies can produce erythema migrans and different genospecies are linked to specific clinical manifestations: *B. burgdorferi* ss is related to arthritis and neuroborreliosis, *B. garinii* is associated with neuroborreliosis, and *B. afzelii* is linked to a chronic skin condition.

Epidemiology

In Europe and the United States, Lyme disease is the most common tick-borne disease. Although Lyme borreliosis is not a novel or emergent disease, an accurate epidemiological description in Europe is impossible given the fact that only a few countries have made this disease obligatory to report. Tick abundance and exposure are related to the disease. Higher Lyme borreliosis risk is associated with occupation (e.g. forestry work and farming), certain activities (e.g. hunting) and age (children aged 5–14 years and adults aged 50–64 years) even if it is no longer considered to be correlated with residency in rural areas (Rizzoli et al., 2011).

Table 2.1.1

The genospecies of *Borrelia burgdorferi* and their tick vectors
(After Allen C. Steere et al., 2004)

	Principal tick vector	**Location**
Three pathogenic species		
Borrelia burgdorferi	*Ixodes scapularis*	Northeastern and norh central US
	Ixodes pacificus	Western US
	Ixodes ricinus	Europe
Borrelia garinii	*Ixodes ricinus*	Europe
	Ixodes persulcatus	Asia
Borrelia afzelii	*Ixodes ricinus*	Europe
Eight minimally pathogenic or nonpathogenic species		
Borrelia andersonii	*Ixodes dentatus*	Eastern US
Borrelia bissettii	*Ixodes spinipalpis*	Western US
	Ixodes pacificus	
Borrelia valaisiana	*Ixodes ricinus*	Europe and Asia
Borrelia lusitaniae	*Ixodes ricinus*	Europe
Borrelia japonica	*Ixodes ovatus*	Japan
Borrelia tanukii	*Ixodes tanukii*	Japan
Borrelia turdae	*Ixodes turdus*	Japan
Borrelia sinica	*Ixodes persulcatus*	China

State of knowledge in Romania

Borrelia has been found in questing ticks in Romania, according to various researches. *B. burgdorferi* sl has been of greatest interest due to its impact on public health. Studies to date have shown a lower average prevalence of *B. burgdorferi* sl in *I. ricinus* ticks compared to the European average (Rauter and Hartung, 2005).

An overall prevalence of 1.4% was determined after testing over 12.000 questing ticks collected from 183 locations across Romania (Kalmar et al., 2013). Another research evaluating three counties in the south and central regions of the country revealed a prevalence of 18% for *B. burgdorferi* (Coipan and Vladimirescu, 2011). *Borrelia* sp. was also identified in 138 of 534 (25.8%) questing *I. ricinus* ticks in Eastern Romania (Raileanu et al., 2017) and in *I. ricinus* ticks collected from different host species: lizards (Foldvari et al., 2009), horses (Ionita et al., 2013), hedgehogs (Dumitrache et al., 2013a), humans (Briciu et al., 2014), dogs and small mammals (Kalmar et al., 2019) and in healthy blood donors (Kalmar et al., 2021). From 2010 through 2018, the National Center for Surveillance and Control of Communicable Diseases recorded 352 confirmed cases of Lyme disease in humans per year.

The annual average of confirmed cases of Lyme disease in humans reported by the National Centre for Surveillance and Control of Communicable Diseases from 2010 to 2018 was 352 cases, but the highest number was recorded in 2018, with 520 confirmed human cases.

2.2. Anaplasmosis

Etiology

The genus *Anaplasma* comprises obligate intracellular bacteria, that can infect several cell types. Actually, *A. marginale, A. bovis, A. ovis, A. platys* and *A. phagocytophilum* are the most important species of the genus (Atif et al., 2021).

Epidemiology

Anaplasmosis is distributed worldwide and is potentially endemic in 43 countries. The prevalence varies among areas, species, breeds, due to the presence of different ticks and diagnostic assays involved. *Anaplasma phagocytophilum* infects humans, domestic and wild animals (Table 2.2.1). Small mammals play an important role in disease transmission and this species is particularly common in northern Europe. *Ixodes, Dermacentor, Haemaphysalis, Amblyomma, Hyalomma*, and *Rhipicephalus* sp. ticks transmit the bacteria which has been found in Asia, Europe, Africa and America (Woldehiwet, 2006).

Anaplasma platys was firstly detected in the blood of a dog in the United States, in 1978. *A. platys* widely infects dogs. It has also been found in deer, cats, cattle and humans (Sainz et al., 2015). *A. bovis* was first described in cattle, in 1936. Since then, the agent has been identified in domestic and wild animals in Spain, Italy, India, China, South Korea, Japan, USA, Brazil, Tunisia and South Africa. *A. ovis* is an important intra-erythrocytic pathogen transmitted by ticks to goats, sheep and small wild ruminants.

The etiological agent of bovine anaplasmosis, *A. marginale*, is distributed worldwide, mainly in tropical and subtropical regions and is transmitted by 17 species of ticks. *R.microplus* is the major vector in countries such as India, South Africa, Brazil, Malaysia and Australia. *I. ricinus* is the main vector in Europe and *D. andesrsoni, D. variabilis* and *D. albipictus* dominate in North America (Atif, 2016).

State of knowledge in Romania

In Romania, some research has been carried out to identify *Anaplasma* sp. in ticks and animals. *A. phagocytophilum* has been the most frequently studied species in Romania. No cases of granulocytic anaplasmosis in people have been documented to yet.

The prevalence of *A. phagocytophilum* in questing ticks has been established in some studies. Matei et al., reported a prevalence of 3.4% by testing of over 10.000 *I. ricinus* ticks, collected from 113 sites and 40 counties. A similar prevalence has been established in ticks collected in the north and center of the country (Matei et al., 2015). *A. phagocytophilum* has been described in ticks collected from deer and goats (Paduraru et al., 2012), cattle and horses in the southeast part of the country (Ionita et al., 2013). Furthermore, few studies has been conducted to detect the human granulocytic anaplasmosis agent in animal tissues. *A. phagocytophilum* has been

found in dogs (Mircean et al., 2012), turtles (Pastiu et al., 2012), hedgehogs (Dumitrache et al., 2013b), wild boar and migratory birds (Sandor et al., 2014).

More recent studies have reported the presence of *A. phagocytophilum* in small mammals (Orders *Rodentia* and *Eulipotyphla*), recording an overall prevalence of 2.53% (Matei et al., 2018) and in different organ samples in wild carnivores, with the highest prevalence (6.3%) in spleen tissue (Matei et al., 2021).

Table 2.2.1

Classified and unclassified *Anaplasma* species infecting different cells, vertebrate hosts and potential vectors (After Atif F. et al., 2021)

***Anaplasma* sp.**	**Infecting cells**	**Vertebrate Hosts**	**Potential Vectors**
A. marginale	Granulocytes	Domestic and wild ruminants, horses,dogs,cats, insectivores, wild swine and humans	*Ixodes,Dermacentor, Hyalomma,Rhipicephalus*
A. centrale	Erythrocytes	Domestic ruminants	*Ixodes,Dermacentor, Rhipicephalus*
A. ovis	Erythrocytes	Domestic and wild ruminants, humans	*Dermacentor, Rhipicephalus, Hyalomma*
A. bovis	Monocytes	Domestic and wild ruminants, small mammals	*Haemaphysalis, Rhipicephalus, Amblyomma*
A. capra	Erythrocytes	Domestic and wild ruminants, humans	*Haemaphysalis*
A. odocoilei	Platelets	Wild ruminants	Not known
A. camelii	Not known	Camels	Not known
A. boleense	Not known	Not known	*Hyalomma*
A. corsicanum	Not known	Domestic ruminants	Not known
A.mediterraneum	Not known	Domestic ruminants	Not known

2.3. Ehrlichiosis

Etiology

Canine monocytic ehrlichiosis is caused by *Ehrlichia canis*, which is transmitted primarily by *Rhipicephalus sanguineus* ticks. Canine ehrlichiosis is a tick-borne disease caused by pathogens from the genera *Ehrlichia* (*E.canis, E. chaffeensis, E. ewingii*). *E. canis* and *E. ewingii* are usual pathogens of dogs that has a worldwide distribution.

Epidemiology

Canine monocytic and granulocytic ehrlichiosis are two important fatal diseases of dogs and also have a public health significance. The worldwide distribution is correlated to the distribution of *R. sanguineus*. All countries bordering the Mediterranean Sea are endemic to *E. canis*. Some studies have indicated that the infectious agent is spreading also to countries north of the Mediterranean like Switzerland and Germany (Gothe, 1998).

Clinical signs can be variable depending on the strain, the immune response of the dog and the presence of other tick-borne infections. Usual signs of ehrlichiosis include pale mucous membranes, epistaxis, ecchymoses, hematuria or melena associated with thrombocytopenia or vasculitis. Co-infections with *Anaplasma* and *Ehrlichia* sp. are common because some species are transmitted by the same vector.

State of knowledge in Romania

In Romania, there are limited studies on *E. canis* infection. However, a study conducted between May 2008 and March 2011 (1146 blood dogs tested samples) revealed an overall seroprevalence of 2.1% (Mircean et al., 2012), *E. canis* infection being recorded only in dogs from Constanta County. This correlates with the distribution of the vector *R. sanguineus* in Romania. *Ehrlichia* sp. was also detected in engorged *I. apronophorus,* two collected from dogs and one from a fox (Andersson et al., 2018a).

2.4. Other bacterial tick-borne pathogens

***Bartonella* species**

Bartonella species are phylogenetically related to *Brucella* species and are facultatively intracellular bacteria belonging to subgroup alpha 2, class *Proteobacteria*.

Long-term intraerythrocytic bacteremia in the host organism is the main characteristic of *Bartonella* sp. infections and the severity of clinical symptoms is associated to the host immunological status (Vayssier-Taussat et al., 2009). Species of this genus are predominantly transmitted via an arthropod bite (body lice for *B. quintana* - the agent of Trench fever, fleas, and ticks for *Bartonella henselae*) (Quebatte and Dehio, 2019).

The *Bartonella* species described to date are capable of infecting a small number of mammalian species. Some hosts, such as cats (*B. henselae, B. clarridgeiae*, and *B. khoelerae*) and rodents (*Myodes glareolus, Apodemus sylvaticus*) can carry at least four different *Bartonella* species (Vayssier-Taussat et al., 2009). The genus *Bartonella* comprises pathogenic species that cause emerging infections in humans and is also a presumed tick-borne agent. Numerous studies have demonstrated the presence of different species in *I. ricinus* ticks (La Scola et al., 2004; Cotte et al., 2008; Quarsten et al., 2015; Sormunen et al., 2016).

In Romania, data concerning *Bartonella* infection are limited. Nevertheless, a recent study reported the detection of the zoonotic *Bartonella vinsonii berkhoffii* in a tick collected from a dog (Andersson et al., 2018b).

Candidatus Neoehrlichia mikurensis

Candidatus Neoehrlichia mikurensis was identified in *I. ricinus* ticks in the Netherlands (Schouls et al., 1999) and has been designated as a novel member of the *Anaplasmataceae* family, as an agent similar to *Ehrlichia* sp. (Kawahara et al., 2004).

The agent was detected in ticks and small wild mammals in Europe and Asia (Rar et al., 2010; Andersson and Raberg, 2011) and was been reported to infect humans, particularly immunocompromised patients in Europe (Fehr et al., 2010; von Loewenich et al., 2010).

For now, there is limited information available on the circulation of *C. N. mikurensis* in Romania. The agent has been identified in *I. ricinus* nymph collected from a patient (Andersson et al., 2014) and another study reported an infection rate of 5.3%, after testing 468 *I. ricinus* ticks (Kalmar et al., 2016). The bacteria was also detected in *I. ricinus* ticks from Tulcea and Iasi counties (Raileanu et al., 2018), with the highest prevalence in Tulcea County.

CHAPTER III
THE MAIN ZOONOTIC VIRUSES TRANSMITTED BY TICKS

The recognized number of major diseases transmitted by ticks has been growing over the past 30 years (Paddock, 2011). Ticks harbor a wide variety of microorganisms, such as viruses, bacteria, nematodes, fungi and protozoa (Jongejan and Uilenberg, 2004). Approximately 160 arboviruses have been found in ticks to date, with about 25% of those linked to human and animal diseases (Kazimirova et al., 2017).

Generally, arboviruses are grouped into nine viral families: one family of DNA viruses (*Asfarviridae*) and eight families of RNA viruses (*Orthomyxoviridae, Flaviviridae, Reoviridae, Nairoviridae, Rhabdoviridae, Nyamiviridae, Phenuiviridae* and *Peribunyaviridae*) (Kazimirova et al., 2017).

Tick-borne viral diseases have a significant and increasing medical and veterinary impact, due to the geographical spread of the vectors and outbreaks in new regions as a result of changes in global socio-economic, climatic conditions and lack of efficient control measures (Labuda and Nuttall, 2004). For most tick-borne viruses, there are currently no effective vaccines or therapeutics to stop the evolution of infections.

3.1. Tick-borne encephalitis virus (TBEV)

Tick-borne encephalitis (TBE) is a severe viral infection of the central nervous system caused by a *Flaviviridae* family enveloped virus. Tick-borne encephalitis virus (TBEV) infection was first reported in humans in Europe in the early 1930s and more than 3.000 cases are reported per year (Knap and Avsic-Zupanc, 2015).

Etiology

The capsid (C), the membrane (M), and the envelope (E) are the three structural proteins that compose the mature virion. The envelope protein is the major antigen that induces the production of neutralizing antibodies (Kaiser, 2016). According to genetic analysis, TBEV has three closely genetically related subtypes: European (Eu), Siberian (Sib) and Far Eastern (FE).

Epidemiology

TBE is endemic in Europe, Siberia, Far-Eastern Russia, Northern China and Japan (Figure 3.1.1). Over the past few decades, endemic regions have expanded and within many endemic areas the number of reported cases increased. Every year, between 10.000 and 15.000 TBE cases (Figure 3.1.1) are reported in Asia and Europe (Dobler, 2010a). TBE is a reportable disease only in 16 countries, but endemic in 27 European countries (Mantke et al., 2011).

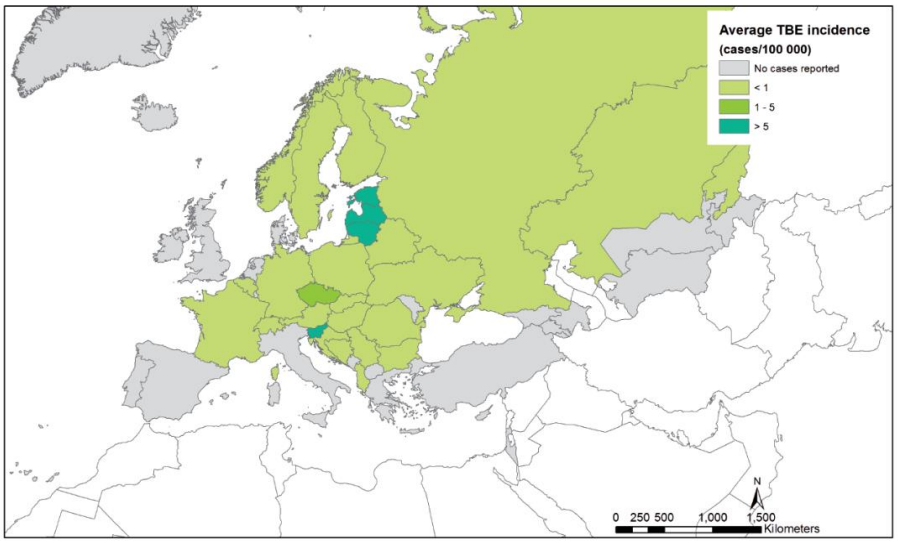


Figure 3.1.1- Average TBE incidence as reported to WHO Centralized Information System for Infectious Diseases (CISID) between 1990-2010 (World Health Organization)

Rodents serve as both hosts and key reservoirs in the viral transmission cycle. Large mammals such as deer, goats, sheep and cattle, have a shorter viremia than small mammals, hence this mode of transmission via ungulates has a limited role in TBEV maintenance (Jaenson et al., 2018). Humans do not play any role in the maintenance of TBEV in nature and they are only accidental hosts. TBEV is transmitted to humans mostly through Ixodid tick bites; the major vector in Europe is *I. ricinus*, while the main vector in Eastern Europe, Russia and Far-Eastern Asia is *I. persulcatus* (Kaiser, 2016). About 1% of all humans TBEV infections are probably acquired by consuming infected unpasteurized milk or milk products from infected livestock, particularly goats (Mansfield et al., 2009). Some cases of laboratory- acquired TBEV infections have been documented in the literature (Avsic-Zupanc et al., 1995).

TBEV infection is most common from April to November, which correlates to tick activity (Dumpis et al., 1999). In all age groups, men are affected more frequently than women (Logar et al., 2006). With the development of tourism, TBE has become a worldwide problem. As a consequence, it should be considered in the differential diagnosis of central nervous system infections, not only in patients living in endemic areas but also in patients living outside of endemic areas with adequate epidemiological background.

Regarding **human infection**, the highest proportion of cases are represented by asymptomatic forms. The incubation period ranges from 2 to 28 days and in the case of oral transmission, the incubation period is reduced to 3 to 4 days. The early phase of the disease is associated with viremia and non-specific signs (fever, headache, myalgia and arthralgia, fatigue, anorexia). In the second phase, about 50% of patients show signs of meningitis, 40% of meningoencephalitis and 10% of patients develop meningoencephalomyelitis (Bogovic and Strle, 2015). The fatality rate for the European subtype is 1-2% and up to 50% of patients have long term sequelae (Bogovic and Strle, 2015).

Ruminants such as goats, sheep and cattle, may be sporadically infected, presenting subclinical manifestations (Holzmann et al., 2009). In dogs, clinical signs are severe, with exclusively fatal outcome. The most common manifestations are hyperthermia, behavioral changes, paralysis, ataxia, myoclonus, vestibular syndrome (strabismus) and nystagmus (Pfeffer and Dobler, 2011).

State of knowledge in Romania

TBE is sporadic in Romania (Figure 3.1.2) and information on its epidemiology is relatively limited. There is no routine screening and the risk of contracting the disease is unknown. Although the first research on the prevalence of arboviruses in Romania was published in 1957 and the first information on regional morbidity was reported in the 1960s, tick-borne encephalitis surveillance has been implemented in 2008 and is carried out in Cluj Public Health Department's counties.

Between 1985 and 1993, a seroepidemiological study was conducted in 19 counties in Romania's northwestern region and in Bucharest, monitoring the seroprevalence of TBEV infection in humans (healthy people and people at risk such as forest workers) and domestic animals (cattle, goats, sheep). Seroprevalence rates in human samples ranged from 1.1% (Constanta County) to 19.4% (Maramures County), with an average 6.5%. A more recent study from 2016, demonstrated that sheep used as sentinels for this disease had significant antibody prevalence (15.02%) in 80% of examined localities (5 counties of Northwestern Romania) (Salat et al., 2017). The most recent data from the National Communicable Disease Surveillance Centre dates from 2013, with three cases reported in Maramureș and Sibiu.

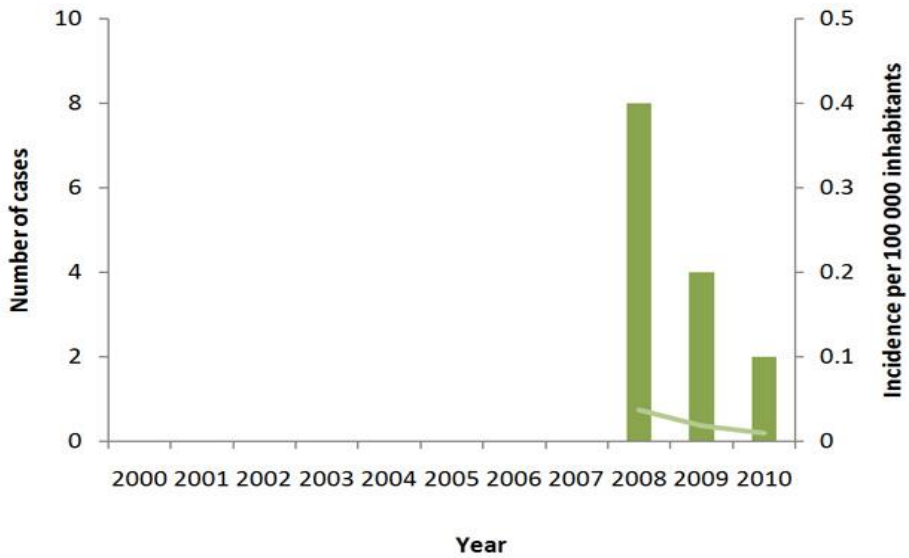


Figure 3.1.2- Number of TBE cases and incidence per 100.000 inhabitants by year in Romania (European Centre for Disease Prevention and Control, 2012)

3.2. Crimean-Congo hemorrhagic fever

Crimean-Congo hemorrhagic fever virus (CCHFV) is a tick-borne virus that causes moderate to severe hemorrhagic disease in humans (up to 40%). The name is derived from the regions where it was first reported (Crimea, in 1945 and Congo, in 1956). CCHFV is transmitted to humans by tick bites. *Hyalomma* species are the principal vector (Tahmasebi et al., 2010). However, it can also be transmitted to humans by contact with infected animals' blood or tissue, including human-to-human transmission in nosocomial situations (Figure 3.2.1) (Chinikar et al., 2013).

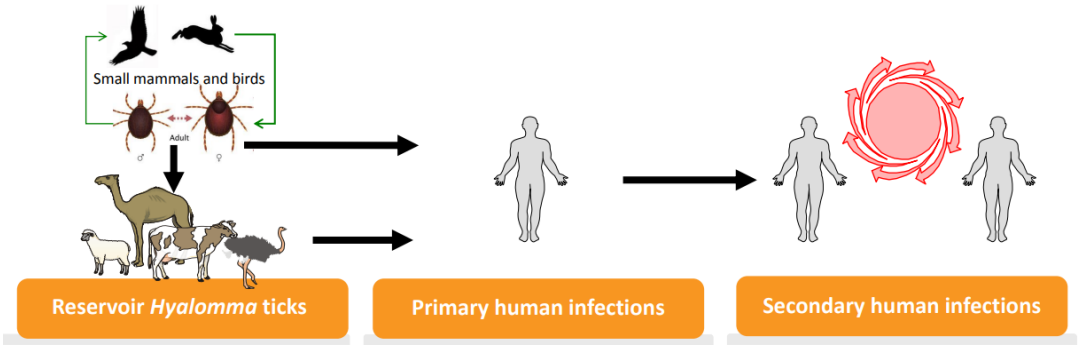


Figure 3.2.1- Crimean-Congo haemorrhagic fever transmission
(World Health Organization)

Etiology

CCHFV is a member of the order *Bunyavirales*, *Nairoviridae* family and *Orthonairovirus* genus. Based on antigenic connections, the 41 species of the genus are divided into 7 serogroups (Whitehouse, 2004). The viral genome is trisegmented and is comprised of single-stranded negative-sense RNA. The small (S) segment has a single open reading frame encoding the nucleocapsid (N) protein. The medium (M) segment encodes a large glycoprotein precursor which is processed into the two transmembrane surface glycoproteins and several non-structural proteins. The large (L) segment encodes a single L protein-RNA polymerase (Figure 3.2.2).

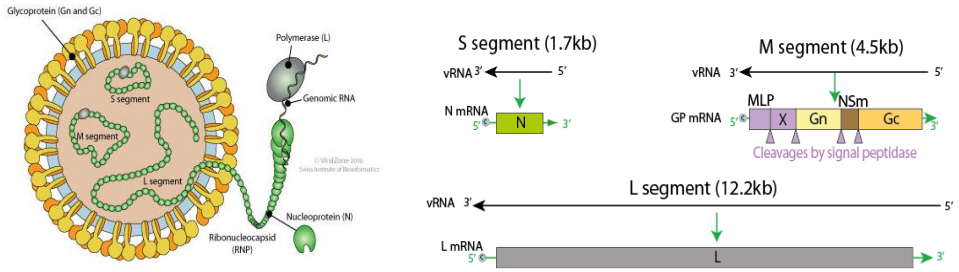


Figure 3.2.2- *Orthonairovirus* genome organization and it's structural and non-structural proteins
(viralzone.expasy.org)

Epidemiology

CCHFV is widely spread around the world: Asia (Al-Abri et al., 2017), Africa (Temur et al., 2021) and Europe (Albania, Bulgaria, Turkey, Greece, Georgia, Russia, Kosovo, Spain) (Maltezou et al., 2010). Until now, CCHF has never been reported in Northern Europe, Australia, or America (Figure 3.2.3).

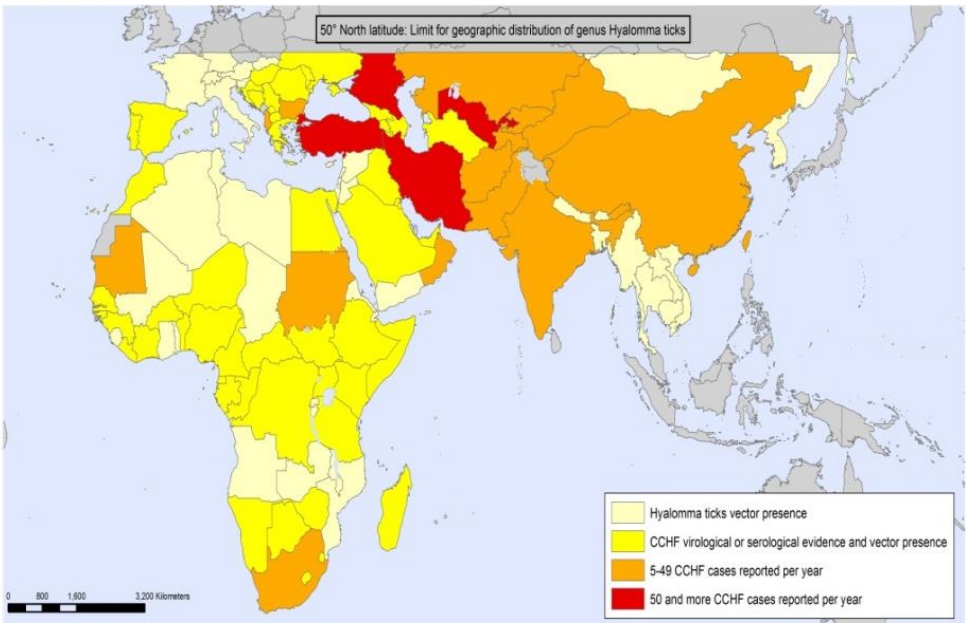


Figure 3.2.3- Geographic distribution of Crimean-Congo haemorrhagic fever
(World Health Organization, 2017)

CCHF in Europe

Between 1953 and 1974, numerous CCHF cases were detected in Bulgaria and the rate fatality was around 17%. During this period, 20 nosocomial infections were reported. Between 1975 and 1996 the number of reported CCHF cases decreased to 11.4%. The CCHFV strains from Bulgaria were found to cluster with other Balkan strains from Kosovo and Albania (Papa et al., 2004). In Northern Greece, the first fatal case of CCHF was discovered in 2008 (Maltezou et al., 2009). According to a survey conducted in Greece between 1981-1988, the seroprevalence of CCHFV was 1% among the population surveyed (Osherovich et al., 2001).

Between 1995-2004, there were three major outbreaks, with 186 serologically confirmed cases (Ahmeti and Raka, 2006). A study suggested that CCHFV was recently introduced to Kosovo, sharing a common ancestor with strains from Turkey (Emmerich et al., 2018). In 2010, CCHFV was discovered in *Hyalomma* ticks in Spain (Estrada-Pena et al., 2012). In August 2016, two autochthonous CCHF cases were identified for the first time (Ramirez de Arellano et al., 2017).

In humans and other mammals, the infection is systemic and CCHFV can be found in blood, body fluids, and other tissues. In humans, horizontal transmission from mother to child has been confirmed (Saijo et al., 2004). The disease has not been linked to airborne transmission

(Saijo et al., 2004). The two most common routes of human infection are direct transmission by contact with infected tissues or tick bites. In livestock, the infection is usually asymptomatic or may occasionally result in mild fever (Swanepoel et al., 1985).

State of knowledge in Romania

To date, no case of CCHFV has been diagnosed in humans in Romania. Only a few serological studies with limited data regarding the CCHFV circulation are available. In 2012, Ceianu et al. tested 471 sheep serum samples from different localities in Tulcea County, obtaining a prevalence of 27.8% for IgG antibodies against CCHFV (Ceianu et al., 2012). However, another study from 2015 (Răileanu C. et al., 2015), reported an overall prevalence of 74% IgG antibodies among 90 domestic ruminants from Tulcea and Constanța counties.

3.3. Other tick-borne flaviviruses

Louping-ill virus belongs to the viral complex spread by *I. ricinus* ticks in Western Europe and is classified as a *Flavivirus* (Table 3.3.1). According to current knowledge, Louping-ill virus is prevalent on the British Island, Ireland, the Iberian Peninsula, Turkey, Bulgaria, and possibly in Japan. In humans, the disease has a biphasic course. The first phase is characterized by fever, retro-orbital pain and headache. In the second phase, nervous symptoms appear in the form of meningoencephalitis or paralytic poliomyelitis. Infection can also be seen in animals in enzootic areas, with sheep and cattle affected after an incubation period of 6-18 days. The dominant clinical signs are fever, motor incoordination, tremors, hypersalivation and lethargy (Dobler, 2010b).

Kyasanur Forest virus produces haemorrhagic fever in India, where rodents and monkeys are common hosts for the virus. The vectors for this virus are nymph ticks of the genus *Haemaphysalis* (Table 3.3.1) (Tandale et al., 2015). In humans, the fever can be followed by hemorrhagic symptoms such as gastrointestinal bleeding or bloody sputum and haemoptysis. In animals, only monkeys have been found to have clinical illness after infection. Langurs and bonnet monkeys develop a severe disease similar to humans (mortality rates of up to 85%). No clinical illness has been detected so far in other wild or domestic animals (cattle, sheep, goats, dogs and cats) (Dobler, 2010b).

Omsk haemorrhagic fever virus causes acute viral infection in the lake areas of western Siberia (Omsk, Novosibirsk, Kurgan and Tyumen) where the natural reservoir is represented by rodents and the tick vector can be *Dermacentor reticulatus, Dermacentor marginatus*, *Dermacentor pictus* and *I. persulcatus* (Table 3.3.1). The disease in humans is manifested by clinical signs of fever, migraine, dizziness, oral vesicular lesions, severe muscle pain, coughing and prostration that can last 1-2 weeks with the possibility of worsening of the general condition to pneumonia, meningitis, severe haemorrhages of the respiratory, digestive and genital systems, nephrosis, with death occurring in 2-10% of cases (Dobler, 2010b).

The causative agent of **Powassan encephalitis** is an RNA virus of the family *Flaviviridae*, genus *Flavivirus*, which is spread throughout the northern hemisphere (including Canada, the United

States, and Russia), transmitted by *D. andersoni, D. variabilis, Ixodes cookie*, and *Ixodes scapularis*. After an incubation period of 8 to 34 days, Powassan encephalitis virus infects humans causing fever, mydriasis, disorientation, vomiting, prostration, dyspnoea, and spastic paresis. Meningoencephalitis and meningitis are possible complications. Animals may show a subclinical course, but horses and wild animals may develop viremia and neurological signs (Dobler, 2010b).

Table 3.3.1

Tick-borne viruses of the family *Flaviviridae*, genus *Flavivirus*
(After P. A. Nuttall, 2004)

Virus group, species and subtype	Main tick vector species	Geographical distribution
Mammalian tick-borne group		
Kadam virus	*R. pravus*	Saudi Arabia, Uganda
Kyasanur Forest disease virus	*H. spinigera*	India
Alkhurma virus	*Unknown*	Saudi Arabia
Langat virus	*I. granulatus*	Malaysia
Louping ill virus	*I. ricinus*	Ireland, England, Scotland, Wales, Spain, Turkey
Omsk haemorrhagic fever virus	*D. reticulatus*	Western Siberia
Powassan virus	*I. cookei*	Canada, USA, Russia
Royal Farm virus	*Argas hermanni*	Afghanistan
Tick-borne encephalitis virus		
European subtype	*I. ricinus*	Northern Europe
Far Eastern subtype	*I. persulcatus*	Northern Asia
Siberian subtype	*I. persulcatus?*	Siberia
Seabird tick-borne group		
Gadgets Gully virus	*I.uriae*	Australia
Meaban virus	*O. maritimus*	France
Saumarez Reef virus	*O. capensis, I. eudyptidis*	Australia
Tyuleniy virus	*I. uriae*	Russia, USA, Norway
Mosquito-borne viruses		
West Nile virus	*O. maritimus, Argas hermanni Hyalomma spp.*	Russia,Azerbaijan, Turkmenistan

3.4. Recently identified tick-borne arboviruses

Several novel viruses have been discovered in arthropods during the last decade, and their characterization has provided new insights into RNA viral diversity. Tick-borne virus studies have primarily focused on arboviruses, that can affect both invertebrates and vertebrates and are responsible for severe human and animal diseases (Kazimirova et al., 2017).

Jingmen tick virus (JMTV) is a recently identified virus that shows an unexpected connection between segmented and unsegmented RNA viruses. JMTV was first identified in ticks from Jingmen city, in China, in 2010 (Qin et al., 2014). The JMTV genome comprises four segments of linear, positive-sense single-stranded RNA (segments S1, S2, S3, and S4, respectively). The segments S1 and S3 encode nonstructural proteins, while the segments S2 and S4 encode structural proteins. The segments S1 and S3 are related to the nonstructural protein genes of classic flaviviruses of the family *Flaviviridae*, the remaining two segments (S2 and S4) share no homology with viral sequences of any known viruses (Qin et al., 2014). JMTV is one of the most recently discovered viruses to provide an unexpected link between segmented and unsegmented RNA viruses.

JMTV has been identified in arthropods and mammals, including cattle and monkey sampled from Asia, Africa, Europe, and America, having an extraordinary diversity and a global geographic distribution. Very importantly, JMTV has been found in humans suffering from hemorrhagic fever in Kosovo (Emmerich et al., 2018). Human febrile illness related to JMTV and JMTV-like virus was also described in China (Jia et al., 2019). JMTV-infected patients were identified by high-throughput sequencing of skin biopsies and blood samples. The patients presented had painful eschar at the site of tick bite, lymphadenopathy, headache and asthenia. Also, the patients showed laboratory abnormalities such as a rise of AST and ALT levels and a low neutrophil count. All this data reveals the importance of Jingmenviruses in both virus evolution and public health.

Tacheng tick virus 2 (TATV2) is a new virus segmented negative-stranded RNA linear genome that missed the M segment coding for the viral glycoprotein. TATV2 is an emerging tick-borne virus that is a member of the genus *Uukuvirus*, in the *Phenuiviridae* family. TATV2 was identified in *Dermacentor marginatus* ticks from China and was also detected in one patient's blood with a history of tick bite (Dong et al., 2021) proving the potential risk for human health. TATV2 was also found in *R. sanguineus* ticks from Turkey (Brinkmann et al., 2018), Romania, (Bratuleanu et al., 2022a) and more recently in Southeastern Kazakhstan. These results also indicate the importance of this virus, which, for the moment, circulates in Eurasia.

CHAPTER IV
NEXT GENERATION SEQUENCING

In recent years, Next-generation Sequencing (NGS) approaches have revolutionized genomic medicine and enabled rapid diagnosis of several diseases. These approaches are widely used for pathogen detection in several infectious diseases and recent studies shed light on the viral diversity of ticks. NGS technologies are divided in two types (Table 4.1) (Shendure and Ji, 2008).

The first generation sequencing is represented by Sanger sequencing while the second generation sequencing technologies refer to the newest technologies developed in the NGS environment (Metzker, 2010). They are characterized by the need to prepare amplified sequencing banks, before starting the sequencing of amplified DNA clones (Mardis, 2008). In addition, there are the third generation sequencing technologies that are sequencing technologies appeared recently (Pareek et al., 2011).

Compared to the second generation, these technologies are classified as „Single Molecule Sequencing Technology" because they can make sequencing a single molecule without the necessity to create the amplification libraries and that are capable of generating longer reads in a shorter time, at much lower costs.

Table 4.1

Summary of NGS platforms
(After Kchouk M et al., 2017)

PLATFORM	YEAR
First generation	
ABI 3730 Sanger	2002
Second Generation	
454 pyrosequencing	2005-2014
Illumina	2011-2014
SOLiD	2011-2013
Ion Torrent	2011-2015
Third Generation	
PacBio	2011-2014

4.1. Illumina sequencing

The most used technology in the NGS market is Illumina technology approach, a sequencing by synthesis. The first step is represented by randomly fragmentation of the DNA samples into sequences and adapters are ligated to both ends of each sequence. These adaptors are then attached to complementary oligonucleotides fixed to a solid plate (Figure 4.1.1A). In the second step, each sequence attached to the solid plate is multiplied by PCR bridge amplification, which creates multiple identical copies of each sequence (a set of sequences made from the same original sequence is called a cluster). Each cluster contains approximately one million copies of

the same original sequence (Figure 4.1.1B). The last step is to determine each nucleotide in the sequences. Illumina uses the sequencing by synthesis approach that uses reversible terminators (Bentley et al., 2008), in which the four modified nucleotides, sequencing primers and DNA polymerases are added as a mix and the primers are hybridized to the sequences. Polymerases are used to extend the primers using the modified nucleotides. Each type of nucleotide is labeled with a fluorescent specific in order for each type to be unique: the nucleotides have an inactive 3'-hydroxyl group which ensures that only one nucleotide is incorporated. Clusters are excited by laser for emitting a light signal specific to each nucleotide, which will be detected by a coupled-charge device camera and computer programs will translate these signals into a nucleotide sequence (Figure 4.1.1C) (Reuter et al., 2015).

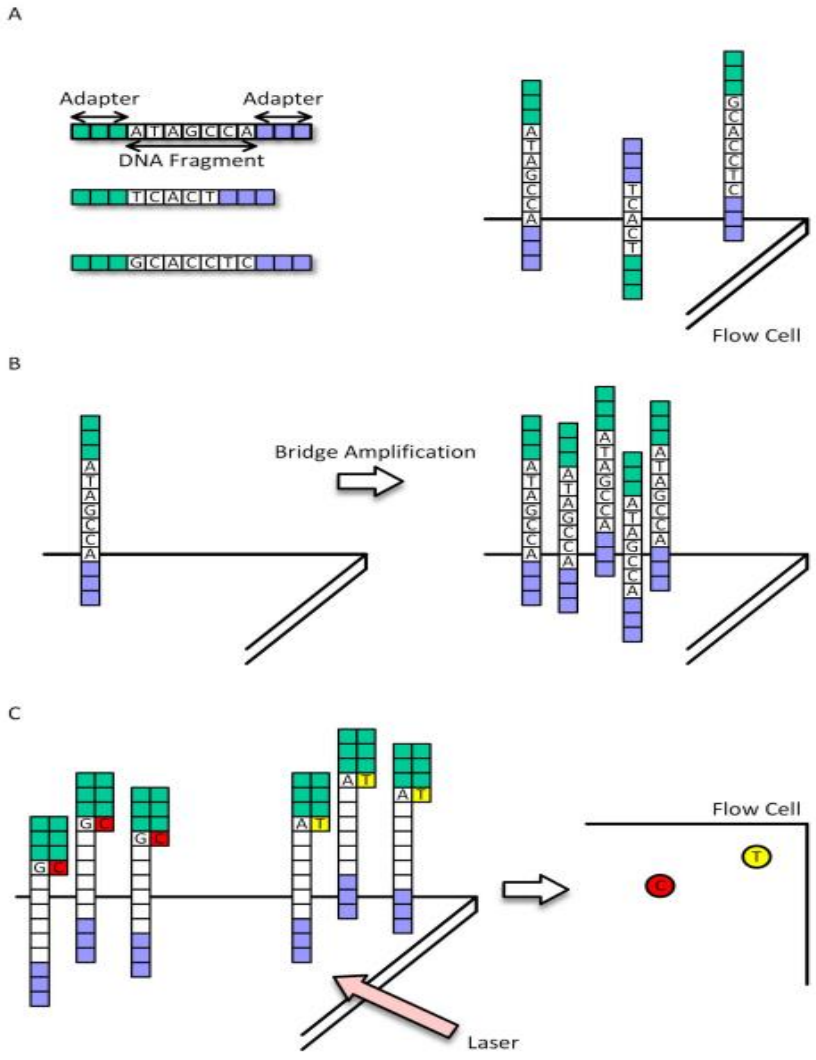


Figure 4.1.1- Illumina sequencing technology
(After Heo and Yun, 2015)

4.2. ABI/SOLiD sequencing

The SoliD sequencing are based on the technology of two-base sequencing, which includes cludes multiple sequencing rounds. The process starts by attaching adapters to the DNA fragments, fixed on beads and cloned by PCR emulsion. An octamer (8 bases, called 8-mer) probe is attached to each sequencing primer, fluorescently labeled due to the complementarity between the di-base of the template and the probe. The beads are placed on a glass slide and the 8-mer are ligated to DNA fragments and the color emitted by the label is registered. The output format is color space which is the encoded form of the nucleotide where four fluorescent colors are used to represent 16 possible combinations of two bases. The sequencer repeats this ligation cycle and each cycle the complementary strand is removed and a new sequencing cycle starts. The cycle is repeated until each base is sequenced twice. The color space data can then be decoded given prior knowledge of the leadingbase (Mardis, 2008).

4.3. Roche/454 sequencing

The 454 sequencing is a technology introduced in 2004, using pyrosequencing. DNA samples are randomly fragmented and each fragment is attached to a bead whose surfaces carries primers. In order to make each bead contain thousands of copies of the initial fragment, a PCR is conducted. Next, beads are arrayed into picotiter plate wells that fix each. In pyrosequencing, each incorporation of a nucleotide releases pyrophosphate, initiating a series of downstream reactions that produce light by luciferase. At each cycle during sequencing, a single type of nucleotide is added and the incorporation of the nucleotide, complementing the next base in the fragments on beads, releases light that is detected by a coupled-charge device camera (Liu et al., 2012).

4.4. Life Technologies Ion Torrent

Ion Torrent sequencing was introduced in 2010, being similar to 454 pyrosequencing technology but it does not use fluorescent labeled nucleotides. This technology uses a chip that has many wells on its surface, and each has a bead with multiple identical fragments. The chip is flooded with one type of nucleotide at each cycle. When a base is incorporated with a fragment in the bead, a hydrogen ion is released, changing the pH of the solution. The chip has Ion sensors for each well and used to detect how many nucleotides are incorporated (Loman et al., 2012).
The big advantages of this type of sequencing is the read lengths that are longer that the other sequencers and the much faster sequencing time (two up to eight hours) (Reuter et al., 2015).
The main disadvantage is the difficulty of interpreting the homopolymer sequences, that produces insertion and delation error.

4.5. Pacific Biotechnology SMRT

The most used thrid-generation sequencing technology was developed by Pacific Biosciences, by using SMRT (single molecule real-time) approach. It is considered a third-generation sequencing (TGS) technology because it does not need any amplification, before sequencing. A structure composed by SMRT cells containing microfabricated nanostructures, called zeromode waveguides (ZMWs) is used. ZMWs are wells of tens of nanometers fabricated

in a metal film that are deposited onto a glass substrate (McCoy et al., 2014; Rhoads and Au, 2015). The wavelength of the light excited from the bottom of the ZMW cannot pass through because the ZMV is very small and the light can penetrate the lower 20-30 nm of the ZMW. A molecule of a single-stranded DNA template and a DNA polymerase at the bottom are available for each ZMW. Nucleotides attached with four corresponding fluorescent dye molecules are introduced as the DNA polymerase performs the synthesis of DNA. Light is produced while a nucleotide is held in the detection volume by the incorporation process and after that is recorded in a movie format.

4.6. Oxford Nanopore

Oxford Nanopore produces a new generation of DNA/RNA sequencing technology. The first commercial machine was introduced in 2012. Nanopore is a nanoscale hole formed from proteins or synthetic material. An ionic current passes across a nanopore by setting up a voltage on the nanopore membrane. If a single-stranded DNA sequence passes through the pore, a characteristic disruption appears in the ionic current. Measuring the current makes it possible to identify the nucleotide in question (Liu et al., 2012).

PART II
PERSONAL CONTRIBUTIONS

CHAPTER V
DESCRIPTION OF THE ORGANIZATIONAL AND INSTITUTIONAL FRAMEWORK WHERE THE RESEARCH WAS DEVELOPED

Research activities were carried out in:

- Regional center of advanced research for emerging diseases, zoonoses and food safety (Rovetemerg), University of Life Sciences, Iasi, Romania
- Faculty of Veterinary Medicine, Iasi, Romania
- Pathogen Discovery Laboratory, Institut Pasteur, Paris, France

Considering the importance of tick-borne diseases, a collaboration has been established between the Regional center of advanced research for emerging diseases, zoonoses and food safety (Rovetemerg), Iasi, Romania and Pathogen Discovery Laboratory, Institut Pasteur, Paris, France.

The research performed in this thesis was conducted between 2018-2022. Serological studies performed on dogs, small ruminants, and entomology activities (genus level identification and storage of collected ticks) were performed at the University of Life Sciences, Iasi, Romania.

From September 2019 to May 2022, the PhD work was carried out at the "Pathogen discovery" laboratory, Institute Pasteur, Paris, France.

The objective of this laboratory is to discover, characterize and demonstrate the imputability of new or unexpected infectious agents in clinical syndromes of unknown etiology, including agents of zoonoses.

At Institut Pasteur were performed all the activities that required the presence of facilities in a BSL-3 (biosafety level 3 laboratory), such as RNA extractions from tick pools or serum samples and molecular biology activities (RT-PCR, qPCR, genome finishing of identified viruses), NGS libraries, NGS sequencing, interpretation and bioinformatics analysis of the obtained data and Sanger sequencing.

The viral isolation step on cell cultures and embryonated eggs was conducted in the laboratory "Molecular Genetics of RNA Viruses" Institut Pasteur, Paris, France.

CHAPTER VI
OUTLINE AND OBJECTIVES OF THE THESIS

The ability of ticks to transmit a wide range of microbial pathogens, combined with their promiscuous feeding and geographical range expansion, makes them a substantial threat to animal and human health. Among ixodid ticks, tick-borne diseases are transmitted mainly by the following families: *Ixodes, Haemaphysalis, Hyalomma, Amblyomma, Dermacentor, Rhipicephalus*, and *Boophilus.*

Tick microbiome consists of communities of bacteria, viruses and eukaryotes, in which several pathogens coexist in the commensal flora. Infectious agents of medical importance comprise Crimean-Congo hemorrhagic fever virus (CCHFV), tick-borne encephalitis virus (TBEV), severe fever with thrombocytopenia syndrome virus (SFTSV), Kyasanur Forest disease virus (KFDV), Alkhurma virus (ALKV) and Heartland virus (HRTV); and species of bacteria within the genera *Anaplasma, Borrelia, Coxiella, Ehrlichia, Francisella, Rickettsia* and parasites as *Theileria*. Similarly, several tick-borne viruses also threaten the health of livestock: these include Nairobi sheep disease virus (NSDV), Africa swine fever virus (ASFV) and Louping ill virus (LIV), among others.

Little is known about ticks microbiome despite the growing number of studies in the last few years. However, taking advantage of the rapid development of next-generation sequencing (NGS) methods, many new viral sequences have been identified in ticks of different species spread in diverse parts of the world.

Romania, due to its geopolitical location at the Eastern border of the European Union, is an epidemiologically important territory and it may represent a continuous risk of infectious diseases emergence. Currently, tick-borne infections in our country are still under evaluation to determine their epidemiological status. There is a need for surveillance programs since tick-borne infectious agents can easily be spread throughout the continent.

The main objectives of thesis were:

- To assess the seroprevalence of the main vector-borne infections in animals in South-Eastern Romania. To this purpose, three seroepidemiological surveys were carried out; The first eroepidemiological survey aimed to identify IgG antibodies against *Borrelia* spp., *A. phagocytophilum* and *E. canis* in dogs from public shelters and in dogs examined in veterinary private clinics diagnosed with babesiosis, from Braila and Iasi counties. The second seroepidemiological survey monitored the detection of IgG antibodies against *A. phagocytophilum* in domestic and hunting dogs from Southern Transilvania. The latest seroepidemiological survey aimed to detect IgG antibodies against Crimean-Congo haemorrhagic fever virus in sera of small ruminants.
- To survey the circulation of Crimean-Congo haemorrhagic fever virus in Romanian ticks using molecular biology techniques. For this purpose, Real Time RT-qPCR based on SybrGreen with specific primer sets for the detection of all the six known CCHFV genotypes in questing and engorged ticks were used.
- To identify novel arboviruses transmitted by ticks. For this purpose, NGS techniques were first used to characterize the microbiome diversity of ticks collected from poorly investigated areas of Romania, followed by the search for antibodies against viral proteins in exposed animals using high-throughput serological technique (LIPS).

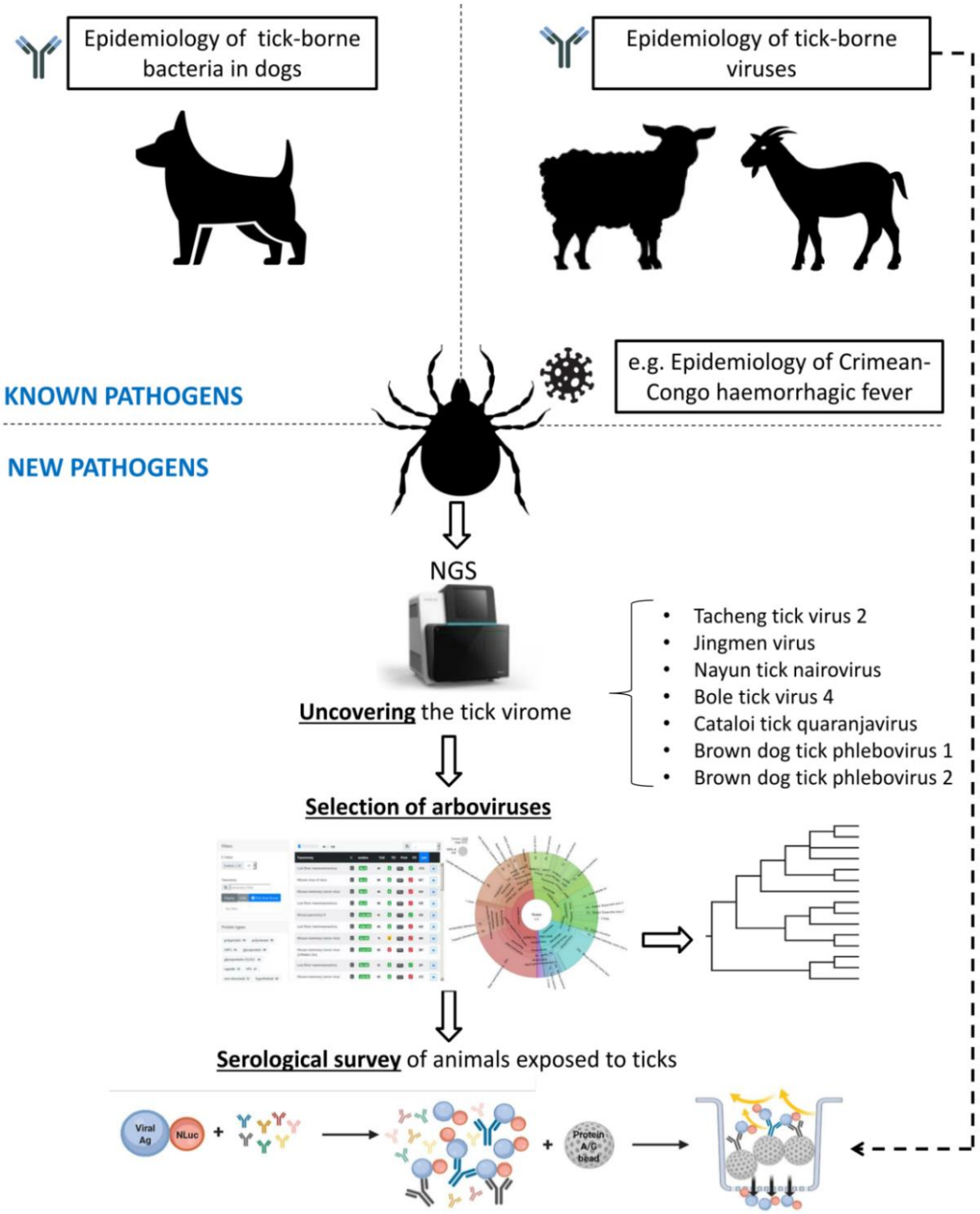


Figure 6.1- Schematic representation of the thesis objectives (original)

CHAPTER VII
SEROEPIDEMIOLOGICAL AND MOLECULAR INVESTIGATION REGARDING THE MAIN PATHOGENS TRANSMITTED BY TICKS TO ANIMALS

7.1. Serological surveys regarding bacterial tick-borne diseases

7.1.1. Serological survey of Lyme disease, anaplasmosis and ehrlichiosis in dogs in two counties from South-Eastern Romania

Study objectives

There is not yet sufficient data on vector-borne diseases in Romania. Serological studies in dogs as sentinels of *Borrelia* spp., *A. phagocytophilum* and *E. canis* provide a valuable perspective in the identification of possible risk areas for humans. The aim of this study was to evaluate the presence of IgG antibodies against *B. burgdorferi*, *A.phagocytophilum* and *E.canis* in dogs in Eastern Romania, in 2019.

Introduction

In recent years, canine vector-borne diseases (CVBD) have become a subject of interest (Parashar et al., 2016). *Anaplasma phagocytophilum, Borrelia burgdorferi* and *Ehrlichia canis* are the most common CVBDs in dogs, representing an increasing problem in veterinary medicine.

In Europe, Lyme disease represents a significant health risk for dogs. Dogs seem to be the most efficient sentinels, as well as a useful animal model for investigating Lyme disease in humans (Krupka and Straubinger, 2010). Lameness due to myositis or arthritis is the most common symptom in dogs, and it usually occurs several weeks or months after infection. However, because active infection does not always induce disease, most dogs do not present clinical signs.

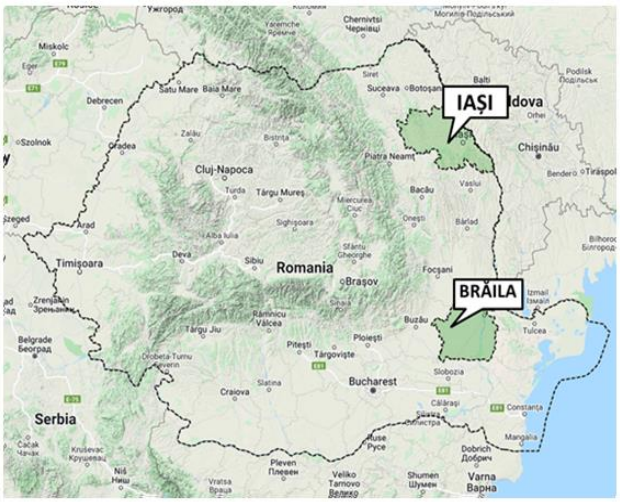
Ixodes ricinus ticks transmit *A.phagocytophilum*, which causes granulocytic anaplasmosis. The first dog cases were recorded in 1982 in the United States and in 1988 in Europe (Madewell and Gribble, 1982). Clinical manifestations usually comprise lethargy, anorexia, lameness, fever, thrombocytopenia, hypoalbuminemia, lymphopenia (Lester et al., 2005) and elevated liver enzyme levels (Chirek et al., 2018). In Romania, Matei et al. (2015) have reported a prevalence in *Ixodes ricinus* of 3.4% for *A. phagocytophilum* after screening more than 10.000 ticks using molecular biology techniques (Matei et al., 2015).

E. canis is transmitted by *R. sanguineus*, which causes monocytic ehrlichiosis (Groves et al., 1975). The infection was first reported in Algeria in 1935. Depression, pyrexia, pale mucosa, epistaxis and melena are the most frequent clinical signs in dogs that might suggest ehrlichiosis. Epistaxis is the most common symptom of ehrlichiosis suspicion among them (Parashar et al., 2016).

Materials and methods

Between March and May 2019, a total of 92 dog serum samples were obtained from two counties in Eastern Romania (Iași and Braila) (Figure 7.1.1.1). Details concerning gender, age, origin, and living conditions were registered for each dog. In the study were included dogs aged between 4 months and 16 years. No formal randomized selection of dogs was applied and none of them were vaccinated against Lyme disease.

The samples belonged to dogs from public shelters and from private veterinary clinics. Dog sera were screened using commercial enzyme-linked immunosorbent assays for specific IgG class antibodies against *Borrelia* VIsE antigen, *Anaplasma phagocytophilum* and *Ehrlichia canis* (Euroimmun 23560 Lübeck, Germany), respectively.



County	No of samples		
	Males	Females	Total
Iași	41	31	72
Brăila	12	8	20
Total	53	39	92

Figure 7.1.1.1- Distribution and number of collected serum samples (original)

Results and discussion

Overall, 14 out of 92 tested dogs were detected seropositive for specific IgG antibodies, among which 6 for *Borrelia* spp. (6.5%), 5 for *Anaplasma phagocytophilum* (5.4%) and 3 for *Ehrlichia canis* (3.2%). Simultaneous detection of IgG antibodies against *B. burgdorferi* and *A. phagocytophilum* was registered in 3 dogs (3.2%). One of the three dogs was detected with a borderline result for IgG *E. canis* antibodies. Simultaneous detection of IgG antibodies against *A. phagocytophilum* and *E. canis* was registered in one dog (1%). Details related to age, gender and origin of the seropositive dogs are presented in Table 7.1.1.1. Correlating the obtained results with the gender, the higher seroprevalence was recorded in females (Figure 7.1.1.2).

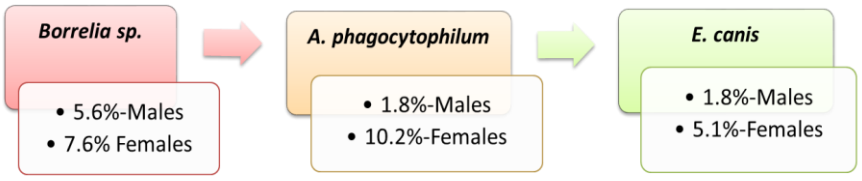


Figure 7.1.1.2- Schematic relationship between gender and registered seroprevalence for *Borrelia* sp., *A. phagocytophilum* and *E. canis*

Table 7.1.1.1

Identification of seropositive dogs from Iasi and Braila counties

Gender and age	County	*Borrelia burgdorferi* IgG antibodies	*Anaplasma phagocytophilum* IgG antibodies	*Ehrlichia canis* IgG antibodies
Female, 12 years	Iași	+	-	-
Male, 1 year	Brăila	+	-	-
Male, 6 years	Iași	+	-	-
Male, 4 years	Iași	+	+	±*
Female, 1 year	Iași	+	+	-
Female, 3 years	Iași	+	+	-
Female, 2 years	Iași	-	+	-
Female, 2 years	Iași	-	+	+
Female, 5 years	Iași	-	-	+
Male, 4 years	Brăila	-	-	+

*Legend: +: Positive; -: Negative; ±: Borderline

The global distribution of vector-borne diseases in dogs has been expanding in recent years. The increased risk of contracting vector-borne diseases is due to a number of factors. Outdoor activity is the major factor. The difference in seroprevalence could be attributed to differences in tasks performed by dogs and therefore the different tick exposure.

Our findings present similar results to previous research on *B. burgdorferi* prevalence in dogs and horses (Kiss et al., 2011). Nevertheless, a more recent study performed by Raileanu et al., reported a lower prevalence of *B. burgdorferi*, in Eastern Romania compared to our results. These findings indicates that the prevalence of *B. burgdorferi* in dogs has increased in the research area. *B. burgdoferi* antibodies had the highest seroprevalence in our research.

The significant number of samples from public shelter dogs, which did not receive any prophylactic repellents or acaricides for protection, could explain these results. Even if the highest prevalence was registered in females, research studies showed that antibody prevalence is not associated with the gender, season or size of the dog (Bhide et al., 2004).

In Europe, several investigations using various diagnostic approaches have reported the occurrence of CVBDs (Cardoso et al., 2012; Alho et al., 2016; Mrljak et al., 2017). In 2015, a study conducted in Bulgaria, showed a similar prevalence of *B. burgdorferi* (2.4%) but a considerably higher prevalence of *E. canis* (21%) and *A. phagocytophilum* (46.1%) (Parashar et al., 2016). Furthermore, the seroprevalence rates registered in dogs from Italy were lower for *A. phagocytophilum* (3.31%) and higher for *E. canis* (16.8%).

These results could be linked to the climatic conditions, which are known to strongly influence the abundance of ticks (Cardoso et al., 2012). Other studies from the Balkan Peninsula

have reported an overall prevalence of CVBDs ranging from 25.7% in Croatia to 25.1% in Albania (18), with Spain (37%) (Miro et al., 2013) and Portugal (66%) having the greatest prevalence rates (Alho et al., 2016).

Conclusions

- Canine vector-borne diseases (CVBD) are a significant concern for veterinarians in Romania. The emergence of parasitic and zoonotic diseases as a result of global warming, increased pet travel, and vector expansion are all likely to have a significant impact on the transmission of CVBD agents in our country.
- Despite the fact that veterinarians inform dog owners about the risk of tick-borne pathogens and tick prophylaxis is used, infections with *B. burgdorferi, E. canis*, and *A. phagocytophilum* are present in Eastern Romania.

7.1.2. Seroepidemiological survey of anaplasmosis in domestic and hunting dogs

Study objectives

Serological surveys performed on dogs for the detection of specific antibodies against *A. phagocytophilum* offer a promising perspective identifying the potential risk regions for humans and animals. This study aimed to investigate the seroprevalence of *A. phagocytophilum* IgG antibodies in dogs from three counties (Iasi, Sibiu, and Tulcea) between 2015- 2019.

Introduction

A. phagocytophilum, also known as *Ehrlichia phagocytophila* or *Ehrlichia equi* is a pathogen of humans, horses and dogs worldwide. *A. phagocytophilum* is a Gram-negative, obligate intracytoplasmic coccus belonging to *Anaplasmataceae* family, *Rickettsiales* order. *A. phagocytophilum* mostly affects neutrophilic and rarely eosinophilic granulocytes (Dumler et al., 2001) and it is transmitted by *Ixodes* ticks*: Ixodes ricinus* in Europe (Blanco and Oteo, 2002), or *Ixodes scapularis* and *Ixodes pacificus* in the United States (Bakken et al., 1994). In dogs, the diagnosis is based on serological detection of antibodies using ELISA, indirect immunofluorescence or rapid tests. PCR detection is regularly used, but the sensitivity of direct detection depends on the stage of infection. However, antibody detection, therefore, remains a method of choice for laboratory diagnosis of canine granulocytic anaplasmosis.

Materials and methods

The population consisted of 92 dogs, of which 17 hunting dogs from Agnita (Sibiu County), 26 dogs kept in shelters in Tulcea County, and 43 dogs from Iasi County (Table 7.1.2.1). Dog's age spectrum varied from 3 months to 13 years. Details regarding age, origin, and living conditions were also collected. The samples were tested using commercial enzyme-linked immunosorbent assays for the detection of IgG anti-*Anaplasma phagocytophilum* antibodies (Euroimmun 23560 Lübeck, Germany).

Table 7.1.2.1

Distribution of the tested dogs by county

County	**Number of samples**		
	Males	Females	Total
Sibiu	12	5	17
Tulcea	20	6	26
Iași	30	19	49
Total	62	30	92

Results and discussion

This study showed an overall prevalence of 14% (13 out of 92 tested dogs were identified as seropositive). Hunting dogs from Agnita (Sibiu County) recorded the highest prevalence, 10 out of 17 (58.8%) dogs were positive. In Tulcea County, 2 out of 26 (7.7%) samples were seropositive and, in Iasi County, where *A.phagocytophilum* IgG prevalence was the lowest, 1 out 49 (2%) samples were detected as seropositive. Correlating the obtained results with gender, 8 out of 62 males (12.9%) and 5 out of 30 females (16.7%) were *A. phagocytophilum* IgG positive. Two dogs from Iași County and one from Sibiu County were identified with a borderline result (Table 7.1.2.2).

Table 7.1.2.2

Table 7.1.2.2- Signalment and origin of the *A. phagocytophilum* IgG positive dogs

Gender and age	**County**	***A. phagocytophilum* IgG antibodies**
Female,13 years	Agnita-Sibiu	+
Female, 7 years	Agnita-Sibiu	+
Female,2 years	Agnita-Sibiu	+
Male, 2 years	Agnita-Sibiu	+
Male, 3 years	Agnita-Sibiu	+
Female, 9 years	Agnita-Sibiu	+
Male, 4 years	Agnita-Sibiu	+
Male, 10 years	Agnita-Sibiu	+
Female, 3 years	Agnita-Sibiu	+
Male, 8 years	Agnita-Sibiu	+
Male, 7 years	Agnita-Sibiu	±
Male, 1 year	Tomesti-Iasi	+
Male, 3 years	Tomesti-Iasi	±
Female, 2 years	Tomesti-Iasi	±
Male,7 years	Tulcea	+
Male, 2 years	Tulcea	+

*Legend: +: positive sample; ±: borderline

The present study demonstrates the presence of canine anaplasmosis in Iasi, Tulcea and Sibiu counties. In Romania, there are few data on granulocytic anaplasmosis in dogs. Mircean et al., analysed 1146 samples collected from 16 counties, from randomly selected dogs, using ELISA SNAP 4DX (IDEXX Laboratories, Inc., Westbrook, ME). This study revealed a lower seroprevalence for *A. phagocytophilum* (5.5%) compared to our findings. These differences can be attributed to the fact that the recent work included hunting dogs living outdoor who received only occasionally antiparasitic treatments.This fact increases the chance of being exposed to vectors. High seroprevalence in the canine population indicates that subclinical *A. phagocytophilum* infection is common in areas where ixodid tick density is high. Dogs infected with vector-borne viruses might be asymptomatic for months or even years, but detecting subclinical infection is critical because those animals can still act as reservoirs for pathogens that infect other hosts, including humans.

Multiple studies have reported the presence of *A. phagocytophilum* in Europe using different diagnostic methods (Kohn et al., 2011; Pantchev et al., 2015; Angelou et al., 2019). A study performed in Bulgaria, showed a much higher seroprevalence (46.1%) compared to our results (Pantchev et al., 2015). Another research from Germany recorded a higher prevalence (43%) after testing 522 dogs (Kohn et al., 2011). These findings could be related to the climatic conditions, which are known to strongly influence the abundance of ticks, the increasing vector exposure depending on animals' age and/or immunological status (Cardoso et al., 2012). Furthermore, the seroprevalence values recorded in dogs in Italy, from 2013 to 2017, was lower for *A. phagocytophilum* (3.31%) (Ebani, 2019). A lower prevalence (2.7%) was reported also in Greece, in 2019.

Conclusions

- Our results suggest that dogs living outdoors and receiving no treatments may be infected with *A. phagocytophilum* and highlight the importance for appropriate antiparasitic treatments. Veterinarians should consider this pathogen in face of suggestive clinical signs and in routine health status checks.
- Because there is little serologic evidence of tick-transmitted diseases in Romania, more serological surveys need to be performed to establish the potential risk of canine anaplasmosis in dog population.

7.2. Serological surveys regarding viral tick-borne diseases

7.2.1. Investigation of tick vectors- Molecular survey of Crimean-Congo hemorrhagic fever virus (CCHFV) in *Rhipicephalus* and *Dermacentor* species ticks from South-Eastern Romania

Study objectives

The objective of this study was to assess the prevalence of CCHFV in *Dermacentor* and *Rhipicephalus* sp. ticks collected from Southern Romania, where previous research reported the presence of CCHFV IgG antibodies in small ruminants.

Introduction

A few prior investigations suggested that the Crimean-Congo haemorrhagic fever virus (CCHFV) was circulating in Romania, but they were based on serological methods. Crimean-

Congo haemorrhagic fever virus (CCHFV), a *Nairoviridae* family orthonairovirus, is the causal agent of a severe human haemorrhagic fever disease characterized by fever, weakness, myalgia, and haemorrhagic symptoms (Bente et al., 2013). The disease, known as Crimean haemorrhagic fever, was first discovered in the Crimean Peninsula in 1944, and the causative agent, which was isolated in 1967, was discovered to be identical to the Congo virus, isolated in 1956 from a febrile child in the Belgian Congo. The average CCHF fatality rate in Africa (22.0%) is lower than in Asia (33.5%) and Europe (33.8%) (Nasirian, 2020).

The main vector is *Hyalomma marginatum* ticks, and the distribution of human cases closely mirrors the distribution of the vector. CCHFV has been detected in more than 30 tick species, including *Dermacentor* and *Rhipicephalus* spp. ticks (Estrada-Pena et al., 2012). Asymptomatic CCHFV infection has been detected in many vertebrate species and appears to be pervasive in both wild and domestic animals. Asymptomatic viremia lasts up to 7–15 days in most vertebrate animal species. CCHFV has been isolated from livestock and small mammals (pigs, horses, donkeys, goats, cattle and sheep) (Maltezou et al., 2010).

In Romania, there is still a lack of information about CCHFV and the virus has never been identified in ticks. Virus detection through real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) is required to establish CCHFV circulation and can be used to identify CCHFV infections in animals and humans, but also in ticks. The significant genetic divergence of the virus has always been a major challenge in developing CCHFV RT-qPCR (Deyde et al., 2006).

Materials and methods

A total of 127 ticks were collected in July 2019 from five sites in Tulcea County, from the environment and domestic ruminants (Table 7.2.1.1). Questing ticks collection was conducted by dragging. Tick sampling was performed only once at each site. Ticks were transferred to the laboratory in vials (inside a sealed secondary container. Standard morphological identification keys were used to identify developmental stages under a stereomicroscope.

After the identification, each tick was cut lengthwise, using sterile scalpels on an ice-cold surface of a Petri dish. Ticks were transferred to Institut Pasteur (Paris, France) and analysed by real time RT-qPCR. To maintain the integrity of RNA, ticks were introduced in RNA later solution (Invitrogen, France), according to best security practices of storing and transporting.

Table 7.2.1.1

Tick collection sites in Tulcea County

Locality	Ticks number	Tick species
Cataloi	18	*Rhipicephalus sp.*
Slava Rusă	27	
Slava Cercheză	17	
Somova	18	
Sat Pescăresc Zărești	47	*Dermacentor sp.*
Total	127	

RNA extraction

Extraction steps were performed in a BSL-3 laboratory. Before extraction, ticks were pooled based on the collection region, resulting in a total of 13 pools (Table 7.2.1.2)
Tick pools were homogenized in 2 mL reinforced tubes MK28-R from Precellys containing 1 ml of TRIzol Reagent for cells. Minilys (Bertin, France) was used to shake the ticks twice or three times for 30 seconds each time. The samples were placed on ice for 1 minute between each shaking step.

The homogenates were utilized to extract total RNA. The lysates were centrifuged for 2 minutes at 4°C at 12.000 g. RNA was purified using Qiagen RNeasy mini kit (Hilden, Germany), including on-column DNAse treatment, and then analyzed with an Agilent Bioanalyzer. The RNA recovered in nuclease-free water was stored at −80°C.

Table 7.2.1.2

Pooling strategy by collection site, tick species and sampling origin

Sample type	Site	Ticks number	Tick genus	Sampling origin
Tick pool	Sat Pescăresc, Zărești	7	*Dermacentor sp*.	Environment
		10		
		10		
		5		
		5		
		10		
	Cataloi	5	*Rhipicephalus sp.*	Sheep
		15		Goats
	Slava Rusă	11		
		16		Sheep
	Slava Cercheză	15		Goats
	Somova	5		Sheep
		13		Goats

Detection of CCHFV in *Dermacentor sp*. and *Rhipicephalus sp*. ticks by Real Time RT-PCR

Specific primer sets for each of the six known CCHFV genotypes, one degenerate primer pair for the detection of all genotypes were used (Sas et al., 2018). These genotypes were assigned to several geographic areas: I – West Africa, II – Central Africa, III – South and West Africa, IV – Asia and Middle East, V- South and East Europe, VI – Europe. Viral RNA was transcribed to cDNA using SuperScript IV Reverse Transcriptase (Invitrogen, France). One µl of DNAc, 0.2 µl of each CCHF-deg primer, 0,2 µl of each genotype-specific CCHF-primer, 10 µl SybrGReen reagent and 8.6 µl H2O were used, in a total reaction volume of 20 µl.

Real time RT-qPCR was performed with Roche LightCycler 96 System (Roche Diagnostics, Germany). The cycling conditions were used as follows: 95°C for 300 s preincubation, followed by 45 cycles at 95°C for 10s (denaturation), 60°C for 10 s (annealing) 72°C for 30 s and 37°C for 30 s (cooling), the annealing temperature being adjusted at 60°C depending on the primers.

Statistical analysis

The upper range of prevalence was calculated using VS Outbreak SurveillanceToolbox. The maximal prevalence of viral infection in ticks was estimated assuming a sensitivity of 100% and a confidence level of 95%.

Results and discussion

The amplification of a 180 bp region from the S segment that codes for nucleoprotein (NP) was targeted to detect CCHFV. Because of its aerosol infectivity, CCHFV is classed as a risk group 4 pathogen with bioterrorism potential. Positive control was not included in the reaction for regulatory reasons. Using a negative control, we have not encountered any false-positive results due to contamination. In all collection sites from Tulcea County, the tested samples were detected as negative for the six genotypes of CCHFV.

The current investigation highlights the absence of CCHFV RNA in *Dermacentor* and *Rhipicephalus* sp. questing ticks and engorged ticks from South-Eastern Romania in 2019, from the same region (Tulcea County) where CCHFV antibodies in animals were previously detected (Ceianu et al., 2012).

Crimean Congo haemorrhagic fever has a wide geographic distribution, with cases reported in many parts of Africa, the Middle East, Europe, and Asia, and outbreaks caused by CCHFV have been recorded in some countries (Chinikar et al., 2010). The disease is endemic in the Balkan Peninsula, with Turkey and Bulgaria reporting the majority of cases. CCHFV has an endemic evolution in Bulgaria (Gergova and Kamarinchev, 2013), which could indicate the virus's circulation in Romania, either by ticks carried by migratory birds or through international livestock trade. In Turkey, migratory birds play a role in the dissemination of Crimean-Congo haemorrhagic fever virus (CCHFV) via attached ticks (Leblebicioglu et al., 2014).

For both spring and fall migrations, the geographic area of our current investigations includes one of the most active bird migration pathways in South-Eastern Europe. CCHFV does not cause clinical disease in livestock. However, domestic animals are very important for the epidemiology of the virus. Sheep have been identified as CCHFV reservoirs in some endemic areas and epidemiologically associated with human infections on various occasions (Humolli et al., 2010; Mostafavi et al., 2013). Small ruminants are not only hosts for adult vectors, but can also multiply the virus and infect other ticks during their short-lived viremia, allowing the virus to spread to new areas via tick-infested and virus-infected livestock movement and trade.

So far, the presence of the CCHFV in Romanian ticks has not been confirmed, and human cases have not been recorded. In 2012, Ceianu et al., tested 471 sheep serum samples from different localities in Tulcea County and obtained a prevalence of 27.8% for IgG antibodies against CCHFV. Nevertheless, a more recent study performed by Raileanu et al., in 2015, reported an overall prevalence of 74% IgG antibodies after testing 90 domestic ruminants (Răileanu C. et al., 2015). The results suggest the seroconversion process occurred before the ticks were attached and the transmission of the virus to vectors was not achieved. Several studies suggest that the viral RNA in attached ticks does not directly indicate transmission to host species. Infected ticks have been identified in seronegative animals and uninfected ticks have been found in seropositive animals (Zeller et al., 1994).

The findings of this study, combined with previously published data, may indicate a CCHFV tick-vertebrate-tick cycle in Romania. *Hyalomma* sp. ticks were not tested, which is a limit of our research. So, this study can be extended by exploring different tick species, areas and seasons of sampling.

Conclusions

- Despite the fact that CCHFV IgG antibodies had been detected in small ruminants from Tulcea County in previous years, CHHFV was not detected in *Dermacentor* and *Rhipicephalus* sp. ticks.
- Even if the species of ticks tested in the present research can transmit CCHFV, the main vector (*Hyalomma marginatum*) has not been tested, which can explain the negative results and represents a limitation of this study.

7.2.2. Investigation of vertebrate hosts-Seroepidemiological and molecular survey of Crimean-Congo hemorrhagic fever virus in small ruminants in Southern Romania

Study objectives

This study aimed to assess the CCHFV seroprevalence in small ruminants in an area where positive serological results have been recorded before (Southern Romania), in 2020. The research was completed by searching for CCHFV RNA in ticks and serum samples collected from small ruminants in the same region of the country.

Introduction

Crimean-Congo haemorrhagic fever is a viral infection that has been recorded in in Asia, Africa, the Middle East and South-Eastern Europe (Bente et al., 2013). In South-Eastern Europe, human cases have been confirmed in Bulgaria, the Republic of Kosovo, Albania, Greece and Ukraine (Whitehouse, 2004; Mertens et al., 2016).

Contrary to humans, domestic animals have no clinical signs of the disease and they act as accidental hosts which amplify and spread the virus. Seroepidemiological surveys in endemic areas suggest that several domestic animals (sheep, goats and cattle) could be asymptomatically infected with CCHFV.

In Romania there is a gap of data concerning the circulation of CCHFV: the main vector is present in the study area (Southern Romania) and some studies showed a high antibody prevalence in animals, although the virus has never been detected in ticks and no human cases were reported to date.

Materials and methods

Sera from 250 sheep and goats, sampled between 2019-2020 were collected in five locations in Tulcea County (Cataloi, Baia, Slava Rusă, Slava Cercheză, Somova) (Table 7.2.2.1). Additionally, 96 sheep serum samples from continental France were used as a reference negative population since there is no evidence of CCHFV circulation in mainland France. Finally, 169 *Rhipicephalus sanguineus* ticks were collected from ruminants investigated from Cataloi location. The detection of antibodies against CCHFV nucleoprotein (NP) 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.

RNA extraction

Ticks and serum samples were transferred to Institute Pasteur (Paris, France) to be tested by Real Time RT-qPCR targeting CCHFV. Tick were introduced in RNA later solution (Invitrogen, France) to ensure the integrity of RNA and virus inactivation, according to best security practices of storing and transporting. Nucleic acids extraction steps were conducted in a BSL-3 laboratory. Ticks were pooled before extraction resulting a final number of 14 pools.

Total RNA was extracted from crushed materials using TRIzol Reagent (Invitrogen, USA) and RNeasy mini kit (Qiagen, Germany) according to the manufacturers' recommendations.

Sixty-one serums samples from Cataloi and Slava Rusa were pooled before extraction, resulting a final number of 7 pools. Total nucleic acids extraction from serums samples was performed using QIAamp Cador kit (Qiagen, Germany) with nuclease pre-treatment.

Determination of tick species

We took advantage of the concomitant sequencing of tick transcriptome and the Barcode of Life Data Systems (BOLD), to determine the species of analyzed ticks, as previously described (Bratuleanu et al., 2022a). Briefly, all trimmed reads were mapped onto the *Ixodidae* BOLD database, de novo assembled after extraction of mapped reads, and submitted to the BOLD Identification System. The identification was confirmed by BlastN. All ticks tested in this study were identified as *Rhipicephalus sanguineus*.

Identification of CCHFV and Pan-*Nairovirus* in *R. sanguineus* and in serum samples by Real Time RT-PCR

Viral RNA was first reverse-transcribed using SuperScript IV Reverse Transcriptase kit (Invitrogen, France) and random hexamers.

Primer sets either specific of each CCHFV genotypes (N=6) or degenerated for the detection of all genotypes were used to screen tick and serum samples as previously described (Sas et al., 2018), except that the PCR was adapted for a real-time SYBR Green format. A pan-Nairovirus PCR system capable to detect 14 representative viruses of the genus, was also used to identify other nairoviruses.

Statistical analysis

Statistical analysis was conducted on commercially available software (SPSS 17.0 IBM), using Chi-square test and Fisher's exact test. Statistical significance was defined as $p<0.05$.

Results and discussion

The global CCHFV antibody seroprevalence rate 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 investigated by Real Time RT-PCR (Table 7.2.2.1).

A significant difference was observed between the reference population from France and Romanian sheep ($p<0.01$).

No statistical seroprevalence difference was observed between sheep and goats from Romania ($p=0.76$). However, a significant difference was enregistred between the five locations ($p <0.01$), (93.1% positivity in animals at Somova and 0% at Baia). No CCHFV RNA was detected in ticks or in small ruminant's sera tested by Real Time RT-PCR. Furthermore, no positive sample was identified using the Pan-Nairovirus PCR system.

This work provides an updated overview of CCHFV seroprevalence in livestock from 5 sites from Southern Romania (Danube Delta region). This area presents a high degree of biodiversity, comprising one of the most active bird migration pathways in South-Eastern Europe. The current findings demonstrated a high seroprevalence and confirm the role of sheep and goats in CCHFV ecology in Romania. CCHFV is endemic in the Balkan Peninsula, with the majority of cases occurring in Turkey and Bulgaria. CCHFV is endemic in Bulgaria and Hungary, countries that are on the border with Romania, suggesting that the virus could potentially circulate in Romania. In certain endemic areas, small ruminants have been recognized as CCHFV hosts and epidemiologically associated to human cases (Gergova and Kamarinchev, 2013; Brinkmann et al., 2018).Our findings revealed seropositive results in small ruminants from four out of five localities, indicating a uniform circulation of the virus in Southern Romania. However, similar seroprevalence rates in domestic animals were also reported in other countries, albeit using in most cases different reagents: Pakistan (36.2%) (Zohaib et al., 2020), Senegal (32.5%) (Mangombi et al., 2020). Lower rates were detected in Greece (25%) (Papa et al., 2014), Kosovo (18.4%) (Fajs et al., 2014), Republic of Macedonia (14.6%) (Mertens et al., 2013), Bulgaria (18.4%) (Christova et al., 2018) and Corsica (9.1%) (Grech-Angelini et al., 2020); whereas an even higher seroprevalence was found in Turkey (57% and 74%) (Mertens et al., 2016).

Table 7.2.2.1

ELISA results of CCHFV antibodies in small ruminants from five localities in Southern Romania

Collection site	**Species**		**Positive/ Tested animals**	***R. sanguineus* ticks**	**Seroprevalence sheep**	**Seroprevalence goats**	**Overall prevalence**
	Sheep	**Goats**					
Cataloi	77	35	54/112	169	31.2%	85.7%	48.2%
Baia	20	0	0/20	N/A	N/A	N/A	N/A
Slava Rusă	9	23	1/32	N/A	11.1%	N/A	3.1%
Slava Cercheză	59	0	13/59	N/A	22%	N/A	22%
Somova	16	13	27/29	N/A	100%	84.6%	93.1%
TOTAL	181	71	95/252	169	29.8% CI 23.2-36.5%	57.7% CI 46.3-69.2%	37.7% CI 31.7-43.7%
Reference population (France)	96	-	6/96	-	6.3% CI 1.4-11.1%	-	-

In Romania, only few serological studies with limited data on the CCHFV circulation are available, that confirm the presence of antibodies in small ruminants (Ceianu et al., 2012; Răileanu C. et al., 2015). These studies showed a seroprevalence between 27.8% and 74%. It is puzzling that such high seroprevalence was recorded without any recorded human cases. This fact is not unique. Some countries record human cases that parallel animal seroprevalence (Yen et al., 1985; Mostafavi et al., 2013) while in other regions, high level of seroprevalence was detected without any evidence for human cases. For example, Bulgaria reported a high seroprevalence in small ruminants in the absence of human cases, in many areas (Christova et al., 2018). While there appears to be an association between the infected ticks and identification of seropositive

animals, viral RNA detection in attached ticks is not linked to seropositivity of the infested host, and vice versa (Zeller et al., 1997). Another possibility is that different CCHFV serotypes with different pathogenicity for humans exist in the field, while not been detected up to now, or, alternatively, that another prevalent nairovirus with antigenic cross-reactivity with CCHFV may circulate. *Rhipicephalus* sp. ticks were collected from small ruminants in one of the locations where we had collected largest number of serum samples in order to complete the research.

First, our findings revealed no connection between ruminant tick infestation (mostly *Rhipicephalus* sp.) and animal seropositivity. *Hyalomma* spp. is the principal CCHFV vector, however ticks of the genus *Rhipicephalus* can also transmit the virus (Zeller et al., 1997). Second, we did not detect any CCHFV-positive ticks. Our findings could be related to the collected tick species, as well as the fact that *Hyalomma* sp. ticks were not tested, which could be considered a study limitation.

Conclusions

- These results indicate the circulation of CCHFV or another antigenically related nairovirus among small ruminants, in Southern Romania.
- Future research should focus on increasing number and diversity of tick species sampled and also expanding the geographic range of surveillance, including NGS to broaden the spectrum of nairovirus detection throughout Romania.

CHAPTER VIII
UNCOVERING THE ROMANIAN TICK MICROBIOME

8.1. Identification of novel viruses with potential relevance for public health in Romanian ticks

Study objectives

The aim of this study was to describe the virome diversity of *Riphicephalus*, *Dermacentor* and *Haemaphysalis* sp. ticks sampled from understudied areas from Romania, in order to extend the knowledge about the diversity of viruses in Eastern Europe. The study was then focused on viruses that could have potential relevance for human and animal health.

Introduction

Ticks are responsible for the transmission of various pathogens, and some tick-borne diseases cause significant health problems for humans and animals. Despite their obvious significance, the composition of tick viral communities and their interactions with pathogens are poorly understood, especially in Eastern Europe, which is (via bird migration) an important hub for animal-arthropod vector exchanges.

Despite the fact that global warming is frequently mentioned as the mechanism that favors the spread of tick-borne diseases, climate is one of many factors (for exemple their population density, the likelihood that they are infected with human pathogens, and the frequency of tick-human contact) that determine which tick species are found in a given geographical region (Estrada-Pena and de la Fuente, 2014).

Tick and tick-borne pathogen distribution are also impacted by a variety of biological and environmental factors, including deforestation and urbanization, that together may favor the spread and establishment of selected vectors in previously tick-free areas (Colwell et al., 2011; Ostfeld and Brunner, 2015).

However, until a decade ago, only a few tick-borne viruses were known, among which flaviviruses were the most characterized (Gould and Solomon, 2008; Hermance and Thangamani, 2017; Yoshii, 2018). The most known tick-borne viruses of medical importance include Crimean-Congo hemorrhagic fever virus (Bente et al., 2013), Kyasanur Forest disease virus (Tandale et al., 2015), tick-borne encephalitis virus, severe fever with thrombocytopenia syndrome virus (Yu et al., 2011), Alkhurma virus (Labuda and Nuttall, 2004) or Heartland virus. Various tick-borne viruses also threaten the health of livestock: Africa swine fever virus (ASFV), Nairobi sheep disease virus (NSDV), and Louping ill virus (LIV), among others (Colwell et al., 2011).

Taking advantage of the rapid development of high throughput sequencing methods in recent years, many new viral sequences have been identified in ticks, increasing the knowledge of tick-borne pathogens. It is the case of Jingmen tick virus-JMTV (*Flaviviridae* family) identified in multiple tick species (Qin et al., 2014; Jia et al., 2019; Kuivanen et al., 2019), rodents, cattle, primates (Ladner et al., 2016; Souza et al., 2018; Jia et al., 2019; Kuivanen et al., 2019) and in bats urine in Cambodia (Temmam et al., 2019a) without any information of related pathologies.

JMTV-like virus (Alongshan virus) was recently discovered in humans with a febrile illness of unknown etiology (Wang et al., 2019) demonstrating how little is known about new tick-borne arboviruses. Indeed, new tick-borne viruses have been identified in recent studies, but the potential zoonotic risk for humans or domestic animals is unknown. For example, among members of the *Nairoviridae* family, that are primarily transmitted by *Ixodidae* and *Argasidae* ticks with natural hosts such as birds, bats, rodents, lagomorphs, or ungulates (Ladner et al., 2016), a novel virus recognized as Nayun tick nairovirus (NTNV) has been identified in dog-infesting *Rhipicephalus sanguineus* Chinese ticks (Xia et al., 2015). This virus belongs to the *Orthonairovirus* genus and is related to the Crimean-Congo haemorrhagic fever virus phylogenetically. In a similar way, among the *Phenuiviridae* family, a novel phlebovirus named Tacheng tick virus 2 (TaTV2) was identified in *Dermacentor marginatus* from China and was also detected in one patient's blood (without current information on TaTV2 pathogenicity for humans), indicating a possible vectorial transmission of this virus (Brinkmann et al., 2018).

The description of these communities increases the knowledge of the diversity of viruses in Eastern Europe and provides a basis for further research about the interrelationship between ticks and tick-borne viruses.

Materials and methods

A number of 506 engorged and questing ticks was collected in 2019 in different locations from Southern Romania (Figure 8.1.1). Ticks were pooled according to collection areas and species, resulting in total of 19 pools for RNA extraction (Table 8.1.2). The 19 pools of ticks were mixed to form 8 NGS libraries and sequencing was carried out on an Illumina NextSeq 500 sequencer in a single-read 1 x 150 bp format to achieve approximately 50 million reads per library.

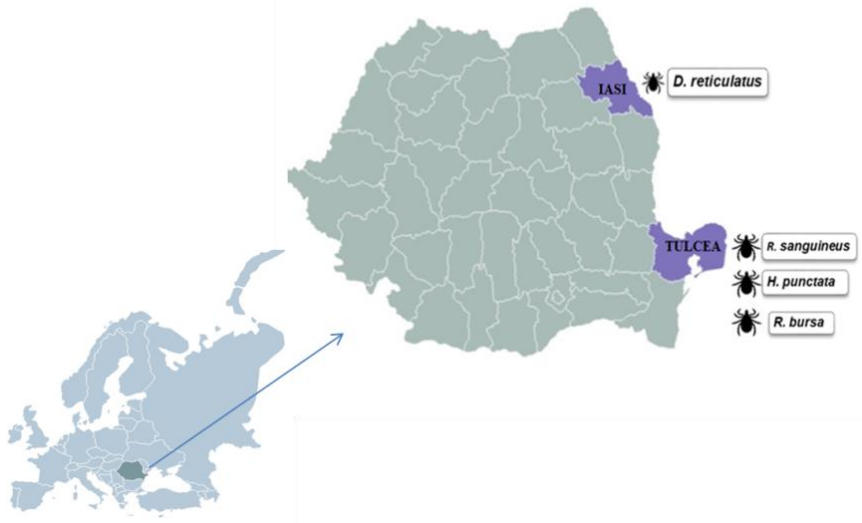


Figure 8.1.1- Sampling sites map of Eastern Romania (original)

The presence of viral RNA was confirmed for relevant viruses by conventional RT-PCR using primers designed from the NGS sequences and the complete genome sequences were obtained by RT-PCR and Sanger sequencing after designing specific primers targeting the identified viruses. In order to explore the potential presence of endogenous viral sequences into tick genomes for relevant viral species identified by NGS, all tick samples were screened by PCR by omitting the reverse transcription step.

Virus assignation

A total of 423668224 raw reads were processed with an in-house bioinformatics pipeline including quality check and trimming, based on AlienTrimmer package (Criscuolo and Brisse, 2014), reads normalization,using BBnorm program, de novo assembly using Megahit tool (Li et al., 2015b), and ORF prediction of contigs and singletons. A BLAST-based similarity search was conducted for all contigs and singletons against the comprehensive and curated protein Reference Viral database (RVDB-prot) (Bigot et al., 2019) followed by a BlastP-based verification of the accuracy of the viral taxonomic assignation against the whole protein NCBI/nr database.

A final BLASTN-based verification was performed against NCBI/nt to confirm that no better hit was obtained with non-coding sequences present in NCBI/nt. The quantification of abundance of each viral taxon was obtained by summing the length, in nucleotides, of all sequences being associated to this taxon, weighted by the k-mer coverage of contigs.

Phylogenetic analyses

Flaviviridae (containing unclassified Bole tick virus 4 and Jingmenviruses) and *Bunyavirales* (*Nairoviridae* and *Phenuiviridae*) phylogenetic analyses were perfomed as follows: the complete nucleotide sequences of Jingmenviruses or the complete amino-acid RNA polymerase or nucleoprotein sequences were retrieved from GenBank® and aligned with Multiple Alignment using Fast Fourier Transform (MAFFT) aligner under auto mode (Katoh et al., 2019).

Alignments were manually cured and the best amino acid or nucleotide substitution models that fitted the data were determined with ATGC Start Model Selection (Lefort et al., 2017), using the corrected Akaike information criterion, and were GTR+G for Jingmenviruses nucleotide phylogenies (whatever the segment considered) and LG+G for amino-acid *Flaviviridae* and *Bunyavirales* phylogenies. Phylogenetic trees were constructed using PhyML method implemented through the NGPhylogeny program (Lemoine et al., 2019) in accordance with the selected substitution model. Nodal support was evaluated using the aBayes parameter.

Results

Most sequences identified in this study were assigned to Eucaryota (ranging from 7% to 61%, depending to the library sample), viruses (0.4-84%) and bacteria (9-68%), according on the tick species considered (Table 8.1.1).

The host spectrum of RNA viruses revealed some differences between the 4 species of analyzed ticks: *D. reticulatus, R. sanguineus, H. punctata and R. bursa* (Figure 8.1.2A). In *D. reticulatus, R. sanguineus* and *H. punctata* questing ticks, respectively, 47%, 67% and 93% of viral reads were assigned to unknown host viruses, while most viral reads (97%) detected in

engorged *R. bursa* represented arboviruses (JMTV being predominantly detected) able to dually infect vertebrate and invertebrate hosts.

This may suggest that arboviruses found in engorged ticks partly reflect the blood virome of sheep and goats from which these ticks fed. In a similar way, the pattern of hosts for DNA viruses was comparable for *D. reticulatus, R. sanguineus* and *H. punctata* questing ticks, with a majority of DNA viruses infecting bacteria (57%, 46% and 53%, respectively) followed by vertebrate-infecting viruses, but differed from the host spectrum of DNA viruses of engorged *R. bursa* that mainly infect invertebrates (Figure 8.1.2B).

Regarding the viral sequences, over 96% were attributed to RNA viruses while few other related viral sequences were detected (1.3% DNA viruses and 2.1% unknown viruses. The most significant viral sequences belonged to the families *Flaviviridae, Phenuiviridae* and *Nairoviridae* (Figure 8.1.3).

Table 8.1.1

Number of reads provided by Kraken2 tool and the distribution of these reads according to viruses

Library ID	Ticks number	Sampling origin	Site	No. of raw reads	No. total of Kraken2 reads	Viruses
IASI20	7	Environment	IASI	54 500 645	181259	1721 (0.9%)
IASI21	10	Environment	IASI	45 974 122	129917	1144 (0.9%)
IASI22	10	Environment	IASI	42 420 208	123191	2281 (2%)
IASI23	5	Environment	IASI	62 115 189	181832	2178 (1%)
IASI50	379	Environment	IASI	45 695 030	75464	663 (0.9%)
TULCEA49	10	Environment	TULCEA	89 613 642	329590	2930 (0.9%)
TULCEA1	80	Sheep/ goat	TULCEA	49 898 354	1201578	1005342 (84%)
TULCEA47	5	Environment	TULCEA	87 951 679	611147	2194 (0.4%)

Table 8.1.2

Pooling strategy for RNA extraction and library preparation

Sample type	Site	Ticks number	Ticks species determined by NGS	NGS libraries	Sampling origin
1. Tick pool	Hlincea, Iași	7	*Dermacentor reticulatus*	Iasi20	Environment
2. Tick pool	Hlincea, Iași	10	*Dermacentor reticulatus*	Iasi21	Environment
3. Tick pool	Hlincea, Iași	10	*Dermacentor reticulatus*	Iasi22	Environment
4. Tick pool	Hlincea, Iași	5	*Dermacentor reticulatus*	Iasi23	Environment
5. Tick pool	Cetățuia, Iași	28	*Dermacentor reticulatus*	Iasi 50	Environment
6.. Tick pool	Hlincea, Iași	57	*Dermacentor reticulatus*		Environment
7. Tick pool	Hlincea, Iași	55	*Dermacentor reticulatus*		Environment
8. Tick pool	Hlincea, Iași	76	*Dermacentor reticulatus*		Environment
9. Tick pool	Hlincea, Iași	82	*Dermacentor reticulatus*		Environment
10.Tick pool	Ezăreni, Iași	81	*Dermacentor reticulatus*		Environment
11.Tick pool	Sat Pescăresc, Tulcea	10	*Haemaphysalis punctata*	Tulcea49	Environment
12.Tick pool	Cataloi, Tulcea	5	*Rhipicephalus bursa*	Tulcea1	Adult sheep
13.Tick pool	Cataloi, Tulcea	15	*Rhipicephalus bursa*		Adult goat
14.Tick pool	Slava Rusă, Tulcea	11	*Rhipicephalus bursa*		Adult goat
15.Tick pool	Slava Rusă, Tulcea	16	*Rhipicephalus bursa*		Adult sheep
16.Tick pool	Slava Cercheză, Tulcea	15	*Rhipicephalus bursa.*		Adult goat
17.Tick pool	Somova, Tulcea	5	*Rhipicephalus bursa*		Adult sheep
18.Tick pool	Somova, Tulcea	13	*Rhipicephalus bursa*		Adult goat
19.Tick pool	Sat Pescăresc, Zaresti, Tulcea	5	*Rhipicephalus sanguineus*	Tulcea47	Environment

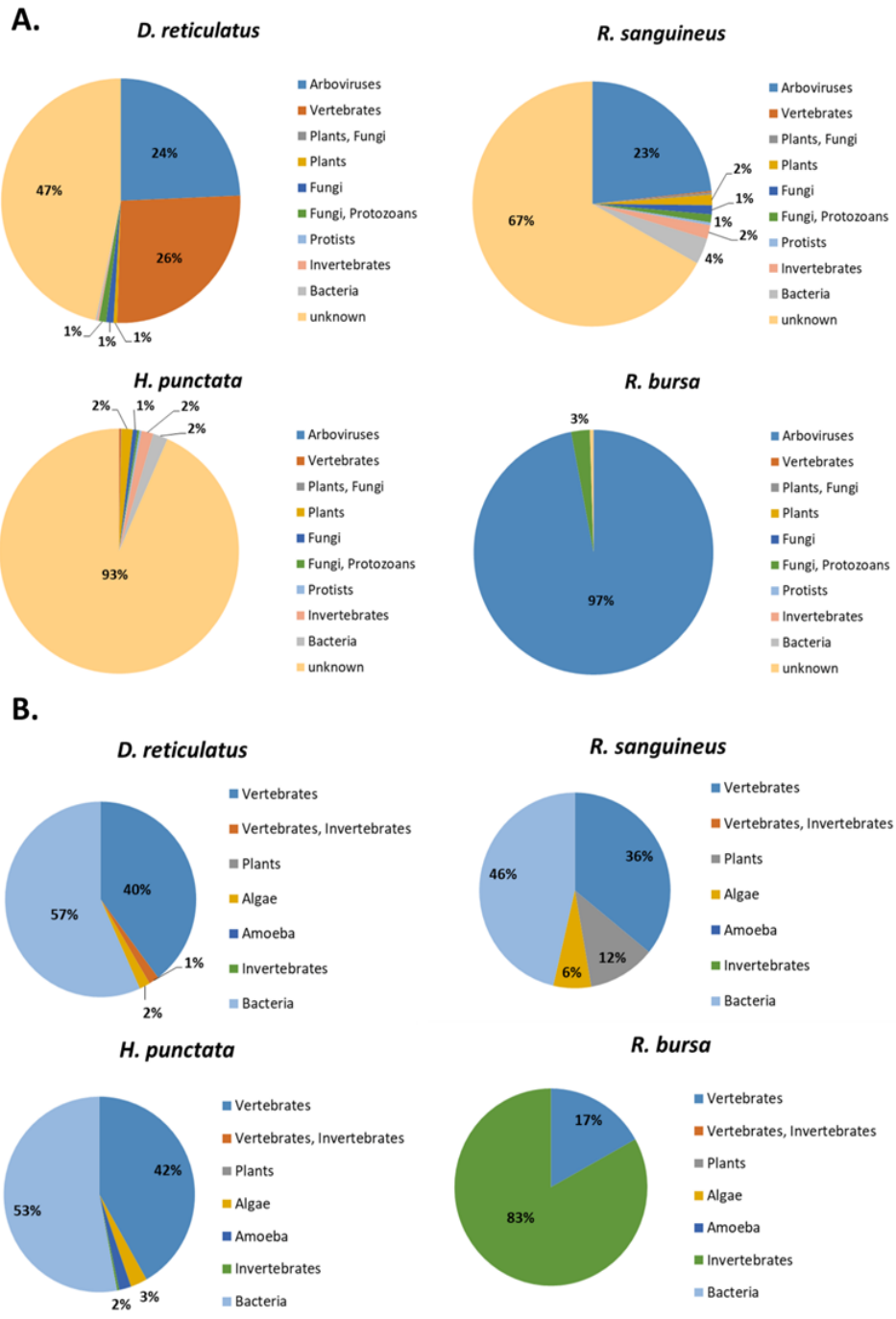


Figure 8.1.2- Classification of detected viruses by host spectrum. Viruses were classified according to the known host of the family they belong: arboviruses (dual arthropod and vertebrate hosts); vertebrates; plants; fungi; algae; amoeba; protozoans; protists; invertebrates; bacteria or unknown host. (a) RNA viruses (b) DNA viruses. (*D. reticulatus*, *R. sanguineus* and *H. punctata*- questing ticks; *R. bursa*-engorged ticks)

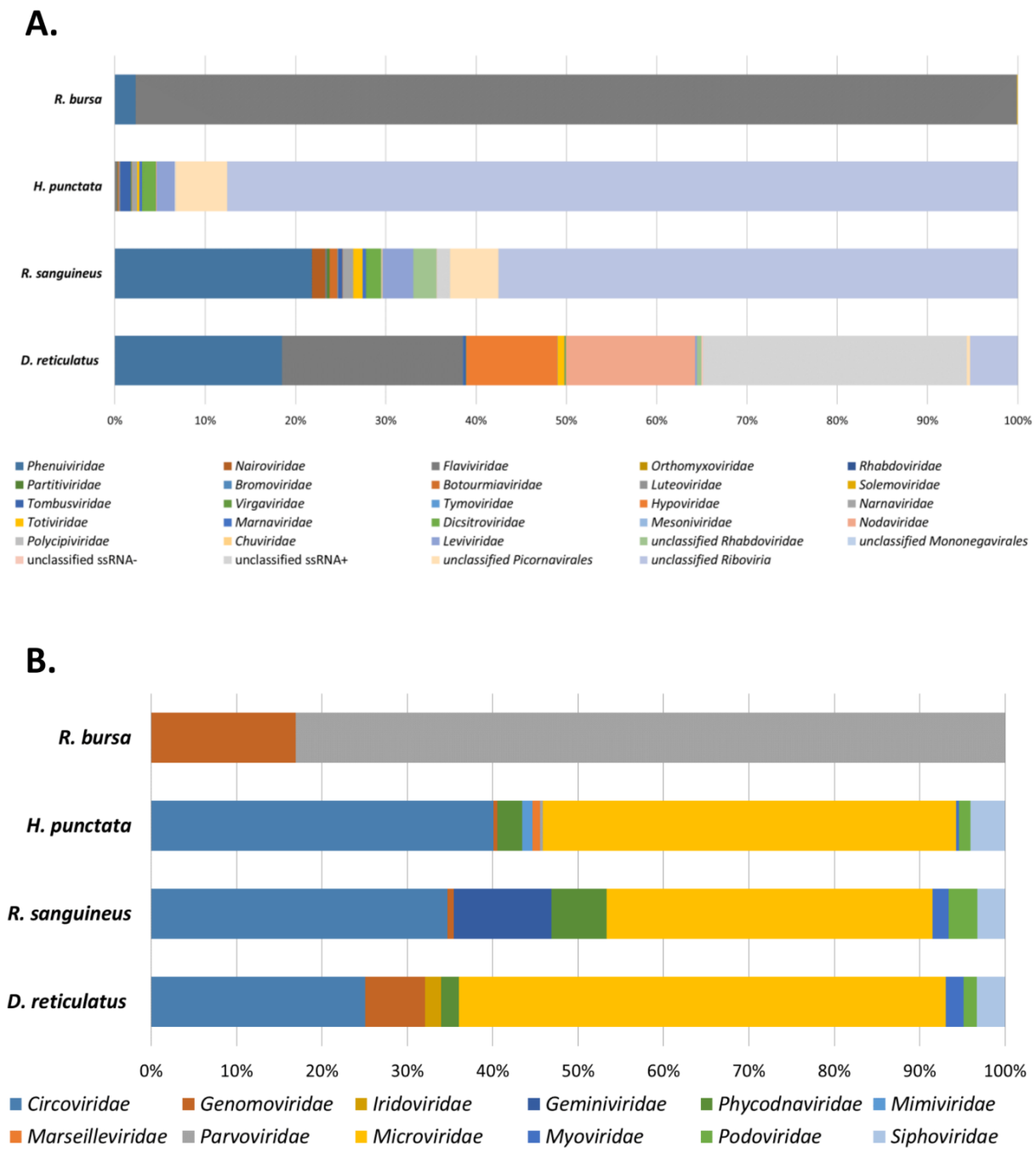


Figure 8.1.3- Classification by viral family according to tick species detected in Romanian ticks in (a) in 29 RNA families (b) in 12 DNA families. (*D. reticulatus*, *R. sanguineus* and *H. punctata*- questing ticks; *R. bursa*-engorged ticks)

The reads belonging to the *Flaviviridae* family were assigned to Jingmen tick virus (JMTV), identified in engorged *R. bursa* ticks. The complete genome of JMTV was obtained directly from the sequencing reads and contigs and showed an amino-acid identity ranging from 98.03% to 99.26%, depending on the segment considered, with its closest tick-borne Turkish isolate relative. Phylogenetic analyses performed placed Romanian JMTV isolate in a clade that includes tick-borne and human isolates originating from Eastern Europe (Turkey and Kosovo) suggesting a possible ability of Romanian JMTV to infect mammals. Nevertheless, this later does not belong to the clade of Alongshan virus, that is to date the unique member of Jingmenviruses that has been confirmed to be responsible of human pahologies (Figure 8.1.5).

Another virus linked to the *Flaviviridae* family was Bole tick virus 4 (BTV4) found in questing ticks from Iasi County (*D. reticulatus*) and in ticks from Tulcea County (*R. sanguineus* and *H. punctata*). BTV4 presented an amino-acid identity ranging from 78.16% to 85.28% with BTV4 previously identified in *H. asiaticum* ticks from China and phylogenetic analyses positioned BTV4 from Romania in a clade comprising tick-borne isolates from Thailand and the Caribbean, belonging to *R. sanguineus* and *H. asiaticum* from China (Figure 8.1.4).

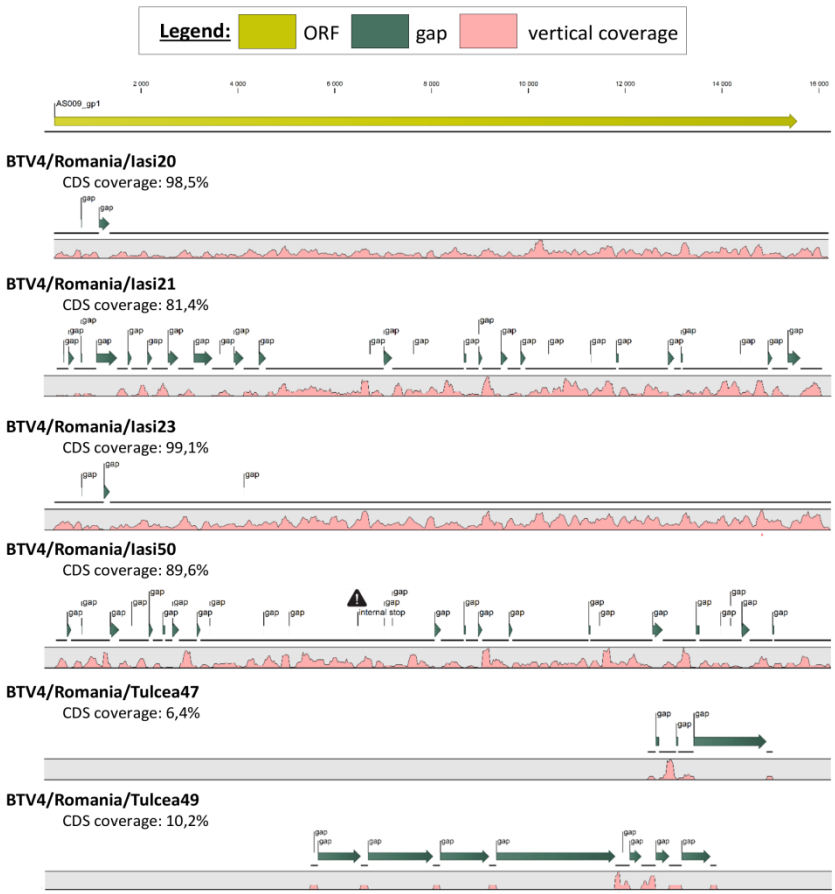


Figure 8.1.4- Schematic organization of BTV4 identified in *D. reticulatus*, *R. sanguineus* and *H. punctata* from Romania. The open reading frames (ORFs) are noted with yellow arrows, the gaps in dark green and genome coverage is indicated in pink

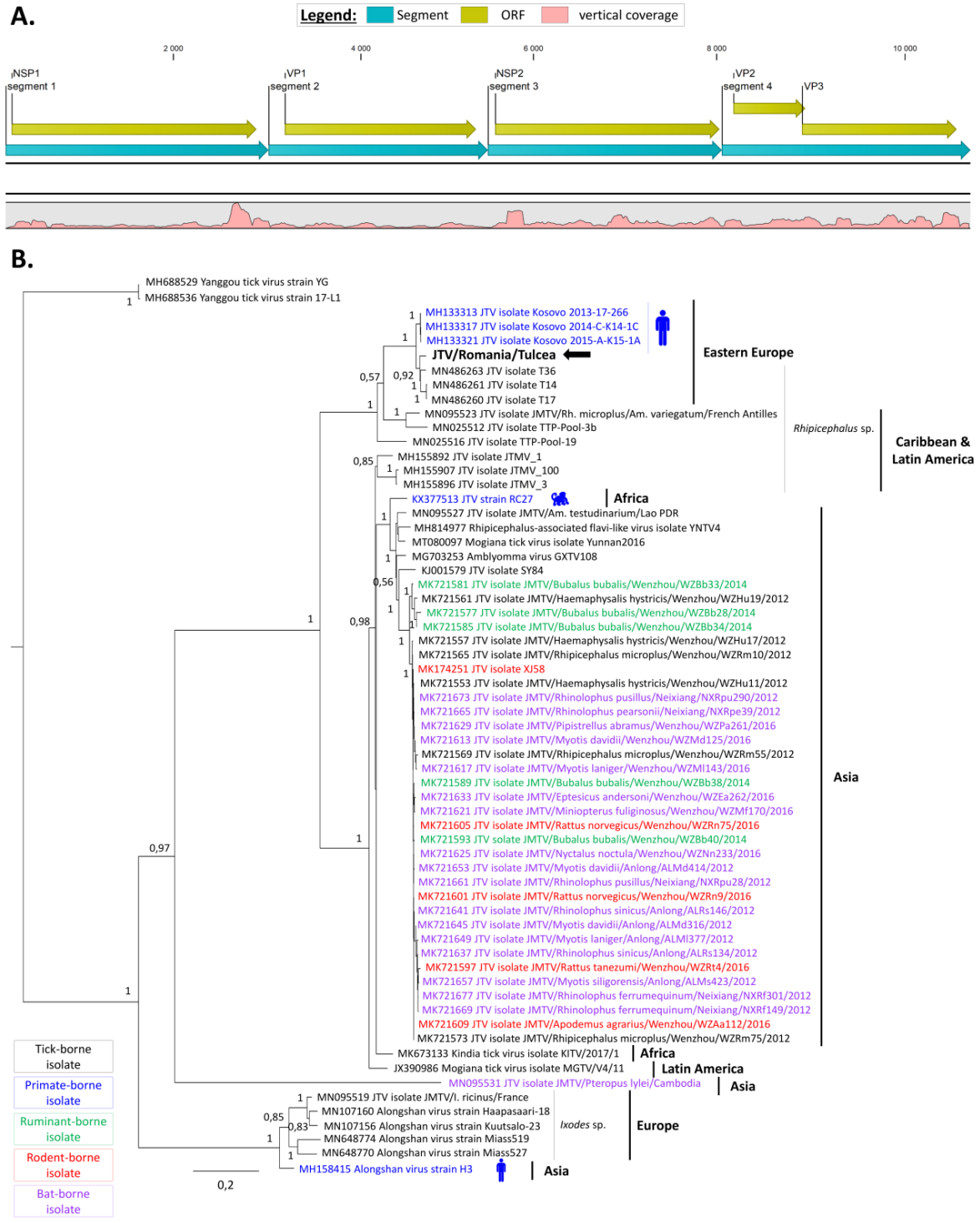


Figure 8.1.5- Jingmen tick virus. (a) Schematic organization of JMTV/Romania/Tulcea genome detected in *R. bursa*. The open reading frames (ORFs) are marked with yellow arrows, and genome coverage in pink. Segmented viruses were presented as concatenated sequences for better clarity (blue arrows represent the different segments).
(b) Phylogenetic relationship of JTV/Romania/Tulcea segment 1 identified in Romanian *R. bursa* with others tick-borne (black), primate-borne (blue), ruminant-borne (green), rodent-borne (red) and bat-borne (purple) Jingmenviruses

Sequences from three different phleboviruses were identified, according on the tick species and the area considered. Sequences assigned to the *Phenuiviridae* family were represented by Tacheng tick virus 2 (TaTV2), Brown dog tick phlebovirus 2 (BDTPV2) and Changping tick virus 1 (CPTV1), all negative sense bi-segmented ssRNA viruses lacking the M segment coding for the viral glycoprotein (Figure 8.1.6). Phylogenetic analyses on the RNA-dependent RNA-polymerase protein sequence positioned the Romanian phleboviruses in the Uukuvirus genus, *Phenuiviridae* family. Romanian TaTV2 clustered in a phylogenetic group comprising tick-borne isolates from Eastern Europe, respectively from Anatolia/Turkey (*R. sanguineus*) and isolates from ticks (*D. marginatus*) and humans from China, indicating a zoonotic potential of Romanian TaTV2.

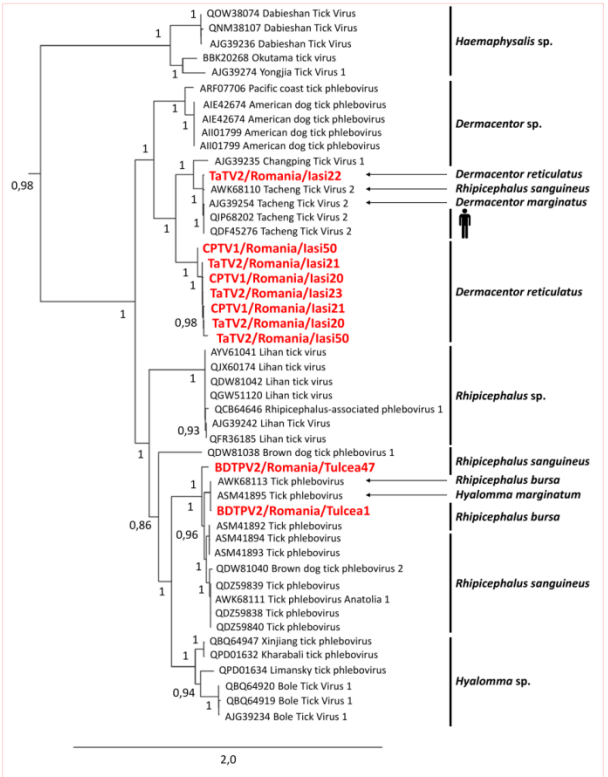


Figure 8.1.6- Phylogenetic relationship of TaTV2, CPTV1 and BDTPV2 RNA-dependent RNA polymerase detected in *D. reticulatus*, *R. bursa* and *sanguineus*) ticks with others viral families (*Nairoviridae*, *Hantaviridae* and *Peribunyaviridae*) among the *Bunyavirales*

Romanian CPTV1 positioned in the same clade as Romanian TaTV2 but clustered in a distinct group, different from other isolates originating from China, suggesting a possible geographical specificity of these viruses. Romanian BDTPV2 has been grouped into a separate clade from TaTV2 and CPTV1. It was placed in a clade apparently limited to tick phleboviruses that were mainly identified in *H. marginatum* or *Rhipicephalus* (*bursa* or *sanguineus*) ticks from Turkey or Trinidad and Tobago. In the *Nairoviridae* family, sequences of a distantly nairovirus-related virus were identified, named Nayun tick nairovirus (NTNV). NTNV was firstly detected in *Rhipicephalus* sp. ticks from China. Romanian NTNV differed from its Chinese counterpart by presenting 73% of amino-acid identity in the S segment.

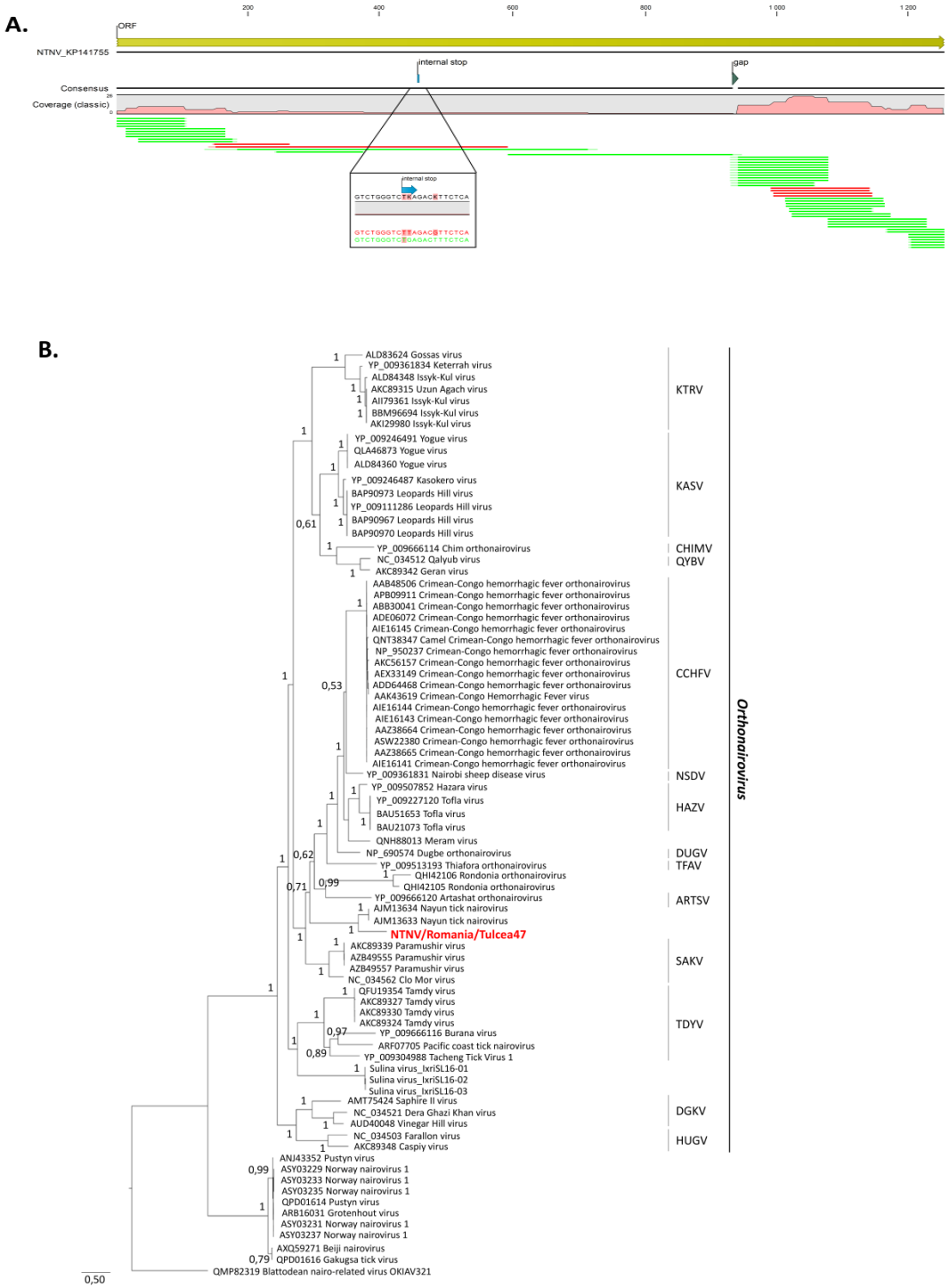


Figure 8.1.7- A) Schematic organization of NTNV detected in *R. sanguineus*. The open reading frames (ORFs) are marked in with yellow arrows, the gaps are marked in dark green, genome coverage is makred in pink, and the internal stop is indicated with a blue arrow. B) Phylogenetic relationship of NTNV S amino-acid sequence with other orthonairoviruses

Sanger sequencing carried to fill the gaps between NGS reads revealed an internal stop in one of the two Sanger sequences, compared with the Chinese sequence reference. Romanian and Chinese NTNV situated at the root of arbo-nairoviruses infecting mammals (CCHFV or NSDV) (Figure 8.1.7), either indicating that NTNV represent a novel virus species within the Orthonairovirus genus or, as it was proposed for phleboviruses (Matsuno et al., 2013) it could constitute an ancestral sequence reflecting the origin of the Orthonairoviruses.

Discussion

Importance of Romanian tick-borne viruses for human and animal health

In the present study, many viral families were identified, including viruses infecting plants, invertebrates and bacteria, using a viral meta-transcriptomic analysis and especially, viruses belonging to three viral families known to comprise arboviruses (*Flaviviridae*, *Phenuiviridae* and *Nairoviridae*). Members of these families were previously detected in other tick species and genera, suggesting different degrees of host permissiveness of these viruses that could reflect their ability to ensure interspecies transmission: invertebrate and even vertebrate.

It's the example of Jingmen tick virus, a newly identified segmented ssRNA+ virus originally detected in *R. microplus* in the Jingmen region of China's Hubei province, in 2010. Because of its potential to infect a wide variety of tick vectors (*Haemaphysalis* sp., *Ixodes sp*., or *Dermacentor* sp.) and mammals (bats, rats, cattle, and primates) and cause disease in humans, this virus has recently raised animal and public health concerns (Wang et al., 2019). We detected JMTV in engorged *R. bursa* (small ruminants from Tulcea County), showing that ruminants may play a significant role in the Jingmenvirus life cycle in Romania, as previously suggested in China, Brazil, and Trinidad and Tobago (Maruyama et al., 2014; Qin et al., 2014). Additionaly, the phylogenetic positioning of Romanian JMTV, close to human Kosovar isolates with a history of tick bite (Emmerich et al., 2018) questioned on the ability of Romanian JMTV to infect humans.

These findings pave the way for more research into the epidemiology and potential clinical impact of JMTV in ruminants and humans in the area, as well as evaluating the virus's risk of spreading to other areas, such as via migratory birds.

In a similar way, the identification of Tacheng tick virus 2 in *D. reticulatus* in northwestern China that was phylogenetically similar to a human clinical isolate with a history of tick bite (Dong et al., 2021) raised concerns about the risk of novel arboviruses being carried by Romanian ticks. Recent studies (not yet published) have confirmed this hypothesis: 6 out 25 patients were positive to S segment of TaTV2 and their common clinical symptoms included headache, fever, asthenia, vomiting, myalgia and rush. Also, TaTV2 was identified in the blood samples of sheep, cattle, wild badger, gerbils spleen samples and of tick eggs.

Changping tick virus 1 and Brown dog tick phlebovirus 2 were found in the same *Phenuiviridae* family and clustered in the *Uukuvirus* genus. Because these viruses have only been discovered in ticks so far (Li et al., 2015a; Sameroff et al., 2019), their ability to infect multiple hosts is unknown. Romanian CPTV1 was exclusively found in *D. reticulatus* from Iasi County, while two variants of BDTPV2 grouped in two separate subclades were found in *R. sanguineus* and *H. punctata* from Tulcea County, indicating that these viruses are specialized to their tick hosts.

BTV4 is a virus not yet recognized by the International Committee for Taxonomy of Viruses of the *Flaviviridae* family. Different Romanian BTV4 strains with a high degree of genome conservation were detected in four *D. reticulatus* pools from Iasi County, and also in R. *sanguineus* and *H. punctata* from Tulcea County. This suggests that very closely related BTV4 strains infect multiple tick species from various geographic areas, as has been previously reported for *H. asiaticum* from China (Shi et al., 2016) and *R. sanguineus* from Thailand (Temmam et al., 2019b) or Trinidad and Tobago (Sameroff et al., 2019). This high degree of genome conservation observed between different tick species from many environments and geographical regions is puzzling and questions on the origin of BTV4. More studies are necessary to establish the origin of BTV4 and its ability to infect vertebrate hosts, if we consider that tick co-feeding onto the same vertebrate host could explain the the ability of this virus to infect numerous tick species.

The identification of Nayun tick nairovirus-related S sequences in *R. sanguineus* ticks from Tulcea County is also concerning. Only sequences corresponding to the S segment of the Nayun tick nairovirus were detected in this study, which questions the presence of an exogenous virus in the tick pool. The detection of an internal stop in one of two Sanger sequences suggests the presence of two populations of viral RNAs: one coding for the full length of NTNV nucleoprotein, and the other presenting an internal TGA stop codon that could result in the possible presence of an endogenous viral element in the genome of *R. sanguineus.*

Conclusions

In the present study, new viruses belonging to the families *Flaviviridae, Phenuiviridae* and *Nairoviridae* were identified for the first time in Eastern Europe, more precisely in Romania by high throughput sequencing.

- JMTV recently raised animal and public health concern because of its ability to infect a wide variety of tick vectors, mammals and humans. In this study, 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, as previously suggested in different parts of the world.
- Phylogenetic analyses placed Romanian TaTV2 in a clade with ticks and humans isolates from China, indicating that this virus may have a major significance for human and animal health. The pathogenicity of this virus for humans and animals was later confirmed, TaTV2 being detected in tick bite patients, sheep, cattle, badgers, gerbils and *Hyalomma asiaticum* tick egg batches, in 2021, in China.
- Other viruses not yet known to if they are pathogenic or not (Bole tick virus 4, Nayun tick nairovirus) have been identified and further studies are needed to determine their importance for human and animal health.

Microbiome data

The read number was normalized by the total number of reads in each sequencing run and performed Principal Component Analysis (PCA) on the entire dataset to evaluate the variance between the different *D. reticulatus* runs and the other tick species. PCA significantly separated a cluster corresponding to the five *D. reticulatus* sequencing runs from those of the three other tick species allowing us to pool all *D. reticulatus* sequencing data into a single analysis (Figure 8.1.8).

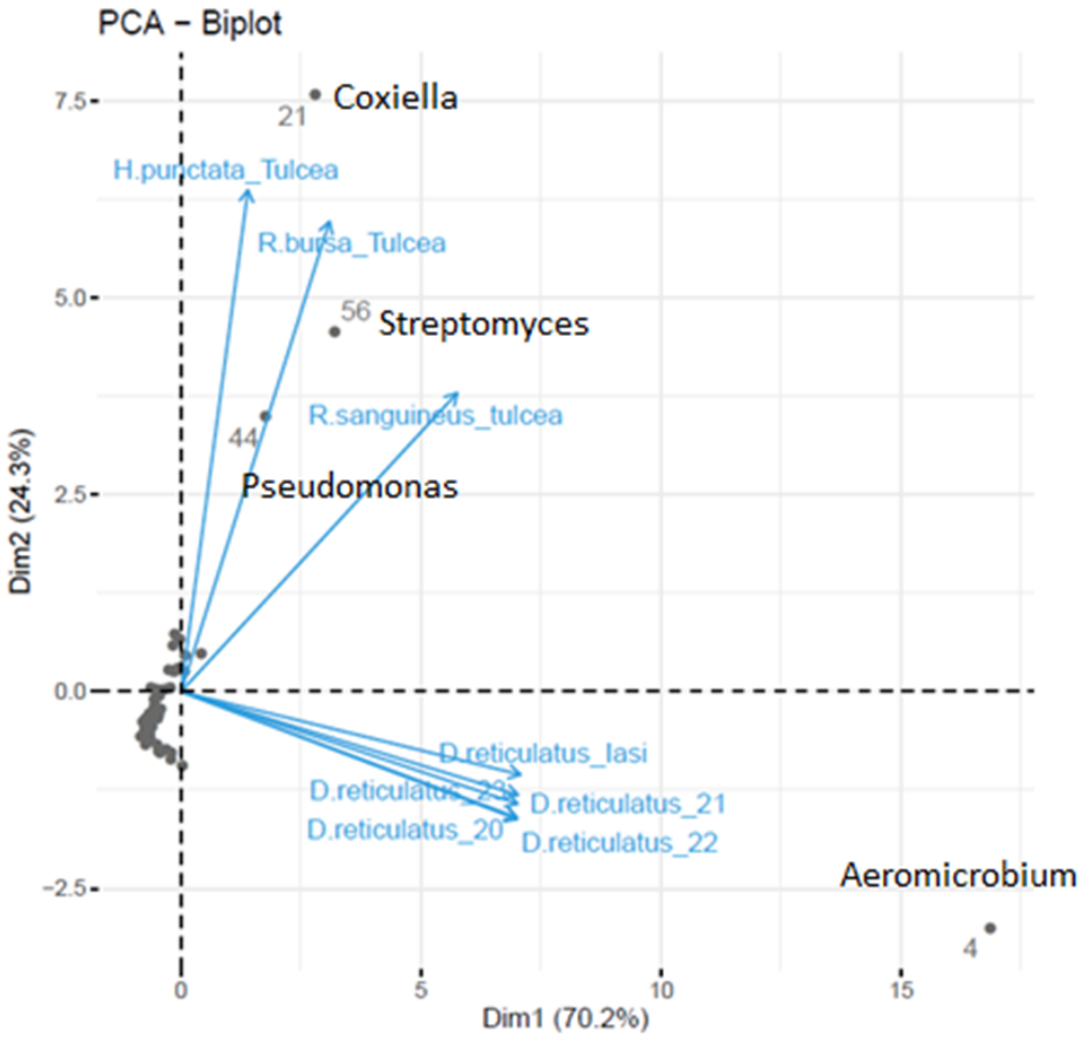


Figure 8.1.8- Principal Component Analysis (PCA)-biplot representation: separation of D. *reticulatus* cluster (libraries D_20 to D_23 and D_Iasi) from those of the three other tick species (*H. punctata; R bursa; R. sanguineus* from Tulcea)

We used metatranscriptomics analysis to describe the microbiome of Romanian ticks collected from Iasi and Tulcea counties in addition to virome analysis to assess the presence of commensal flora or the putative presence of bacterial pathogens carried by *D. reticulatus*, *H. punctata*, *R. bursa*, and *R. sanguineus*. As part of the tick microbiome, we detected some previously described ambient bacteria, such as *Aeromicrobium erytherum*, *Leadbetterella bysophilla*, and *Streptomyces*.

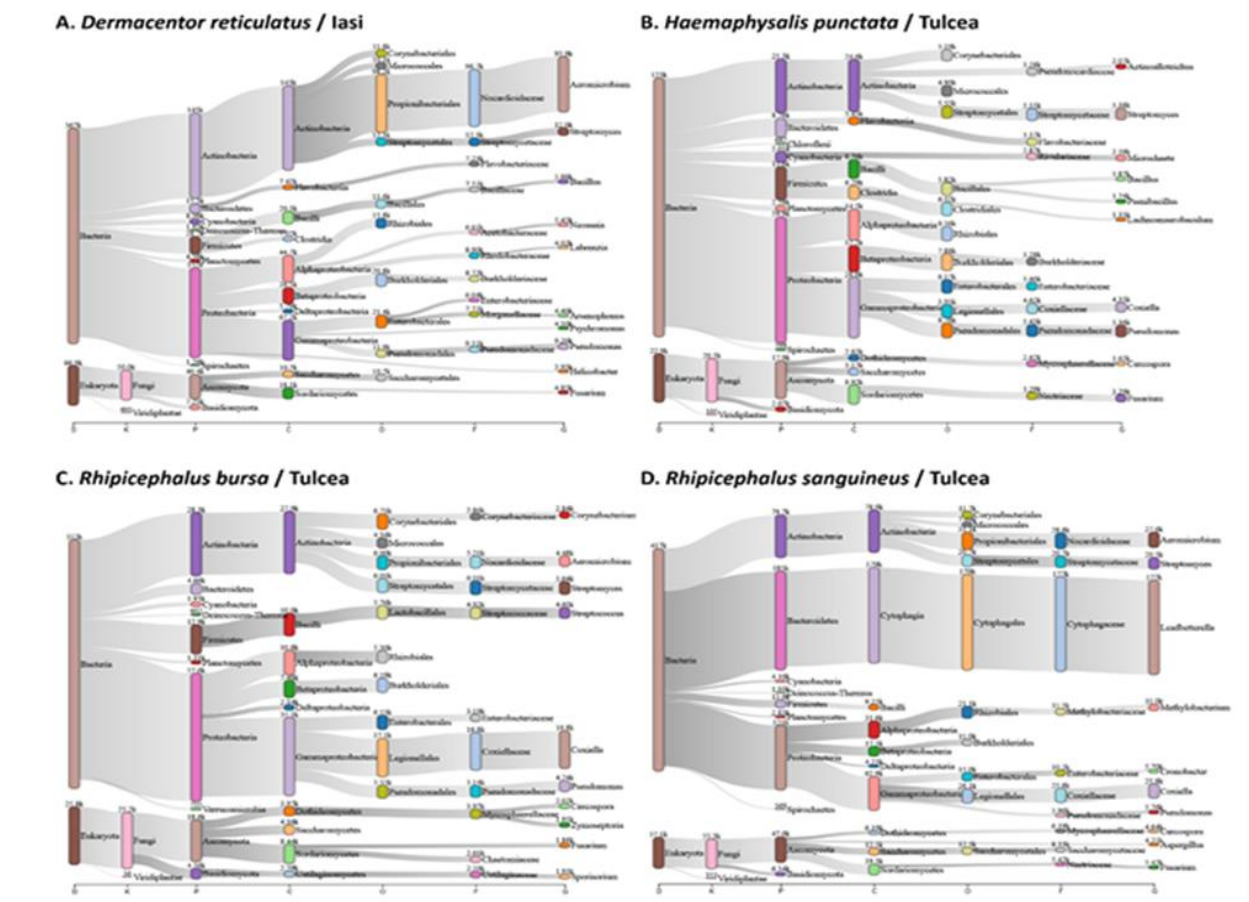


Figure 8.1.9- Representation of Romanian tick microbiome using Sankey diagrams based on Kraken2 analyses

The information was synthetized by a heatmap representation, restricted to the most abundant bacterial genera detected by more than 1000 reads per genus and per tick species. Within the bacterial genera identified, certain bacterial species deserve special mention because of their abundance or they are known or suspected pathogens for vertebrates (Figure 8.1.9).

Coxiella was the most frequently detected microorganism, being represented by *Candidatus Coxiella mudrowiae*, a Coxiella-like tick endosymbiont (Tsementzi et al., 2018). *Candidatus Coxiella mudrowiae* was found in all four tick species, being the most abundant in *R. sanguineus* from Tulcea County with a horizontal genome coverage of 31% (Figure 8.1.10).

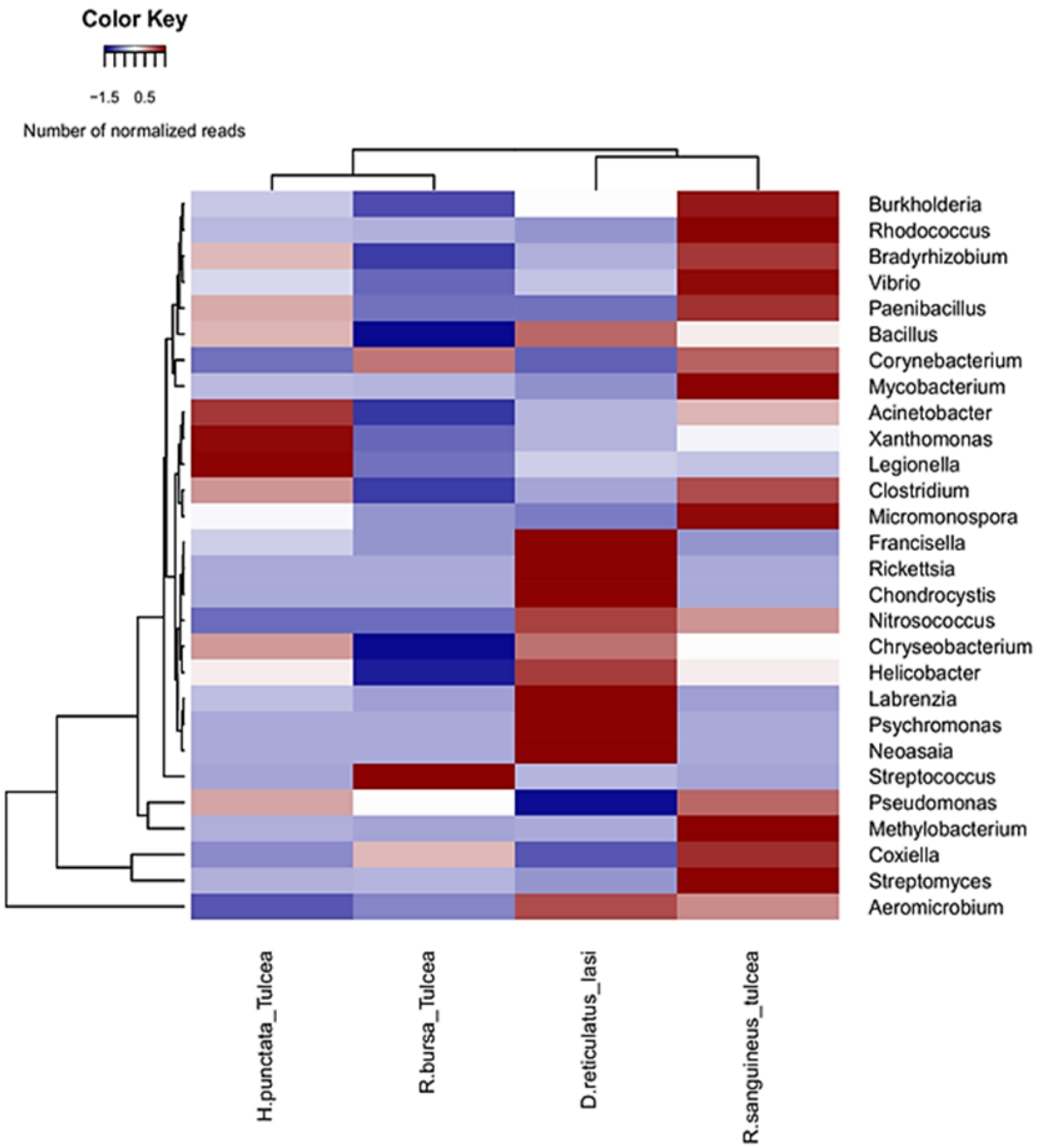


Figure 8.1.10- Heat map representation of the main bacterial genera in *D. reticulatus*, *R. bursa*, *R. sanguineus* and *H. punctata* collected from South-Eastern Romania. Rows are top bacterial genera and colums are the species of ticks

The agent of Q fever, *Coxiella burnetti*, was not found. *Francisella persica*, which was reclassified from *Wolbachia persica* in 2016, was another bacterial species found specifically in *D. reticulatus*, followed by *Rickettsia raoultii* (Larson et al., 2016). *Rickettsia raoultii* was first discovered in 1999 in *Rhipicephalus pumilio* and *Dermacentor nuttalli* from the former Soviet Union (Rydkina et al., 1999), and has been linked to human infection (Dong et al., 2019), indicating a possible zoonotic potential.

Rickettsia raoultii was only identified in *Dermacentor* sp. ticks in our investigation, as in previous studies (Mediannikov et al., 2008; Wen et al., 2014; Duscher et al., 2016; Karbowiak et al., 2016; Rudolf et al., 2016).

In Europe, *D. reticulatus* seems to be the most competent vector for *R. raoultii* (Mierzejewska et al., 2015). Tick-borne lymphadenopathy in humans (TIBOLA) is caused by this pathogen, which was previously discovered in Romanian feeding *Dermacentor* sp. ticks (Ionita et al., 2013) indicating their involvement in *R. raoultii* transmission.

8.2. Identification of a novel *Quaranjavirus* and other viral families in *Ixodidae* ticks from Danube Delta

Study objectives

Little is known about viruses transmitted by the ticks in Romania. Since ticks are known vectors for potentially pathogenic and non pathogenic viruses and the virome of ticks is understudied in Southern Romania (Danube Delta region), the aim of the current study was to identify viruses associated with *Rhipicephalus* ticks collected from small ruminants using metagenomics.

Introduction

Ticks are hematophagous arthropod vectors that transmit a variety of human and animal pathogens. Since these pathogens are generally transmitted by specific tick species, they are often limited to geographic areas where the arthropods are present. Furthermore, the introduction of animals from different regions and also migratory birds expand tick distribution, exposing new populations in remote areas (Ogden et al., 2013).

Despite reports that recently described tick-borne disease-causing agents (Tacheng tick virus 2, Jingmen virus) and novel viruses belonging to viral families encompassing tick-borne arboviruses (Nayun tick nairovirus, Bole tick virus 4, Sulina virus) circulate in the region (Tomazatos et al., 2021; Bratuleanu et al., 2022a), the virome of ticks in this area remains understudied.

Romania is among the most biologically diverse countries in Europe, with sutable conditions for the establishment of tick-borne virus outbreaks, especially in the Southern half of the country, comprising Danube Delta region. Danube Delta preserved a higher biodiversity and constitutes a major hub for birds migration from Africa and Asia, leading to a significant risk of introduction of animal pathogens, including zoonotic and vector-borne agents.

Multiple studies have used metagenomics to investigate viral communities in different tick species and have in these identified viruses associated with a broad range of animals, plants and insects. For exemple, viruses belonging to *Quaranjavirus* genus, *Orthomyxoviridae* family are known as argasid tick-borne viruses and some viruses in this genus have the ability to infect humans but little is known about their pathogenicity. Recent studies have reported novel quaranjaviruses-like, for example, sequences belonging to new quaranjaviruses-like were identified in *Ixodes uriae* (Pettersson et al., 2020) in the Artic, *Amblyomma dissimile* (Sameroff et al., 2021*)* removed from wild animals, or in *Haemaphysalis hystricis* ticks collected in Japan (Kobayashi et al., 2021).

In a similar way, among the *Phenuiviridae* family, two novel phleboviruses, Brown dog tick phlebovirus 1 (BDTPV1) and Brown dog tick phlebovirus 2 (BDTPV2) (Sameroff et al., 2019) presenting similarity in both the S and L segments to known phleboviruses but with an apparently missing M segment were identified.

Because these viruses have only been identified in ticks so far, their ability to infect vertebrates is unknown. In addition to the viral families that are known to contain tick-borne viruses, new families have recently been discovered. One exemple is the *Chuviridae* family, associated with various species of ticks from different regions of the world (Brinkmann et al.,

2018; Souza et al., 2018; Sameroff et al., 2019; Temmam et al., 2019b; Gondard et al., 2020). For now, miviruses have been predominantly identified in arthropods.

Materials and methods

A number of 169 *Rhipicephalus sanguineus* ticks were collected in Cataloi village, Tulcea County, in October 2020 from small ruminants. Ticks were pooled resulting in a total of 14 pools (9 up to 16 ticks/pool) for RNA extraction. The 14 pools of ticks were mixed to form one NGS librarie and sequencing was carried out on an Illumina NextSeq 500 sequencer in a single-read 1 x 150 bp format to achieve approximately 65 million reads. The presence of viral RNA for Cataloi tick quaranjavirus (CTQV), Brown dog tick phlebovirus 1 (BDTPV1), Brown dog tick phlebovirus 2 (BDTPV2) and Cataloi mivirus (CTMV) was confirmed by RT-PCR for all the 14 tick pools. In order to explore the potential presence of integrated viral sequences into tick genomes for relevant viral species identified by NGS, tick samples were also screened by PCR by omitting the reverse transcription step.

Results

Three viral families were identified: *Phenuiviridae* (41%), *Chuviridae* (36%) and *Orthomyxoviridae* (22%).

***Phenuiviridae* and *Chuviridae*-related viruses**

Phlebovirus is the only known viral genus in the *Phenuiviridae* family that can infect vertebrates, including humans and domestic animals**.** The reads belonging to the *Phenuiviridae* family detected in our study were assigned as Brown dog tick phlebovirus 1 (BDTPV1) and Brown dog tick phlebovirus 2 (BDTPV2), all negative sense bi-segmented ssRNA viruses that missed the M segment coding for the viral glycoprotein. The polymerase segment of Romanian BDTV2 presented an amino-acid identity of 96.33% with its closest tick-borne Caribbean isolate relative (QDW81040), while the nucleoprotein segment showed a lower amino-acid identity (93.88%), and a coverage of 99.57% for the L segment and 93.91% for the S segment. Romanian BDTPV1 presented a horizontal coverage of 98.62% (L segment) and 93.87% (S segment); and amino-acid identity ranged from 96.79% (L segment) to 92.55% (S segment) with its closest relative BDTPV1 found in Trinidad and Tobago (QDW81039).

Phylogenetic analysis conducted on the amino-acid sequences, placed Romanian phleboviruses within the genus *Uukuvirus*. Tick-borne isolates from Trinidad and Tobago were situated in a clade with Romanian BDTPV2. Romanian BDTV2 clustered in a highly supported distinct clade (posterior probability of 1), distinct from other strains also originating from Southern Romania (the same region as the current study), revealing the presence of two distinct BDTPV2 strains in the same area and a possible geographical and/or host specificity of these viruses. Romanian BDTV2 belongs to a clade that appears to be limited to tick phleboviruses primarily detected in *Rhipicephalus* (*bursa* or *sanguineus*) ticks (Figure 8.2.1).

The viral RNA for BDTPV1 and BDTPV2 was confirmed by RT-PCR and all tick pools tested for the presence of EVE were negative, which confirm that these sequences were not endogenous to the ticks.

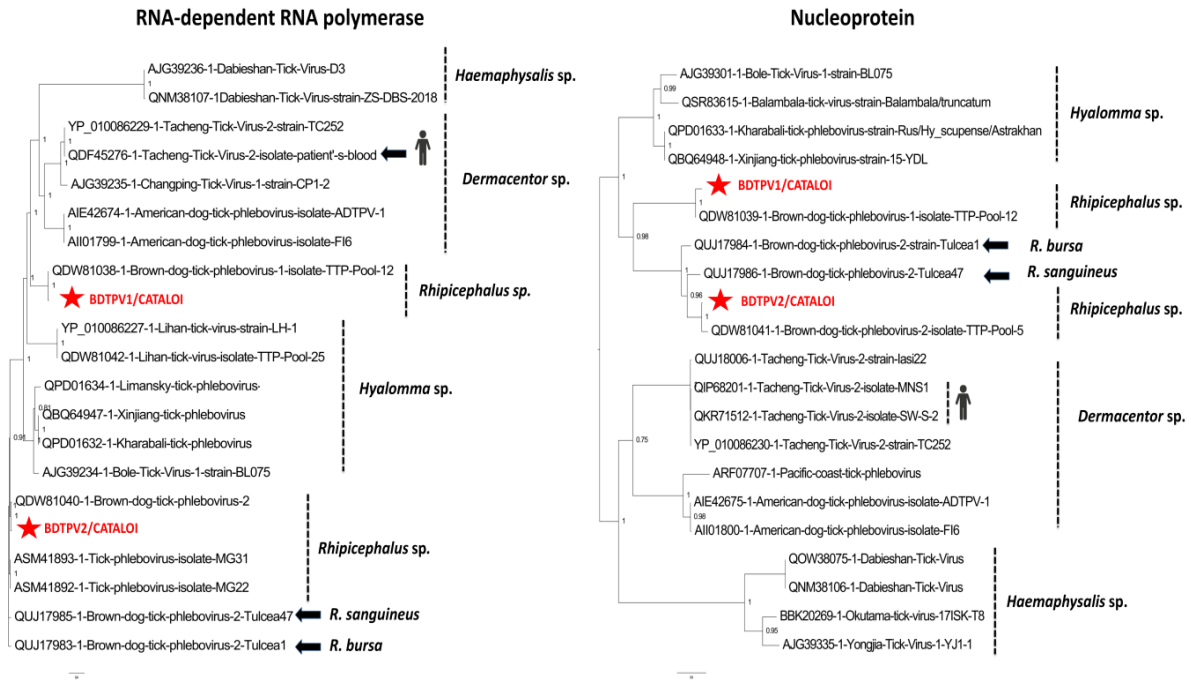


Figure 8.2.1- Phylogenetic relationship of NP segment and RdRp segment of BDTPV1 and BDTPV2 identified in Romanian *Rhipicephalus* ticks with other viruses among the *Phlebovirus* genus

Sequences belonging to *Chuviridae* family were also detected. *Chuviridae* is a new recognized viral family among the Jingchuvirales, infecting a wide variety of invertebrate hosts (Crustacea, Nematoda, Insecta, Myriapoda, Arachnida, and Ixodida).

The complete genome of Cataloi mivirus (CTMV) detected in the current study has three ORFs: the first ORF codes for a large protein of 2156 amino-acids corresponding to the RdRp segment; ORF2 codes for glycoprotein segment 683 amino-acid and ORF3 codes for the nucleoprotein (NP) of 527 amino-acid. CTMV presented an amino-acid identity ranging from 83% for the nucleoprotein segment to 94% for the RdRp gene with its closest tick-borne Chinese isolate detected in *R. turanicus* (QYW06785) and a genome coverage of 100%.

According to phylogenetic analysis performed on the RdRp sequences, CTMV was placed in a specific clade comprising *Rhipicephalu*s sp. ticks isolates from China, Trinidad and Tobago and Thailand, indicating that these miviruses may have a tick vector specialization (Figure 8.2.2). RT-PCR confirmed the presence of CTMV viral RNA, and all tick pools tested for EVE were negative, indicating that these sequences were not endogenous to the ticks.

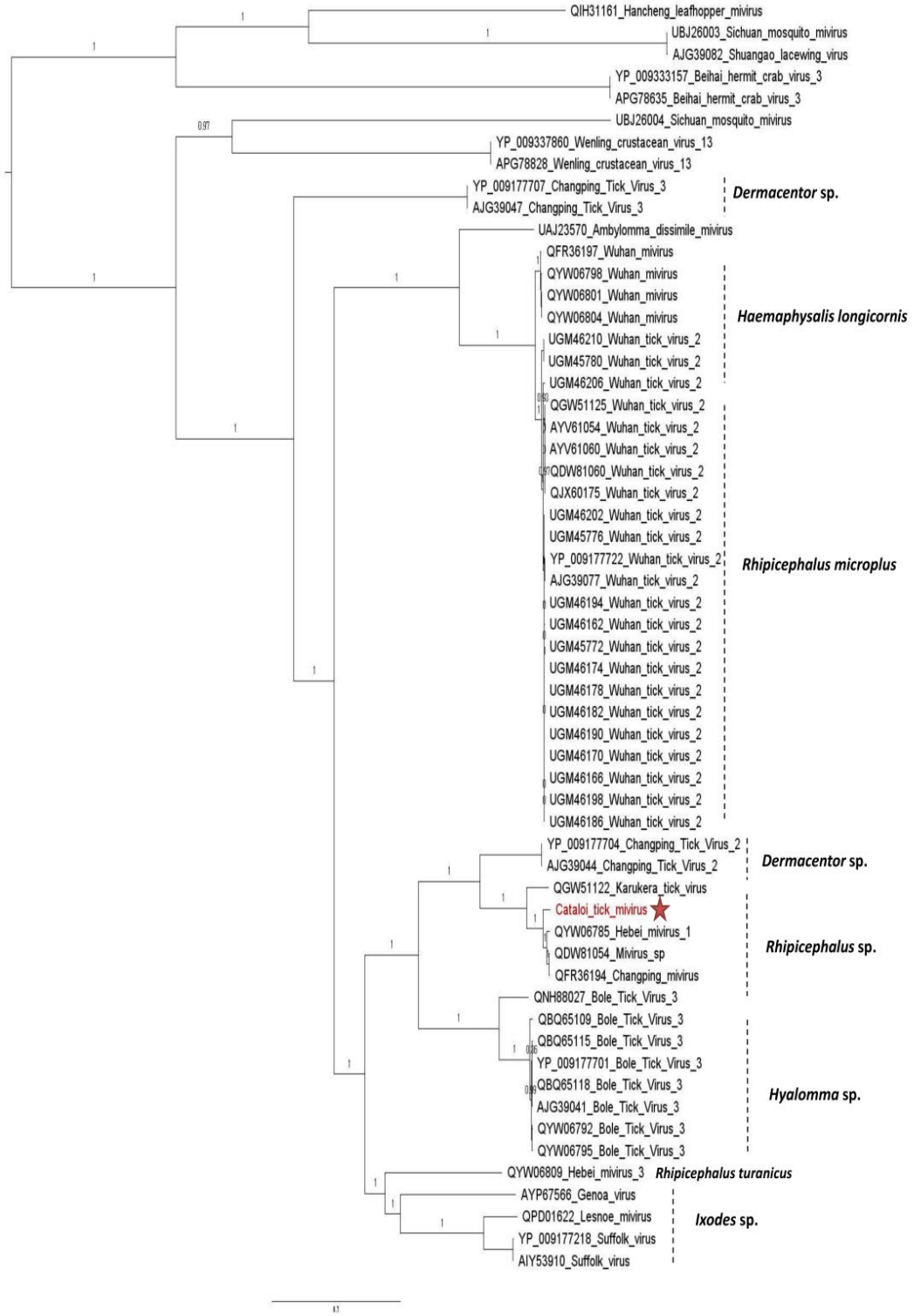


Figure 8.2.2- Phylogenetic relationship of RdRp segment of CTMV detected in *Rhipicephalus* sp ticks with other viruses among the *Chuviridae* family

***Orthomyxoviridae* family**

Within the *Orthomyxoviridae* family, sequences of a new quaranjavirus, named Cataloi tick quaranjavirus (CTQV) was detected. CTQV sequences comprise five segments with similarity to known quaranjaviruses: within the HA, PA, PB2, and NP segments, CTQV was most closer to Granville quaranjavirus (GQV), a novel quaranjavirus detected in *A. dissimile* ticks removed from iguanas. Contrary with other segments that were similar to GQV, PB1 protein was closer to Zambezi tick virus 1 (ZaTV-1), a highly divergent quaranjavirus found in *Rhipicephalus* ticks from Mozambique. Additional segments for ZaTV-1 were not available in the databases. The Viral RNA for CTQV was confirmed by RT-PCR and all tick pools tested for the presence of EVE were negative (Bratuleanu et al., 2022b)

CTQV is positioned differently depending on the segment, according to phylogenetic analysis performed on the five segments. In general, CTQV clustered with quaranjaviruses and related viruses, which are distributed across different geographical regions. Whitin the PA, PB2, HA and NP segments, CTQV formed a separate branch, and was placed in a clade with other recently tick-borne quaranjaviruses detected in ixodid ticks from Caribe (Granville virus) and Japan (Ohshima virus). CTQV was phylogenetically close to ZaTV-1 in PB1 protein (Figure 8.2.4). Attempts to isolate CTQV on Vero cells and embryonated eggs inoculated with CTQV positive tick pools were unsuccessful.

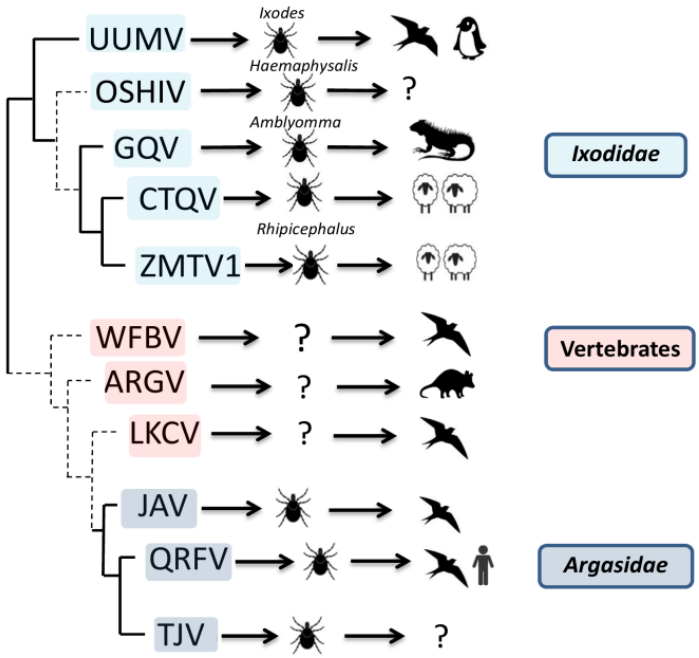


Figure 8.2.3- Schematic representation of *Quaranjavirus* relationship with their corresponding vectors and associated vertebrate hosts. Continuous lines represent constant phylogenetic clustering across the different segments; dashed lines correspond to different phylogenetic positioning among the segments

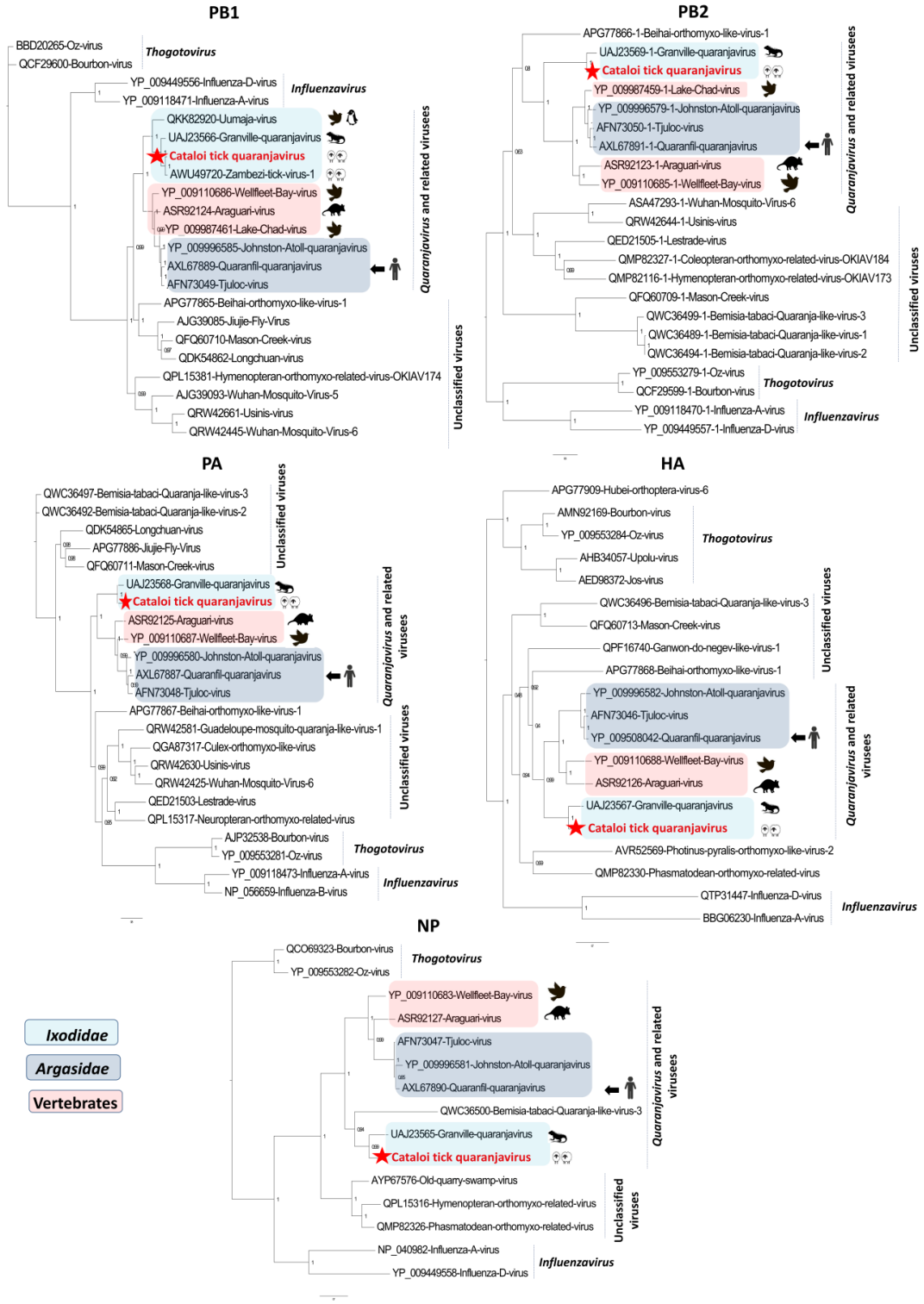


Figure 8.2.4- Phylogenetic analysis of CTQV PB1, PB2, PA, NP and HA segments with other viruses from the the *Orthomyxoviridae* family. In light blue: *Ixodidae*-related quaranjaviruses; in dark blue: *Argasidae*-related quaranjaviruses, in pink: vertebrates related quaranjaviruses

Discussion

In this study, we used a viral meta-transcriptomic analysis to identify viruses associated with *Ixodidae* ticks collected in the Danube Delta Biosphere Reserve (DDBR), Europe's second biggest wetland. Identifying viruses at higher risk of emergence, requires first understand the virus's ecological cycle, which includes its probable reservoir hosts, hematophagous arthropod vectors (in the case of arboviruses), and vertebrate recipient animals (Temmam et al., 2014).

There are few investigations on the occurrence of quaranjaviruses in hard-ticks around the world, and little is known about the viruses' molecular pathogenesis. To our knowledge, this is the first report on the identification of a quaranjavirus-like virus related with ixodid ticks in Eastern Europe region.

The Quaranjavirus genus (*Orthomyxoviridae* family) is a family of negative-sense segmented viruses with a single-stranded genome and, six segments (polymerase acidic protein-PA, polymerase basic protein 1-PB1, polymerase basic protein 2-PB2, hemagglutinin protein-HA, nucleocapsid protein-NP and matrix protein-M). ICTV recognizes two virus species in the genus *Quaranjavirus:* Johnston Atoll (JAV) virus and Quaranfil virus (QRFV), which are both transmitted to birds primarily by argasid ticks. In Egypt (Al-Khalifa et al., 2007), South Africa (Sang et al., 2006), Afghanistan, Nigeria (Kemp et al., 1975) Kuwait, Iraq, Yemen (Converse and Moussa, 1982), and Iran, several strains of QRFV have been discovered from ticks and seabirds (Sureau and Klein, 1980).

Quaranjavirus-like sequences have also been identified in hard ticks in more recent studies: for example, sequences of a novel quaranjavirus, tentatively designated Granville quaranjavirus (GQV), were identified in *A. dissimile* ticks removed from iguanas (Sameroff et al., 2021). Related viruses were also detected in *Rhipicephalus* sp. ticks from Mozambique (Cholleti et al., 2018), *H. hystricis* ticks from Japan (Kobayashi et al., 2020) and *I. uriae* ticks from Sweden (Pettersson et al., 2020). Because these viruses have only been identified in ticks so far, their ability to infect vertebrates is unknown.

CTQV was identified in engorged *Rhipicephalus* sp. ticks, which can infect a variety of domestic and wild hosts, including cats, rats, birds, cattle, horses, goats, and humans (Estrada-Pena and Jongejan, 1999; Walker et al., 2000; Dantas-Torres et al., 2006). CTQV belongs to a phylogenetic clade that appears to be limited to *Ixodidae* and separate from argas-related quaranjaviruses. Because of the apparent diversity of vertebrate hosts (iguanas, small ruminants, and seabirds), CTQV and other *Ixodida*e-associated quaranjaviruses are considered to harbor a tick specificity, independently of the hosts on which ticks feed (Figure 8.2.3).

It's still questionable if ixodid-associated quaranjaviruses can replicate in their vertebrate hosts. Similarly, CTQV detected in Eastern Romania placed in a clade with viruses from various geographical biotopes, including Trinidad and Tobago, Mozambique, and Sweden, indicating that no geographical specificity shapes the evolution history of these viruses and supporting the hypothesis about the vector specificity of these viruses.

Conclusions

- This is the first detection of a quaranjavirus-like virus associated with ixodid ticks in Eastern Europe region.
- This study represents a first step for a more comprehensive overview of the quaranjaviruses diversity in ixodid ticks and contributes to the knowledge of the diversity of viruses transmitted by ticks in poorly studied areas from Romania.
- Future studies are necessary to isolate the virus and determine the pathogenic potential of CTQV and other viruses for animals and humans health, such serological surveillance of sheep from which ticks have been collected, to determine the arbovirus status of CTQV (Chapter 8.3).

8.3. Luciferase immunoprecipitation systems (LIPS)- Based Serological Screening of small ruminants sera

Assays capable to detect antibody responses to infectious agents have both diagnostic and prognostic purposes. Neverthless, one major limitation of the most common enzyme-linked immunosorbent assay (ELISA) is the need to detect pathogen-specific antigens or epitopes that induce antibody responses in a significant number of individuals. Such tests often show cross-reactivity and uses denatured proteins. LIPS test developed by Burbelo et al., allows the recovery of viral antigens under native conditions using expression system in mammalian cells that allows the glycosylation of viral antigens, limiting therefore this issue (Burbelo et al., 2007). This method can be easily and quickly introduced to study different antigens with little or no change in methodology. A major advantage of this test is the use of native antigens that contain many non-linear immunoreactive epitopes.

LIPS demands a minimum of assay optimization and due to its simplicity, high-quality data can usually be produced using LIPS in less than two weeks for any given antigen, starting from cloned antigens. The most time-consuming steps are cloning and generation of the appropriate plasmid expression vector containing the nanoluciferase-viral antigen fused protein. Once these plasmids are generated, single or multiple antigens can be rapidly tested.

Method Principle

The LIPS assay is a liquid phase immunoassay allowing high-throughput serological detection of antigen-specific antibodies and is based on the use of antigens made of viral proteins (or domains) fused to nanoluciferase (nanoluc). The Nanoluc-antigen fusion protein is recognized by antigen-specific antibodies, and antigen-antibody complexes are captured by protein A/G-coated beads that preferentially recognize the Fc region of the IgG antibodies (Figure 8.3.1).

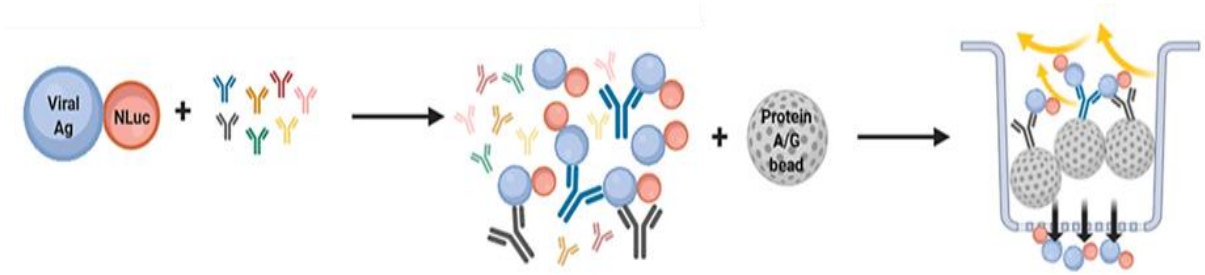


Figure 8.3.1- LIPS method principle

Objectives

The aim of this study 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.

Materials and methods

Small ruminants sera

A total of 396 blood samples were collected between 2019-2021 from small ruminants from six different villages in a rural area whitin Tulcea County (Danube Delta region). Details regarding the number of serum samples and the collection sites are presented in Table 8.3. These sera were obtained with the approval of the animal owners. In addition, 50 sera from healthy sheep from France were screened for presence of antibodies against CTQV as a likely non-exposed group control (kindly provided by Stephan Zientara & Emmanuel Breard, Anses, Maisons-Alfort, France). LIPS assay was performed as previously described (Temmam et al., 2019b) with no diluted sera. Residual background was calculated as the mean of results from 10 negative controls (without serum), and the positivity threshold was defined as the mean of these controls + 3 standard deviations.

Table 8.3

Ruminants serum samples collected from Tulcea County

Year of collection	Collection site	Species	Number of samples
2019	Baia	Sheep	19
	Cataloi	Goat	28
		Sheep	28
	Slava Cercheză	Sheep	59
	Somova	Sheep	26
2020	Baia	Goat	6
	Baia	Sheep	6
	Cataloi	Sheep	21
	Slava Rusă	Goat	23
		Sheep	9
2021	Cataloi	Sheep	106
	Mihail Kogalniceanu	Sheep	10
	Slava Cercheză	Sheep	54
TOTAL		**396**	

Antigen design

To increase the probability of detecting specific antibodies to CTQV and to minimize cross-reactions, the predicted ectodomain of CTQV haemagglutinin was targeted (Figure 8.3.2). Swiss Model was used to model the structure of the viral protein and to compare it to known structures deposited onto the PDB database (https://swissmodel.expasy.org/interactive) (Arnold et al., 2006).

Synthetic genes coding for CTQV ectodomain was ordered from GenScript with codon usage optimized for protein expression in mammalian cells, and cloned in the pcDNA3.1(+) vector. An exogenous signal peptide was added to ensure efficient protein secretion. The nanoluc was added to the carboxy-terminal end of the construct, or spaced by a 3-residues linker.

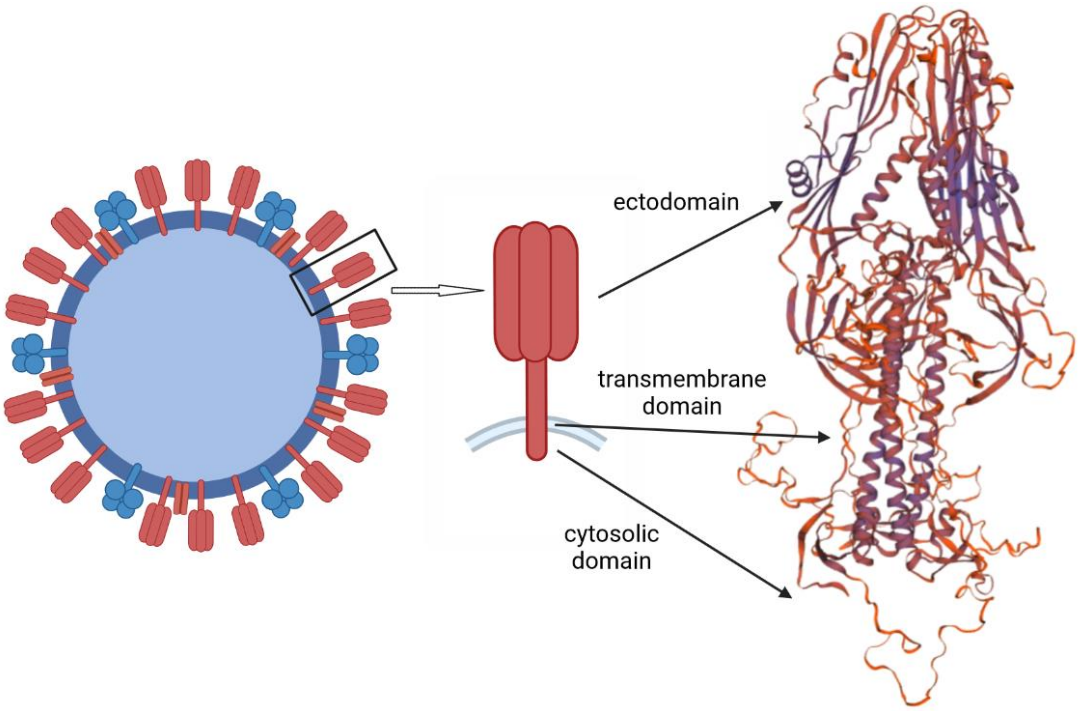


Figure 8.3.2- Schematic representation of antigen design for Cataloi tick quaranjavirus (original)

Expression of recombinant proteins

HEK-293F cells were grown in suspension and transfected with ExpiFectamine 293 reagent (ThermoFisher) according to the manufacturer's recommenfations. Recombinant proteins were harvested at day 4 in the culture supernatant. Luciferase activity was quantified with a Centro XS^3 LB 960 luminometer (Berthold Technologies, France).

LIPS assay

LIPS assay was performed as previously described (Temmam et al., 2019b), except that 10^8 LU of antigens and 10 µL of non-diluted sera were engaged per reaction.

Statistical tests

Statistical analyses were conducted with GraphPad Prism 8 (GraphPad Software, San Diego). The signal-to-noise light unit (LU) ratios between each group of sera were compared using the Kruskal-Wallis ANOVA and Dunn's multiple comparisons tests with the French cohort used as reference.

RESULTS & DISCUSSION

The transmission of CTQV by *R. sanguineus* ticks to the small ruminants exposed to tick bites was evaluated by LIPS targeting the predicted ectodomain of CTQV haemaglutinin. 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 as 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 (Figure 8.3.3). Since we do not know if this virus is able to infect vertebrates, we could not obtain any positive control and therefore we were not able to define an adapted positivity threshold and assess the analytical sensitivity of LIPS assay for the targeted antigen. 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.

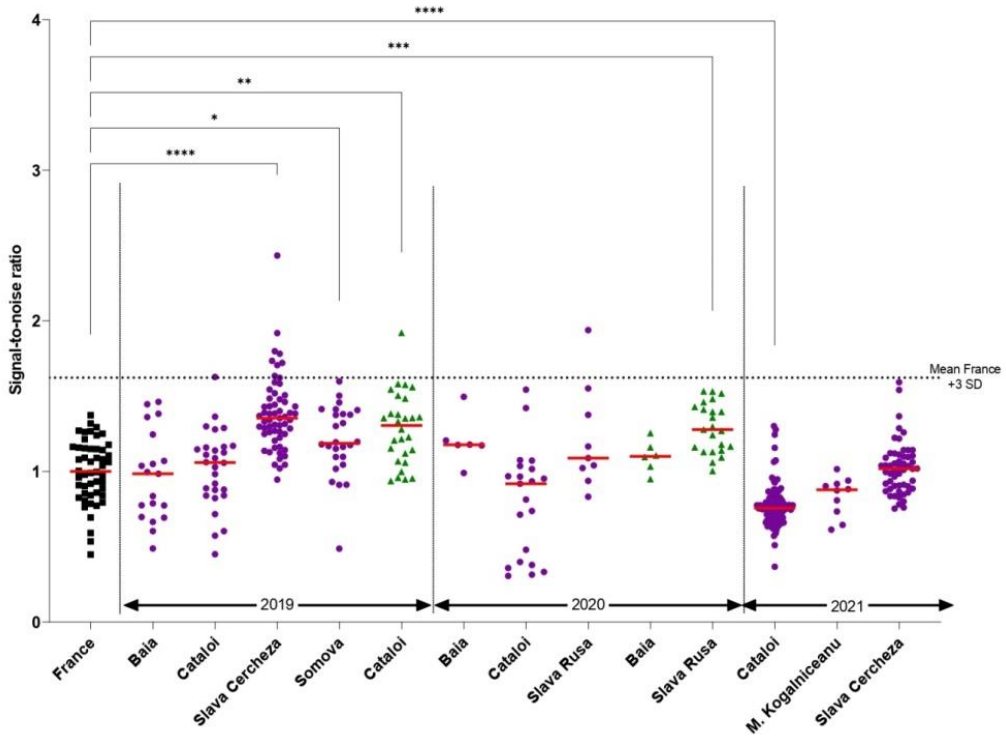


Figure 8.3.3- Luciferase activity (in LU/ml) distribution of measures after luciferase immunoprecipitation system (LIPS) performed in small ruminants. In black- sheep from France; in purple-sheep from Romania; in green- goats from Romania

CHAPTER IX
FINAL CONCLUSIONS

The results obtained after pursuing the studies on tick-borne infections in ticks from Eastern Romania allowed the formulation of the following conclusions:

- The serologic profile in dogs is an important factor that may be used **to assess the risk** of *Borrelia spp., A. phagocytophilum* and *E. canis* for **humans and animals**. It has been observed that in most cases, humans are developing Lyme disease in areas where the disease is prevalent in dogs.

- Our findings present similar results compared to the values registered in other studies undertaken in our country on *B. burgdorferi* prevalence in dogs and horses. Nevertheless, a previously conducted study performed in Eastern Romania, reported a prevalence for *B. burgdorferi* of 1.1%. These findings indicates that in recent years, **the prevalence in dogs has increased in the research area**.

- The serological study performed on dogs for the detection of antibodies anti-*A. phagocytophilum* demonstrates the presence of canine anaplasmosis in Iasi, Tulcea and Sibiu counties, with the **highest seroprevalence in hunting dogs from Sibiu County**. These results suggest that dogs living outdoors and receiving no treatments may be infected with *A. phagocytophilum* and highlight the importance for appropriate antiparasitic treatments.

- Despite the fact that the presence of the CCHF virus has yet to be confirmed in Romania, several research performed in Romania, may indicate that the **virus is circulating in small ruminant flocks**.

- Even if the species of investigated ticks (*Rhipicephalus* and *Dermacentor* sp.) can transmit CCHFV, the main vector (*Hyalomma marginatum*) has not been tested, which can explain the negative results obtained by molecular survey.

- The global CCHFV antibody seroprevalence rate in sheep and goats from Tulcea County was 37.7%. This work provides an updated overview of CCHFV seroprevalence in livestock from five sites from Southern Romania (Danube Delta region) and **support the role of sheep and goats in CCHFV ecology** in Romania.

- **These findings also indicate the circulation of CCHFV or another antigenically related nairovirus among small ruminants, in Southern Romania**. Future research should focus on increasing number and diversity of tick species sampled and also

expanding the geographic range of surveillance, including NGS to broaden the spectrum of nairovirus detection throughout Romania.

- In order to extend the knowledge about the diversity of viruses in Eastern Europe, several species of ticks collected from Iasi and Tulcea counties were investigated. In this work, **new viruses** belonging to the families ***Flaviviridae, Phenuiviridae*** and ***Nairoviridae*** were identified for the **first time** in Eastern Europe, more precisely in Romania, by **high throughput sequencing**.

- Because of its ability to infect a wide variety of tick vectors, mammals, and humans, **Jingmen tick virus** (JMTV) has recently raised animal and public health concerns. JMTV was found in engorged ticks from sheep and goats in this study, suggesting that **ruminants may play a role in the Jingmenvirus life cycle in Romania**, as has been reported in other regions of the world.

- For the first time in Romania, **new pathogens have been identified** such as Romanian **Tacheng tick virus 2** (TaTV2). TaTV2 was placed in a clade with ticks and humans isolates from China, indicating that this virus may have a major significance for human and animal health.

- The **pathogenicity** of **TaTV2** for **humans and animals** was later **confirmed**, being **detected** in **tick bite patients, sheep, cattle, badgers, gerbils** and ***Hyalomma asiaticum*** **tick egg batches**, in 2021, in China.

- Other viruses not yet known to if they are pathogenic or not (**Bole tick virus 4**, **Nayun tick nairovirus**) have been identified and further studies are needed to determine their importance for human and animal health.

- Regarding microbiome data, ***Coxiella*** was the most frequently detected microorganism, being represented by ***Candidatus Coxiella mudrowiae***, followed by ***Rickettsia raoultii***, causing tick-borne lymphadenopathy in humans (TIBOLA).

- The **NGS** analysis of *Rhipicephalus sanguineus* ticks from Danube Delta region unvealed the **first identification** of a **quaranjavirus-like virus** related with **ixodid ticks** in **Eastern Europe,** named **Cataloi tick quaranjavirus.** This study represents a first step for a more comprehensive overview of the quaranjaviruses diversity in ixodid ticks and contributes to the knowledge of the diversity of viruses transmitted by ticks in poorly studied areas from Romania.

- **The transmission** of **Cataloi tick quaranjavirus** (CTQV) by ***R. sanguineus*** ticks to the **small ruminants** exposed to tick bites was evaluated by **LIPS assay**. Only eight sheep sera collected in Slava Cercheza (Tulcea County) 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.

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Annex 1- Abreviation list

ALKV	Alkhurma virus
ALT	Alanine aminotransferase
ASFV	Africa swine fever virus
AST	Aspartat aminotransferase
BDTPV1	Brown dog tick phlebovirus 1
BDTPV2	Brown dog tick phlebovirus 2
Blast	Basic Local Alignment Search Tool
BlastN	Basic Local Alignment Search Tool-Nucleotides
BlastP	Basic Local Alignment Search Tool-Proteins
BOLD	Barcode of Life Data Systems
Bp	Base pair
BSL-3	Biosafety level-3
BTV4	Bole tick virus 4
CCHFV	Crimean-Congo hemorrhagic fever virus
CISID	Centralized Information System for Infectious Diseases
CPTV1	Changping tick virus 1
CTMV	Cataloi mivirus
CTQV	Cataloi tick quaranjavirus
CVBD	Canine vector-borne diseases
DDBR	Danube Delta Biosphere Reserve
DNA	Deoxyribonucleic acid
DNAc	Complementary deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EVE	Endogenous viral elements
GQV	Granville quaranjavirus
HA	Hemagglutinin protein
HRTV	Heartland virus
ICTV	International Committee on Taxonomy of Viruses
IgG	Imunoglobulina G
JMTV	Jingmen tick virus
KFDV	Kyasanur Forest disease
LIPS	Luciferase immunoprecipitation systems
LIV	Louping ill virus
MP	Matrix protein
NCBI	National Center for Biotechnology Information
NGS	Next-generation Sequencing
NP	Nucleoprotein
NSDV	Nairobi sheep disease virus

NT	Nucleotides
NTNV	Nayun tick nairovirus
OHSIV	Ohshima virus
ORF	Open Reading Frame
PA	Polymerase acidic protein
PB1	Polymerase basic protein 1
PB2	Polymerase basic protein 2
PCA	Principal Component Analysis
pcDNA	Plasmid cloning DNA
PCR	Polymerase chain reaction
RdRp	RNA-dependent RNA-polymerase protein
RNA	Ribonucleic acid
RT-PCR	Reverse transcription-polymerase chain reaction
RT-qPCR	Real-time quantitative reverse transcription polymerase chain reaction
SFTSV	Thrombocytopenia syndrome virus
Sl	Sensu lato
SMRT	Single molecule real-time
Ss	Sensu stricto
ssRNA+	Positive single-stranded RNA
TATV2	Tacheng tick virus 2
TBEV	Tick-borne encephalitis virus
TBP	Tick-borne pathogens
TGS	Third-generation sequencing
TIBOLA	Tick-borne lymphadenopathy
WHO	World Health Organization
ZaTV-1	Zambezi tick virus 1
ZMW	Zeromode waveguides

Annex 2- Figures list

Annex 3- Tables list

Annex 4- List of published papers

In ISI journals (first author)

PhD field (3)

1. **Bianca Elena Bratuleanu**, Sarah Temmam, Delphine Chrétien, Béatrice Regnault, Philippe Pérot, Christiane Bouchier, Thomas Bigot, Gheorghe Savuța, Marc Eloit, 2021- The virome of *Rhipicephalus*, *Dermacentor* and *Haemaphysalis* ticks from Eastern Romania includes novel viruses with potential relevance for public health, Transboundary and Emerging Diseases, 2021;00, Pag 1-17 https://doi.org/10.1111/tbed.14105. **IF 5.05**.

2. **Bianca Elena Bratuleanu**, Sarah Temmam , Sandie Munier, Delphine Chrétien, Thomas Bigot, Sylvie van der Werf, Gheorghe Savuta, Marc Eloit, 2022. Detection of *Phenuiviridae*, *Chuviridae* Members, and a Novel *Quaranjavirus* in Hard Ticks From Danube Delta. Front Vet Sci. 2022 Apr 13; 9:863814. doi:10.3389/fvets.2022.863814 PMID: 35498749; PMCID: PMC9044029, Pag 1-9. **IF 3.41**.

3. **Bianca Elena Bratuleanu**, Adriana Anita, Sarah Temmam, Anca Dascalu, Luciana Crivei, Andreea Cozma, Philippe Pourquier, Gheorghe Savuta, Marc Eloit, Dragos Anita, 2022. Seroprevalence of Crimean Congo Hemorrhagic Fever among small ruminants from Southern Romania.Vector-Borne and Zoonotic Diseases. **IF 2.13**.

ISI Abstracts (first author)

PhD field (1)

1. **Bianca Elena Bratuleanu**, Sarah Temmam, Delphine Chrétien, Béatrice Regnault, Philippe Pérot, Christiane Bouchier, Thomas Bigot, Gheorghe Savuța, Marc Eloit, 2021. Caractérisation des communautés virales dans des tiques de Roumanie : nouveaux virus a potentiel zoonotique ? Virologie, Vol 25, supplément, avril 2021, Pag 57-58, **IF 0.5**.

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PhD field (2)

1. **Bianca Elena Bratuleanu**, Adriana Aniţă, Mihaela Anca Dascălu, Dragos Aniță, Gheorghe Savuța, 2019. Evidence of canine vector-borne diseases in two counties from Eastern Romania. Rev Rom Med Vet (2019) 29 | 4: 87-90.

2. **Bianca Elena Bratuleanu**, Delphine Chretien, Béatrice Regnault, Sarah Temmam, Philippe Perot, Adriana Aniță, Dragos Aniță, Gheorghe Savuța, Marc Eloit, 2020. Survey of Crimean-Congo haemorrhagic fever virus in *Rhipicephalus* and *Dermacentor* species ticks from South-Eastern Romania. Rev Rom Med Vet (2020) 30 | 3: 57-62.

In BDI journals (first author/coauthor)

PhD field (2)

1. **Bianca Elena Bratuleanu**, Adriana Aniţă, Dragos Aniţă, Luanda Oșlobanu, Anca Dascalu, Gheorghe Savuța, 2021. A survey on anaplasmosis in dogs from three counties of Romania. LUCRĂRI ŞTIINŢIFICE MEDICINĂ VETERINARĂ VOL. LIV(1), 2021, TIMIŞOARA

2. Ratoi I.A. (Anton), Cozma A.P, Dascalu M.A., Crivei L.A, **Bratuleanu B.E.**, Oșlobanu L., Aniţă D., Aniță A., Savuta G., 2019. Bolile transfrontaliere la animale- o permanentă provocare! Lucrări Științifice- Medicină Veterinară, Universitatea Agrară de Stat din Moldova, vol. 54, pag 58-68.

Communications in international conferences

1. **Bianca Elena Bratuleanu**, Sarah Temmam, Delphine Chrétien, Béatrice Regnault, Philippe Pérot, Christiane Bouchier, Thomas Bigot, Gheorghe Savuța, Marc Eloit- Caractérisation des communautés virales dans des tiques de Roumanie : nouveaux virus a potentiel zoonotique (**poster** and **oral presentation**), Les XXIIIemes Journées Francophones de Virologie, 26-27 avril, 2021.

2. **Bianca Elena Bratuleanu**, Sarah Temmam , Sandie Munier, Delphine Chrétien, Thomas Bigot, Sylvie van der Werf, Gheorghe Savuta, Marc Eloit, 2022. Detection of *Phenuiviridae*, *Chuviridae* members, and a novel *Quaranjavirus* in hard ticks from Danube Delta (**poster**). Journées departementale de Virologie, Institute Pasteur, Paris.

Oral presentations

1. **Bianca Elena Bratuleanu**, Gheorghe Savuța. Identification of novel viruses with potential relevance for public health in ticks from South-Eastern Romania. Simpozionul Stiintific Studentesc, USAMV Iasi, sectiunea Scoli doctorale, 18 noiembrie 2021- **Premiul I**.

2. **Bianca Elena Bratuleanu**, Gheorghe Savuța. Detection of *Phenuiviridae, Chuviridae* members, and a novel *Quaranjavirus* in hard ticks from Danube Delta. Simpozionul Stiintific Studentesc, USAMV Iasi, sectiunea Scoli doctorale, 14 aprilie 2022- **Premiul II**.

3. **Bianca Elena Bratuleanu,** Cristian Răileanu, Gheorghe Savuța- Noi agenți patogeni cu potențial zoonotic identificați la căpușe. A XIV-a Conferinţă Naţională de Microbiologie şi Epidemiologie, 6 noiembrie 2021.

4. **Bianca Elena Bratuleanu**, Adriana Aniţă, Dragos Aniţă, Luanda Oșlobanu, Anca Dascalu, Gheorghe Savuța, 2021. A survey on anaplasmosis in dogs from three counties of Romania. 3rd International Conference on Sustainable Development, 08 – 09 October 2020, Timisoara.