ABSTRACT

Ticks are parasite arthropods, obligatory blood-feeding with a global importance in terms of medical and veterinary importance. To date 896 species have been identified of ticks, spread from the sub-Arctic zone to the Equator. From these only 10% have the ability to transmit diseases or to cause damage by way of nutrition (anemia, dermatitis and poisoning), (Jongejan and Uilenberg, 2004). Ticks feed on the blood of mammals (including humans), birds and reptiles, and are capable of transmitting a greater variety of viruses, bacteria and protozoa, than any other group of blood-sucking arthropods (Dennis and Piesman, 2005). In terms of medical importance, they are second only to mosquitoes (Sonenshine, 1991). Interest has increased worldwide for vector-borne diseases in the context of their ascendancy incidence (Walker, 1998; Telford and Goethert, 2004).

The doctoral thesis **„Epidemiology and diagnosis of tick-transmitted zoonotic diseases"** contains 213 pages and is structured in accordance with current legal provisions into two main parts: the first part is entitled **„Current state of knowledge"** being structured in 59 pages and the second part **„Personal contributions"** 116 pages.

„Current state of knowledge" includes four chapters that summarizes information from the literature on the thesis subject and they are used later in the second part **„Personal contributions"** as references for the interpretation and discussion of results.

The first chapter entitled **„Bibliographic data about the *Ixodidae* ticks"** is divided into 6 chapters and summarizes the literature about phylogenetic classification of ticks, morphology and general anatomy, life cycle, ecology and behavior, species of ticks present in Romania and control of tick populations. There are currently 896 species and subspecies of ticksrecognized, that are spread worldwide (Guglielmone et al., 2010). In Romania, have been identified 27 species of ticks, including 25 species belonging the *Ixodidae* family and two species belonging to *Argasidae* family (Coipan et al., 2011).

The second chapter „**Medical and veterinary importance of ticks**" describes the vector role of ticks in the transmission of pathogens with the identification of main species of ticks

present in Romania. It also describes the reservoir role of ticks. Ticks in addition to diseases caused by transmission of pathogens can have toxic actions causing paralysis, allergies and dermatitis. In last years, ticks saliva has been the subject of various studies, because of interest inthe identification and isolation of biomolecules with vasodilator, anti-inflammatory, immunosuppressive and anticoagulant activity (Oliveira et al., 2010).

Chapter III entitled „**Tick-borne pathogens**" describes the most important pathogens transmitted by ticks. Bacteria transmitted by ticks belong to two main groups: Spirochete and Proteobacteria and 10 genera: *Borrelia, Anaplasma, Ehrlichia, Neorickettsia, Aegyptionella, Rickettsia, Wolbachia, Bartonella, Francisella* and *Coxiella.* Besides bacteria, ticks transmit many viruses, the most important being: tick-borneencephalitis virus and Crimean-Congo haemorrhagic fever virus. Also, ticks are incriminated in the transmission of parasites of the *Babesia* and *Theileria* genus. Moreover they can becoinfected with various pathogens that can lead to increased severity of diseases, selection by increasing virulence and difficulties in establishing diseases diagnosis and treatment (Thomas et al., 2001; Yochay Regev et al., 2004). *Ixodes* ticks can be infected simultaneously with *Borrelia burgdorferi*, *Babesia microti*, *Anaplasma phagocytophilum*, *Bartonella henselae* and other pathogens (Goodman et al., 2005).

The last chapter of the first part „**Tick-transmitted zoonotic diseases to humans and animals in Romania**"provides informations on tick-borne diseases in Romania. The presence of the etiologic agent of Lyme disease, *B. burgdorferi s.l.* was reported for the first time in our country over 20 years ago (Crace et al., 1988). Since then, scarcelyin 2011 appeared the first studies on the prevalence of *B. burgdorferi s.l.* in its vector, *Ixodes ricinus* (Coipan and Vladimirescu, 2011). Surveillance of Lyme disease in humans has been carried outsince 2009 and since then the number of cases increased exponentially from year to year. Boutonneuse fever was described in Romania for the first time in 1910 and the first outbreak erupted in 1948 in Bucharest and Dobrogea (Teodorovici, 1978). A systematic monitoring plan for boutonneuse fever was implemented in the year 2000. This disease is endemic in the south-east of the country, with most human cases having being recorded in Bucharest and the surrounding area, and also in Dobrogea. For tick-borne encephalitis surveillance is carried out only in the counties subordinated of DSP Cluj and annual number of reported cases is very small. In 2012 the first data on the presence of Crimean-Congo hemorrhagic fever virus was published in Romania, following a serological survey conducted in sheep from Tulcea county (Ceianu et al., 2012).

The second part of the thesis „**Personal contributions**" is divided into five chapters, each following an established structure and ends with the general conclusions.

Although in Europe and other Romania neighboring countries, a large number of studies have been undertaken on the presence and distribution of tick-borne pathogens, there remains little information concerning these, even though the main vector*Ixodes ricinus*tick is widely distributed (Mihalca et al., 2012). In this context the present study had as objectives:

- seroprevalence evaluation of tick-borne diseases in domestic animals that serve as a reservoir for pathogens transmitted by ticks with the identification of risk areas for human and animal health;
- collection of ticks from vegetation and animals from risk areas and identification by means of molecular biology techniques of pathogens incriminated in producing zoonotic infections in humans and animals;
- achievement of metagenomic profil for microbiome present in questing *Ixodes ricinus* ticks collected from Romania, with a modern sequencing technique: NGS -Next Generation Sequencing.

Chapter VI „**Seroepidemiological investigations in zoonotic infections transmitted by questing *Ixodes ricinus* ticks**" is divided into two chapters. The first chapter made a serological screening for antibodies against the tick-borne encephalitis virus on domestic animals that serve as hosts. The investigations were conducted on a total of 180 serum samples collected during 2010-2011 from three species of domestic animal (cattle, sheep and goats) from 6 counties of eastern Romania (Bacău, Botoșani, Galați, Iași, Suceava and Vrancea). Samples were analyzed by immunoassay test TBEV Ig EIA - competition immunoassay test for detection of all types of tick-borne encephalitis virus antibodies in human and animal sera, produced by Test - Line Clinical Diagnostics Ltd., Czech Republic. Of 180 samples analyzed, 3.33% (6/180) were positive for tick-borne encephalitis virus. Seroprevalence was statistically similar between the 6 counties ($p = .51$). Depending on the animal species tested, of the 180 samples analyzed for tick-borne encephalitis virus, seropositivity was 5.79% (4/69) in goats, 2.19% (2/91) in cattle and 0% (20/20) insheep (Cojocaru et al., 2011). Investigations conducted revealed the existence of possible outbreaks in the east of Romania, a region considered free of tick-borne encephalitis. The results are important, because consumption of raw milk from goats, sheep and cattle may be associated with the risk of infection with TBE.

The second chapter presents a seroepidemiological survey on dogs, the main reservoir of *R. conorii*, using available serological tests for surveillance of boutonneuse fever on dogs and with the identification of risk areas for human beings.The investigations were conducted in 2010-2011 on a total of 92 blood samples collected from dog shelters (Tulcea and Brăila) and dog households (Brăila). Dog sera were analyzed using a commercial enzyme-linked immunosorbent assay (*Rickettsia conorii* Canine IgG ELISA produced by Euroclone Spa - Life Sciences Division, Italy) for the detection of specific antibodies of the immunoglobulin G (IgG) class against *Rickettsia conorii*. Following serological testing, 43.48% (40/92) of the dogs examined had IgG antibodies - *Rickettsia conorii*. The highest prevalence was recorded in Buzău 47.5% (19/40). The high prevalence (43.48%) for IgG antibodies - *Rickettsia conorii* founded in this study indicates that the animals were heavily exposed to infection with *Rickettsia conorii* and is higher than the seroprevalence founded in Sardinia (26.1%), (Segura-Porta et al. , 998) and Portugal (38.5%) (Levin et al., 2012) but lower than that reported in Israel (81%), (Harrus et al., 2007). This study is the first serological survey carried out in Romania for antibodies against *Rickettsia conorii* in canine population (Cojocaru and Savuța, 2013). These results suggest that the serological tests performed in dogs can be useful and sensitive in identifying areas at risk for boutonneuse fever.

Chapter VII „**Identification of *Borrelia burgdorferi s.l.* in questing *Ixodes ricinus* ticks**„ aimed to estimate the prevalence of *B. burgdorferi s.l.* in a population of *I. ricinus* ticks collected from vegetation from an area in which the outdoor recreational activities (picnic, gathering mushrooms, hunting) are carried out and the potential risk of contracting Lyme disease is determined. Ticks were collected by means of dragging method from Luncani (46°36'32"N, 26°45'45"E) a locality situated in Bacău county. DNA extraction was done by the alkaline lysis method with ammonium hydroxide. Identification of *Borrelia burgdorferi s.l.* was performed by PCR and identification of *Borrelia* genospecies was done with RFLP technique. Out of a total of 202 ticks investigated by PCR, 2.5% (5/202) of ticks were positive for *Borrelia burgdorferi s.l.* After RFLP's, 0.99% (2/202) of tested samples were positive for *Borrelia garinii* and 1.49% (3/202) samples were positive for *Borrelia afzelii*.

The main objective of chapter VIII „**Identification of pathogens transmitted by *Ixodes ricinus* ticks collected from vegetation (*questing* ticks or unfed ticks) and in ticks collected from animals (fed ticks)**„ aimed to evaluate infection of *I. ricinus* ticks with *Borrelia* spp., *Anaplasma* spp., SFG *Rickettsia* spp., *Babesia* spp., *F. tularensis* and *Bartonella* spp. During

April-September 2011 a total of 530 ticks were collected, of which 38.11% (202/530) were collected from different animal species and 61.89% (328/530) were collected from vegetation. DNA was extracted using the kit Genomic DNA from Tissue (Macherey-Nagel). Identification of pathogens was performed by PCR and the presence of genospecieswasachieved by sequencing.

Detection of DNA pathogens in fed *I. ricinus* ticks

A total of 202 fed *I. ricinus*ticks were collected from different animal species from four counties from eastern Romania: Galați, Bacău, Iași and Neamț. Of these 19.81% (40/202) were collected from goats, 65.35% (132/202) were collected from deer, 10.89% (22/202) were collected from dogs, 0.99% (2 / 202) from cats and 2.97% (6/202) from cows. Among the 202 ticks, 86.63% (175/202) showed a 320 bp amplified fragment of the *I. ricinus* 16SrRNA gene. Among the 175 ticks examined, 0.6% (1/175) were positive for *Borrelia burgdorferi s.l.*, 17.1% (30/175) were positive for *Rickettsia* spp., 5.14% (9/175) were positive for *Bartonella* spp., 4% (7/175) were positive for *Francisella tularensis*, 24% (42/175) were positive for *Anaplasma* spp. and 0.6% (1/175) for *Babesia* spp. Globally 50.86% (89/175) of analyzed ticks were infected with at least one pathogen.

Detection of DNA*Borrelia* spp. Among the 175 samples analyzed by PCR, only one female (0.6%) collected from deer from Bacău county presented a 226 bp amplified fragment of the 23S-5S gene space of *B. burgdorferi s.l.* The amplicon analysis in GenBank showed a maximum identity of 99% with *Serratia proteamaculus.*.

Detection of DNA *Rickettsia* spp. Of the 175 samples analyzed by PCR, 30 (17.1%; 30/175) showed a 380 bp amplified fragment of the *glt*A gene belonging to SFG *Rickettsia* spp, resulting in a prevalence rate of 19.04% in females, 3.33% in males and 26.3% in nymphs. Eighteen PCR fragments were successfully sequenced, generating 18 nucleotide sequences, of which 9 were found to be related to the *Rickettsia monacensis* with 99-100% similar nucleotide. The other 9 sequences were releated to *Rickettsia helvetica* with 98-100% nucleotide matches. The two species of rickettsia were found in three places: Galați (11.1%), Bacău (11.5%) and Neamț (33.3%).

Detection of DNA*Bartonella* sp. Among the 175 samples analyzed by PCR, 9 (5.14%) had a 356 bp amplified fragment of the *glt*A gene of *Bartonella* sp. Samples were successfully sequenced. Two sequences were similiar 98-99% with *Bartonella* sp isolated from the blood of *Apodemus sylvaticus* in Staffordshire, UK. The other sequence was found to be 98% related to

Bartonella sp. isolated from bats in Kenya. The remaining 6 sequences were similar in a proportion of 84-85% with a microorganism that lives in the soil and contributes to the process of digestion, *Acinetobacter* sp.

Detection of DNA *Francisella tularensis*. Of the 175 samples analyzed by PCR, 7 samples were positive, resulting in an overall prevalence of 4%. All ticks that had a positive response were females. The samples were successfully sequenced. All 7 amplicons from the analysis in GenBank were found to be related to *Francisella tularensis*.

Detection of DNA *Anaplasma* spp. Of the 175 ticks analyzed by PCR, 42 ticks were positive and all were females. Of the 42 amplicons, 41 were successfully sequenced and a comparation in GenBank revealed a sequence related to *Ehrlichia muris* with a similarity of 99% and 2 other sequences were related with *Anaplasma phagocytophilum* with 99-100% nucleotide matches. The remaining 38 sequences were found to be related to a symbiont belonging *Anaplasmataceae* family, *Candidatus Midichloria mitochondrii* with 99-100% nucleotide matches.

Detection of DNA *Babesia/Theileria* spp. Of the 175 samples analyzed by PCR, only one (0.6%) nymph collected from deer presented a 359 bp amplified fragment 18S rRNA gene of *Babesia/Theileria* spp.The sample was successfully sequenced and was found to be related to the *Babesia* sp.EU1.

Detection of DNA pathogens in *questing I. ricinus* ticks

A total of 328 unfed ticks (58 females, 160 males and 114 nymphs) were collected from vegetation from 5 counties from eastern Romania: Bacău, Iași, Galați, Neamț and Suceava. Females were analyzed individually, males and nymphs were analyzed in pools of 5 and 10 ticks. A total of 99 samples were analyzed, 58 females, 29 pools with males and 12 pools with nymphs. All the 99 samples were positive for *I. ricinus*.

Detection of DNA *Rickettsia* spp. *Rickettsia* spp. was identified in 10.1% (10/99) of the analyzed samples. Only adult ticks were found to be infected. Among adults, 10.34% (6/58) of females and 13.79% (4/29) of males pools were positive. The samples were successfully sequenced, resulting 10 nucleotide sequences from which 5 sequences have been related to *Rickettsia monacensis* with 99-100% similar nucleotides and 5 similar nucleotide sequences were related to *Rickettsia helevtica* with 99-100% similar nucleotides.

Detection of DNA *Francisella* spp. *Francisella* spp. DNA was amplified in 3 (3.03%) females collected from Bacău. All the samples were successfully sequenced and all were found to be related to *Francisella tularensis*.

Detection of DNA *Anaplasma* spp. Of 99 samples analyzed, 6 (6.06%) samples were positive. The six amplicons were successfully sequenced, and all sequences have been found to be related to *Candidatus Midichloria mitochondria*.

Our research extends to the geographical distribution of tick-borne pathogens in Romania and supports the conclusion that they are important vectors for pathogens in suburban forests. We showed that in the study area, the *Ixodes ricinus* ticks collected from vegetation and animals are infected with: *Anaplasma phagocytophylum*, *Ehrlichia muris*, *Rickettsia helvetica*, *Rickettsia monacensis*, *Francisella tularensis*, *Bartonella spp.*and *Babesia* sp.EU1 (Păduraru et al., 2012).

The following results obtained were deposited in GenBank following nucleotide sequences: 3 *Rickettsia helvetica* sequences (*glt*A gene) (access number in GenBank **JX040636, JX040637, JX040638**), 3 *Rickesttsia monacensis* sequences (*glt*A gene) (access number in GenBank **JX003686, JX040639, JX040640**), 1 sequence of *Ehrlichia muris* (16S rRNA gene) (access number in GenBank **JX040641**) and 1 *Babesia* sp EU1 sequence (access number in GenBank 18S rRNA gene) (access number in GenBank **JX040642**).

Chapter IX „**Research on microbiome present în questing *Ixodes ricinus* ticks collected in Romania by using NGS”** is divided in four chapters and were analysed 544 questing ticks collected from 4 counties (Bacău, Galaţi, Suceava and Iaşi) from the eastern part of Romania. Collection sites were between 26 and 388 m altitude. From 544 ticks, 81.06% (441/544) were nymphs and 18.93% (103/544) were females. Ticks were divided into pools depending on collection site and development stage.

In the first chapter „**Identification of tick encephalitis virus in questing *Ixodes ricinus* ticks**” aimed at the detection of tick-borne encephalitis virus amplifies a 68-bp fragment of the 3 non-coding regions by real-time RT-PCR by protocol developed by Schwaiger and Cassinotti (2003). Of 544 *Ixodes ricinus* ticks (441 females and 103 nymphs) analyzed by real time RT-PCR for detection of tick-borne encephalitis virus, all samples were negative. In Europe, the prevalence of TBE infection in ticks varies between 0.1-5% (Oehme et al., 2002; Randolph, 2001) but these values are subject to yearly fluctuations. Borman et al. (2004) following a study

conducted in 1995 has found in adult ticks collected from vegetation that there was a tick-borne encephalitis prevalence of 26.6% and in 2002 in the same region the prevalence was only 5%.

The second chapter „**Identification of infectious agents transmitted by questing *Ixodes ricinus* ticks**"aimed to identify pathogens transmitted by ticks. A total of 544 ticks questing (441 nymphs and 103 females) were collected from vegetation. Following PCR analysis they did not show any evidence of a positive response *Borrelia* spp., *Bartonella* spp., *Francisella tularensis* and *Babesia* spp..

Detection of DNA *Anaplasma* spp. A set of primers was used that link '5 regions of 16S rDNA gene from numerous members of *Anaplasmataceae* family and closely related with rickettsial agents. Thus 58.33% (21/36) from pools of analyzed ticks showed an amplification product with this primer set. Depending on the stage of tick development, prevalence was 16.7% (6/18) in nymphs and 41.7% (15/18) in females. The samples were successfully sequenced, generating 21 sequences of nucleotides. Of the 21 sequences, 16 were related to *Candidatus Midichloria mitochondria* (99-100%) and 4 sequences have been found to be related to *Wolbachia* sp. (99-100%) and the last sequence was related to „*Candidatus Neoehrlichia mikurensis*".

Detection of DNA *Rickettsia* spp. From 36 pools of ticks analyzed by PCR, 19 showed a 380 bp fragment of the gltA gene of SFG *Rickettsia* spp. Of the 19 pools that have shown a positive response, six pools were with nymphs resulting in a prevalence of 16.7%, and 13 ticks were females with a prevalence of 36.1%. All the samples were successfully sequenced, 16 sequences were related to *Rickettsia helevtica* with 99-100% similar nucleotide sequences and 3 were related to *Rickettsia monacensis* with 99-100% similar nucleotides.

The last chapter „**Metagenomic profile of the microbiome associated with questing *Ixodes ricinus* ticks**" is divided into two parts. In the first part the 36 pools of ticks were reorganized into 20 pools of ticks (10 pools of nymphs and 10 pools of females) which were inoculated into 20 immunocompromised mice. Three days after mice inoculation, blood was collected on EDTA and leukocytes and plasma were recovering. Mixing occurred after extraction of nucleic acids from plasma and white blood cells, and a plasma pool and a leukocytes pool obtained. The two pools were subjected to NGS.

Analysis of NGS results from plasma and leukocytes

Following NGS, 274.452.408 readings for plasma and 281.285.878 reads for leukocytes were obtained. After bioinformatics analysis of the reads, which involves the removal of

duplicate readings, the sequences as part of the host genome, the adapters - needed in NGS and as well as the other stages of filtration, remained from the initial reads 40.215.193 reads for plasma and 38.081 readings for leukocytes. Readings were assembled resulting 1.450.530 contigs for plasma and 38.081 contigs for leukocytes. After comparation of contigs with the nucleotide sequences existing in GenBank, were identified 32.001 contigs from plasma and 2.019 contigs for leukocytes.

Identification of bacteria. In plasma and white blood cells 30 genera of bacteria were identified. Most the types of bacteria were in the Proteobacteria Phylum followed by Actinobacteria Phylum, Firmicutes Phylum and others. Microorganisms from soil and water such as: *Bacillus* spp., *Frankia* spp., *Clostridium* spp., *Streptomyces* spp., etc., as well as bacteria known to be pathogenic to animals: *Bordetella* spp., *Burkholderia* spp., *Mycobacterium* spp., *Rhodococcus* spp. were detected.

Among the tick-borne bacteria identified were bacteria of the genera: *Borrelia*, *Anaplsma*, *Ehrlichia*, *Rickettsia*, *Francisella* and *Bartonella*. With a few exceptions the vast majority of bacteria have been identified only in the sample of plasma.

Identificationof parasites. The nucleotide sequence identified as belonging to the parasites transmitted by ticks was found only in plasma. In total 5 contigs were found from *Babesia* genus and 12 contigs from *Theileria* genus.

Analysis of NGS results obtained from pooled ticks

The NGS analysis of pooled ticks revealed 135.538.902 raw reading. After bioinformatics analysis remained 7.149.453 readings. These were assembled and resulted in 97.960 contigs. After comparation of contigs with the nucleotide sequences existing in GenBank, 272 contigs were associated with viruses, 5733 contigs with bacteria and 4908 contigs with parasites.

Identification of bacteria. After analysis in GenBank of bacteria contigs were identified 37 bacterial genera. As in the case of bacteria found in plasma and white blood cells, most of them belong to the Phylum *Proteobacteria*, followed by the Phylum *Actinobacteria*, *Firmicutes* and others. Among the pathogenic bacteria transmitted by ticks, coding region sequences belonging to the genera *Anaplasma*, *Ehrlichia*, *Orientia*, *Rickettsia*, *Francisella*, *Borrelia*, *Bartonella* and *Coxiella* were found.Other organisms detected in the pool of ticks were bacteria from soil and water: *Magnetospirillum*, *Sorangium*, *Bacillus*, *Frankia*, *Clostridium* și

Streptomyces. In addition, animal pathogenic bacteria has been identified: *Bartonella*, *Brucella*, *Bordetella*, *Burkholderia*, *Mycobacterium* and *Rhodococcus*.

Identification ofviruses.Comparison of nucleotide sequences in GenBank revealed 137 contigs belonging to the families: *Bunyaviridae*, *Reoviridae*, *Rhabdoviridae* and *Togaviridae*family. Most of the contigs (102 contigs) belong to the *Bunyaviridae* family and of these 61 contigs belong to *Nairovirus* genus, 3 contigs belong to *Orthobunyavirus* genus and 38 contigs belong to *Phlebovirus* genus.

Identification of parasites. After comparation in GenBank of contigs obtained by NGS and after reads assemblation, 430 contigs in different percentages of homology with 6 parasites genera were found to be similar: *Babesia, Theileria, Plasmodium,Cryptosporidium, Neospora* and *Toxoplasm*a.