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RESEARCHES REGARDING ECOBIOLOGY AND EPIDEMIOLOGY OF HARD TICKS IXODIDAE ATTACK – VECTORS OF LYME DISEASE IN ROMANIA

SUMMARY OF Ph.D. THESIS

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ABSTRACT

Ticks are obligatory blood-sucking arthropods with a great medical and veterinary importance worldwide. There are known over 907 tick species, distributed on all continents from Subarctic area to Ecuator. 10% from all ticks have the capacity of transmitting or producing diseases due to their way of feeding (Jongejan și Uilenberg, 2004). They are feeding with the blood of mammals (including humans), birds and reptiles. They are capable of transmitting a great variety of viruses, bacteria and protozoa, being on the first position from this point of view (Dennis și Piesman, 2005). Regarding medical importance, they are preceded only by mosquitos (Sonenshine, 1991). Ticks are incriminated also for other medical problems such as anemia, dermatitis and poisoning. Worldwide the interest in vector-borne diseases is increasing in the context of the ascendancy of their occurrence (Walker 1998, 2005; Telford și Goethert 2004).

Ticks involvement in Lyme disease was observed for the first time by the swedish dermatologist Afzelius in 1909, when he noticed the pathognomonic skin lesion, erythema migrans, which he associated with the bite of Ixodes ricinus. Lyme disease ecology studies became possible when Burgdorfer et al. (1983) were able to isolate the etiologic agent called Borrelia burgdorferi, in I. dammini. Since then information about Lyme disease became more numerous. Borrelia burgdorferi, the agent that causes Lyme disease is transmitted to humans and animals by Ixodes ticks during feeding. The most important vector species in Europe is Ixodes ricinus (Parola et al., 2005; Zygner et al., 2008).

There are many studies showing epidemiological data on the distribution of B.burgdorferi in Europe. In Romania, however, there is little information about the epidemiology spirochetei causing Lyme disease, this despite the fact that the vector, Ixodes ricinus is widely distributed (Coipan and Vladimirescu, 2011, Mihalca et al., 2012). The presence of B.burgdorferi in Romania was reported more than 20 years ago (Crăcea et al., 1988). In 2011, Coipan and Vladimirescu detected three B.burgdorferi s.l. genospecies in Ixodes ricinus ticks: B.afzelii, B.garinii and B.valaisiana. In a study conducted 10 years ago, Hristea et al. (2001) evaluated the seroprevalence Borrelia infection in healthy blood donors and foresters. Recently, Kiss et al. (2011) conducted a
serological surveillance in dogs and horses in several counties in Romania. Information on the prevalence of infection with *B. burgdorferi* in unfed ticks also have a limited character (Coipan, 2010; Coipan and Vladimirescu, 2011).

In this context this paper has as general objectives the following:

Establish community structure of ticks parasitising on wild animals in Romania and associations between species of ticks and their hosts.

Establishing the main species of questing ticks, from forest habitats in Romania.

Determination of *Borrelia burgdorferi* sensu lato prevalence in unfed ticks, collected by the "flag" in various locations in Romania, using PCR.

Assessing the prevalence of disease vector *Ixodes ricinus* ticks parasitic on *Erinaceus roumanicus* in Romania

Establish the seroprevalence of some vector-borne disease in dogs Romania and identify the role that this species plays in the ecobiology of the studied pathogens.

The first part summarizes the information in the literature on the systematics and evolution of Ixodidae ticks, their ecobiology, the vector role of Ixodidae ticks and some data on major diseases transmitted by them. This first part is divided into four chapters. The first chapter presents data on the origin of ticks, their phylogenetic classification, the number of species and recent changes that have occurred in systematic nomenclature and classification of species of Ixodidae. The second chapter is a description of Ixodidae ticks biology and ecology, and an overview of the factors affecting their population dynamics. The third chapter is devoted to vector role of these ticks. Provides a summary of factors influencing vector capability, necessary conditions for the manifestation of this attribute and the host specificity related information and its use. The last chapter of the first part is devoted to the main vector-borne diseases treated in the thesis. It has general information about clinical manifestations and etiopathogenesis in borreliosis, anaplasmosis, ehrlichiosis and heartworm disease.

The second part of the thesis, the personal researches, is divided into six chapters, each of them following a well defined structure. In the end there are presented the general conclusions and the cited literature. The reference list includes 402 titles.

**Chapter 1** aimed to determine the structure of communities of ticks parasitising on wild animals in Romania and associations between species of ticks and their hosts.
From a public health perspective, our research aimed to provide data to help improving the prediction of possible shots of outbreaks or epidemics of diseases and training vector control strategies for such situations.

The only study regarding Ixodidae ticks diversity in Romania was published more than 45 years ago (Feider, 1965). Under this and subsequent reports on the presence of new species (Mironescu, 1966, Georgescu, 1968), in Romania there are 25 valid species of ticks (taking into account updating taxonomic made by Guglielmone et al., 2010). Moreover, most of the information about the preferences of the host, host association and geographic distribution are not current and are available in formats that do not allow easy access. Between February 2004 and October 2011, 4745 of ticks were collected from 597 individuals of 58 species (Mammalia, Aves, Reptilia) from different regions of Romania. Ticks were separated on developmental stages and sex for adults and species were identified based on morphological characters, using identification keys.

From the 58 examined species there were collected ticks from 19 species.

We found 58 new host-ticks associations: 20 for *Ixodes ricinus*, 1 for *Laprionophorus*, 6 for *Larboricola*, 2 for *Lhexagonus*, 9 for *Lredikorzevi*, 1 for *I.trianguliceps*, 2 for *I.vespertilionis*, 2 for *Haemaphysalis punctata*, 1 for *H.sulcata*, 2 for *H.concinna*, 1 for *D.marginatus*, 4 for *Rhipicephalus sanguineus* s.l., 1 for *R.bursa* şi 6 for *Hyalomma marginatum*.

In Chapter 2 the aim was to identify and count the ticks parasitic species from *Vulpes vulpes* in Romania. Red fox (*Vulpes vulpes*) is the most common predator globally, populating almost all habitats in the Northern Hemisphere forests, mountains, deserts (Letkov et al., 2006). Of the many species of wildlife that can be hosts for ticks, carnivores are important hosts for endophile ticks because they spend many hours of the day in nests, burrows or caves, shelters that the ticks remain active. These animals are hosts for exophile ticks as *Ixodes ricinus* (Santos-Silva et al., 2011). Knowledge communities ticks that infest wildlife can help evaluate and possibly even minimize the impact of these arthropods on health and survival of species considered vulnerable (Lorusso et al., 2011).
The study was conducted between November 2010 and May 2012. There were examined a total of 357 foxes, from 12 counties in Romania. From parasitized animals, all ticks were collected independently of species and/or developmental stage.

Of the 357 foxes examined, 156 (43.7%) presented infestation with various species of tick. We identified 5 species of ticks: *Ixodes hexagonus* (n = 113, 72.44%), *Ixodes ricinus* (n = 45, 28.84%), *Ixodes crenulatus* (n = 12, 7.7%), *Dermacentor marginatus* (n = 11; 7.05%) and *Haemaphysalis punctata* (n = 1, 0.64%). We collected and identified 5,753 ticks. The most prevalent development stage was larvae (91.01%, n = 5,236), followed by adults (13.5%, n = 285) and nymphs (3.86%). Regarding adults, *I. ricinus* was the most frequently encountered species (71.86%).

This study indicates that foxes in Romania harbour a number of relevant potential vector species of ticks. The information provided can help to understand the ecology of ticks that parasitize wild carnivorous and can be a basis for studies on the epidemiology of vector diseases.

Besides the ecological significance of the obtained data, the high prevalence of tick parasitism on foxes, coupled with the increasingly emergence of this species in the synanthropic environment as a consequence of the increasing number of individuals within populations may increase the incidence of infestation by ticks to humans and domestic animals and may increase the likelihood of transmission of vector diseases.

The purpose of Chapter 3 was to investigate populations of ticks parasitising on species of hedgehog in Romania, *Erinaceus roumanicus* and provide epidemiological data on the presence in the body of pathogens ticks of two major pathogens with public health implications, *Borrelia burgdorferi* sensu lato and *Anaplasma phagocytophilum*.

Exotic animals, including hedgehogs, are increasingly among pets. Although benefits of having pets are indisputable, yet their presence is associated with exposure to certain risks related to the ability to transmit different pathogens, some of them with zoonotic nature (Rosen and Jablon, 2003). Given the wide range of species of ectoparasites and high levels of parasites and their behavior anthropophil, hedgehogs play an important role in the epidemiology of vector diseases transmitted by ticks (Földvár et al., 2012). Most published data about ixodid ticks that feed on hedgehogs and their role in the complex cycle of tick-borne diseases, refer to the species *Erinaceus romanicus*.
while information about *Erinaceus roumanicus* are extremely limited, despite the generous geographical distribution (Dziemian et al., 2010).

The study was conducted in two consecutive spring seasons in 2011 and 2012, respectively. 57 hedgehogs were examined from different counties in Romania. To facilitate the examination, the animals were anesthetized using the inhalation technique the recommended procedure for insectivorelor (West et al., 2007). Each animal was examined in order to identify possible tick infestation. From parasitized animals, all ticks were collected independently of species and / or developmental stage. Genomic DNA extraction was performed for each individual sample. The processed material is the tick preserved in ethanol. For this purpose we used the method of extraction with 1.25% ammonium hydroxide (Cadenas F. Moran et al., 2007; Coipan and Vladimirescu, 2010). Identification of *Borrelia burgdorferi* s.l. and *Anaplasma phagocytophilum* was performed by PCR and the identification of involved *Borrelia* genospecies was done by RFLP technique.

Of 57 examined hedgehogs, 29 were infested with ticks of various species. Most animals (n = 24) were infested with *Ixodes ricinus* species, which also was the most represented species number (n = 959). We identified on five hedgehogs, other 5 species of ticks as follows: *Dermacentor marginatus, Haemaphysalis punctata, Hyalomma marginatum, Rhipicephalus rossicus, Rhipicephalus sanguineus*. Each of this five species of parasites were represented by an individual.

Regarding parasitism of the species *Ixodes ricinus*, have the following values: prevalence in relation to developmental stage: larvae 16.5% (158/959, 95% CI: 14.2% - 19.0%), 55.3% nymphs (530 / 959, 95% CI: 52.1% -58.4%), females 21.8% (209/959, 95% CI: 19.2% -24.6%) and males 6.5% (62/959, 95% CI: 5.0% -8.3% ).

The overall prevalence of infection with *Borrelia burgdorferi* in ticks was 0.4% (4/959, 95% CI: 0.1-1.1%). Positive samples were recorded at 3 nymphs and one male. Of the 24 hedgehog species infested with *Ixodes ricinus* species, two had positive ticks infected with *Borrelia burgdorferi* sensu lato (8.3%, 2/24, 95% CI: 1.1-28.0). In all four positive cases the involved genospecies was identified as *Borrelia afzelii*.

Regarding the infection with *Anaplasma phagocytophilum*, 115 of the total of 959 ticks were positive (12%, CI 95%: 1.3-4.2%). This ticks were parasitising on eight
hedgehogs (34.8%, 8/23, IC 95% : 16.4-57.3). The highest prevalence of A. phagocytophilum DNA was recorded in nymphs (19.1%, 101/530, 95% CI: 15.9-22.7). Two hedgehogs (8.3%; 2/24, 95% CI: 1.1-28.0) had positive tick infestations and B. burgdorferi sl and A. phagocytophilum positive ticks.

A single tick of the total analyzed was infected with both investigated pathogens 0.1% (1/959, 95% CI: 0.0-0.7%).

This is the first study to evaluate the prevalence of Anaplasma phagocytophilum infection and Borrelia burgdorferi sl in ticks collected from Erinaceus roumanicus in Romania. The results confirm the presence of both investigated pathogens, presents specific epidemiological data and stresses the importance of disease surveillance in the context of vector-borne diseases and the risk they pose to human health.

The main objective of Chapter 4 was to establish the structure of the main questing ticks species from forest habitats in Romania.

The study was carried out between 01.05.2010-18.06.2011. 13,771 ticks were collected by using the flagging method (method adapted from that described by Estrada-Peña, 2001), of 188 localities in all 41 counties from Romania. The locations for collection were randomly chosen within forest habitats, in order to achieve a uniform geographical distribution. Deciduous margins and mixed forests with abundant vegetation were preferred. Tick species were identified in the laboratory.

The 13,771 ticks collected by flagging were classified into 11 species as follows: Ixodes ricinus (n = 11 965, 86.9%), Dermacentor marginatus (n = 1310, 9.5%), Haemaphysalis punctata (n = 354 , 2.6%), H. concinna (n = 89, 0.6%), H. sulcata (n = 35, 0.3%), H. parva (n = 11, 0.1%), Hyalomma marginatum (n = 2, 0.02%), D. reticulatus (n = 2, 0.02%), I. crenulatus (n = 1, 0.007%), I. hexagonus (n = 1, 0.007%) and I. laguri (n = 1; 0.007%). I. ricinus was the dominant species (p <0.001).

Of the 188 sites studied, I. ricinus was present in 180 (97.7%, 95% CI: 91.8-98.1), being the most widespread species (p = 0.0001). In the 75 settlements I. ricinus was the only species identified (41.7%, 95% CI: 34.4-49.2), while in 73 sampling points was the dominant species, accounting for over 70% of local ticks communities. A second prevalent species was D. marginatus followed by H. punctata and H. concinna. The other
species identified were found in only a few locations. However *D. marginatus*, *H. punctata*, *H. sulcata* and *H. concinna* were dominant species in several locations.

This study, the first of this kind and of this magnitude carried out in Romania, confirms the position that that *I. ricinus* has it in Europe as the most common tick species with questing behavior type. *I. ricinus* tick abundance, host specificity extremely low, the high frequency of parasitism in humans and significantly vector capacity correlated with the results obtained in this study suggest that this species is the most important arthropod vector in Romania.

In the current conditions of globalization intensive and major climate changes are urgently needed studies on the biology, vector capacity, invasive behavior and ecology of the most important vectors of the arachnoentomology field (Mehlhorn et al., 2011).

Chapter 5 had as a purpose to determine the prevalence of *Borrelia burgdorferi* sensu lato in unfed ticks, collected by the "flagging" method in various locations in Romania, using PCR method.

By estimating the prevalence of *Borrelia burgdorferi* s.l. in *Ixodes ricinus* ticks, which constitutes the main vector species of this pathogen in Europe, is an indicator of the risk of infection with *Borrelia* for the investigated area (Cisak et al., 2006). Tick infection prevalence may vary depending on the region under study, stage of development and the method used (Maetzel et al., 2005).

Between 01.05.2010-20.08.2011 13,918 ticks were collected using the flagging method (method adapted from that described by Estrada-Peña, 2001), from 167 localities in all 41 counties of Romania. The locations were selected in randomly. Were studied and processed by methods of molecular biology, only ticks of the species *Ixodes ricinus*. Genomic DNA extraction was performed for each individual sample. The processed material is the tick preserved in ethanol. For this purpose we used the method of extraction with 1.25% ammonium hydroxide (Cadenas F. Moran et al., 2007; Coipan and Vladimirescu, 2010). Identification of *Borrelia burgdorferi* s.l. was performed by PCR.

Of the total of 13,918 ticks, 12,221 were identified as *Ixodes ricinus*. Staging structure is as follows: larvae 4.46% (n = 545), nymphs 42.35% (n = 5176), females 26.78% (n = 3273) and 26.40% males (n = 3227). The highest prevalence was recorded in 8.9% Neamt County. Values in other counties ranged from 0.4% (Cluj county) and
4.9% (Arad county). In 12 counties found no positive samples were found. In this study we evaluated the prevalence of infection with *Borrelia burgdorferi* sensu lato taking into account the stage of development.

The total prevalence of infection with *Borrelia burgdorferi* sl in unfed *Ixodes ricinus* ticks in Romania is 1.4%. The highest prevalence was recorded at in females 3.2%, in general adult being the most infected developmental stage.

*Borrelia* infection risk depends on the density of ticks and their infection with *Borrelia*. This is the first comprehensive study on the prevalence of *Borrelia burgdorferi* in unfed *Ixodes ricinus* ticks from Romania, thus constituting a very useful database for assessing disease risk.

The prevalence of infection with *B.burgdorferi* sl presented in this study are comparable with prevalence rates in other European countries. Epidemiological studies of vector-borne disease must be continuous, taking into account climate change taking place that can affect vector ecology, but also hosts and pathogens.

Chapter 6 objectives were to determine seroprevalence and geographical distribution of *D.immitis*, *A.phagocytophilum*, *E.canis* and *B.burgdorferi* in dogs in Romania and to determine risk factors (age, sex, race, living environment and their prophylactic) associated with the presence of the antigens or the antibodies.

Vector-borne diseases of dogs have a wide geographical spread in areas where existing climatic conditions allow the development of arthropods (mosquitoes and ticks). Pathogens transmitted by vectors, such as *Dirofilaria immitis*, *Anaplasma phagocytophilum*, *Ehrlichia canis* and *Borrelia burgdorferi* in dogs can cause serious diseases. Their role in human and animal health has become increasingly prominent in recent decades, which makes evident the need for information about the prevalence and distribution of vector-borne diseases.

The study was conducted between May 2008 and March 2011. 1146 blood samples from dogs were randomly collected (guard, pets, housing, community and hunting) from 25 counties. Demographic information and data on the use of prophylactic treatment with substances having acaricide / insecticide effect for each dog were obtained using a questionnaire.
All samples in this study were tested using a SNAP® rapid diagnostic kit 4Dx® (IDEXX Laboratories, Inc., Westbrook, ME), which is based on an enzyme immunoassay (ELISA), which highlights antibodies against *A.phagocytophilum*, *B.burgdorferi* and *E.canis* and *D.immitis* antigens. Technology has been specified by the manufacturer.

The total number of seropositive dogs to any of the four pathogens investigated was 129 (11.3%). The overall seroprevalence was as follows: *A.phagocytophilum* 5.5%, *D.immitis* 3.3%, *E.canis* 2.1% and *B.burgdorferi* 0.5%. Coinfection with *E.canis* and *A.phagocytophilum* were recorded in two dogs (0.2%). There were no significant statistically differences regarding region of origin in infections with *A.phagocytophilum* and *B.burgdorferi*, but in terms of *D.immitis* infections and *E.canis*, they were significantly most prevalent in the southeastern region (p <0.001).

This study indicates that in Romania, the dogs are at risk of contacting major vector-borne pathogens, as emphasized by the relatively high prevalence of both tick-borne pathogens and mosquito-borne pathogens.

Geographical distribution of positive cases suggests the presence of foci in the south gupate west of the country respectively, for *D.immitis* and south-east (Constanta county) for *E.canis*. Infection with *A.phagocytophilum* and *B.burgdorferi* had a homogeneous distribution, with a tendency to concentrate in the central regions of positive cases to *B.burgdorferi*. For *D.immitis*, *A.phagocytophilum* and *E.canis*, the presence of prophylactic treatment was a risk factor associated with infection. Another risk factor was the group of dogs (stray dogs were more susceptible to infection with *D.immitis*, dogs from shelters to *E.canis* and hunting dogs to *B.burgdorferi*).

Due to lifestyle and increased likelihood of contact with ticks, dogs have an increased risk of infection with *B.burgdorferi*, so they become a true marker of risk of exposure for humans (Duncan et al., 2004, Little et al., 2010).

This is the first study that presents data on the seroprevalence of infection with *A.phagocytophilum* and *E.canis* in dogs in Romania and the largest epidemiological study of vector-borne diseases in our country.


28. COIPAN EC, VLADIMIRESCU AF, 2010, First report of Lyme disease spirochetes in ticks from Romania (Sibiu County), Exp Appl Acarol, 52, 193-197.
