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DETECTION OF THE CO-INFECTIONS WITH BORRELIA, ANAPLASMA, AND EHRLICHIA SPECIES IN DOGS WITH BABESIOSIS IN NORTH-EASTERN ROMANIA

DETECTAREA CO-INFECȚIILOR CU BORRELIA, ANAPLASMA ȘI EHRLICHIA LA CÂINII INFESTAȚI CU BABESIOZĂ ÎN NORD-ESTUL ROMÂNIEI

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ABSTRACT | REZUMAT

Vector-borne diseases are an important threat to animals and human health, due to their zoonotic potential, increasing the global prevalence. In this study, we collected blood samples from dogs confirmed with canine babesiosis, and the ticks attached were removed and stored until morphological identification and DNA extraction. The aim of this study was the screening for the possible co-infections with bacterial tick-borne pathogens, using PCR protocols to detect: *Borrelia* spp., *Anaplasma* spp., and *Ehrlichia* spp. in blood and tick samples. Of the 66 dogs, 15 (22.7%) were positive for *Borrelia burgdorferi s.l.*, 0 for *Anaplasma* spp./*Ehrlichia* spp. and 2 (3.03%) for *Candidatus Midichloria mitochondrii*. 2 dogs (3.03%) were co-infected with *Borrelia burgdorferi s.l.* and *Candidatus Midichloria mitochondrii*. Morphologically we identified a total of 99 ticks as *Ixodes ricinus* (48.5%) and *Dermacentor reticulatus* (51.5%), which were distributed in pools according to the following criteria: canine patient, species, and developmental stage (gender). The most common pathogen species detected in ticks was *Candidatus Midichloria mitochondrii* (25.8%) without clinical significance, followed by *Borrelia burgdorferi s.l.* (12.9%) and co-infection *Borrelia burgdorferi s.l.* + *Candidatus Midichloria mitochondrii* (6.5%). The results of this study show the importance of identifying co-infections in tick-infested dogs and the need for prevention protocols of ticks infestations in dogs.

Keywords: co-infections, canine babesiosis, borreliosis, ticks, *Candidatus Midichloria mitochondrii*

Bolile transmise de vectori reprezintă o amenințare importantă pentru animale și sănătatea umană, datorită potențialului lor zoonotic, crescând prevalența la nivel global. În acest studiu, am recoltat probe de sânge de la câini confirmați cu babesioză canină, iar căpușele atașate au fost îndepărtate și păstrate până la identificarea morfologică și extracția ADN-ului. Scopul acestui studiu a fost screening-ul pentru posibilele co-infectii cu agenți patogeni bacterieni transmiși de căpușe, folosind protocoale PCR pentru a detecta: *Borrelia* spp., *Anaplasma* spp. și *Ehrlichia* spp. în probe de sânge și căpușe. Din cei 66 de câini, 15 (22,7%) au fost pozitivi pentru *Borrelia burgdorferi s.l.*, 0 pentru *Anaplasma* spp./*Ehrlichia* spp. și 2 (3,03%) pentru *Candidatus Midichloria mitocondrii*. 2 câini (3,03%) au fost co-infectați cu *Borrelia burgdorferi s.l.* și *Candidatus Midichloria mitocondrii*. Din punct de vedere morfologic am identificat un total de 99 de căpușe ca *Ixodes ricinus* (48,5%) și *Dermacentor reticulatus* (51,5%), care au fost distribuite în eșantioane după următoarele criterii: pacient canin, specie și stadiu de dezvoltare (gen). Cea mai frecventă specie patogenă detectată la căpușe a fost *Candidatus Midichloria mitochondrii* (25,8%) fără semnificație clinică, urmată de *Borrelia burgdorferi s.l.* (12,9%) și co-infecția *Borrelia burgdorferi s.l.* și *Candidatus Midichloria mitocondrii* (6,5%). Rezultatele acestui studiu demonstrează importanța identificării co-infecțiilor la câinii infestați cu căpușe și necesitatea unor protocoale de prevenire a infestărilor cu căpușe la câini.

Cuvintele cheie: co-infecții, babesioză canină, borelioză, căpușe, *Candidatus Midichloria mitochondrii*

Ticks are large-bodied arthropods, bloodsucking, and non-permanent ectoparasites that feed on small or large mammals, birds, humans, reptiles, and amphibians in all regions of the world, particularly in subtropical and tropical regions (2, 3). In Europe, companion animals are parasitized by various tick species of the genera *Ixodes, Dermacentor, Haemaphysalis, Rhipicephalus,* or *Hyalomma* (7) and many of these species are also very important for public health because they play the role as of reservoirs or sentinel hosts (7, 41). After mosquitoes, ticks are the most common blood-feeding parasites (33) with the possibility of transmission protozoa, bacteria, or viruses.

There is a strong indication of research interests in

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vector-borne diseases (VBDs) in humans, and animals is because pets are traveling with owners in areas at risk (7, 39). The movements provide ideal conditions for the circulation of pathogens so that over the last decades, the transport (by air, train, sea, or road) has increased with intense movements of companion or production animals (5).

Global warming affects arthropod vector density, vectorial capacity, and geographical distribution (5). VBDs are considered dynamic systems with complex ecology, which tend to adapt continually to environmental changes (44).

It is extremely important to identify the vector ticks and their transmission pattern of the pathogens for the development and implementation of control strategies in the target geographical region (2). One of the strategies is to develop epidemiological models, being necessary three key elements: biomathematics (to study the dynamics of vectors and pathogens), satellite mapping (to monitor the emergence and distribution of diseases related to vegetation and human activities), and climate modelling (to follow parasite activity caused by meteorological trends) (5).

Lyme borreliosis is an infection with zoonotic potential caused by the tick vector-borne bacterium *Borrelia burgdorferi* sensu lato complex (23, 40, 45), transmitted by *Ixodes ricinus* and *Ixodes persulcatus* in Europe (40, 41). For Lyme borreliosis, the spiralled bacteria belonging to the Order *Spirochaetes* are responsible (35). The most common three European pathogenic species of *Borrelia burgdorferi* sensu lato that affects dogs and humans are *B. afzelii*, *B. garinii,* and *B. burgdorferi* sensu stricto (39).

In 2016 has been reported in hard ticks from Europe a species of *Borrelia*, *Borrelia miyamotoi*, not belong to the *B. burgdorferi* (s.l.) species complex. *Borrelia miyamotoi* are usually transmitted by *Argasidae* soft ticks but in Europe have been reported some human cases with *B. miyamotoi* infection, transmitted by *Ixodes* ticks (40).

Most studies conducted in Romania are on canine babesiosis (18, 20) or co-infections with other pathogens (4, 10, 19).

Anaplasma phagocytophilum and *Anaplasma platys* are the gram-negative bacteria that cause canine anaplasmosis, and the pathogen for human granulocytic anaplasmosis is *A. phagocytophilum* (23, 24), identified mainly in central and northern Europe (38). In dogs, *A. platys* has been diagnosticated in the Mediterranean basin, including Romania (38). A study published in 2017 by Matei et al. identified *A. platys* infection in puppies from Romania, being the hypothesis of vertical transmission of the pathogen (29). Three species of *Ehrlichia* cause canine ehrlichiosis: *Ehrlichia canis, Ehrlichia ewingii,* and *Ehrlichia chaffensis* but only two of them (*E. ewingii and E. chaffensis*) are human zoonotic pathogens (21, 24). In Europe, the only species isolated in dogs are *E. canis* (38).

Several studies serological (8, 22, 30) and molecular (4, 31) were performed in Romania regarding to detect the occurrence of tick-borne pathogens or the co-infections identified in dogs' blood, therefore the aim of the study was to detect the possible co-infections with *Borrelia*, *Ehrlichia,* and *Anaplasma* in dogs co-infected with babesiosis in north-eastern Romania.

METHODS AND MATERIALS

Animals and samples

From March 2018 to November 2019, 66 dogs that were confirmed with babesiosis (10) at the Clinics of Veterinary Medicine Faculty of Iasi (Romania) were included in this study. During the general clinical examination of the dogs, the attached ticks were removed and placed in separate tubes with 70% ethanol, each receiving a unique number for species identification using standard taxonomic keys (12, 13). The identified ticks from each dog were divided into pools based on species, sex, and developmental stage. Whole blood samples were collected from a cephalic vein and stored at -20°C until DNA extraction. Both blood and tick samples were screened for the possible co-infections with the bacterial tick-borne pathogens *Borrelia* spp., *Anaplasma* spp., and *Ehrlichia* spp. using the following PCR protocols described by Sainz et al. (2015), Cisak et al (2012), and Massung et al. (2007).

DNA extraction, amplification, and sequencing

The samples (blood and ticks) were transferred to the University of Federico II, Department of Veterinary Medicine and Animal Production, in Naples, Italy, where DNA extractions and molecular techniques were performed, to identify the species of *Borrelia, Anaplasma,* and *Ehrlichia* (Table 1). The DNA was extracted using a commercial kit (Dneasy Blood & Tissue Kits, Qiagen, Germany) according to the manufacturer's instructions. The ticks were completely lysed after an incubation of 13-14 hours.

The PCR protocol for identification *Borrelia* spp., described by Cisak E. et al in 2012 using Fla1 and Fla 2 primers specific for the Fla gene sequence fragment. The thermal profile used was 95°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 54°C for 45 seconds, 72°C for 45 seconds, then the final expansion to 72°C for 3 minutes (9).

To identify *Ehrlichia*/*Anaplasma* species, partial mitochondrial sequences of 16S genes were generated and analysed using PER1 and PER2 primers (Table 1). The thermal profile used was 95°C for 10 minutes, followed by 40 cycles of 94°C for 60 seconds, 52.4°C for 45 seconds, 72°C for 60 seconds, then expansion final

Table 1

The primers used in the PCRs

Pathogen	Primers	Reference
Borrelia spp.	**Fla1** (5'-AGAGCAACTTACAGACGAAATTAAT-3') **Fla2** (5'-CAAGTCTATTTTGGAAAGCACCTAA-3')	9, 38
Anaplasma spp./ *Ehrlichia* spp.	**PER1** (5`-TTTATCGCTATTAGATGAGCCTATG-3) **PER2** (5`-CTCTACACTAGGAATTCCGCTAT-3)	15, 27

Table 2

Results of pathogen species (*Borrelia* spp., *Anaplasma*/*Ehrlichia* spp.) identified in the blood samples from dogs

No. samples	*Borrelia burgdorferi s.l.*		*Anaplasma* spp. /*Ehrlichia* spp.		*Candidatus Midichloria mitochondrii*		Co-infection *Borrelia burgdorferi s.l.* + *Candidatus Midichloria mitochondrii*	
66	15	(22.7%)	0	(0%)	2	(3.03%)	2	(3.03%)

Table 3

The prevalence of positive dogs with Borrelia *burgdorferi s.l.* included in study

	Positive dogs with Borrelia *burgdorferi s.l.*	Prevalence (%)
Age categories		
< 3 years	6/28	21.4%
4-7 years	3/15	20%
7-12 years	5/19	26.3%
> 12 years	1/4	25%
Gender		
Male	12/41	29.3%
Female	3/25	12%
Total dogs included	**15/66**	**22.7%**

at 72°C for 10 minutes (15, 27).

Amplicons were introduced into the agarose gel (Bio-Rad, Madrid, Spain) with ethidium bromide (2%) and the PCR products were purified (DNA Purification Kit ZYMO) and sequenced with forward and reverse primers (Eurofins, Martinsried, Germany). Sequencing results were analysed with the Chromas version 2.6.6 software (www.technelysium.com.au). DNA sequence comparisons were achieved by BLAST (www.ncbi.nlm.nih.gov).

RESULTS AND DISCUSSIONS

Blood samples

Blood samples (n = 66), presented in Table 2, were collected from dogs of different breeds and aged between 3 months and 13 years. 15/66 (22.7%) dogs were positive for *Borrelia burgdorferi s.l.*, 0/66 for *Anaplasma* spp./*Ehrlichia* spp. and 2/66 (3%) for *Candidatus Midichloria mitochondrii*. The co-infection with *Borrelia burgdorferi* s.l. and *Candidatus Midichloria mitochondrii* was identified in 2/66 (3%) dogs.

Table 3 presents data regarding the age and gender of the dogs included in our study. The highest prevalence was reported at the age category under 3 years, 6/28 (21.4%), followed by the category 7-12 years - 5/19 (26.3%). Regarding gender, 29.3% (12/41) of dogs infected with *Borrelia burgdorferi s.l*. were male and only 12% (3/25) were female.

Ticks

As presented in Table 4, a total of 99 adult ixodid ticks were collected from dogs during the clinical examination. The ticks were identified as *Ixodes ricinus* (48.5 %; 48/99) and *Dermacentor reticulatus* (51.5%; 51/99), 66.7% were engorged females and 33.3% males. The identified ticks were distributed in pools according to the following criteria: canine patient, species, sex, and developmental stage. The PCR assays were performed for 3 pathogens and the result are shown in Table 5. The most common pathogen species detected in ticks was *Candidatus Midichloria mitochondrii* (25.8%)

Table 4

Identification of tick samples collected from dogs

Identified tick species	Number of ticks		Developmental stage				Positive pools
			Larva	Nymph	Adult		
					Males	Females	
Dermacentor reticulatus	51	(51.5%)	0	0	17	34	2
Ixodes ricinus	48	(48.5%)	0	0	16	32	2
TOTAL	99		-	-	33 (33.3%)	66 (66.7%)	4

Table 5

Results of pathogen species and co-infections identified in ticks pools

Pathogen species	Positive pools/Total pools		Pools according tick species	
			Ixodes ricinus	*Dermacentor reticulatus*
Borrelia burgdorferi s.l.	4/31	12.9%	2	2
Anaplasma spp./*Ehrlichia* spp.	0/31	0%	0	0
Candidatus Midichloria mitochondrii	8/31	25.8%	6	2
Co-infection *Borrelia burgdorferi s.l.* + *Candidatus Midichloria mitochondrii*	2/31	6.5%	1	1

without clinical significance, followed by *Borrelia burgdorferi s.l.* (12.9%) and the co-infection with *Borrelia burgdorferi s.l.* and *Candidatus Midichloria mitochondrii* (6.5%). *Candidatus Midichloria mitochondrii* was identified in 6 pools of female ticks *Ixodes ricinus* and 2 pools of female ticks *Dermacentor reticulatus*.

Lyme disease remains a complex zoonosis and the diagnosis is challenging (36). Humans are less exposed to ticks than dogs. However, in Europe are being reported annually approximately 85.000 cases of Lyme borreliosis in humans (43). Similarly, in a study published by Manciuc et al. (2019), cases of Lyme disease in humans have begun to decline in recent years. According to the same study, in 2016 in Romania were reported 688 suspicious cases, of which 4.1% were reported in Iasi (25).

In 33/34 of European studies that detected *B. burgdorferi* in *Ixodes ricinus* ticks using PCR techniques, have demonstrated that this tick species is the major transmitting source of this pathogen in this continent, with 17.7% prevalence (16). Molecular identification of *B. burgdorferi* in ticks collected from dogs showed a prevalence of 15% in Denmark (42), 7.9% in Latvia (32) and 0.4% in 64 Italian provinces (46).

Almost all the studies from Romania have used serological tests to identify *B. burgdorferi*. In the questing ticks, the prevalence of *Borrelia* species was 26.35% in Cluj (6), 25.8% in Eastern Romania (37), 18.6% in Western Romania (17). The outcome of the present study showed the following pattern: 22.7% of the babesiosis-positive dogs were co-infected with *B. burgdorferi* and 12.9% of their ticks were DNA positive for the same pathogen, hence, Iasi could be an endemic area for *B. burgdorferi* infection. To establish the endemicity of this area, further studies are needed to be performed using a large size population of dogs for the screening of *B. burgdorferi* infection.

Anaplasma spp. and *Ehrlichia* spp. were identified in Romania in dogs (4, 28) as well as in ticks (20) but neither *Anaplasma* spp. nor *Ehrlichia* spp. DNA was identified in the present study.

The most common member of the microbiome of the *Ixodes ricinus* tick is *Candidatus Midichloria mitochondrii*, a gram-negative alphaproteobacterium that belongs to the order *Rickettsiales* (11). The prevalence of *Candidatus Midichloria mitochondrii*, the symbiont found in the ticks appears to be higher in females and lower in males (1), similar to the results from the present study. Indeed, it has been shown that the presence of *Candidatus Midichloria mitochondrii* is abundant in various cell types of the ovary, including oocytes (26). Moreover, in the study published by Vozmediano et al. (2021) regarding the genetic diversity of *Rickettsiella* symbionts in *Ixodes ricinus* ticks in Europe, *Candidatus Midichloria mitochondrii* was reported in Sweden (62.1%), United Kingdom (48.7%), Germany (36.4%), the Netherlands (73.6%), Belgium (77.9%), and Italy (68.9%). Another study confirmed the co-infection of *Borrelia* burgdorferi and *Candidatus Midichloria mitochondrii* in the ticks with the highest

prevalence in Germany (100%), Italy (86.3%), Sweden (61%), Belgium (20.7%), the Netherlands (15.5 %), and the United Kingdom (13.5%) (14). Paduraru et al. (2012) demonstrated the presence of *Candidatus Midichloria mitochondrii* DNA in 30/147 of *Ixodes ricinus* ticks collected from roe deer and goats in the Eastern part of Romania (Galați and Bacău) (34).

CONCLUSIONS

The results of this study showed the importance of identifying co-infections in tick-infested dogs and the need for enhanced monitoring of prevention protocols of ticks' infestations in dogs. The *Anaplasma* and *Ehrlichia* species were not detected in the analysed blood and tick samples. The first report of the symbiote *Candidatus Midichloria mitochondria* in the blood of dogs but also in the ticks collected from them, confirms its presence, thus requiring further investigation to better understand the role and possible implications on animals and public health. The presence of borreliosis in dogs confirmed with babesiosis, as well as the identification of the bacterium in ticks, reinforces to alert the veterinary practitioners and the public health authorities for the risk of this zoonotic disease. Co-infections might complicate the diagnosis and should be further studied in companion animals.

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