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Prevalence of *Babesia canis*, *Borrelia burgdorferi* sensu lato, and *Anaplasma phagocytophilum* in hard ticks collected from meadows of Lubelskie Voivodship (eastern Poland)

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Abstract

The aim of the study was to assess the distribution of *Anaplasma phagocytophilum, Borrelia burgdorferi* sensu lato, and *Babesia canis* in adult females and males of *Ixodes ricinus* and *Dermacentor reticulatus* ticks, inhabiting meadows near large forest complexes throughout the Lubelskie Voivodship (eastern region of Poland). Ticks were collected using the flagging method. Among 720 ticks collected, 506 were identified as *D. reticulatus*, and 214 as *I. ricinus*. DNA of *B. canis* and *B. burgdorferi* s.l. was detected in 21.3% and 0.6% of *D. reticulatus* ticks, respectively. In *I. ricinus* ticks, DNA specific to *B. burgdorferi* s.l. and *A. phagocytophilum* was detected in 5.6% and 10.3%, respectively. Co-infections of *B. burgdorferi* s.l. and *A. phagocytophilum* were found in two *I. ricinus* ticks. These results indicate that the Lublin region is an area at risk of tick-borne diseases of humans and animals, which must be considered in clinical practice.

Key words: *Anaplasma phagocytophilum*, *Babesia canis*, *Borrelia burgdorferi* sensu lato, ticks, vector-borne diseases.

Introduction

Hard ticks are ectoparasites of humans and animals, with a worldwide distribution. They are vectors of many viral, rickettsial, bacterial, and protozoan pathogens. Depending on the geographical region, the invasion of ticks in farms and companion animals, as well as in humans, results in the risk of various tick-borne diseases. *Dermacentor reticulatus* and *Ixodes ricinus* are the most common tick species recorded on the territories of eastern Poland. Less frequently, *I. apronophorus, I. trianguliceps, I. hexagonus, I. crenulatus, Haemaphysalis punctata*, and *Rhipicephalus rossicus* are reported (3, 4, 12, 16, 18). *I. ricinus* is the most prevalent tick species in Poland, which can transmit *Borrelia burgdorferi* sensu lato and *Anaplasma phagocytophilum,* the aetiological agents of Lyme disease and granulocytic anaplasmosis (7). *D. reticulatus* is considered to be an important vector of some species of the *Babesia* genus, being responsible for human and animal babesiosis (2, 16). These ticks can also transmit *Borrelia burgdorferi* s.l.

Tokarz *et al.* (13) claim that a single tick bite may lead to polymicrobial infections. Therefore, molecular investigations of specific patterns of mixed infections within individual ticks inhabiting different ecosystems

may provide valuable epidemiological data during the formulation and implementation of prevention strategies for animal and human health.

The primary objective of the analyses was to assess the distribution of *Anaplasma phagocytophilum* and *Borrelia burgdorferi* s.l., as well as *Borrelia burgdorferi* s.l. and *Babesia canis* mixed infections in adult females and males of *I. ricinus* and *D. reticulatus* ticks, inhabiting meadows near large forest complexes, in different parts of the Lubelskie Voivodship (eastern region of Poland).

Material and Methods

During the tick activity season (between March 2012 and October 2012), ticks were collected from meadows in various parts of the Lubelskie Voivodship (Lublin, Leczna, Pulawy and Swidnik districts). The meadows were situated near large forest complexes, rich in small and large mammal fauna being hosts for the various developmental stages of the ticks. Between spring and autumn, the meadows were used for grazing cattle.

Ticks were collected using the flagging method, which involved sweeping the vegetation with a 1 × 1 m white cloth attached to a 1.5 m long bamboo pole. After the cloth was swept over the plants several times, its surface was viewed and ticks attached were transferred with forceps to tubes (17). The field studies were performed on sunny days only. No tick collections were undertaken when the grass was wet after rainfall. Ticks were collected three times.

The larvae, nymphs, adult females, and males of *I. ricinus* and *D. reticulatus* ticks were collected. Approximately 15-20 collected nymphs and larvae together were an insufficient amount, so the studies were performed on adult ticks. Among the 720 adult ticks collected, 506 were identified as *D. reticulatus* (184 males and 322 females). The remaining 214 ticks were identified as *I. ricinus* (76 males and 138 females) on the basis of the key developed by Siuda (10).

All ticks were kept at -70°C until the isolation of DNA of pathogens studied. Before DNA extraction, the ticks were washed in 70% ethanol and sterile water, and homogenised in 100 µL of PBS with a sterile pestle. DNA was extracted from individual ticks using the Genomic Mini kit (A&A Biotechnology, Poland) according to the manufacturer's instructions, preceded by 6 h digestion with Proteinase K. The efficiency of the DNA isolation was confirmed by electrophoresis in a 1.5% agarose gel. Isolated DNA was stored at -70°C (19).

Lysates from *I. ricinus* were used to detect DNA of *B. burgdorferi* s.l. and *A. phagocytophilum*. PCR was performed according to the method described by Skotarczak *et al.* (11) and Adaszek *et al.* (1) with the primers: SC1 (5′-GCT GTC AGT GCG TCT TAA-3′) and SC2 (5′-CTT AGC TGC TGC CTC CGT A-3′) used to amplify the 16S rDNA gene fragment of *B. burgdorferi* s.l., and EHR 521 (5′-5’-TGT AGG CGG TTC GGT AAG TTA AAG -3′) and EHR 747 (5′- GCA CTC ATC GTT TAC AGC GTG -3′) enabling amplification of the 16S RNA gene fragment of *A. phagocytophilum*. The expected product sizes were about 300 bp for *B. burgdorferi* s.l. and about 247 bp for *A. phagocytophilum*. As a positive control, DNA of *Borrelia burgdorferi* s.l. and DNA of *A. phagocytophilum* obtained from National Reference Center for Borreliae of the Max von Pettenkofer Institute of Ludwig Maximilian University Munich were used.

Lysates from *D. reticulatus* were used to detect DNA of *B. canis,* and *B. burgdorferi* s.l. PCR for *B. canis* was performed according to the method described by Adaszek *et al.* (2) with the primers BAB GF 2 (5′- GTC TTG TAA TTG GAA TGA TGG -3′) and BAB GR 2 (5′- CCA AAG ACT TTG ATT TCT CTC -3′) used to amplify the 18S rDNA gene fragment of the piroplasmas. The expected product size was about 559 bp. As a positive control, DNA isolated from blood of dogs infected with *B. canis* was used. PCR amplification was performed using a programmable thermocycler (Biometra, Goettingen, Germany).

The size of the PCR products was analysed by electrophoresis in a 1.5% agarose gel stained with ethidium bromide. The products were purified using QIAquick spin columns (Qiagen) and eluted in 50 µL of Tris 10 mM, pH 7.6. DNA sequencing was performed on both strands using the same primers employed for PCR at DNA Sequencing and Synthesis Service of the Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland. DNA sequences were assembled and edited using SeqMan (DNAStar, Lasergene, USA), and MegAlign (DNAStar, Lasergene, USA) alignments to the published *B. canis canis* 18S rRNA gene EU622792, EU622793, *A. phagocytophilum* 16S RNA gene GU183908, and *B. burgdorferi* s.l. 16S RNA gene DQ111061.

Results

The ticks were collected from meadows, which were overgrown by vegetation typical for ecosystems composed of dense shrub-land and short trees and bushes that have developed around and within the habitat.

DNA of *B. canis* was amplified from 108 of the 506 (21.3%) samples of *D. reticulatus* ticks. Thirty two selected product sequences showed 100% similarity within the 18S rDNA partial sequence EU622792 of *B. canis canis* isolated in previous studies from dogs in eastern Poland, and eight selected product sequences showed 99% similarity with EU622793.

Borrelia DNA was detected in 12 out of the 214 (5.6%) samples of *I. ricinus* and in three of the 506 (0.6%) *D. reticulatus* ticks. These 15 products were

sequenced, and the sequences showed 99% similarity within the 16S rDNA partial sequence of *B. afzelii* (DQ111061).

Anaplasma DNA was detected in 22 of the 214 (10.3%) *I. ricinus*. Sequences analysis demonstrated 100% similarity with the 16S RNA partial gene of *A. phagocytophilum* (GU183908).

The distribution of the pathogens in ticks collected in four studied districts of Lubelskie Voivodship is presented in Table 1. Ticks infected with *B. canis* were most frequently noticed in Lublin district and its two eastern neighboring districts (Leczna and Swidnik). Much less - only five ticks infected with the protozoan, were reported in the Pulawy district. Different results were obtained for *A. phagocytophilum* infection. As many as 45% of ticks infected with rickettsia was recorded in Pulawy district. This percentage was twice lower in Swidnik and Leczna districts and up to five times lower in the district of Lublin. The percentage of ticks infected with *B. afzelii* ranged from 13.3% to 40.0%. Spirochetes were demonstrated in two *I. ricinus* ticks and in one *D. reticulatus* from district of Lublin, in five *I. ricinus* and in one *D. reticulates* in Leczna district, and in three *I. ricinus* and one *D. reticulatus* in Pulawy district, while in Swidnik district in two *I. ricinus* ticks.

The frequency of detecting the DNA of *Babesia*, *Borrelia*, and *Anaplasma* in ticks was not related with the gender of the mites. The proportions of males and females infected with the bacteria and protozoa were similar. *B. canis* was detected in 23.2% of males and 20.1% of females of *D. reticulatus*, while *A. phagocytophilum* was noted in 10.5% of males and 10.3% of females of *I. ricinus*. *B. afzelii* was demonstrated in 0.5% of males and 0.6% of females of *D. reticulates,* and in 5.3% of males and 5.6% of females of *I. ricinus* (Table 2).

Co-infections with *B. afzelii* and *A. phagocytophilum* were found in two *I. ricinus* ticks, but no co-infections with *B. afzelii* and *B. canis* were detected in *D. reticulatus* ticks.

Discussion

The studies have shown that *Babesia* protozoa were the most frequent pathogens detected in hard ticks. As shown by clinical observations, canine babesiosis is the most common tick-borne disease in animals in eastern Poland (2). Borreliosis and granulocytic anaplasmosis are diagnosed rarely in small as well as farm animals in this part of our country (1, 8, 15). In other regions of Poland, canine babesiosis is recorded less frequently.

Table 1. Frequency of detection of infected ticks collected in four districts of the Lublin Voivodship

Pathogen	Localisation (District)			
	Lublin	Leczna	Pulawy	Swidnik
B. canis	33/108 (30.6)*	48/108 (44.4)	5/108 (4.6)	22/108 (20.4)
B. afzelii	3/15 (20.0)	6/15 (40.0)	4/15 (26.7)	2/15 (13.3)
A. phagocytophilum	2/22 (9.1)	5/22 (22.7)	10/22 (45.5)	5/22 (22.7)

* number of pathogens detected in ticks in each district/total number of pathogens detected (%)

Table 2. Number of ticks harboured by *Babesia canis*, *Borrelia afzelii*, and *Anaplasma phagocytophilum* DNA

Pathogen	*Dermacentor reticulates*		*Ixodes ricinus*	
	Male	Female	Male	Female
B. canis	46/198 (23.2)*	62/308 (20.1)	–	–
B. afzelii	1/198 (0.5)	2/308 (0.6)	1/19 (5.3)	11/195 (5.6)
A. phagocytophilum	–	–	2/19 (10.5)	20/195 (10.3)

* number of ticks harboured/total number of ticks (%)

This is an effect of the lower prevalence of *B. canis* in vectors, such as *D. reti-culatus*, as confirmed by Zygner *et al*. (19), who detected protozoan DNA in just 11% of *D. reticulatus* mites collected from dogs in Warsaw (central Poland). This rate is almost half of that observed in the present work (21.3%).

The prevalence of *B. afzelii* and *A. phago-cytophilum* in collected hard ticks in comparison with the prevalence of *B. canis*, was rather low. The prevalence of spirochetes in the present work is in agreement with the results obtained by Cisak *et al.* (5) and Wójcik-Fatla *et al.* (17). In these studies, 5.4% and 4.94% of *I. ricinus* ticks collected in eastern Poland harboured *B. burgdorferi* s.l. Similar results were also obtained in central Poland – 6.2% (19), and in Hungary – 5.5% (6). It should be noted that among 506 tested *D. reticulatus* lysates, the presence of spirochete DNA was observed in three samples only. This shows that in comparison to *I. ricinus*, these ticks are of a marginal importance in the transmission of Lyme disease, as confirmed by Siński *et al.* (9) and Wegner *et al.* (14).

The percentage of *I. ricinus* ticks infected with *A. phagocytophilum*, rickettsia registered in this study, was the highest so far, reported in the Lubelskie Voivodship. According to Wójcik-Fatla *et al.* (17), the presence of rickettsial DNA was detected in 4.94% of *I. ricinus* ticks between 2004 and 2006, while in 2012 this rate was 10.3%. This indirectly points to the growing risk of granulocytic anaplasmosis for humans and animals.

Another problem that needs consideration is the risk of mixed infections of ticks with *B. afzelii* and *A. phagocytophilum*, which may be further transmitted to humans and animals. Co-infections considerably hinder the diagnosis of the disease, making it severe and undermining the efficacy of the treatment. Even though, in the present work, such infections were observed only in two *I. ricinus* ticks, and the simultaneous presence of the DNA of *B. canis* and *B. afzelii* was not detected in the tested *D. reticulatus* lysates. Their occurrence should be always considered in the clinical practice, especially if we are dealing with cases of anaplasmosis or borreliosis resistant to therapy.

Our studies revealed the presence of the DNA of *B. canis* protozoa, *A. phagocytophilum* rickettsia, and *B. burgdorferi* s.l. spirochetes in hard ticks. The analysis of the sequences of the obtained amplicons has shown they are highly similar (99%-100%) to the sequences previously published for those pathogens, and published in the gene bank. Isolates of *B. canis* EU622792, EU622793 and *A. phagocytophilum* GU183908 were obtained from dogs and a horse, from the Lublin region, infected with protozoa and rickettsia, respectively (1, 2). In turn, the presence of *Borrelia* spirochetes of high homology to the DQ111061 sequence was recorded near Warsaw. These results indicate the endemic presence of these pathogens in the central and eastern Poland, and that the Lublin region is an area at risk of tick-borne diseases of humans and animals. This must be considered in clinical practice, in particular during the period of ticks' activity.

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