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COEXISTENCE DNA OF *BORRELIA BURGDORFERI* SENSU LATO AND *BABESIA MICROTI* IN *IXODES RICINUS* TICKS FROM NORTH-WESTERN POLAND

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Abstract: The tick *Ixodes ricinus* may carry microorganisms which cause serious human and animal diseases, i.a., the Lyme disease (borreliosis), caused by the spirochaete *Borrelia burgdorferi* sensu lato and babesiosis, induced by the protozoan *Babesia microti*. Both microbe species may co-occur in the same and other species of the genus tick and produce a mixed infection in humans and animals. The major objective of the study was to identify DNA of *B. burgdorferi* and *B. microti* in the *I. ricinus* ticks collected in spring and autumn 1999 from 6 sites in north-western Poland. The microbial DNA was identified with polymerase chain reaction (PCR). The marker used to detect the *B. burgdorferi* s.l. DNA was a fragment of the *fla* gene encoding the protein flagellin, while the *B. microti* DNA was detected with a fragment of the gene encoding 16S rRNA. A total of 550, 1,160, and 385 tick adults, nymphs, and larvae, respectively, were examined. Among the 155 (7.4%) *B. burgdorferi*-infected ticks and the 130 (6.2%) infected with *B. microti*, mixed infection was detected in 0.6% of individuals. The prevalence of coinfection differed between the tick developmental stages. Coinfection was most prevalent (3.1%) in females, males and nymphs being less affected (0.4 and 0.2%, respectively). No coinfection was revealed in the tick larvae. The study described was the first of its kind to be conducted in the former District of Szczecin. For the phenomenon of microbial co-occurrence and related mixed infections to be properly evaluated, the research will be continued.

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Key words: *Borrelia burgdorferi* sensu lato, *Babesia microti, Ixodes ricinus* ticks, PCR.

INTRODUCTION

The *Ixodes* ticks are parasitic Acari feeding on the blood of vertebrates, including humans. They may carry various pathogens and infect their hosts with them. The most important tick in Europe, including Poland, is *Ixodes ricinus* [2]. The spirochaete *Borrelia burgdorfer*i it carries causes borreliosis, called also the Lyme disease after Old Lyme in Connecticut, USA, where the condition was very frequent in the late 1970s. The disease has been known in Europe since at least the 18th century, but was intially misdiagnosed and classified as several different diseases. At present, several thousand cases of borreliosis are recorded in Europe each year. In Poland, for a few years now, each case of borelliasis has had to be reported and the number of cases has been steadily increasing. For example, 784 and 1,850 cases were reported in 1998 and 2000, respectively (State Hygiene Inspection data).

Human babesiosis, caused by *Babesia microti*, was for the first time recorded in 1968 in Massachusetts, USA [21]. A total of 14,000 cases had been reported in USA until 1994 [21] and in 1996 the case rate of human babesiosis for all states within the United States was 524 per 100.00 (Center for Disease Control and Prevention

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case reports). In Europe, the disease was first reported in 1957 from Yugoslavia [3, 19]. The extent of the condition in Europe is not known in detail.

Our earlier studies on ticks collected in north-western Poland showed the presence of *B. burgdorferi* s.l. DNA [12, 17, 18] and three genomic groups, i.e., *B. burgdorferi* sensu stricto, *B. afzelii*, and *B. garinii* [22]. In a later study, we identified DNA of *B. microti* and *B. divergens* [13, 14, 15] and that of the HGE agent as well [10, 16]. This study was aimed at detecting the concurrent presence of DNAs of *B. microti* and *B. burgdorferi* s.l. in various individuals of *I. ricinus.* In addition, we attempted to identify the risk centres of borreliosis and babesiosis transmission from the areas studied.

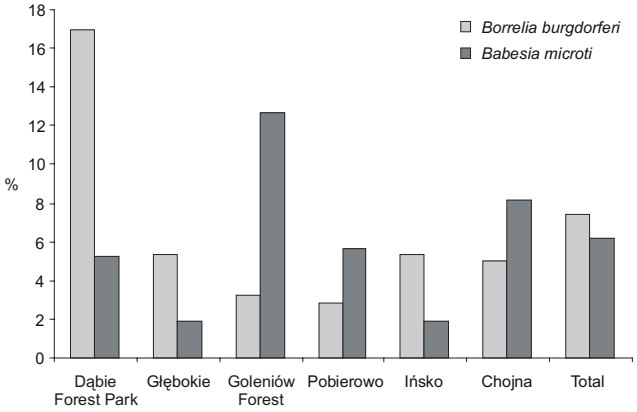

Figure 1. Prevalence (%) of *I. ricinus* infection with spirochaete *B. burgdorferi* s. l. and protozoan *B. microti* at each collection site.

MATERIAL AND METHODS

Collection of ticks. *I. ricinus* ticks were collected in the spring and autumn of 1999 at 6 sites in natural habitats, i.e., forests in the District of Szczecin (Tab. 1) by dragging a 1 m^2 piece of flannel cloth over the vegetation cover. Immediately after collection, the ticks were immersed in 70% ethanol and stored. Sex and developmental stage of each tick were determined. Of the total number of 2,095 specimens collected, 287, 263, 1160, and 385 were classified as females, males, nymphs, and larvae, respectively. Each tick was examined for the presence of *B.burgdorferi* sensu lato and *B. microti* DNAs.

Preparation of tick samples for PCR. *I. ricinus* ticks were processed as described earlier [14, 15]. The ticks were removed from the ethanol solution, air dried, and boiled for 20 min. in 100 µl 0.7 M ammonium hydroxide to release the DNA. After cooling, the vial containing the lysate was left open for 10 min at 96°C for ammonia to evaporate. The tick lysate was either used immediately for PCR or stored at -24°C until used.

Detection of DNAs of *B. microti* and *B. burgdorferi* sensu lato. Each of the tick lysates was used to detect DNAs of both pathogens. For the amplification of the *B. microti* DNA, we used a fragment of a gene encoding the nuclear small-subunit ribosomal RNA (SS-rDNA) as a target. Primers Bab1 - Bab4 (the expected PCR product is 238 bp in size) described by Persing *et al.* [9] were applied. As a positive amplification control in each reaction, we used 1 ng o*f B. microti* merozoite DNA obtained from the University of Warsaw, Poland. Negative amplification control, i.e., 5µl distilled water added to the PCR buffer, was also included in each PCR. The reactions for the detection of the *B. microti* DNA involved initial denaturation (1 min. at 94°C) followed by 35 rounds of temperature cycling (94°C for 1 min., 55°C for 1 min., and 72°C for 2 min.).

Table 1. Frequency (PCR+, %) of *B. burgdorferi* sensu lato (B. b.) and *B. microti* (B. m.) in *I. ricinus* ticks in relation to tick sex, developmental stage, and collection site.

Collection site		Total			Females			Males			Nymphs			Larvae		
		N	PCR positive		N	PCR positive		N	PCR positive		N	PCR positive		N	PCR positive	
			(N)	(%)		(N)	(%)		(N)	(%)		(N)	(%)		(N)	(%)
Dąbie Forest Park	B.b.	518	88	17.0	142	49	34.5	119	7	5.9	166	21	12.7	91	11	12
	B.m.		27	5.2		16	11.3		8	6.7		2	1.2		1	1.1
Goleniów Forest	B.b.	464	15	3.2	52	1	1.9	38	4	10.5	304	10	3.3	70	0	0
	B.m.		59	12.7		21	40.4		5	13.2		29	9.5		4	5.7
Pobierowo	B.b.	252	7	2.8	26	0	0	33	0	0	135	6	4.4	58	1	1.7
	B.m.		14	5.6		1	3.8		8	24.2		5	3.7		0	0
Ińsko	B.b.	432	23	5.3	14	0	0	19	0	0	287	18	6.3	112	5	4.5
	B.m.		8	1.9		0	0		0	0		6	2.1		2	1.8
Chojna	B.b.	220	11	5	30	4	13.3	32	0	0	124	4	3.2	34	3	8.8
	B.m.		18	8.2		6	20		3	9.4		4	3.2		5	14.7
Głębokie	B.b.	209	11	5.3	23	2	8.7	22	0	0	144	8	5.6	20	1	5
	B.m.		4	1.9		0	0		1	4.5		3	2.1		0	0
Total	B.b.	2,095	155	7.4	287	56	19.5	263	11	4.2	1160	67	5.8	385	21	5.5
	B.m.		130	6.2		44	15.3		25	9.5		49	4.2		12	3.1

Table 2. Occurrence of *B. burgdorferi* s. l. and *B. microti* DNAs in adult males and females as well as in nymphs and larvae of *I. ricinus*.

Collection site	Total			Females			Males			Nymphs			Larvae		
	N	PCR positive		N	PCR positive		N	PCR positive		N	PCR positive		N	PCR positive	
		(N)	(%)		(N)	(%)		(N)	(%)		(N)	(%)		(N)	(%)
Dąbie Forest Park	518	6	1.2	142	5	3.5	119	1	0.8	166	-	-	91	-	-
Goleniów Forest	464	2	0.4	52	1	1.9	38	-	-	304	1	0.3	70	-	-
Głębokie	209	-	-	23	-	-	22	-	-	144	-	-	20	-	-
Ińsko	432	1	0.2	14	-	-	19	-	-	287	1	0.3	112	-	-
Pobierowo	252	-	-	26	-	-	33	-	-	135	-	-	58	-	-
Chojna	220	3	1.4	30	3	10	32	-	-	124	-	-	34	-	-
Total	2,095	12	0.6	287	9	3.1	263	1	0.4	1,160	2	0.2	385	-	-

To amplify the *B. burgdorferi* sensu lato DNA, we used the primers FLA1 and FLA2 (5' AGA GCA ACT TAC AGA CGA AAT TAA T 3' and 5; CAA GTC TAT TTT GGA AAG CAC CTA A 3') we constructed, in the conserved regions of the *fla* gene. In each PCR run, DNA of *B. burgdorferi* s.l., strain Bo-148c/2 was used as the positive control and distilled water as the negative. The samples were initially denatured for 3 min. at 95°C and 35 cycles performed (94°C for 30 s, 54°C for 45 s, 72°C for 45 s). The PCR products were examined on 2% agarose gels in 1 × TRIS-buffer. To minimise contamination, the reagent setup, extraction and sample addition, and the PCR and sample analysis were performed in 3 separate laboratories.

RESULTS

In the spring and autum of 1999, a total of 2,095 *I. ricinus* individuals (550 adults, 1160 nymphs, and 385 larvae) were collected at 6 sities in the District of Szczecin. The average number of ticks per collection site was 349 (ranging from 252–518). In the entire collection, the nymphs occurred at the highest frequency (n = 1160; 55.3%), followed by larvae (n = 385; 18.3%), females (n = 287; 13.6%), and males (n = 263; 12.5%).

All ticks were examined for the presence of *B. burgdorferi* s.l. and *B. microti* DNAs by means of PCR. The DNA of *B. burgdorferi* s.l. was identified in ticks collected at all 6 sites (Tab. l). The prevalence of infection in the 2,095 *I. ricinus* individuals examined was found to vary, depending on the collection site, from 2.8% in Pobierowo to 17.0% in the Dąbie Forest Park (Tab. 1).

Among the 2,095 ticks examined, the *B. burgdorferi* s. l. DNA was detected in 155 individuals, i.e., 7.4%; the prevalence of infection in females, males, nymphs, and larvae was 19.5, 4.2, 5.8, and 5.5, respectively (Tab. 1).

The *B. microti* DNA was identified in *I. ricinus* from all the sites visited (Tab. 1). The prevelance of infection ranged from 1.9% in Ińsko and Głębokie to 12.7% in the Goleniów Forest (Tab. 1). Of the 2095 *I. ricinus* individuals examined, 130 (6.2%) tested positive for the presence of *B. microti* DNA, 44 (15.3%), 25 (9.5%), 49 (4.2%), and 12 (3.1%) of them being identified as females, males, nymphs, and larvae, respectively (Tab. 1).

The spirochaete *B. burgdorferi* s.l. and the protozoan *B. microti* were found to co-occur in 12 (0.6%) individuals of the 155 (7.4%) ticks infected with the former and the 130 (6.2%) ticks infected with the latter pathogen (Tab. 2, Fig. 1). The prevalence of coinfection was developmental stage-dependent: females proved to be most affected (3.1%), followed by males (0.4%) and nymphs (0.2%), no co-infection being recorded in the larvae. Coinfection was absent from the 2 sites showing the lowest *B. microti* prevalence (Tab. 2, Fig. 1).

DISCUSSION

Human migrations, intensified during the last century, resulted in a growing importance of ticks as pathogen vectors. In response, dramatic progress has been made in broadening the knowledge of tick biology and the diseases they carry, particularly the tick-borne encephalitis, various forms of borreliosis, babesiosis, and human granulocytic and monocytic ehrlichiosis.

A phenomenon that has caused growing concern is human coinfection with *B. microti* and other tick-borne pathogenic agents, particularly *B. burgdorferi* [4]. Serosurveys allowed Benach *et al.* [1] and Krause *et al.* [5, 6] to estimate that as many as 13% of borreliosis patients in *Babesia*-endemic areas were coinfected with *B. microti*. In New York State, 23% of patients with *B. microti* infection had evidence of concurrent borreliosis [8]. Initial symptoms in each separate infection, i.e., babesiosis and borreliosis, are not specific. Both in babesiosis and in the Lyme disease, patients report fever, fatigue, and other flu-like symptoms. Symptoms of the *B. microti–B. burgdorferi* coinfection are more severe, the coinfection being fatal in some cases. The *B. burgdorferi* DNA is much more persistent in the blood of the coinfection-affected patients than in those infected by a single pathogen only [6]. Krause *et al.* [6] emphasise that the therapy applied to coinfection-affected patients has to differ from that used for those with Lyme disease.

So far, ticks have been tested for the presence of more than one pathogenic agent mostly in USA [4, 20, 21]. In Europe, Schouls *et al.* [11] studied the co-occurrence of the *B. burgdorferi* and human granulocytic ehrlichiosis agent DNAs in Dutch and Leutenegger *et al*. [7] and in Swiss populations of *I. ricinus*.

Telford *et al.* [20] reported the coprevelance rate of *B. burgdorferi* and the HE agent as 4%, no simultaneous infection with *B. burgdorferi* and *B. microti* being observed in *I. scapularis* in Massachusetts (USA). Verde *et al.* [21] conducted a similar study in New Jersey (USA) where 10 out of 100 ticks examined proved coinfected with 2 of those 3 pathogens.

In our study, the DNAs of *B. burgdorferi* s.l. and *B. microti* were found in ticks from all the 6 sities visited (Tab. 1, 2). For this reason, each of those sites poses a risk of being a centre of borreliosis and babesiosis transmission. The number of ticks housing the *B. burgdorferi* s.l. DNA was higher than that of ticks with the *B. microti* DNA (Tab. 1); 12 out of the 155 *B. burgdorferi* s.l-affected ticks proved also to be *B. microti* vectors (Tab. 2, Fig. 1). As the pathogens' DNAs were detected in the adult ticks, nymphs, and larvae, human contact with any developmental stage of *I. ricinus* is potentially hazardous. This is the first study of this kind to be carried out in the former District of Szczecin. For the phenomenon of microbial co-occurrence and related mixed infections to be properly evaluated, the research will be continued.

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