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IXODES RICINUS AS A VECTOR OF *BORRELIA BURGDORFERI SENSU LATO, ANAPLASMA PHAGOCYTOPHILUM* AND *BABESIA MICROTI* IN URBAN AND SUBURBAN FORESTS

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Stańczak J, Gabre RM, Kruminis-Łozowska W, Racewicz M, Kubica-Biernat B: *Ixodes ricinus* as a vector of *Borrelia burgdorferi sensu lato, Anaplasma phagocytophilum* and *Babesia microti* in urban and suburban forests. *Ann Agric Environ Med* 2004, **11**, 109–114.

Abstract: In the suburban and urban forests in the cities of Gdańsk, Sopot and Gdynia (northern Poland), *Ixodes ricinus* ticks should be considered as the vector of pathogenic microorganisms that may cause significant diseases in wild and domestic animals and humans. These microorganisms include etiologic agents of Lyme disease, human anaplasmosis (HA) and babesiosis: *Borrelia burgdorferi sensu lato*, *Anaplasma phagocytophilum* and *Babesia microti*, respectively. DNA extracts from 701 ticks collected in 15 localities were examined by PCR for the simultaneous detection of these 3 pathogens. Overall, 14% were infected with *A. phagocytophilum* followed by 12.4% with *B. burgdorferi s.l.* and 2.3% with *B. microti*. In total, the percentage of infected females (32.9%) was 2.4 times higher than in males (13.7%) and 3.2 times higher than in nymphs (10.3%). Among adult ticks (n = 303), 8.3% were dually infected with *A. phagocytophilum* and *B. burgdorferi s.l.*, 2.0% with the agent of human anaplasmosis and *B. microti* and 0.3% with borreliae and *B. microti*.

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INTRODUCTION

Known in Poland mainly as a vector of tick-borne encephalitis, *Ixodes ricinus* was subsequently recognised as a vector of Lyme borreliosis [45], a multisystemic zoonosis caused by pathogenic spirochetes belonging to the species *Borrelia burgdorferi sensu lato*. Further investigations provided evidence that it is also involved in transmission of *Anaplasma phagocytophilum* [13, 40, 41], obligate intracellular rickettsiae that invade granulocytes of various mammalian species and are causative agent of human anaplasmosis (HA), formerly known as human granulocytic ehrlichiosis (HGE). Recently, *I. ricinus* was proved to carry *Babesia microti* and *B. divergens* [17, 34], intraerythrocytic protozoal pathogens, the agents of babesiosis.

This most commonly observed tick species in Poland is responsible for the majority of tick bites in humans. Its infection with *B. burgdorferi s.l.* and *A. phagocytophilum* seems to be frequent in the different woodland areas [13,

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35, 36, 40, 45]. However, to date, little is known about the occurrence of these two microrganisms and babesiae in ticks in forested, urban environments in Poland. Studies conducted in 1993–1995 showed that *I. ricinus* was common and numerous in urban and suburban forests of the cities of Gdańsk, Sopot and Gdynia (northern Poland) [46]. In this expanding urban agglomeration, commonly called the Tri-City, newly-built housing estates are localised frequently in wooded settings. This is followed by increasing contacts of inhabitants with previously undisturbed environments and produces risk of exposures to ticks living there. As the presence of *I. ricinus* may lead to establish foci of different zoonoses, the aims of our present study were:

a) to investigate the prevalence of *B. burgdorferi s.l.*, *A. phagocytophilum* and *B. microti* in ticks collected in the recreational, forested areas of the Tri-City;

b) to estimate the frequency of mixed infections; and

c) to evaluate the risk of acquiring infection for residents and visitors from infected ticks.

MATERIAL AND METHODS

Tick collection. Questing *I. ricinus* were collected from April–September 2001 by flagging lower vegetation in 15 different sites localised in the urban and suburban forests of Gdańsk (n = 6), Sopot (n = 2) and Gdynia (n = 7). In the laboratory ticks were separated by stage and than preserved in 70% ethanol at room temperature until analysis.

DNA isolation. Extraction of DNA was carried out by lysis of crushed ticks in ammonium hydroxide (NH_4OH) [30]. Adult ticks were processed individually while nymphs were pooled. Pools contained from 2–6 specimens, depending on the number of nymphs collected at the particular site. The majority of pools (n = 72/83) consisted of 5 specimens, while the other 10 of 2 (n = 3/83), 3 (n = 3/83), 4 (n = 2/83) and 6 nymphs (n = 2/83), respectively. Obtained lysates were kept at -20ºC for further investigation by the polymerase chain reaction (PCR).

Amplification of DNA of *B. burgdorferi s.l.* Primers FL6 and FL7 were used to amplify a 276 bp fragment of the flagellin gene of this borreliae species [28]. PCR was performed as described previously [39]. Positive (*B. burgdorferi sensu stricto* strain B 148c/2) and negative (double distilled water /DDW/ in place of template) controls were run with each PCR reaction.

Amplification of DNA of *A. phagocytophilum*. A set of primers EHR 521 and EHR 747, designated to amplify a 247 bp fragment of the 16S rDNA of *A. phagocytophilum*, (formerly *E. phagocytophila*, *E. equi* and HGE agent) [25], was used in the PCR tests. The conditions of PCR were as described earlier [13]. Negative and positive controls were used in each set of PCR reactions. In our previous investigation [13], HGE-1-infected HL60 cells extracted from the IFA assay (MRL Diagnostics, USA) served as positive control. This time, we used tick lysates from positive reactions obtained in the investigations mentioned above and confirmed by the analysis of sequences of the PCR product. They showed only 2 nucleotide differences from the DNA of *A. phagocytophilum* amplified from *I. ricinus* in France (GenBank; gi: 4102574). Negative controls used DDW.

Amplification of DNA of *B. microti*. For *B. microti* a nested PCR was performed with outer primers bab1 and bab4, and inner primers bab2 and bab3 [26], targeting specific fragment from a gene encoding the nuclear small sub-unit ribosomal RNA (SS-rDNA). The primer sets amplify 238 bp and 154 bp fragments, respectively.

Primary reactions used 2.5 µl of genomic DNA as template in a total volume of 25 µl reaction mixture that contained: 0.625 U (0.125 µl) *Taq* polymerase (Gibco), 2.5 µl of 10 × PCR reaction buffer, 0.75 µl of 50 mM $MgCl_2$ (final concentration 1.5 mM) (Gibco), 0.5 µl of 2.5 mM dNTPs mixture (final concentration 0.05 mM) (MBI Fermentas, Lithuania), 1 µl of each 10 µM primer (final concentration 0.4 µM) and 16.625 sterile DDW. DNA of *B. microti* merozoites extracted from mouse blood (kindly provided by Prof. Edward Siński, Department of Zoology, University of Warsaw) was used as positive controls and DDW in place of template as negative controls. Samples were incubated for 1 min in 94ºC and then thermally cycled 35 times at 94ºC for 1 min, 60ºC for 1 min and 72ºC for 2 min. Final extension lasted 7 min at 72ºC.

Nested amplification used 1µl of the primary PCR product dissolved 1:10 as a template in a total volume of 25 µl, as described above, and the primers bab2 and bab3, yielding a 154 bp fragment internal to the reaction product of the first PCR run. For the inner reaction, the same conditions as described for the primary amplification were used, but DNA was amplified for 30 cycles. Nested amplification was found to be necessary because of low sensitivity of the initial reaction of tick templates in comparison with positive control samples, and occurrence of unspecific bands.

All PCR reactions were carried out in Perkin Elmer GeneAmp PCR System 2400 and 9700 thermal cyclers. Amplification products were analysed after electrophoresis in a 2% agarose gel stained with ethidium bromide.

Data evaluation. Statistical analysis of the prevalence of infection levels was performed with Pearson's chi^2 test using Yates' correction when the numerical force of subgroup was < 10; p values < 0.05 were considered statistically significant. Calculation was done using Statistica 6.0. software (StatSoft Inc., USA).

RESULTS

In total, 701 (398 nymphs, 139 male and 164 female) *I. ricinus* were collected and examined for infection with the agents of human anaplasmosis, Lyme borreliosis and babesiosis. Overall, 14% ticks were infected with

A. phagocytophilum, 12.4% with *B. burgdorferi* and 2.3% with *B. microti*, respectively (Tab. 1).

The prevalence of *A. phagocytophilum* infection in *I. ricinus* in particular sites ranged from 0–27.6%. The highest proportion of infected ticks, 19.2%, was noted in the Gdańsk forests, followed by the forests of Gdynia and Sopot with the rate of infection of 11.7% and 5.1%, respectively (Tab. 1). These differences were statistically significant ($p = 0.001$). Ticks harboured *B. burgdorferi* occurred at the 13/15 collection sites and the infection rate varied there between 4.3–20.9%. The frequency of infection in Gdańsk (12.9%) and Gdynia (13.7%) was comparable ($p = 0.77$), being 2.5–2.7 fold higher than that observed in Sopot (5.1%) (Tab. 1). However, the differences were not statistically significant ($p = 0.11$). Ticks carried *B. microti* were noted at 10/15 localities (Tab. 1). Percentage of infected specimens ranged there from 0.6–8.7%. The overall prevalence of infection in the areas of Gdańsk, Sopot and Gdynia were 3.1%, 2.6% and 1.5%, respectively, and did not differ significantly ($p = 0.38$).

In the case of *B. burgdorferi* and *B. microti* infection, nymphs showed approx. 3 times lower positivity rates (7.0% and 1.3%) compared to adult stage (19.5% and 3.6%, respectively), and an approx. 15 fold lower level of infection (2.0% *vs.* 29.7%) in the case of *A. phagocytophilum* infection (Tab. 2). However, percentage of infected nymphs was estimated at the minimal level provided that each positive pool contained just one infected nymph, thus the actual values are probably higher and the differences in infection levels between nymphs and adult ticks slightly lower.

Among adults, females and males differ significantly in rates of infection by either *A. phagocytophilum* (47.6% and 8.6%) ($p < 0.001$) and *B. burgdorferi* (24.3% and 13.7%, respectively) ($p < 0.03$) while the prevalence of babesial infection was comparable for both stages (4.3% and 3.0%) ($p = 0.78$) (Tab. 2).

Table 2. Number (percentage) of questing nymphs and adult *I. ricinus* collected in the urban and suburban forests of Gdańsk, Sopot and Gdynia, infected with *B. burgdorferi*, *A. phagocytophilum* and *B. microti*.

Tick stage	No. tested	No. (%) ticks infected with		
		B. burgdorferi	*A. phagocytophilum*	*B. microti*
Adults	303	59 (19.5)	90 (29.7)	11 (3.6)
male	139	19 (13.7)	12 (8.6)	6 (4.3)
female	164	40 (24.3)	78 (47.6)	5 (3.0)
Nymphs	398	28 (7.0)	8 (2.0)	5 (1.3)
Total	701	87 (12.4)	98 (14.0)	16 (2.4)

Table 1. Prevalence of *B. burgdorferi s.l.*, *A. phagocytophilum* and *B. microti* in *Ixodes ricinus* ticks in particular collection sites of the Tricity forests in 2001.

Collection site (city district)		n	No. (%) infected ticks*		
			B. burgdorferi s.l.	*A. phagocytophilum*	*B. microti*
Gdańsk	Wrzeszcz I	67	14 (20.9)	16 (23.9)	2 (3.0)
	Wrzeszcz II	22	1 (4.5)	3 (23.1)	0 (0.0)
	Stogi	29	3 (10.3)	8 (27.6)	2 (7.0)
	Sobieszewo	40	4 (10.0)	11 (27.5)	1 (2.5)
	Otomin	85	12 (14.1)	9 (10.6)	3 (3.5)
	Firoga	44	3 (6.8)	8 (18.2)	1 (3.1)
Subtotal		287	37 (12.9)	55 (19.2)	9 (3.1)
Sopot	Brodwino	31	2 (6.5)	3 (9.7)	0 (0.0)
	Świemirowo	47	2 (4.3)	1 (2.1)	2 (4.2)
Subtotal		78	4 (5.1)	4 (5.1)	2 (2.6)
Gdynia	Chwarzno	158	24 (15.2)	19 (12.0)	1 (0.6)
	Marszewo	5	0 (n.c.)	2 (n.c.)	1 (n.c.)
	Witomino	70	10 (14.3)	3 (4.3)	0 (0.0)
	Leszczynki	18	3 (16.7)	2 (11.1)	0 (0.0)
	Redłowo	3	0 (n.c.)	0 (n.c.)	1 (n.c.)
	Obłuże	23	4 (17.4)	3 (13.0)	2 (8.7)
	Łężyce	58	5 (8.6)	10 (17.2)	0 (0.0)
Subtotal		336	46 (13.7)	39 (11.7)	5 (1.5)
TOTAL		701	87 (12.4)	98 (14.0)	16 (2.3)

n - number tested; * - Infection rate of ticks in particular collection site was calculated when number of collected ticks ≥ 10; n.c. - not calculated.

Table 3. Infection and co-infection of adult *I. ricinus* ticks with *Anaplasma phagocytophilum*, *Borrelia burgdorferi s.l.* and *Babesia microti*.

Tick stage	n	Number of (%) ticks infected with single species			Number of (%) ticks infected with mixed species			None species
		Anaplasma phagocytophilum	*Borrelia burgdorferi s.l.*	*Babesia microti*	*Anaplasma phagocytophilum* + *Borrelia burgdorferi s.l.*	*Anaplasma phagocytophilum* + *Babesia microti*	*Borrelia burgdorferi s.l.* + *Babesia microti*	
Females	164	53 (32.3)	19 (11.6)	1 (0.6)	21 (12.8)	4 (2.4)	0 (0.0)	66 (40.3)
Subtotal			73 (44.5)			25 (15.2)		
Males	139	6 (4.3)	14 (10.1)	3 (2.2)	4 (2.9)	2 (1.4)	1 (0.7)	109 (78.5)
Subtotal			23 (16.5)			7 (5.0)		
Total	303	59 (14.5)	33 (10.9)	4 (1.3)	25 (8.3)	6 (2.0)	1 (0.3)	175 (57.7)
Subtotal			96 (31.7)			32 (10.6)		

In the majority of adults (n = 96/303, i.e. 31.7%), infections with single pathogenic species were observed, although co-infections were also detected (n = 32/303, i.e. 10.6%). Twenty five ticks (8.3%) had dual infection with *A. phagocytophilum* and *B. burgdorferi* with higher prevalence in females (12.8%) than in males (2.9%). Six ticks (2.0%) were co-infected with the agent of human anaplasmosis and *B. microti*, and one (0.3%) male tick was infected with *B. burgdorferi* and *B. microti* (Tab. 3).

DISCUSSION

The suburban and urban forests of the Tri-City agglomeration consist primarily of beech trees or, rarely, planted pines and spruces. Sparse patches of forests grow on dry (oak-hornbeam forest) or marshy (alder-ash riparian forest) ground. Diversity of habitats and a wide range of vertebrate tick hosts create suitable conditions for development and survival of *I. ricinus*. The occurrence of deer is especially important as the density of large hosts, on which adult females feed to produce next generation, seems mainly to determine the abundance of this tick species [18]. We confirmed that densities of *I. ricinus* in the Tri-City forests are relatively high and found ticks to be infected with *B. burgdorferi s.l.*, *A. phagocytophilum* and *Babesia microti*.

The overall level of tick infection with *B. burgdorferi s.l.* (12.4%) is comparable with the positivity rate (7.2–12.8%) noted there during previous investigations in 1994–1995 [46] and shows that Lyme borreliosis focus is well established in the studied area. It is also in agreement with data from the urban and suburban biotopes of other Polish and European cities. In Poland, positive ticks were found in the city of Katowice (4–12.3%) [27], Warsaw (19.2–31%) [33] and Poznań (9.5–34.6%) [24]. Borreliae infection was reported in England in 5–12% ticks from 2 London parks [12], in 7.9–9.7% *I. ricinus* from the different habitats in Prague (Czech Republic) [29] and in 12.8–15% ticks from the park forests and pericentral areas of the city of Košice (Slovakia) [23].

Contrary to the well-documented data on the occurrence of *B. burgdorferi s.l.* in urban, forested environment, reports concerning the prevalence of *A. phagocytophilum* and *B. microti* in such habitats are still rare. In Poland, the agent of human anaplasmosis has so far been observed in 3.3% and 20.5% ticks collected in the forests surrounding 2 summer resorts, the town of Krynica Morska (northern Poland) and the village of Białowieża (north-eastern Poland) [41], and in 1.4% ticks from the suburban forests of the city of Szczecin (north-western Poland) [35]. In comparison, in different woodland areas in northern Poland, the level of infection among ticks varied between 7.7–38.5% [40]. The result obtained in this study (14%) is in agreement with that given above.

Demonstration of *B. microti*-infected ticks (2.3%) in the Tri-City forests confirm recent findings that *I. ricinus* can be also involved in circulation of *B. microti* in Europe [7, 11, 34] where to date tick infection rates with babesiae have been calculated at 7.4% in Slovenia [7] and 6.2% in north-western Poland [34]. These data and detection of anti-*B. microti* antibodies in 1.8–5.4% of people exposed to ticks in some regions of Germany [15, 44] and Switzerland (1.5%) [8] support the suggestion that human exposure to this pathogen may occur more often in Europe than has been recognised [8, 43].

The prevalence of *B. burgdorferi* and *B. microti* infection in questing ticks increased approx. 3-fold from nymphal to adult stage, while the prevalence of infection with *A. phagocytophilum* showed an approx. 15-fold increase. Our results confirm similar observations by Levin *et al.* [21]. In their opinion, such dissimilarities between 2 pathogens suggests that their natural cycles differ. They share the same species of vector, but the principal amplifying hosts are not the same. Thus, small mammals, mainly rodents are recognised as competent reservoir hosts both for *B. burgdorferi s.l.* and *B. microti* [9, 16, 43] while large wild mammals, such as roe deer (*Capreoplus capreolus*) in Europe [2, 22, 42] are considered as potential reservoirs for *A. phagocytophilum*.

The phenomenon of mixed infection noted in the present study has already been noted. Coexistence of *B. burgdorferi* and *B. microti* was observed in 0.6% of ticks from north-western Poland [37] while *B. burgdorferi* and *A. phagocytophilum* in 5–16.7% *I. ricinus* from north-eastern Poland [13, 40]. The latter type of dual infection

seems to be frequent in *Ixodes* spp. It has been noted in different European countries, in the USA and China with various prevalence: 0.7–28.2% [3, 4, 5, 6, 14, 18, 19, 20, 31]. Moreover, Skotarczak *et al.* (38) in *I. ricinus* in Poland noted for the first time even triple infection with *B. burgdorferi s.l.*, *A. phagocytophilum* and *B. microti*.

People also may acquire concurrent infections as a consequence of a single tick bite [1]. Thus, local physicians should consider the possibility of co-infection with different pathogens for those who declare a tick bite and/or develope Lyme borreliosis or flu-like symptoms. More attention should be paid to the problem and health authorities should take preventive steps, first of all by providing advice to people on methods of avoiding tick attacks, in recognising attached tick and their proper removal.

CONCLUSIONS

Knowledge of the distribution of *I. ricinus* and estimation of infection level of ticks with the etiologic agents of Lyme disease, HGE and babesiosis can be helpful in preventing the transmission of these emerging zoonosis to humans. Results presented in this paper confirm that *B. burgodrferi s.l.*, *A. phagocytophilum* and *B. microti* circulate in the suburban and urban forests of the Tri-City agglomeration and indicate a potential risk for the residents of the cities and their surrounding areas, as well as for visitors, to contract these tick-borne agents.

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