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Evidence for a risk of tick-borne infection in the city parks of Vilnius, Lithuania

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Thirty-six adult ticks collected in the parks of the Lithuanian capital Vilnius in the spring of 2005 were identified as *Ixodes ricinus* (L.) and studied for the presence of live pathogens by darkfield microscopy, of exoskeleton anomalies by light stereomicroscopy, and of several tick-borne pathogens by PCR. One of the ticks was positive for three pathogens simultaneously: *Ehrlichia muris* (monocitic ehrlichiosis agent), *Borrelia afzelii*, and *Borrelia garinii*; one was dually infected by both species of *Borrelia*. In addition, ticks positive for several species of *Borrelia* were detected: *B. afzelii* (n = 5), *B. garinii* (n = 2), some other species of *B. burgdorferi* sensu lato (n = 2), *Ehrlichia muris* (n = 1). The total proportion of infected ticks was 27.8% (10 of 36), of which 20% (2 of 10) were multiply infected; 47.2% of ticks had exoskeleton anomalies, and such specimens had more multiple infections (35.3% versus 21%). One triply infected tick was found only among the anomalous specimens. Live spirochaetes prevailed among the anomalous specimens: 35.3% (6/17) vs. 5.3% (1/19). The difference was statistically significant: chi square was equal 5.166, $P < 0.05$. In conclusion, even this small sample size reveals several important tick-borne pathogens in the newly detected focus in the centre of Vilnius, transmitted by *I. ricinus*. It suggests that people visiting these parks are exposed to the risk of tick-borne infection, especially when resting on the grass.

Key words: park, tick *Ixodes ricinus,* exoskeleton anomalies, tick-borne infection prevalence

INTRODUCTION

Ticks of the genus *Ixodes*, which have a circumpolar distribution within the forested areas of the Northern hemisphere, are well known as vectors of bacteria belonging to the groups of Spirochetales (genus *Borrelia*), Rickettsiales (genera *Ehrlichia* and *Anaplasma*), piroplasmids (protists of the genus *Babesia*) and flaviviruses. However, in Western Europe the main vector for tick-borne pathogens, *Ixodes ricinus* (L.) has become common not only in wild-forested areas, but also in green patches near and inside towns and especially in their park areas. It is necessary to stress that in the Baltic countries Latvia and Estonia and in North-western Russia there are large areas where *I. ricinus* and *Ixodes persulcatus* Schulze coexist (Ecker et al., 1999; Haglund et al., 2003; Charrel et al., 2004; Golovljova et al., 2004) and, parasitizing on similar hosts, are able to exchange various pathogens. In town parks, especially in those distributed in the centre or near a city, favourable conditions exist for *I. ricinus* ticks. These ticks are very often infected with tick-borne pathogens and can thus be dangerous for the park's visitors, especially when resting on the grass.

In a recreational park in south-western Ireland, *I. ricinus* ticks infected by *Borrelia burgdorferi* sensu lato were numerous on the edges of paths and roads. Gray et al. (1999), who described this phenomenon, concluded that the main source of infection for ticks was not rodents, but birds. Bašta et al. (1999) hold a similar opinion, because in the Prague (Czech Republic) urban parks of Petřin, Vitkov and Stromovka where ticks were regularly collected, the authors found mainly *B. garinii* which is transported by birds, and fewer *B. afzelii* associated with rodents. The infection rate was high; every tenth tick was infected.

In another study, *I. ricinus* ticks were collected in a green suburban area of Chisinau, Moldova, in the spring of 2005 by Koči et al. (2007) who revealed not only 5 species of *B. burgdorferi* s.l. (again mainly (42%) *B. garinii*, commonly found in birds), but also *Anaplasma phagocytophilum*, an agent of a disease in which human blood granulocytes are affected.

Alekseev et al. (2003) worked in a suburb forested recreation zone 28 km from the centre of St. Petersburg. This study revealed 7 different pathogens of viral, bacterial and protozoan origin (maximum 3 simultaneously in one *I. persulcatus* female). These authors

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found one *I. persulcatus* female quickly moving in the grass, providing evidence for the possibility of exposure of humans to ticks in urban parks. Even on comfortable benches near green grassplots, it is necessary to be careful. Thrushes, starlings and other birds can also spread ticks (Alekseev et al., 2001).

Pollution can also affect the spread of disease, and cities are commonly polluted with heavy metal ions, cadmium among them, from industrial and automobile exhaust. As proven previously (Alekseev, Dubinina, 1996; Dubinina et al., 2004; Alekseev et al., 2007), ticks in polluted areas become more dangerous as vectors and have some exoskeleton deformations which may contribute to their identification.

The main source of cadmium for rodents, granivorous birds, hosts of tick larvae and nymphs, is vegetation which can arise from polluted soil. Within the last few years, the literature regarding the influence of heavy metals on the growth, development and size of different kinds of vegetation is steadily increasing (Гладков, 2007).

The above data prompted us to investigate ticks collected in Vilnius parks for the presence of tick-borne pathogens and of exoskeleton anomalies. Furthermore, the purpose was to confirm whether the possible anomalies can be linked to multiple infections of *I. ricinus* whatever the region.

MATERIALS AND METHODS

Ticks. Adults and nymphs of *I. ricinus* were collected by flagging in the Lithuanian capital Vilnius parks (Vingis and Bukčiai) located in the central part of the city, in 2005, from April 15 till May 02. The total fortnightly yield of collected ticks was not great: 62 specimens were collected, among them 23 nymphs (37%) and 39 adults. Only adults were investigated for the presence of anomalies and pathogens. Exoskeleton anomalies were revealed by light stereomicroscopy.

Pathogen detection methods. The hind part of adult specimens was dissected, and live spirochaetes were observed and calculated in 200x microscope dark fields in a drop of saline solution.

Adults, fixed in 70% ethanol, were transferred to St. Petersburg to observe specimens with anomalies of their exoskeletons and then to investigate them using PCR with species-specific primers to identify whether they were infected by *B. afzelii*, *B. garinii*, *E. muris, A. phagocytophilum* or *Babesia microti*. Tick-borne encephalitis was not investigated in this study.

Preparation of ticks for pathogen identification. Adult ticks were rinsed in 70% alcohol, then dried, frozen in liquid nitrogen and crushed individually. To every sample, 200 µl sterile H_2O, 3 µl transfer-RNA solution and 200 µl 4 M guanidine thiocyanate were added, followed by 10 min of incubation at 0 °C. After incubation, 200 µl of phenol : chloroform : isoamyl alcohol (25 : 24 : 1) solution was added. Samples were frozen at –30 °C and then centrifuged for 4–5 min at 10,000× g. The supernatant was transferred into a polypropylene tube and mixed with 1/10 vol. of 2 M sodium acetate (pH 4.5) and an equal volume of isopropyl alcohol. The samples were frozen in liquid nitrogen, then thawed, and centrifuged for 10 min at 20,000× g. The supernatant was removed. RNA/DNA pellets were washed with 0.5 ml of 75% ethanol, dried and redissolved in 150 µl H_2O. Samples were used for PCR either immediately or after storage at –70 °C.

PCR assay. Polymerase chain reactions (PCR) were performed in a volume of 25 µl using a PerkinElmer 9600 thermal cycler. Each PCR mix contained 200 µM each of dNTP, 2 U Taq polymerase, 0.5 µM of each primer and 10 µl of template DNA. The primers are listed in Table 1. The primers for *Borrelia* species, *A. phagocytophilum* and *E. muris* / *E. chaffeensis* were supplied by DNA Technology (original method of "Helix" enterprise, St. Petersburg, Russia). For *Babesia microti* / *Babesia odocolei* and for TBEV detection, the primers were from ASB "Vector-Best" (Novosibirsk, Russia). dNTP, Taq-polymerase, RT-set, and buffers were from "DNA-Technology" (Russia) and MBI "Fermentas" (Lithuania).

For *B. burgdorferi* s.l. a three-step cycling program was used: an initial 1-min denaturation at 94 °C, 35 cycles of 30-s denaturation at 94 °C, 40-s annealing at 59 °C, 40-s polymerization at 72 °C, and a final 3-min extension at 72 °C. Identical procedures were used for the other *Borrelia* species, but with annealing temperatures of 51 °C for *B. burgdorferi* sensu stricto, 48°C for *B. afzelii*, and 50 °C for *B. garinii*. The PCR cycles for other microbes were as follows:

for *Ehrlichia* sp. – an initial 1-min denaturation at 94 °C, 35 cycles of 30-s denaturation at 94 °C, 40-s annealing at 52 °C, 40-s polymerization at 72 °C, and 3-min extension at 72 °C;

Table 1. **List of primers**

Primer pairs	Nucleotide sequence	Amplicon size (bp)	Target species/gene
F	5′-ATG TTG AAA TCT CAA GCT ATG AAG-3'	501	*Borrelia burgdorferi*
R	5′-CTG TAG GCT ATT TTG AAT TGC AAG-3'		*s.l.*/16S RNA:
F	5′-CCT ACA TCA ACC TTA AGT TGC T-3'	293	Borrelia
R	5′-GCC AAG AGA AAT TGT TGT AAA TC-3'		*afzelii*/16S RNA
F	5′-CAA GCT CAG CTG CTG ATG CA-3'	287	Borrelia
R	5′-GCC AAG AGA AAT TGT TGT AAA TC-3'		*garini*/16S RNA
F	5′-ACA ATT TCA AGC ACC ACT GAA-3'	286	Borrelia burgdorferi
R	5′-GCC AAG AGA AAT TGT TGT AAA TC-3'		*s.s.*/16S RNA
F	5′-AGG AAG CGT AAT GAT GTC TAT GG-3'	553	Anaplasma phagocyto-
R	5′-TCC CAT CGA TAC TAG GGT AAG AGA-3'		*philum*/16S RNA:
F	5′-ATC TGT TTA TTA TTT GCA GCA A-3'	587	Ehrlichia muris Ehrlichia
R	5′-GAG ATA TTG TTT TAT TAT AGA TAG-3'		*chaffeensis*/16S RNA
PIRO-A	5′-AAT ACC CAA TCC TGA CAC AGG G-3'	438/408	*Babesia microti Babesia odocolei*/
PIRO-B.	5′-TTA AAT ACG AAT GCC CCC AAC-3'		nss-rDNA

for *Babesia* sp. – an initial 1-min denaturation at 94 °C, 35 cycles of 30-s denaturation at 94 °C, 40-s annealing at 60 °C, 40-s polymerization at 72 °C, and 3-min extension at 72 °C.

All samples that were PCR-positive with Bab-1 and Bab-4 primers, indicating the presence of *Babesi* sp., were tested with PIRO-A and PIRO-B primers; 438-bp-sized fragments were visualized in electrophoresis. *Babesia microti* can be distinguished from *Babesia odocolei*, *Babesia divergens* and *Babesia capreoli* by the PCR restriction fragment length polymorphism (RFLP) assay. *Babesia microti* is characterized by digestion of the 438-bp amplicon by the *Hinf* I enzyme producing two fragments, 356 and 81 bp in length, and by resistance to digestion by *Bst* E II (Armstrong et al., 1998; Duh et al., 2001).

Statistical methods. Pearson's chi-square test (STATISTICA for Windows 6) was used to estimate the significance of differences between infection prevalence in anomalous and normal ticks. A p value of 0.05 or less was considered significant.

RESULTS

Thirty-six specimens were studied for the presence of exoskeleton anomalies and infestation by live spirochaetes. In 17 of 36 ticks (47.2%) anomalies were revealed. Among them (Table 2), 6 specimens contained spirochaetes (35.3%), whereas among 19 normal specimens only 1 (5.3%) was infested by this microorganism (the chi-square value between the absolute values of figures 5.166, $P < 0.05$). Among 7 positive ticks, 6 were males and only 1 was female. The number of spirochaetes only once exceeded 10 (in one male it was 28).

There were no coincidences between the detection of live spirochaetes and the presence in the same specimens of pathogenic borreliae or other agents. The latter were never discovered in the ticks in which live spirochaetes were revealed by darkfield microscopy.

Neither *B. burgdorferi* s.s. nor *A. phagocytophlum* or *Bab. microti* were found in the small group of ticks studied. Nevertheless, 10 of 36 ticks contained microorganisms pathogenic to man. Thus, nearly every third tick was protentially dangerous. Among this lot of ticks, *B. burgdorferi* s.l. was found only twice: once as a single infection and one time together with *E. muris*. It is not possible to exclude that at least one of these species could be infected by *Borrelia lusitaniae* whose pathogenicity and distribution has been observed in Europe but was not studied here. PCR analysis revealed *B. afzelii* in 7 ticks (5 times as a monoinfection and 2 in mixed ones), *B. garinii* was detected 4 times and in 2 of them together with other pathogens. Two times the agent of human monocytic ehrlichiosis, *E. muris,* was revealed, once alone and another time together with both mentioned above species of borreliae. Not only spirochaetes, but also agents pathogenic for man were more common among anomalous ticks.

The difference between the prevalence of spirochaetes in anomalous ticks versus normal ones was 2.8 times greater, whereas the prevalence of tick-borne pathogens in anomalous ticks versus normal specimens was only 1.8 times, but, nevertheless, it was visible. Triple-infected ticks were observed only among the anomalous group of ticks (*E. muris*, *B. afzelii* and *B. garinii*). A comparison of differences using the chi-square index concluded that the difference in both cases was statistically significant (chi-square 5.055 and 5.166, P in both cases <0.05).

DISCUSSION

Even with a small sample size, we can conclude that infected ticks *I. ricinus* are common in the centres of small and large cities. In addition, pollution might be greater inside urban territories than outside of them. For example, on the Curonian Spit, part of which belongs to Russia and another part to Lithuania, in the national park territory, the number of anomalous tick was 42.5% in 2005, whereas in Vilnius parks in the same year and the same season of collection its number was somewhat higher (47.2%) (Table 2). Like in other green patches (for example, in Chishinau), the same two most common *Borrelia* species were found, but it was quite possible that by using other primers more representatives of the *B. burgdorferi* s.l. group could be revealed. In both places, human ehrlichiosis agents were found, but in Moldova the human granulocytic ehrlichiosis agent occurred both alone and as a mixed infection, whereas in Vilnius only the human monocytic ehrlichiosis agent *E. muris* was detected. In the St. Petersburg suburb forested recreation zone, among 147 infected *I. persulcatus* ticks (1.412 specimens were studied) one third of them had multiple infections. In Vilnius, 2 out of 10 (20%) of collected ticks were either dually or triply infected. Thus, the same trend is apparent despite the very small number of ticks studied.

Interestingly, in Finland on the Kokkola archipelago (Alekseev et al., 2007) which is far from any capitals or towns 40% (12 of 30) of ticks, had exoskeleton anomalies. There, the more commonly found *I. persulcatus* contained tick-borne pathogens in 63.3% of cases (19 out of 30, and 12 were multiply infected). This difference is understandable since in the wild the sources of reservoirs of tick-borne pathogens are greater than in towns or their suburbs. Nevertheless, the described phenomena have more similarities than differences.

Table 2. **Prevalence of live spirochaetes and other tick-borne agents in anomalous and normal *Ixodes ricinus* ticks collected in the city parks of Vilnius**

Ticks		Microorganisms and No. of the number of observations								
Characteristic	No.	Probable apathogenic		Pathogenic for man						
				Monoinfection			Dual	Triple	Prevalence	
		abs.	%	Ba	Bg	Em	Ba + Bg	Ba + Bg + Em	abs.	%
Anomalous	17	6/17	35.3	3	2	0	0	1	6/17	35.3
Normal	19	1/19	5.3	2	0	1	1	0	4/19	21.0
Chi-square		5.166		Chi-square between ratios					5.055	
p		<0.05		p					<0.05	

Ba – *Borrelia afzelii*, **Bg** – *Borrelia garinii*, **Em** – *Ehrlichia muris*.

In conclusion, even this small sample size reveals several important tick-borne pathogens in the centre of Vilnius, suggesting that people visiting these parks are exposed to the risk of tick-borne infections transmitted by *I. ricinus*, especially while resting on the grass.

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UŽSIKRĖTIMO ERKIŲ PLATINAMOMIS LIGOMIS RIZIKOS ĮRODYMAS VILNIAUS MIESTO PARKUOSE

S a n t r a u k a

Ištirtos trisdešimt šešios *Ixodes ricinus* (L.) erkės, 2005 m. pavasarį surinktos dviejuose Vilniaus parkuose dėl užsikrėtimo spirochetomis, egzoskeleto anomalijų ir keletos erkių platinamų ligų sukėlėjų. Vienoje iš erkių aptikta trys sukėlėjai: *Ehrlichia muris* (monocitinės erlichiazės sukėlėjas), *Borrelia afzelii* ir *Borrelia garinii*. Be to, tirtose erkėse nustatyta *B. afzelii* (n = 5), *B. garinii* (n = 2), kiti *B. burgdorferi* sensu lato grupės genotipai (n = 2), *Ehrlichia muris* (n = 1). Iš viso rasta 27,8% (10/36) infekuotų erkių, dviejose iš jų (20%) nustatyta daugiau nei vienas ligų sukėlėjas; 47,2% erkių turėjo egzoskeleto anomalijas ir tokios erkės buvo labiau infekuotos (35,3 prieš 21%) ligų sukėlėjais. Triguba infekcija nustatyta tik tarp anomalių erkių. Gyvos spirochetos taip pat dažniau aptiktos anomaliose erkėse: 35,3 (6/17) prieš 5,3% (1/19). Rezultatai statistiškai patikimi. Nedidelės apimties tyrimai patvirtina, kad *I. ricinus* erkės, rastos Vilniaus parkuose, yra kelių užkrečiamųjų ligų sukėlėjos ir tai yra rizikos veiksnys parkų lankytojams.

Raktažodžiai: parkas, *Ixodes ricinus*, egzoskeleto anomalijos, erkių platinamų patogenų paplitimas