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MOLECULAR EVIDENCE FOR *ANAPLASMA PHAGOCYTOPHILUM* AND *BORRELIA BURGDORFERI* SENSU LATO IN *IXODES RICINUS* TICKS FROM EASTERN SLOVAKIA

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Abstract: *Ixodes ricinus* ticks (20 males, 20 females and 20 nymphs) collected in Košice, Slovakia were examined for the presence of *Anaplasma phagocytophilum* and *Borrelia burgdorferi* sensu lato (s.l.) by PCR. 38.3% of the tested ticks carried single infection of *B. burgdorferi* s.l. and 8.3% were infected with *A. phagocytophilum*. Double infection of both pathogens was detected in 5% of tested ticks. These results indicate that both *B. burgdorferi* s.l. and *A. phagocytophilum* co-circulate in the enzootic sites of Eastern Slovakia and may cause co-infection in humans.

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INTRODUCTION

Over the last decades, due to the climatic and urban changes in the environment, ticks and tick-borne diseases have became an emerging problem in temperate regions in Europe [7]. In Slovakia, the most common tick-transmitted bacterial disease is Lyme borreliosis with the incidence of 12.5 cases per 100,000 inhabitants [3]. The causative agent of Lyme borreliosis is spirochete forming a complex of species: *B. burgdorferi* sensu lato (s.l.) that is characterized by a high level of genetic heterogeneity. The following genospecies have been detected in different areas of Slovakia: *B. afzelii*, *B. garinii*, *B. burgdorferi* sensu stricto (s.s.), *B. valaisiana* and *B. lusitaniae* [6, 9, 15].

Recently, *Anaplasma (Ehrlichia) phagocytophilum*, another important bacterial pathogen transmitted by *Ixodes ricinus* ticks, claims the attention of public health professionals in Europe. Based on phylogenetic analyses, *A. phagocytophilum* belongs to the newly-reorganized genus *Anaplasma* which also encopasses the former *Ehrlichia* equi and humane granulocytic ehrlichiae (HGE) agent [5]. It is a Gram-negative, intracytoplasmic bacteria that infects granulocytes. In Europe, it has been detected in ticks [1, 8, 10, 11] animals [11] as well as patients [12].

The main aim of this study was to evaluate if *Anaplasma phagocytophilum* is present in *Ixodes ricinus* ticks collected from suburban park of the town of Košice in Eastern Slovakia where Lyme borreliosis is highly endemic.

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Table 1. Infectious prevalence of *A. phagocytophilum* and *B. burgdorferi* s.l. in *I. ricinus* ticks collected in a suburban Košice park.

	I. ricinus gender and stage			
	Males	Females	Nymphs	Total
	n (%)	n (%)	n (%)	n (%)
Total examined ticks	20	20	20	60
A. phagocytophilum positive only	2 (10)	3 (15)	0 (0)	5 (8.3)
B. burgdorferi s.l. positive only	7 (35)	8 (40)	8 (40)	23 (38.3)
A. phagocytophilum + *B. burgdorferi* s.l. positive	2 (10)	1 (5)	0 (0)	3 (5)

MATERIAL AND METHODS

Collection of ticks. In spring 2002, 63 ticks (20 males, 20 females and 23 nymphs) were collected by flagging the vegetation in a hornbeam deciduous suburban forest near an apartment complex area in the eastern part of Košice (latitude 48° 59' 5" N and longitude 14° 28' 5" E). Ticks were immediately immersed in 70% ethanol.

DNA extraction. *I. ricinus* DNA was obtained using DNA easy tissue kit (Qiagen, Valencia, CA) according to a previously described modified protocol [2].

PCR. For all PCR reactions, MasterTaqDNA polymerase kit (Eppendorf, Westbury, N.Y.) was used. A total of 2.5 µl of template DNA were added to a PCR master mix containing 10.4 µl of deionized water, 5 µl of 5 X TaqMaster PCR Enhancer, 2.5 µl of 10 X Taq buffer (with 15mM Mg^{2+}), 1.5 µl of a 25mM solution of $Mg(OAc)_2$, 0.1 µl of Taq DNA Polymerase (5U/µl), 0.5 µl of dNTP-mix (10mM) (Eppendorf) and 1.25 µl of each primer (10pmole/µl) (Invitrogen, Frederick, MD). 620bp fragment of the tick mitochodrial cytochrome *b* gene was amplified from each sample to comfirm the presence of tick DNA [2, 4]. Only positive samples were further examined.

Detection of *A. phagocytophilum*. Primers EHR 521 (5´-´TGT AGG CGG TTC GGT AAG TTA AAG-3´) and EHR 747 (5´-GCA CTC ATC GTT TAC AGC GTG-3´) were used to amplify the 247bp fragment of 16rDNA from *A. phagocytophilum*. PCR was performed according to the previously described protocol of Pancholli *et al.* [13].

Detection of *B. burgdorferi* s.l. Primers IGSa (5'-CGA CCT TCT TCG CCT TAA AGC -3') and IGSb (5'- AGC TCT TAT TCG CTG ATG GTA -3') were used to amplify the 250bp fragment of 5S-23S *B. burgdorferi* s.l. intergenic spacer region. PCR conditions were as described by Derdáková *et al.* [4]. The PCR products were electrophoresed on a 2% agarosed gel, stained with ethidium bromide and visualized with a UV transilluminator.

Positive and negative control. DNA of *I. ricinus* tick infected with *B. burgdorferi* s.s. was used as a positive control for *B. burgdorferi* s.l., and as a positive control for *A. phagocytophilum*, DNA of a *I. scapularis* tick infected with HGE agent was used. Two negative controls were used: (i) DNA of noninfected nymph from a laboratory colony and (ii) DNA, RNA free water instead of DNA template.

RESULTS

The PCR analysis targeting the tick mitochondrial gene cytochrome *b* was carried out on 63 *I. ricinus* ticks collected in suburban forest in the eastern part of Košice. 60 examined ticks (20 females, 20 males and 20 nymphs) yielded positive PCR results and only these ticks were further investigated by PCR for the presence of *A. phagocytophilum* and *B. burgdorferi* s.l. *A. phagocytophilum* DNA was detected in 8 (13.3%) of tested ticks. Only adult ticks (4 males and 4 females) were positive. Single infection with *A. phagocytophilum* was detected in 2 (10%) males and 3 (15%) females. None of the tested nymphs carried *A. phagocytophila*. In contrast, 8 (40%) nymphs, 8 (40%) females and 7 (35%) males carried *B. burgdorferi* s.l. only. 2 males (10%) and 1 (5%) female were simultaneously infected with both pathogens (Tab. 1).

DISCUSSION

I. ricinus represents the most common tick in broad-leaved and mixed forests of Central Europe. It is an important vector of Tick-Borne Encephalitis virus, *B. burgdorferi* s.l., *A. phagocytophilum* and *B. microti*. In this preliminary study, we report for the first time that *A. phagocytophilum*, the causative agent of the HGE, is present in ticks from eastern Slovakia. This finding is not surprising since *A. phagocythophilum* had already been detected in ticks collected in western parts of Slovakia [14]. The 13.3% positivity for *A. phagocytophilum* detected in our study is comparable to results obtained by Greszuck *et al.* [8] in northeastern Poland, where 16% of ticks were infected. The authors of the above-mentioned study reported significantly higher infectious rate in adults (19.5%) than in nymphs (1.4%). This is in accordance with our results. However, in Norway the prevalence of HGE agent was highest in nymphs [10]. This may indicate different enzootic cycles of *A. phagocytophilum* in reservoir hosts from different geographic areas. Co-infections with *B. burgdorferi* s.l. was detected in 3 ticks. Double infections with these two pathogens were previously reported by other authors [8, 10] and indicates the possible co-infection of patients with both agents. In previous studies, the overall prevalence of *B. burgdorferi* s.l. in *I. ricinus* from the same area during three consecutive years was 16.9% [15]. We report higher infectious prevalence of *B. burgdorferi* s.l. (43.3%). The difference between the findings can be explained by the use of different methods of dark-field microscopy versus PCR in our case.

The results of this pilot study show the presence of *A. phagocytophilum*, as well as confirm the high prevalence

of *B. burgdorferi* s.l. in *I. ricinus* ticks in Eastern Slovakia. This finding should be noted by physicians, especially in suspected Lyme borreliosis patients with a history of tick-bite and clinical symptoms but negative laboratory findings. Since the co-infection of both pathogens was detected, it is possible that patients may suffer from more than one tick-borne infection.

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