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The First Detection of *Babesia* EU1 and *Babesia canis canis* in *Ixodes ricinus* Ticks (Acari, Ixodidae) Collected in Urban and Rural Areas in Northern Poland

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A b s t r a c t

Ixodes ricinus, the most commonly observed tick species in Poland, is a known vector of such pathogenic microorganisms as TBE viruses, *Borrelia burgdorferi sensu lato*, *Anaplasma phagocytophilum*, *Rickettsia helvetica*, *Babesia divergens* and *B. microti* in our country. Our study aimed to find out whether this tick can also transmit other babesiae of medical and veterinary importance. DNA extracts of 1392 ticks (314 nymphs, 552 male and 526 female ticks) collected in urban and rural areas in the Pomerania province (northern Poland), were examined by nested PCR for the detection of *Babesia* spp., using outer primers: 5-22F and 1661R, and inner primers: 455-479F and 793-772R, targeting specific fragment of 18S rRNA gene. Overall, at least 1.6% ticks were found to be infected with babesial parasites. In the case of nymphs, the minimal prevalence was 0.6%, and it was approx. 3-times lower than in adults (1.9%). Percentages of infected males and females were comparable (2.0% *vs.* 1.7%). Sequences of 15/22 PCR-derived fragments of 18S rRNA gene demonstrated 100% similarities with the sequence of *Babesia* EU1 (proposed name *B. venatorum*) (acc. no. AY046575) (n = 13) and with *B. canis canis* (acc. no. AY321119) (n = 2), deposited in the GenBank database. The partial 18S rDNA sequences of *Babesia* EU1 and *B. c. canis* obtained by us from *I. ricinus* have been deposited in GenBank, accession nos. GQ325619 and GQ325620, respectively. The results obtained suggest the possible role of *I. ricinus* as a source of microorganisms, which have been identified as agents of human and canine babesiosis, respectively, in Europe. To our knowledge this is the first report on the occurrence of *Babesia* EU1 and *B. c. canis* in *I. ricinus* in Poland.

K e y w o r d s: *Babesia canis canis*, *Babesia* EU1, *Ixodes ricinus*, canine babesiosis, human babesiosis, Poland

Introduction

Ixodes ricinus is a widely distributed tick species in Europe, including Poland, where serves as a vector and reservoir of various pathogens causing diseases in animals and humans. It may transmit such emerging infectious diseases like: Lyme borreliosis, human granulocytic anaplasmosis, and babesiosis. The latter is caused by piroplasms of the genus *Babesia*, tick-transmitted obligatory parasites of mammalian red blood cells. Of them, mainly two species are responsible for human babesiosis. In the North America, the disease is predominantly caused by *B. microti*, a rodent parasite transmitted by *I. scapularis* ticks, while in Europe most cases have been attributed to *B. divergens*, a cattle parasite, transmitted by *I. ricinus.*

The first demonstrated, fatal case of human babesiosis in the world was reported in Europe, in 1957, in an asplenic man in the former Yugoslavia (Škrabalo and Deanovič, 1957). Since then, approximately 400 confirmed cases of human babesiosis have been reported in the United States (Kjemtrup and Conrad, 2000). In Europe the disease is considerably less prevalent and for over 50 years just about 40 cases have been noted (Homer *et al.*, 2000), including two human infections with *B. microti* recently reported in patients from Switzerland and Germany (Meer-Scherrer *et al*., 2004, Hildebrandt *et al.*, 2007).

However, during the last decade of the XXth century, new species of *Babesia* and *Babesia*-like microorganisms have been identified as potential infectious agents. In North America, these pathogens include: *B. duncani* (Conrad *et al.*, 2006), previously referred to as strains WA1-and CA1 (Persing *et al.*, 1995, Quick *et al.*, 1993, Herwaldt *et al.*, 2004), and the MO1 type parasites (Herwaldt *et al.*, 1996). In Europe, the new

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Babesia EU1 (proposed name *B. venatorum*) was isolated for the first time from two asplenic patients in Austria and Italy (Herwaldt *et al*., 2003), and later reported also in patient from Germany (Häselbarth *et al*., 2007). Its competent vectors are *I. ricinus* ticks (Becker *et al.*, 2009) while cervids, primarily roe deer, are considered as potential reservoir hosts (Bonet *et al*., 2007, Duh *et al.*, 2005).

While no cases of human babesiosis have been so far reported in Poland, canine babesiosis is a growing veterinary problem both in our and other European countries. The disease is primarily caused by *B. gibsoni* and *B. canis*, which is divided into three subspecies namely *B. canis vogeli*, *B. canis rossi* and *B. canis canis* (Uilenberg *et al.*, 1989). Among them, the latter is responsible for most infections in dogs throughout Europe (Adaszek and Winiarczyk, 2008, Duh *et al.*, 2004, Földvári and Farkas, 2005), where is transmitted mainly by the hard tick *Dermacentor reticulatus* (Földvári *et al.*, 2007, Rar *et al.*, 2005b, Zahler *et al.*, 2000). In Poland, canine babesiosis is frequently noted in dogs between the Vistula River and the San River basin (north-eastern, eastern and south-eastern areas) (Adaszek and Winiarczyk, 2008, Sobczyk *et al.*, 2005), which is the distribution range of *D. reticulatus* in our country. The majority of cases noted by us in the Tri-City agglomeration (Gdańsk, Sopot and Gdynia, northern Poland) (data not published) also concern dogs travelling with their owners to the areas where *D. reticulatus* is common. However, in a few cases, dogs suffering from babesiosis did not leave the Pomerania province, where the meadow ticks do not occur. Thus, they might acquire infection only by *I. ricinus* bite.

As to date there has been no reports that *I. ricinus* ticks can take part in circulation of *B. canis canis* in nature, a survey was carried out to detect this and other babesial parasites in ticks from urban and rural recreational areas in northern Poland.

Experimental

Materials and Methods

Tick sampling. In April – September 2008, ticks were collected by flagging lower vegetation in three different collection sites localised in urban forests of the Tri-City agglomeration (Gdynia – Kolibki, Gdynia – Chwarzno, Gdańsk – Jaśkowa Dolina) and in one site in a wooded, recreational area in the vicinity of the village Sulęczyno (Kaszuby region) (northern Poland).

In the laboratory, ticks were identified by species (based on morphological characteristics) and stage of development, killed by rapid immersion in a hot water and preserved in 70% ethanol for further investigations.

DNA extraction. An ammonium hydroxide (NH_4OH) method was used to extract DNA from crushed nymphs and adult *I. ricinus* (Rijpkema *et al.*, 1996). All adult ticks were processed separately, while nymphs individually or in pools of 2–3 specimens. The obtained lysates were stored at –20°C until examined.

The quality of isolated samples was confirmed by PCR with primers specific to 28S rRNA gene of genus *Ixodes*.

Amplification of DNA of *Babesia* spp. For *Babesia* spp., a nested PCR was performed with outer primers: 5-22F and 1661R, and inner primers: 455-479F and 793-772R, targeting specific fragment of 18S rRNA gene. An outer primer pair amplified nearly full-length gene, while an inner primer pair was originally designed to amplify an approximately ~340-bp fragment from *B. gibsoni* (Asian serotype), *B. canis vogeli*, *B. canis rossi* and *B. canis canis* (Birkenheuer *et al.*, 2003). Primary reaction used 2 µl of a tick template in a total volume of 20 µl reaction mixture, while nested amplification used 1 µl of the primary PCR product in a total volume of 20 µl.

Dog blood samples positive for *B. c. canis*, confirmed by the analysis of sequences of the PCR products, and double distilled water were used as positive and negative controls, respectively. The conditions of PCR and nested PCR were as described earlier (Birkenheuer *et al.*, 2003). All PCR reactions were carried out in a GeneAmp® PCR System 9700 (Applied Biosystems 850, Foster City, CA, USA). Obtained PCR products were analyzed after electrophoresis in 2% agarose gel stained with ethidium bromide.

Sequencing of PCR products. The PCR products of chosen positive samples were purified using Clean-Up purification kit (A&A Biotechnology, Gdynia, Poland) and sequencing reaction were carried out using ABI Prism® Big Dye™ Terminator v.3.1 Cycle Sequencing Kit. Then, the obtained products were sequenced with ABI Prism 310 Genetic Analyser (Applied Biosystem 850, Forster City, CA, USA) according to the manufacturer's protocol. Sequences were compared with gene sequences deposited in GenBank database using NCBI BLAST network service.

Results

To detect babesial parasites, a nested PCR was performed with outer primers: 5-22F and 1661R, and inner primers: 455-479F and 793-772R. Although inner primers were originally designed to amplify an approximately ~370-bp fragment 18S rRNA gene from *B. gibsoni* (Asian serotype) and ~340 bp of *B. canis* (Birkenheuer *et al.*, 2003), we proved in our assays that they may also amplify DNA of *Babesia* EU1. The results of these studies are given in Table I.

Table I
Prevalence of infection with *Babesia* spp. in nymphs and adult *Ixodes ricinus* ticks collected in urban and rural forested areas in the Pomerania province (northern Poland) in 2008

Area	Site	Tick stage	No examined	Number / % positive			
				Babesia EU1	*B. canis canis*	*Babesia* spp.	Total
Urban	Gdańsk Jaśkowa Dolina	Adults:	102	0 / 0	0 / 0	0 / 0	0 / 0
		females	41	0 / 0	0 / 0	0 / 0	0 / 0
		males	61	0 / 0	0 / 0	0 / 0	0 / 0
		Nymphs	78	0 / 0	0 / 0	0 / 0	0 / 0
		Subtotal	180	0 / 0	0 / 0	0 / 0	0 / 0
	Gdynia Kolibki	Adults:	422	9 / 2.1	2 / 0.5	4 / 0.9	15 / 3.6
		females	209	5 / 2.4	1 / 0.5	0 / 0.0	6 / 2.9
		males	213	4 / 1.9	1 / 0.5	4 / 1.9	9 / 4.2
		Nymphs	139	0 / 0.0	0 / 0.0	1 / 0.7	1 / 0.7
		Subtotal	561	9 / 1.6	2 / 0.4	5 / 0.9	16 / 2.9
	Gdynia Chwarzno	Adults:	356	2 / 0.6	0 / 0	0 / 0.0	2 / 0.6
		females	176	1 / 0.6	0 / 0	0 / 0.0	1 / 0.6
		males	180	1 / 0.6	0 / 0	0 / 0.0	1 / 0.6
		Nymphs	56	0 / 0.0	0 / 0	1 / 1.8	1 / 1.8
		Subtotal	412	2 / 05	0 / 0	1 / 0.2	3 / 0.7
Urban area – total		Adults:	880	11 / 1.3	2 / 0.2	4 / 0.5	17 / 1.9
		females	426	6 / 1.4	1 / 0.2	0 / 0.0	7 / 1.6
		males	454	5 / 1.1	1 / 0.2	4 / 0.9	10 / 2.2
		Nymphs	273	0 / 0.0	0 / 0.0	2 / 0.7	2 / 0.7
		Total	1153	11 / 1.0	2 / 0.2	6 / 0.5	19 / 1.6
Rural	Sulęczyno	Adults:	198	2 / 1.0	0 / 0	1 / 0.5	3 / 1.5
		females	100	1 / 1.0	0 / 0	1 / 1.0	2 / 2.0
		males	98	1 / 1.0	0 / 0	0 / 0.0	1 / 1.0
		Nymphs	41	0 / 0.0	0 / 0	0 / 0.0	0 / 0.0
		Subtotal	239	2 / 0.8	0 / 0	1 / 0.4	3 / 1.3
Urban and rural area – total		Adults:	1078	13 / 1.2	2 / 0.2	5 / 0.5	20 / 1.9
		females	526	7 / 1.3	1 / 0.2	1 / 0.2	9 / 1.7
		males	552	6 / 1.1	1 / 0.2	4 / 0.7	11 / 2.0
		Nymphs	314	0 / 0.0	0 / 0.0	2 / 0.6	2 / 0.6
			1392	13 / 0.9	2 / 0.1	7 / 0.5	22 / 1.6

In total, 1392 *I. ricinus* (314 nymphs, 552 male and 526 female ticks) were collected in urban and rural areas in the Pomerania province, northern Poland. Altogether, 1262 tick lysates, including individual adults (n = 1078) and nymphs (n = 103), as well as 32 pools containing two nymphs (n = 64) and 49 pools consisting three nymphs (n = 147), were examined for the presence of *Babesia* spp. Of these, specific DNA (Fig. 1) was identified in 22 samples (1.7%), *i.e.* in 20 adult *I. ricinus* and in two pools of nymphs.

There were no significant differences between the percent of infected males and females (2.0% *vs.* 1.7%). In case of nymphs, assuming that only one of them in each positive pool was infected, the minimal prevalence was 0.6%, and it was approx. 3-times lower than in adults (1.9%). Overall, at least 1.6% ticks were found to be infected with babesial parasites.

In urban forests, *Babesia* spp. was detected in two sites in the city of Gdynia: Kolibki (2.9%) and Chwarzno (0.7%), while in the city of Gdańsk, in Jaśkowa Dolina, no infected specimens were reported. In total, 1.6% infection level in urban area was slightly higher than infection rate noted in rural environment, near Sulęczyno village – 1.3%. The highest percentage of infected ticks was observed in April – 2.9%, and then gradually decreased to 1.6% in May and June, 1% in July and 0% in August and September.

Fifteen of 22 positive samples were sequenced. Analysis of nucleotide sequences of 340 bp obtained from the 18S rRNA gene revealed that 13 of them showed 100% similarity to *Babesia* EU1 (acc. no. AY046575), while two others were 100% homologous to *B. canis canis* (acc. no AY072926, AY321119) deposited in the GenBank database.

The GenBank accession numbers for the partial sequences we generated of the 18S rRNA gene for the babesial organisms are as follows: *Babesia* EU1 – GQ325619, *B. canis canis* – GQ325620.

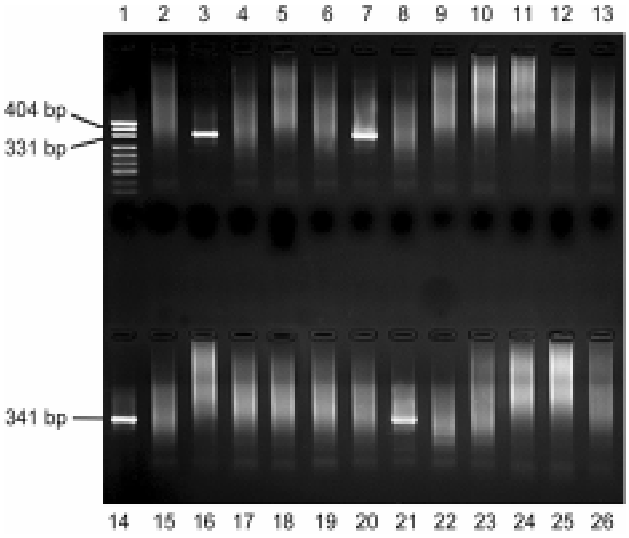

Fig. 1. Agarose gel electrophoresis analysis of nested PCR products obtained with inner primer set targeting 18S rRNA gene of *Babesia* spp. (455-479F and 793-772R).

Lane 1 – pUC19/MspI marker, lane 2 – negative control, lane 3 and 14 – positive controls (*Babesia canis canis* isolated from dog), lane 4–13 and 15–26-tick samples. Lane 7 and 21 – positive samples, confirming the presence of *Babesia* sp DNA.

Discussion

It has been already documented that in Europe *I. ricinus* harboured *B. divergens* and *B. microti* infections (Blaschitz *et al.*, 2008, Casati *et al.*, 2006, Duh *et al.*, 2001, Foppa *et al.* 2002, Wielinga *et al.*, 2009). These two pathogens were also detected in ticks in our country (Siński *et al.* 2006, Skotarczak and Cichocka, 2001, Stańczak *et al.*, 2004). This is the first study reporting the occurrence of a new species – *Babesia* EU1 (proposed name *B. venatorum*) in *I. ricinus* ticks in Poland. Moreover, we reported also for the first time the detection of *B. canis canis*, the parasite associated primarily with *Dermacentor reticulatus,* in the same tick species.

Overall, *Babesia* spp. DNA was detected in 1.6% of the examined *I. ricinus.* The 13/15 partial sequences of the 18S rRNA gene generated from ticks showed to be identical to the corresponding gene of EU1 (AY046575) isolated from two patients in Austria and Italy (Herwaldt *et al.*, 2003). The two gene sequences showed 100% homology to *B. c. canis*, isolates from infected dogs from Warsaw area (AY321119) (Sobczyk *et al.*, 2005) and Croatia (AY072926) (Cacciò *et al.*, 2002), and 99.4% identity to *B. c. canis* sequence (AY 649326) found in *D. reticulatus* from western Siberia, Russia (Rar *et al.*, 2005a), due to a GA → AG inversion status at position 150 and 151. According to the presence of inversion and the restriction pattern, Adaszek and Winiarczyk (2008) proposed to classify European *B. c. canis* isolates in two group: group A – GA variant, cut by HincII restriction enzyme and group B – AG variant, uncut when digested. *Babesia c. canis* detected by us in *I. ricinus* belongs to the group A.

So far *D. reticulatus,* which occurs in the east and central region of our country, has been considered as a main vector of canine babesiosis both in Poland and other European countries, where is frequently found on dogs. For instance, 64.6% ticks collected in Warsaw veterinary clinics from dogs presented for veterinary care, were identified as *D. reticulatus* (Zygner and Wędrychowicz, 2006). Of them, *B. c. canis* was detected in 9.5% male (13/137) and 11.9% female (29/244) ticks (Zygner *et al.*, 2008). Moreover, in *D. reticulatus* (n = 144) originating from dogs from Budapest and other locations in Hungary at least 29.9% samples were positive (Földvári *et al.*, 2007). On the other hand, babesial DNA was detected in 0.3% unfed adults in Germany (Naucke, 2007) and in 3.6% ± 2.0% questing adult *D. reticulatus* collected in Novosibirsk and Omsk regions in western Siberia, Russia (Rar *et al.*, 2005a). In our study, questing *I. ricinus* ticks were infected with *B. c. canis* only in 0.1%. However, even such a low infection level indicates that they can play a role in the circulation of the etiological agent of canine babesiosis in areas where meadow ticks do not occur, including Pomerania province (northern Poland).

Moreover, sheep ticks are competent vectors of *Babesia* EU1 (Bonnet *et al.*, 2007). It has been recovered from *I. ricinus* from Slovenia (2.2%) (Duh *et al.*, 2005), The Netherlands (0.9%) (Wielinga *et al.*, 2009) and Switzerland (Casati *et al.*, 2006), but there have been no evidence that this species occurs in our country. Thus, we demonstrated, for the first time in Poland the natural infection of *I. ricinus* ticks with EU1. The calculated overall minimal infection level was 0.9%. *Babesia* EU1 was detected in ticks collected in rural and urban sites. In both areas a wide range of vertebrate tick host occurred. The presence of roe deer (*Capreolus capreolus*) is especially important as this species is considered the main vertebrate reservoir of EU1. A survey conducted in Slovenia showed that 21.6% roe deer tested were infected with EU1 (Duh *et al.*, 2005), and this rate was comparable to 23% noted in *C. capreolus* in France (Bonnet *et al.*, 2007). Roe deer are important hosts both for nymphs and female *I. ricinus* ticks (Adamska, 2008, Tälleklind and Jaenson, 1997), which may acquire infection during feeding on infected animal. *Babesia* EU1 is transmitted transtadially and transovarially by ticks, thus they may serve also as reservoirs of pathogen (Bonnet *et al.*, 2007). The prevalence of *Babesia* EU1 infection in questing ticks collected by us increased approx. 3-fold from nymphal to adult stage, that suggests that adults acquired parasites when feeding as nymphs on infected host, the most probably being roe deer.

Babesia spp. were detected in *I. ricinus* collected both in the rural area and in two localities in the for-

ests of the city of Gdynia, where the anthropopression was relatively low. However, no infected ticks were found in the third urban site – Jaśkowa Dolina in the city of Gdańsk, characterised by intensive degradation of this area by the human activity.

The overall infection rate in ticks from urban and rural areas were comparable (1.6% *vs*. 1.3%). It seems that diversity of habitats and a wide range of vertebrate tick host in the suburban and urban forests of the Tri-City agglomeration create as suitable conditions for development and survival of *I. ricinus* as forests in rural area. So far it has been demonstrated that ticks occurring there were infected with *Borrelia burgdorferi* s.l., *Anaplasma phagocytophilum* and *B. microti* (2.3%) (Stańczak *et al.*, 2004). Our study showed that these ticks may also harbour *Babesia* EU1 and *B. c. canis*.

The results of this study confirm the existence of natural foci of human and canine babesiosis in rural (Kaszuby region) and urban forests of Tri-City (the Pomerania province), and indicate a potential risk for people and their dogs living or visiting these areas to contract *Babesia* EU1 and *B. c. canis,* respectively. Although, taking into consideration the low percent of infected ticks (~0.9% *vs.* ~0.1%) the risk of transmission of both pathogens is not significant, they should be added to the list of potentially dangerous microorganisms transmitted by ticks in Poland. Further systematic sampling is needed because knowledge of their prevalence in *I. ricinus* ticks and distribution is insufficient.

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