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ORIGINAL ARTICLE

Leptospirosis as a tick-borne disease? Detection of *Leptospira* spp. in *Ixodes ricinus* ticks in eastern Poland

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Abstract

A total of 836 unfed *Ixodes ricinus* ticks were collected from 2 forested areas of the Lublin region in eastern Poland. Of these, 540 ticks were collected in area 'A', exposed to flooding from the Vistula river, while the remaining 296 ticks were collected in suburban area 'B', not exposed to flooding. Ticks were examined by nested-PCR for the presence of DNA of *Leptospira* spp. and of *Borrelia burgdorferi* sensu lato, including its genospecies. The presence of the *Leptospira* spp. DNA was found in the examined specimens of *Ixodes ricinus*. The infection rate was much greater in area 'A' exposed to flooding, compared to unexposed area 'B' (15.6% vs. 1.4%, $p<0.0001$). A significant difference was noted in the case of all developmental stages. For the total results, the prevalence of *Leptospira* spp. in nymphs (16.9%) was two-fold greater ($p<0.01$) than in females and males (7.9% and 7.1%, respectively). The total prevalence of *B. burgdorferi* sensu lato in examined ticks amounted to 24.3%. Altogether, the genospecies *Borrelia burgdorferi* sensu stricto was detected most often. No correlation was found to exist between the presence of *Leptospira* spp. and *B. burgdorferi* sensu lato in the examined ticks, which indicates that the detection of *Leptospira* in ticks was not due to a false-positive cross-reaction with DNA of *B. burgdorferi*. In conclusion, this study shows for the first time the presence of *Leptospira* spp. in *Ixodes* ticks and marked frequency of the occurrence of these bacteria in ticks. This finding has significant epidemiological implications by indicating the possibility of the transmission of leptospirosis by *Ixodes ricinus,* the commonest tick species in Europe and most important vector of numerous pathogens.

Key words

Leptospira spp., *Ixodes ricinus*, *Borrelia burgdorferi*, PCR, eastern Poland

INTRODUCTION

Leptospirosis is regarded as the most widespread zoonosis in the world, and represents a re-emerging health problem because of the increasing incidence in humans and domestic animals [1, 2, 3, 4, 5, 6, 7, 8]. The disease is caused by thin, motile spirochetes belonging to the genus *Leptospira*, comprising at least 13 pathogenic and 6 saprophytic species. The bacteria usually survive in the renal tubules of rodents and many other wild and domestic mammals [1, 9]. Humans become infected most commonly through occupational, recreational, or domestic contact of skin with the urine of infected animals, either directly or via contaminated water or soil. Depending on the species, the immune status of the host, and many other known and unknown factors, the disease can run as a mild, flu-like illness or a severe infection able to cause serious multiorgan or systemic disorders leading to death [1, 2, 3, 4]. With global climate change, extreme weather events such as cyclones and floods are expected to occur with increasing frequency and greater intensity, and may potentially result in an upsurge in the incidence of the disease, as well as the magnitude of outbreaks of leptospirosis [4, 5, 10].

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The aim of the presented study was to investigate the prevalence of *Leptospira* spp. in *Ixodes ricinus* ticks living on the territories of 2 rural communities of the Lublin province of eastern Poland: community 'A' – situated in the western part of the province on the Vistula river, and exposed to floods, and community 'B' – situated near the city of Lublin in the central part of the province, and not exposed to floods. The study was carried out within a bigger project on the incidence of *Leptospira* in humans, domestic and wild mammals, ticks, water, and soil [9]. Although ticks were last associated with the transmission of leptospirosis over 50 years ago [11, 12, 13], the significant role of *Ixodes ricinus* in the transmission of numerous pathogens [14, 15, 16, 17, 18] was recognized as important enough to undertake the survey. In order to exclude the possibility of false-positive reactions with *Borrelia burgdorferi*, the ticks were also tested for the presence of this pathogen.

MATERIALS AND METHODS

Collection of ticks. A total of 836 unfed *Ixodes ricinus* ticks (290 females, 280 males, and 266 nymphs) were collected during the spring/summer seasons in 2011 and 2012 from the forested areas of 2 rural communities of the Lublin region in eastern Poland. Of these, 540 ticks (146 females, 155 males, and 239 nymphs) were collected on the territory of the rural community 'A', exposed to flooding from the Vistula river,

while the remaining 296 ticks (144 females, 125 males, and 27 nymphs) were collected on the territory of the suburban rural community 'B', not exposed to floods. Ticks were collected by dragging a woollen flag over the lower vegetation at the peripheral and inner parts of deciduous and mixed forests. Collected ticks were placed in glass tubes with 70% ethanol for further investigation.

DNA isolation. Bacterial DNA was isolated from ticks after removal from alcohol by boiling in 0.7 M ammonium hydroxide, according to Rijpkema et al. [19] and stored at -20 °C.

PCR assay. The nested-PCR method was used to identify DNA of *Leptospira* spp. and *Borrelia burgdorferi*.

***Leptospira* spp. DNA identification.** The gene fragment of LipL32 lipoprotein was used as a genetic marker to detect *Leptospira* spp. DNA [20]. Identification was carried out using a pair of primers (Eurogentec, Seraing, Belgium): Amu1 (5'- CGC GCT GCA GTT ACT TAG TCG CGT CAG AAG-3') and Amu2 (5'- CGC GGT CGA CGC TTT CGG TGG TCT GCC AAG c-3') for amplification of the fragment of LipL32 gene. For seminested PCR reaction the primers Amu2 and AmuN (5'-CTA TGT TTG GAT TCC TGC-3') were used. First PCR reaction in final volume of 25 µl contained: 0.625 U (0.125 µl) of *Taq* DNA polymerase (Qiagen, USA), 1 × PCR buffer (2.5 µl) containing 15 mM $MgCl_2$ (Qiagen, USA), 2.5 µl 2 mM dNTPs (final concentration 0.2 mM) (Fermentas, Vilnius, Lithuania), 1.25 µl of 10 µM of each Amu1 and Amu2 primers, 14.875 µl nuclease-free water (Applied Biosystems Inc., USA) and 2.5 µl of matrix DNA from tick isolates. The reaction was performed in a C1000 Thermal Cycler (BioRad) and consisted of the initial denaturation (3 min at 94 °C) and 35 cycles; each of which included the proper denaturation (30 sec at 94 °C), primers annealing (30 sec at 55 °C), and elongation (60 sec at 72 °C), followed by the final elongation (7 min at 72 °C). Electrophoresis was performed in 2% agarose gels in standard conditions. The gels were stained with ethidium bromide and read under UV light. 756 bp-long electrophoresis strips were considered positive. As the positive control, thermally-inactivated suspensions of following strains were used: *Leptospira interrogans* serovar Icterohaemorrhagiae, *Leptospira kirschneri* serovar Grippotyphosa, *Leptospira borgpetersenii* serovar Tarassovi, and *Leptospira interrogans* serovar Pomona. The negative control, instead of matrix DNA, was nuclease-free water.

Amu2 and AmuN primers were used in reamplification. 25 µl of the reaction mixture contained: 1.25 U (0.25 µl) of *Taq* DNA polymerase (Qiagen, USA), 1 × PCR buffer (2.5 µl) (Qiagen, USA), 1.25 µl of 2 mM dNTPs, final concentration 0.1 mM (Fermentas, Vilnius, Lithuania), 1.25 µl of 10 µM of each primer, 16.0 µl nuclease-free water (Applied Biosystems Inc., USA) and 2.5 µl of matrix DNA from first PCR, 10 × diluted in nuclease-free water. The time-temperature profile of the reaction was identical with the previous one, with the exception of the primers annealing, performed at the temperature of 58 °C. The reaction products were detected in 2% agarose gels in standard electrophoresis conditions. After ethidium bromide staining, the strips were read under UV light. Samples with a 574 bp-long strip were considered positive.

***Borrelia burgdorferi* DNA identification.** *Borrelia burgdorferi* sensu lato (s.l.) identification was carried out by detection of a fragment of *fla* gene sequence, as described earlier[21, 22], using a pair of primers (Eurogentec, Seraing, Belgium): Fla1 (5'- AGA GCA ACT TAC AGA CGA AAT TAA T-3') and Fla2 (5' CAA GTC TAT TTT GGA AAG CAC CTA A 3'). The reaction mixture (20 µl) contained 0.5 U (0.1 µl) of *Taq* DNA polymerase (Qiagen, USA), 1 × PCR buffer (2 µl) containing 15 mM $MgCl_2$ (Qiagen, USA), 0.5 µl 2 mM dNTPs, final concentration 0.05 mM (Fermentas, Vilnius, Lithuania), 0.8 µl of 10 µM of each Fla1 and Fla2 primer, 13.8 µl of nuclease-free water (Applied Biosystems Inc., USA), and 2 µl of matrix DNA from tick isolates. The reaction was performed in a C1000 Thermal Cycler (BioRad) and consisted of the initial denaturation (3 min at 95 °C) and 35 cycles; each of them included the proper denaturation (30 sec at 94 °C), primers annealing (45 sec at 54 °C), and elongation (45 sec at 72 °C), followed by the final elongation (7 min at 72 °C). Electrophoresis was performed in 2% agarose gels in standard conditions. The gels were stained with ethidium bromide and read under UV light. 482 bp-long electrophoresis strips were considered positive. The positive control was strain *B. burgdorferi* s.l. Bo-148c/2. The negative control, instead of matrix DNA, was nuclease-free water.

All tick lysates in which the presence of *B. burgdorferi* s.l. was detected were examined for the presence of 3 pathogenic *Borrelia* genospecies by nested-PCR reaction [23]. The reaction was done using the specific pairs of primers (Eurogentec, Seraing, Belgium) for each genospecies: *Borrelia garinii* (BG1: 5'- AAT CTA TTC TCT GGC GAA -3' and BG3: 5 -' GGA GAA TTA ACT CCA CCC -3'), *Borrelia afzelii* (BA1: 5'- ATG TTG CAA ATC TTT TTG -3' and BA2: 5'- TAG CAG GTG TTG GTT GCT -3') and *Borrelia burgdorferi* sensu stricto (BB1: 5'- AAT CTT TTC TCT GGT GAG -3' and BB2: 5'- GAG CTC CTT CCT GTT GAA -3'). The nested-PCR reaction was done in final volume of 20 µl which contained the following mix of reagents: 0.5 U (0.1 µl) of *Taq* DNA polymerase (Qiagen, USA), 1 × PCR buffer (2 µl) (Qiagen, USA), 0.5 µl 2 mM dNTPs (final concentration 0.05 mM) (Fermentas, Vilnius, Lithuania), 1.2 µl of 10 µM of primers, 13 µl of nuclease-free water (Applied Biosystems Inc., USA) and 2 µl of matrix DNA from the first PCR reaction (10 × diluted in nuclease free-water). The reaction was performed in a C1000 Thermal Cycler (BioRad) under the following conditions: initial denaturation at 94 °C for 60 sec and 30 cycles; each of which included the proper denaturation (30 sec at 94 °C), primers annealing (30 sec at 58 °C for *B. afzelii* and B.b. sensu stricto and 30 sec at 60 °C for *B. garinii*), and elongation (60 sec at 72 °C), followed by the final elongation (7 min at 72 °C). The reaction products were detected in 3.5% agarose gels in the standard electrophoresis conditions. After ethidium bromide staining, the strips were read under UV light. The samples with a 76 bp-long strip for *B. burgdorferi* sensu stricto, 103 bp-long strip for *B. afzelii* and 125 bp-long strip for *B. garinii* were considered positive.

Statistical analysis. The data were analysed by Student's t-test and χ^2 test with Yates correction, with the use of STATISTICA for Windows v. 5.0 package (StatSoft Inc., Tulsa, Oklahoma, USA).

RESULTS

The presence of the *Leptospira* spp. DNA was found by the use of nested-PCR in the examined specimens of *Ixodes ricinus*. The infection rate was much greater in area 'A', exposed to floods, compared to the unexposed area 'B' (15.6% vs. 1.4%, p<0.0001). A significant difference was noted in the case of all developmental stages (Tab. 1). For the total results, the prevalence of *Leptospira* spp. in nymphs (16.9%) was two-fold greater (p<0.01) than in females and males (7.9% and 7.1%, respectively).

The prevalence of *Borrelia burgdorferi* sensu lato and its genospecies depending on stage and area of collection are presented in Tables 2 and 3, respectively. The total prevalence of *B. burgdorferi* sensu lato in examined ticks amounted to 24.3%. The prevalence did not differ in the individual stages of ticks (Tab. 2), but was significantly greater in area 'B', not exposed to floods, compared to area 'A' (Tab. 3). The incidence of the infections with only one genospecies was similar to the frequency of mixed infections with 2-3 genospecies. Altogether, the genospecies *Borrelia burgdorferi* sensu stricto was detected most often.

Table 4 shows that, except for male ticks, no significant correlation was found to exist between the presence of *Leptospira* spp. and *Borrelia burgdorferi* in *Ixodes ricinus*, thus suggesting a specific detection of the *Leptospira* DNA in examined ticks.

Table 1. Prevalence of infection with *Leptospira* spp. in *Ixodes ricinus* ticks

Area	Stage	Total ticks examined (N)	Total ticks infected (number, percent)
'A'	Females	146	22 (15.1%)***
	Males	155	17 (11.0%)**
	Nymphs	239	45 (18.8%)*
	Total	540	84 (15.6%)***
'B'	Females	144	1 (0.7%)
	Males	125	3 (2.4%)
	Nymphs	27	0
	Total	296	4 (1.4%)
Total: 'A' + 'B'	Females	290	23 (7.9%)
	Males	280	20 (7.1%)
	Nymphs	266	45 (16.9%)++
	Total	836	88 (10.5%)

*–***significantly greater compared to area 'B'; *p<0.05; **p<0.01; ***p<0.0001;
++significantly greater compared to females and males (p<0.01).

Table 4. Correlation between infection of ticks with *Leptospira* spp. and *Borrelia burgdorferi*
(Tables 2 × 2, χ^2 with Yates correction)

Stage	Number of ticks examined (N)	Significance of the correlation (χ^2, p-value)
Females	290	χ^2=1.39; p=0.2377
Males	280	χ^2=6.06; p=0.0138
Nymphs	266	χ^2=1.08; p=0.2997
Total	836	χ^2=0.05; p=0.8195

DISCUSSION

More than 50 years ago, Burgdorfer [11, 12], Krepkogorskaya and Rementsova [13] suspected that ticks transmitted *Leptospira* spp., but since then, to the best of our knowledge, they have no longer been considered as vectors of this bacterium. Burgdorfer [11] demonstrated the transmission of *Leptospira pomona* by the argasid tick *Ornithodoros turicata* from experimentally-infected hamsters to guinea pigs. In another study [12], this author has shown that the ixodid ticks *Dermacentor andersoni* and *Amblyomma maculatum*, infected artificially by ingestion of a heavy suspension of

Table 2. Numbers of *Ixodes ricinus* ticks infected with individual genospecies of *Borrelia burgdorferi* sensu lato depending on stage

Stage	Total ticks examined (N)	Ticks infected with individual genospecies of *Borrelia burgdorferi* sensu lato (number, percent of the total)								Total ticks infected with *Borrelia burgdorferi* sensu lato
		Infected with 1 genospecies			Infected with 2 genospecies			Infected with 3 genospecies	Genospecies not identified	
		B. b. s.s.	*B. afzelii*	*B. garinii*	*B.b.*s.s. + *B. afzelii*	*B.b.*s.s. + *B. garinii*	*B. afzelii* + *B. garinii*	*B.b.*s.s. + *B. afzelii* + *B. garinii*		
Females	290	18 (6.2%)	10 (3.4%)	3 (1.0%)	16 (5.5%)	21 (7.2%)	2 (0.7%)	3 (1.0%)	1 (0.3%)	74 (25.5%)
Males	280	9 (3.2%)	11 (3.9%)	9 (3.2%)	3 (1.1%)	32 (11.4%)	0	4 (1.4%)	1 (0.4%)	69 (24.6%)
Nymphs	266	6 (2.3%)	25 (9.4%)	4 (1.5%)	2 (0.7%)	13 (4.9%)	1 (0.4%)	4 (1.5%)	5 (1.9%)	60 (22.5%)
Total	836	33 (3.9%)	46 (5.5%)	16 (1.9%)	21 (2.5%)	66 (7.9%)	3 (0.4%)	11 (1.3%)	7 (0.9%)	203 (24.3%)

*B.b.*s.s.=*Borrelia burgdorferi* sensu stricto.

Table 3. Numbers of *Ixodes ricinus* ticks infected with individual genospecies of *Borrelia burgdorferi* sensu lato depending on area

Area	Total ticks examined (N)	Ticks infected with individual genospecies of *Borrelia burgdorferi* sensu lato (number, percent of the total)								Total ticks infected with *Borrelia burgdorferi* sensu lato
		Infected with 1 genospecies			Infected with 2 genospecies			Infected with 3 genospecies	Genospecies not identified	
		B. b. s.s.	*B. afzelii*	*B. garinii*	*B.b.*s.s. + *B. afzelii*	*B.b.*s.s. + *B. garinii*	*B. afzelii* + *B. garinii*	*B.b.*s.s. + *B. afzelii* + *B. garinii*		
'A'	540	24 (4.4%)	26 (4.8%)	12 (2.2%)	4 (0.7%)	31 (5.7%)	2 (0.4%)	0	6 (1.1%)	105 (19.4%)
'B'	296	9 (3.0%)	20 (6.8%)	4 (1.3%)	17 (5.7%)	35 (11.8%)	1 (0.4%)	11 (3.7%)	1 (0.4%)	98 (33.1%)*
Total	836	33 (3.9%)	46 (5.5%)	16 (1.9%)	21 (2.5%)	66 (7.9%)	3 (0.4%)	11 (1.3%)	7 (0.9%)	203 (24.3%)

*B.b.*s.s.=*Borrelia burgdorferi* sensu stricto; *Percent significantly greater compared to area 'A', (p<0.05).

L. pomona in Verwoert's medium, may transmit infection by feeding on wean guinea pigs. Parallelly, Krepkogorskaya and Rementsova [13] isolated 2 strains of *Leptospira grippotyphosa* from 35 homogenates of *Dermacentor marginatus* ticks collected from cattle in Kazakhstan.

The presented study shows for the first time the presence of *Leptospira* spp. in *Ixodes* ticks, and the marked frequency of the occurrence of these bacteria in ticks belonging to this genus, in particular in those collected in the area exposed to flooding. This finding has significant epidemiological implications by indicating the possibility of the transmission of leptospirosis by *Ixodes ricinus,* the commonest tick species in Europe and most important vector of numerous pathogens [14, 15, 16, 17, 18]. In the areas exposed to flooding, ticks may serve as a reservoir enabling the persistence of leptospiras in the interval periods between subsequent floods.

The prevalence of *Borrelia burgdorferi* in collected ticks was high (24.3%), being 2-4 times greater compared to our earlier studies on the territory of Lublin province [23, 24]. The proportion of ticks infected with 2 genospecies was similar to that infected with 1 genospecies (10.8% vs. 11.3%), being distinctly greater than the proportion found in the year 2006 [23], but similar to that noted in the year 2008 [24]. Altogether, the genospecies *Borrelia burgdorferi* sensu stricto was detected most often, which was similar to the results obtained in 2006 [23], but not to those obtained in 2008, when *B. afzelii* was the commonest genospecies [24].

Lack of correlation between the presence of *Leptospira* spp. and *Borrelia burgdorferi* sensu lato in the examined *Ixodes ricinus* ticks indicates that the detection of *Leptospira* in ticks was not due to a false-positive cross-reaction with DNA of *B. burgdorferi*.

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