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PREVALENCE OF *BORRELIA BURGDORFERI* SENSU LATO IN *IXODES RICINUS* TICKS (ACARI, IXODIDAE) IN DIFFERENT POLISH WOODLANDS

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Abstract: In 1996-1998, a total of 2285 *Ixodes ricinus* ticks (1063 nymphs, 637 males, 585 females) were collected from vegetation from 25 different localities in the 8 Polish provinces throughout the country. Ticks inhabited all 25 collection sites. The average number of ticks per collection site was 91.4 ± 13.7. All 2285 ticks were examined for *Borrelia burgdorferi* sensu lato (s.l.) presence, of which 1333 specimens from 3 provinces were tested by routine indirect immunofluorescence assay (IFA) using polyclonal antibody PAB 1B29. The remaining 952 specimens from 5 provinces were examined by polymerase chain reaction (PCR), using FL6 and FL7 primers. The overall infection rate in ticks estimated by these 2 methods was 10.2%. Nymphs showed lower positivity rate (6.2%) as compared to adult ticks (14.9% in females and 12.4% in males). The highest percentage of infected *I. ricinus* ticks (37.5%) was noted in the Katowice province while the lowest (4.1%) in the Białystok province. In particular collection sites, infection rates varied from 0–37.5%. The obtained results confirmed that *B. burgdorferi* s.l. is present throughout the distributional areas of *I. ricinus* in Poland and that a prevalence of spirochete-infected ticks may be high in some locations.

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INTRODUCTION

Lyme borreliosis, a multisystemic zoonosis, is acquired by the bite of a tick of the *Ixodes ricinus* complex infected with spirochetes of the species *Borrelia burgdorferi* sensu lato. In Poland, the first human cases of the disease were described in the middle of 1980s [9] from Western Pomerania, while the etiologic agent of the disease, *B. burgdorferi*, was detected in 1993 [20, 24] in larval, nymphal and adult *I. ricinus* collected both from vegetation and small rodents. Since then, borreliae-infected ticks were observed in at least 21 out of 49 former Polish provinces* [11]. Data on the prevalence of such ticks could be obtained by one of the indirect methods, as well as by measurements of the abundance of *I. ricinus* and seroprevalence studies, of the assessment of the risk areas of Lyme borreliosis [14].

The aim of our study was to make a survey of the occurrence of *B. burgdorferi* sensu lato in selected *Ixodes ricinus* population from various parts of Poland, from the north to the south of the country. Results obtained can serve as a basis for further, extensive and more detailed epidemiological investigations.

* Since the 1st of January 1999 a new administrative division of Poland has reduced the number of provinces from 49 to 16.

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Figure 1. Location of sites surveyed in Poland.

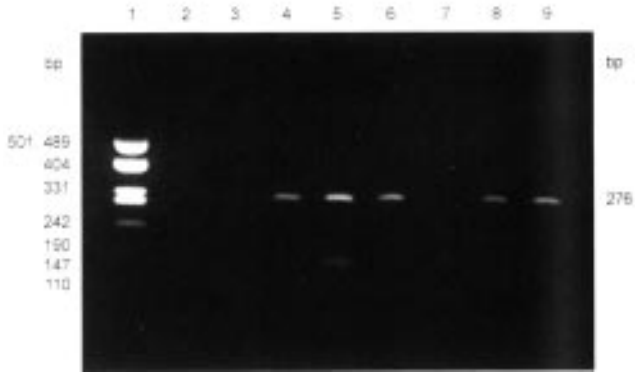

Figure 2. PCR (FL6/FL7) amplification of *B. burgdorferi sensu lato* of *I. ricinus* lysates demonstrated by agarose gel electrophoresis after ethidum bromide staining. Lane 1: M1 marker; Lane 2: DDW negative control; Lane 3: NH_4OH negative control; Lane 4: positive control of *B. burgdorferi* sensu stricto, stain Bo-148c/2 (276 bp); Lane 5-6 and 8-9: tick midguts with positive amplification; Lane 7: tick midgut with negative amplification.

MATERIALS AND METHODS

Study areas and sampling methods. Unfed nymphs and adults of *I. ricinus* were collected during spring/ summer seasons of 1996–1998 by flagging lower vegetation in the 8 Polish provinces (Fig. 1). All study sites were located in woodlands which consist mainly of mixed deciduous and coniferous forests, for instance: the Białowieża Primeval Forest (*Puszcza Białowieska*), the Biebrza River Swamps (*Biebrzańskie Błota*), the Niepołomice Primeval Forest (*Puszcza Niepołomicka*), the Kozłowiecki Landscape Park (*Kozłowiecki Park Krajobrazowy*), the Ojców National Park (*Ojcowski Park Narodowy*), and the suburban Wola Forest (*Las Wolski*). Ticks were collected at the edge of forests, along the forest paths, in the surroundings of the forest parking lots and picnic areas. Ticks were removed from the cloth and placed separately in 2 ml vials. In the laboratory on the same day they were prepared for immunofluorescence assay (IFA) or killed with hot water (ca. 70°C) and preserved in 70% ethanol for further investigation by polymerase chain reaction (PCR).

Detection of *Borrelia burgdorferi* sensu lato. Two methods of spirochete detection were chosen for our purpose: indirect immunofluorescence assay (IFA) and polymerase chain reaction (PCR).

1. Indirect immunofluorescence assay (IFA). Ticks collected in the Białystok, Olsztyn and Elbląg provinces in 1996 were examined for spirochetes by routine IFA, as previously described [1, 24]. The anti-*B. burgdorferi*, strain 1B29 polyclonal antibody (PAB 1B29) was used as a first antibody and FITC-labelled goat anti-rabbit IgG antibody as the second. Positive control preparations, prepared with *B. burgdorferi* strain B 31 (bioMérieux) were examined with each set of slides assayed.

2. Polymerase chain reaction (PCR). Ticks collected in the Lublin, Kraków, Katowice (1997), Słupsk and Bydgoszcz provinces (1998) were analysed by PCR:

a) Preparation of tick samples for PCR. Ticks were processed for PCR by lysis in ammonium hydroxide. All specimens were removed from alcohol and air dried on filter paper. Then each adult individually and nymphs individually, or in pools of 2–7 specimens, were immersed in 100 µl of 0.7 M NH_4OH and crushed with pipette tips. The suspensions were boiled for 15–20 min in a heating block in a sealed vial. Then caps were opened and heating was extended for another 10 min to remove ammonia and reduce the volume to 50 µl. The lysates were then stored at –20°C [19].

b) DNA amplification and identification. PCR was performed according to Picken [17], using the oligonucleotide primers: FL6 (5' TTC AGG GTC TCA AGC TTG CAC T 3') and FL7 (5' GCA TTT TCA ATT TTA GCA AGT GAT G 3') in conserved regions of the *fla* gene of *B. burgdorferi*. Six different polymerases: *Taq* Shark II (DNA Gdańsk), *Taq* DNA (Epicentra), *Taq* DNA (Gibco), *Taq* DNA (Promega), *Pfu* DNA (Promega) and *AmpliTaq* DNA (Perkin Elmer) were tested in the preliminary assays and the last one was chosen for further investigation.

PCR was performed in a reaction volume of 25 µl containing 0.625 U of *AmpliTaq* DNA polymerase, 2.5 µl 10x PCR Buffer II, 1.5 µl $MgCl_2$ (stock 25 mM) (Perkin Elmer), 2.5 µl dNTPs mixture (stock 2.5 mM) (Promega), 0.5 µl FL6 (stock 50 mM), 0.5 µl FL7 (stock 50 mM), 15 µl double distilled water (DDW) and 2.5 µl of the processed tick sample. In each PCR run, *B. burgdorferi sensu stricto*, Polish strain Bo-148c/2, was used as a positive control and DDW and NH_4OH as negative ones.

All reactions were carried out in Perkin Elmer GeneAmp PCR System 2400 thermal cyclers. Samples were initially denatured for 1 min at 94°C. Subsequent

Table 1. Infection rates of nymphal and adult *Ixodes ricinus* ticks with *Borrelia burgdorferi sensu lato* in the selected forested areas of 3 north-eastern provinces in 1996, according to IFA results.

Province	Stage	No. examined	Positive	
			Number	%
Białystok	Adults	397	19	5.0
	females	*192*	*13*	*6.8*
	males	*205*	*6*	*2.9*
	Nymphs	170	4	2.4
	Subtotal	567	23	4.1
Olsztyn	Adults	286	39	13.6
	females	*146*	*23*	*15.8*
	males	*140*	*16*	*11.4*
	Nymphs	275	19	6.9
	Subtotal	561	58	10.3
Elbląg	Adults	96	8	8.3
	females	*41*	*4*	*9.8*
	males	*55*	*4*	*7.3*
	Nymphs	109	19	17.4
	Subtotal	205	27	13.2
Total	Adults	779	66	8.5
	females	*379*	*40*	*10.6*
	males	*400*	*26*	*6.5*
	Nymphs	554	42	7.6
	Total	1333	108	8.1

Table 2. Infection rates of nymphal and adult *Ixodes ricinus* ticks with *Borrelia burgdorferi sensu lato* in the selected forested areas of 5 north and south-eastern provinces in 1997-1998, according to PCR results.

Province	Stage	No. examined	Positive	
			Number	%
Słupsk	Adults	80	14	17.5
	females	*39*	*3*	*7.7*
	males	*41*	*11*	*26.8*
	Nymphs	106	3[a]	2.8[b]
		(1×2, 1×4, 20×5)		
	Subtotal	186	17	9.1
Bydgoszcz	Adults	20	2	10.0
	females	*10*	*1*	*10.0*
	males	*10*	*1*	*10.0*
	Nymphs	48	3	6.3
	Subtotal	68	5	7.4
Lublin	Adults	103	31	30.1
	females	*48*	*16*	*33.3*
	males	*55*	*15*	*27.3*
	Nymphs	207	4[a]	1.9[b]
		(1×3, 3×4, 38×5, 1×6)		
	Subtotal	310	35	11.3
Kraków	Adults	208	41	19.7
	females	*93*	*21*	*22.6*
	males	*115*	*20*	*17.4*
	Nymphs	148	14[a]	9.5[b]
		(2×3, 27×5, 1×7)		
	Subtotal	356	55	15.5
Katowice	Adults	32	12	37.5
	females	*16*	*6*	*37.5*
	males	*16*	*6*	*37.5*
	Nymphs	-	-	-
	Subtotal	32	12	37.5
Total	Adults	443	100	22.6
	females	*206*	*47*	*22.8*
	males	*237*	*53*	*21.5*
	Nymphs	509	24[a]	4.7[b]
	Total	952	124	13.0

(No. of pools × No. of specimens given in parentheses); [a] - minimal number of infected nymphs, i.e. one positive tick in each positive pool; [b] - calculated minimum infection rate

cycles were at 94°C for 30 sec (denaturation), 55°C for 30 sec (annealing), and 72°C for 1 min (extension). Forty cycles were performed.

For the analysis of PCR amplification products, 13 µl aliquots of reaction mixtures were applied to 1.5% agarose gels (Sigma) with Tris-Borate-EDTA (pH 8.2) as running buffer and electrophoresed for 30 min at 150 V. DNA bands were stained with ethidium bromide and visualised by UV transillumination. Achieved specific products of 276 base pairs were considered as a positive result (Fig. 2).

RESULTS

Borrelia burgdorferi sensu lato* in host-seeking *Ixodes ricinus. During 1996-1998, altogether 2285 *I. ricinus* (1063 nymphs and 1222 adults) were collected from 25 localities in 8 provinces from the north down to the south of the country. Ticks inhabited all sites studied. The average number of ticks per collection site was 91.4 ± 12.9. In total collection, the contribution of nymphs was the highest (n=1063; 46.5%) followed by males (n=637; 27.9%) and females (n=585; 25.6%). All ticks were examined for *B. burgdorferi* s.l. presence. The ticks (n=1333) collected in the 3 north-eastern provinces in 1996 were examined by IFA while the ticks collected in the remaining 5 northern and south-eastern provinces in 1997-1998 were examined by PCR (n=952). In IFA, both adult and nymphal ticks were tested individually. In PCR, all adults and 48 of collected nymphs were examined individually and majority of nymphs (n=461) in pools. Out of 95 pools tested, 85 consisted of 5 specimens, four of 4 specimens, three of 3 specimens, one of 2, one of 6 and one of 7 specimens. We assumed that a single pool should consist of 5 specimens. There were 9 pools with different numbers of nymphs, however, as we did not want to merge ticks from different collection sites. The average pool consisted of 4.9 specimens.

The positivity rate in the case of ticks originating from 3 north-eastern provinces and examined by IFA came out to 8.1%. In the case of nymphs, males and females, it was 7.6, 6.5 and 10.6%, respectively (Tab. 1).

By PCR, 103 single and 21 pooled samples of ticks collected in the 5 northern and south-eastern provinces were found to be positive for *B. burgdorferi* s.l. Provided that in each positive pool one infected nymph was present, the minimal infection level was calculated. The total positivity rate in the case of all PCR-examined ticks

Table 3. Prevalence of *B. burgdorferi*-infected ticks in particular study sites.

Province	Site	Detection method	*Ixodes ricinus*		
			No. tested	No. infected	% infected
Białystok	1. Biebrza River Swamps	IFA	67	0	0.0
	2. Białowieża National Park	IFA	59	0	0.0
	3. Grudki	IFA	52	0	0.0
	4. Nowosady	IFA	92	8	8.7
	5. Budy	IFA	189	10	5.3
	6. Podolany	IFA	108	5	4.6
Olsztyn	7. Lidzbark Warmiński	IFA	48	5	10.4
	8. Rychnowo	IFA	61	8	13.1
	9. Frąknowo	IFA	114	26	22.8
	10. Napiwoda	IFA	98	4	4.1
	11. Trękusek	IFA	73	3	4.1
	12. Dajtki	IFA	86	6	7.0
	13. Dywity	IFA	81	6	7.4
Elbląg	14. Orneta	IFA	124	17	13.7
	15. Olkowo	IFA	55	7	12.7
	16. Pasłęk	IFA	26	3	11.5
Słupsk	17. Nowa Dąbrowa	PCR	85	8	9.4
	18. Domanica k. Białego Boru	PCR	101	9	8.9
Bydgoszcz	19. Tleń	PCR	68	5	7.4
Lublin	20. Kozłowiecki Landscape Park	PCR	292	34	11.6
	21. Firlej	PCR	18	1	5.5
Kraków	22. Niepołomice Primeval Forest	PCR	286	33	11.5
	23. Ojców National Park	PCR	28	7	25.0
	24. Kraków – Wola Forest	PCR	42	15	35.7
Katowice	25. Mikołów	PCR	32	12	37.5
Total		IFA/PCR	2285	232	10.2

was 13%. Nymphs were infected in 4.7% while females and males in 22.8 and 21.5%, respectively (Tab. 2).

The overall spirochete infection rate in field-collected *I. ricinus* determined by the two methods (IFA/PCR) was 10.2% (Tab. 3). Nymphs showed a lower positivity rate (6.2%) as compared to adult ticks (14.9% in females and 12.4% in males).

Prevalence of spirochete-infected ticks in various areas. *B. burgdorferi* s.l. positive ticks were recorded from all 8 provinces examined (Tables 1–3). The prevalence of infection ranged from 4.1% for the Białystok province to 37.5% for the Katowice province. In the other 6 provinces, however, no such significant difference in prevalence could be demonstrated and percentages of infected ticks were 7.4-15.5% (Tables 1-2).

Ticks infected with spirochetes occurred at 22 out of 25 examined sites (Tab. 3). Non-infected ticks were found in sites localised in the Biebrza River Swamps, in the Natural Monument Reservation of the Białowieża National Park, and nearby village of Grudki at the edge of the Białowieża Primeval Forest (Białystok province). The infection rates in ticks from other locations varied; the percentages of infected ticks ranging from 0.1–5.0% were observed in 3 sites, from 5.1–10% in 8 sites, and from 10.1–15% in 7 sites. Moreover, comparatively higher positivity rates were noted in 4 locations: nearby Frąknowo village (22.8%) (Olsztyn province), in the Ojców National Park (25.0%) (Kraków province), in the Wola Forest (35.7%) on the outskirts of Kraków (Kraków province) and in the vicinity of Mikołów (37.5%) (Katowice province) (Tab. 3).

DISCUSSION

Among the different methods for detection of *B. burgdorferi* s.l. in ticks, two: imunofluorescence assay (IFA) and polymerase chain reaction (PCR), are probably the most widely used. IFA is known to be very sensitive, but it can be used only in the case of live tick. In 1996, at the beginning of our investigation, this method was used as we were able either to deliver ticks collected from the

Olsztyn and Elbląg provinces to our laboratory on the same day, or prepare them for IFA in the laboratory lent us by the Mammals Research Institute (Polish Academy of Sciences) in Białowieża during research conducted in the Białystok province. During investigation carried out in south-eastern provinces (1997/1998), however, no such possibilities existed. As it was suspected that too many ticks would die during transportation, we decided to change the method of spirochete detection. We chose PCR that can be easily applied to detect borreliae in tick specimens stored in 70% ethanol [15]. As a target for PCR amplification we chose the flagellin gene that is particularly suitable as the *fla* sequences are known to be highly conserved among *Borrelia* species. Noppa *et al.* [13] reported that the Lyme borreliosis *Borrelia* species showed 94-95% identity to each other.

The positivity rate in the case of ticks examined by IFA was 8.1%: 7.6% in nymphs and 8.5% in adults. It resulted, among other things, from the fact that ticks from 3 out of 16 sites did not harbour any spirochetes. Ticks examined by PCR showed a higher positivity rate – 13.0%. In this case, it should be noticed that in 3/8 collection sites percentages of infected ticks reached 25–35.7%. Regarding different stages, the percentage of infected adult specimens was 22.8% while in nymphs it was 4.7%. For the latter, however, this was calculated on the minimal infection rate as nymphs were tested in pools. Thus, the actual value is probably higher.

We did not intend comparing the sensitivity of IFA and PCR, as in this case each tick should be examined simultaneously by both methods. We consider that each method used was sensitive enough to give results approximating the actual state of ticks' infection. The overall spirochete infection rate in ticks by both methods was 10.2%.

Ixodes ricinus is the most commonly observed and most widely distributed tick species in Poland. Our surveys document that infection of these ticks with *B. burgdorferi* s.l. is frequent and widespread and confirm the presence of Lyme disease foci in all 8 provinces examined.

In Poland, the most extensive studies on the occurrence of *B. burgdorferi* s.l. in ticks were carried out in the north-eastern part of the country in 1993-1996 [24, 25, 27] and then a total of 17469 nymphal and adult *I. ricinus* collected in 81 sites were individually examined by IFA. Spirochetes were found in 6.5, 11.5 and 8.8% ticks originated from the Gdańsk, Olsztyn and Białystok provinces, respectively. Data presented in this paper are comparable to the above reports. We confirm the existence of active Lyme borreliosis foci in the Olsztyn (10.3% infected ticks) and Białystok (4.1% infected ticks) provinces. In the latter, however, the infection level reported by us was twice as low as in 1994 [27]. Such variable percentages of *B. burgdorferi*-infected ticks in the same locations during following years of investigation were also observed in the urban and suburban forests of the cities of Gdańsk, Gdynia and Sopot [26]. The Białystok province is regarded as an endemic region of the disease, especially in the area of the Białowieża Primeval Forest. In the village of Białowieża, for instance, 49.7% of examined inhabitants revealed antibodies against *B. burgdorferi.* Diagnosis of Lyme disease was established in 37.7% persons from this seropositive group [5]. These results, as compared to ours, suggest that those people were frequently bitten by ticks.

In the survey conducted by us in the central-northern region, we reported new foci of the disease in the Elbląg, Słupsk and Bydgoszcz provinces. In the first one, the percentage of ticks infected with *B. burgdorferi* s.l. (13.2%) was comparable to that previously reported for the Olsztyn province (11.5%) [24]. In the Słupsk province, 9.1% of *I. ricinus* ticks were infected and this value was comparable to the infection level of 6.5-6.9% reported in the adjoining Gdańsk [25] and Koszalin [8] provinces. In the Bydgoszcz province, we noted a focus of Lyme borreliosis near the village of Tleń in the Tuchola Forests (*Bory Tucholskie*) (7.4% infected ticks). Among the human cases reported from this province in 1995-1996, at least 59 were noted in that area alone [2].

In central-eastern Poland, in the Lublin province where we noted 5.5-8.5% infected ticks, studies carried out in high risk group of people showed that among forestry workers 16.0% were seropositive [3].

In southern Poland, we conducted surveys in the Kraków and Katowice provinces. In the first area, each of 3 investigated populations of *I. ricinus* contained positive specimens with a rate of infection ranging from 11.5-34.1%. The overall infection level was 15.5%. In earlier studies, Siński *et al.* [20] reported that 19.2% of ticks originated from the same province were borreliae-positive.

Finally, in the Katowice province we examined *I. ricinus* collected near Mikołów and found 37.5% to be infected with *B. burgdorferi.* In 1996, Pet'ko *et al.* [16] examined *I. ricinus* collected in the same vicinity. Minimum infection rates calculated by them were 17.8% for males and 12.7% for females. Moreover, the same authors carried out studies in urban and suburban woods in the city of Katowice. The minimum infection rate of *I. ricinus* nymphs, males and females ranged there from 5–15.5% [16].

As mentioned above, in ticks collected in 2 sites (in the Wola Forest and in Mikołów), percentages of infected specimens exceeded 35%. Those investigations, however, included small numbers of ticks (n=32-42) and probably do not reflect actual infection rates of tick population in those locations. On the other hand, comparable high positivity rates have been already reported in an tick population near Frąknowo (37.5%), the Olsztyn province [24] and in ticks collected in the Zamość province (58.3%) [20].

According to our and other data, infection rates of *I. ricinus* varied in Poland between 0–58.3% [8, 20, 21, 22, 23, 24, 25, 26, 27] and generally are in agreement with those found in neighbouring countries. For instance, studies in Lithuania [12] revealed that 11.5% of adult *I. ricinus* and 1.0% of nymphs out of 3820 collected specimens

were infected with *B. burgdorferi*. In Brandenburg State, Germany, Gupta *et al*. [6] calculated infection rates of 19.4-22.4%, 10.8-21.1% and 10.9-33.3% for nymphal, male and female *I. ricinus*, respectively, while Kahl *et al*. [10] estimated minimal infection rate of 2.5% in nymphs and 5-10% in adult ticks collected in Berlin forests. In Slovakia, the overall prevalence of infected *I. ricinus* was 9.2% (0.8-22.6%) [18] and in Sweden on average, 10% of nymphs and 15% of the adult *I. ricinus* were positive for *B. burgdorferi* spirochetes [7].

Regarding different stages, we found that generally the infection rate was obviously higher in adults (13.6%) than in nymphs (6.2%), and slightly higher in females (14.9%) than in males (12.4%). These findings are in agreement with those of Wegner *et al*. [24, 25, 26], Gupta *et al*. [6], Fingerle *et al*. [4], Gustafson *et al*. [7] and others.

Although we report that *B. burgdorferi* s.l. is present throughout the distributional area of *I. ricinus* in Poland and show a high prevalence of spirochete-infected ticks in some locations of investigated regions, it is not possible to give a clear statement about of the risk of acquiring infection by humans. Too little is known about the seroprevalence among the human population inhabiting areas with existing Lyme borreliosis foci; the actual number of human cases is still unknown, and finally, too little is known about the distribution and pathogenicity of different *B. burgdorferi* genospecies. Further extensive and detailed investigations are therefore necessary to clarify the epidemiology of Lyme disease in Poland.

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