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Borrelia burgdorferi genospecies detection by RLB hybridization in *Ixodes ricinus* ticks from different sites of North-Eastern Poland

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Abstract

Introduction. RLB (Reverse Line Blot Hybridization) is a molecular biology technique that might be used for *Borrelia burgdorferi* sensu lato (sl) DNA detection with genospecies specification. Among *B. burgdorferi* sl genospecies at least 7 are regarded as pathogenic in Europe.

Objective. The aim of the study was to evaluate the frequency of different *Borrelia* genospecies DNA detection in *Ixodes ricinus* ticks in the endemic area of North-Eastern Poland by using RLB.

Materials and method. *Ixodes ricinus* ticks were collected in May – June, from 6 different sites in North-Eastern Poland (Jakubin, Kolno, Grajewo, Suwałki, Siemiatycze, Białowieża) by flagging. Extracted DNA was amplified by polymerase chain reaction (PCR) targeting the intergenic spacer *5S 23S* of *B. burgdorferi* sl. PCR products were hybridised to 15 different oligonucleotide probes for 9 different *Borrelia* genospecies (*B. burgdorferi* sl, *B. burgdorferi* ss, *B. garinii, B. afzelii, B. valaisiana*, *B. lusitaniae*, *B. spielmanii*, *B. bissettii* and *B. relapsing fever-like* spirochetes (*B. myamotoi*)) by RLB.

Results. Borrelia genospecies DNA was detected in 205 *Ixodes ricinus* ticks. Among 14 infected with *Borrelia* ticks, 4 were identified as *B. garinii* and 10 as *B. afzelii*. Higher numbers of infected ticks were noticed in the eastern part of the research area, where large forest complexes dominate. Nymphs appeared to be the most frequently infected tick stage, which has an epidemiological meaning in the incidence of Lyme borreliosis.

Conclusions. The study demonstrated that RLB might be easily used in *Borrelia* DNA detection with genospecies-identification, and indicated the domination of *B. afzelii* and *B. garinii* in ticks from North-Eastern Poland.

Key words

reverse line blotting, *Ixodes ricinus* ticks, *Borrelia burgdorferi*, genospecies-identification, *5S 23S* intergenic spacer

INTRODUCTION

Lyme borreliosis is a disease that can affect the skin, joints, heart, as well as the musculoskeletal and neurological systems. The disease is caused by genospecies belonging to *Borrelia burgdorferi* sensu lato (sl) Gram-negative bacteria complex. In Europe, 7 *Borrelia* genospecies: *B. burgdorferi* sensu stricto (ss), *B. garinii*, *B. afzelii*, *B. valaisiana*, *B. lusitaniae*, *B. spielmanii* and *B. bissettii* transmitted by *Ixodes ricinus* ticks have been detected [1- 4]. Moreover, during the last few years in Europe an additional species – *B. miyamotoi*, was detected in *I. ricinus* ticks [3, 5, 6, 7, 8], and according to Barbour et al. it belongs to another group of *Borrelia* species [9]. *B. miyamotoi* is related to the relapsing-fever spirochete group according to DNA sequences.

In Poland, *Borrelia* spirochaetes are transmitted to humans by *I. ricinus* ticks [8, 10, 11]. North-eastern Poland is an endemic region for Lyme borreliosis, with a high incidence of this disease. Various genospecies of *B. burgdorferi* sl cause different clinical manifestation in patients. *B. burgdorferi* ss is an agent of Lyme arthritis, whereas *B. garinii* is connected with neuroborreliosis and *B. afzelii* with long-lasting skin lesions (ACA – Acrodermatitis Chronica Atrophicans) [12, 13].

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Different molecular methods are used for *Borrelia* genospecies-specification, such as: polymerase chain reaction (PCR) and its various types, RLB (Reverse Line Hybridization) [5, 14, 15, 16, 17, 18, 19], Restriction Fragment Length Polymorphism (RFLP) [13, 20, 21], Random Amplified Polymorphic DNA (RAPD) [22] and DNA sequence analysis [23]. All these methods can be used for *Borrelia* DNA detection in non-human material, such as ticks.

The aim of the presented study was to evaluate the frequency of pathogenic *B. burgdorferi* sl genospecies DNA detection and co-infections in different life stages of ticks collected at different sites in north-eastern Poland by using RLB hybridization and estimation of usefulness of this method in genospecies-identification.

MATERIALS AND METHOD

Ticks collection. Ticks were collected during in May – June 2007 from 6 different areas of North-Eastern Poland (Fig. 1): Jakubin (53°10'N, 23°30'E), Kolno (53°24'N, 21°56'E), Grajewo (53°39'N, 22°27'E), Suwałki (54°07'N, 22°56'E), Siemiatycze (52°26'N, 22°52'E) and Białowieża (52°42'N, 23°52'E) by flagging low vegetation. Collected ticks were placed separately in Eppendorf tubes containing 70% ethanol until DNA extraction and kept at +4°C.

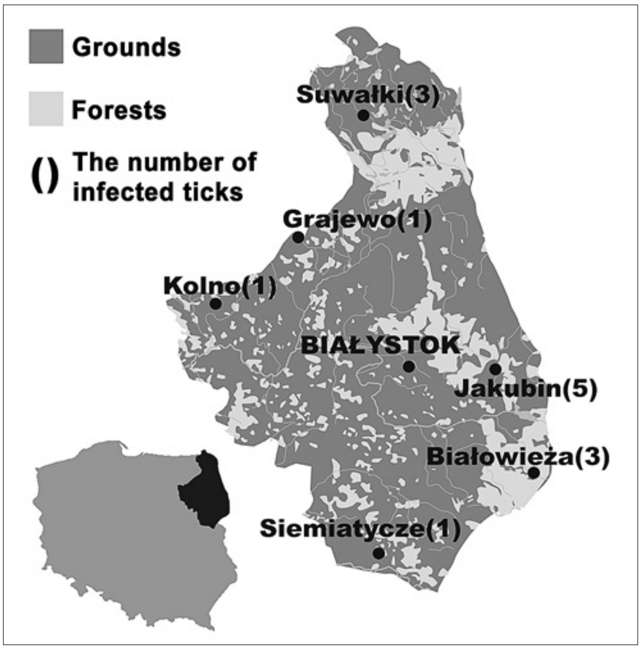

Figure 1. Locality of study sites in North-Eastern Poland and indication of sites with number of infected ticks

DNA isolation. Before DNA extraction, ticks were washed in fresh 70% alcohol. Then, ticks were then incubated at 100 °C in 100 µl of 0.7 M NH_4OH for 15 minutes, cooled immediately, and boiled again for 15 minutes with open vials, as described in Morán Cadenas et al. [17]. Approximately 40 µl extracted DNA were obtained, cooled at room temperature and kept at -20 °C until PCR amplification.

PCR amplification. For PCR amplification, primers B5S-Bor (5`- biotin-GAG TTC GCG GGA GAG TAG GTT ATT-3`) and 23S-Bor (5`-TCA GGG TAC TTA GAT GGT TCA CTT-3`) targeting the intergenic spacer *5S 23S rDNA* of *B. burgdorferi* were used [14]. A mixture (total volume 50 µl) with 10 µl of extracted DNA, buffer × 10 containing 1.5 mM $MgCl_2$ (Qiagen, Switzerlad), 1 µl 10 mM dNTPs, 1 µl 10 mM of each primer, and 0.15 1 µl (5U/µl) Taq DNA polymerase (Qiagen, Switzerland) was used. The PCR was processed as described by Morán Cadenas et al. [17] and Burri et al. [15] and conducted in a Whatman Biometra Tgradient Thermocycler 96 (Göttingen, Germany). The programme for amplification was as follows: initial denaturation at 94 °C for 3 min, 40 cycles (denaturation at 94 °C for 20s, annealing at 52 °C for 30s, extention at 72 °C for 30s), and final extention at 72 °C for 7 min. In all reactions positive controls (isolates of: *B. burgorferi* ss – strain B31, *B. garinii* – strain NE11 and *B. afzelii* – strain NE632) and negative controls (redistilled water replaced DNA isolates) were included. The size of amplification products for intergenic spacer *5S 23S* was approximately 410 base pairs.

RLB hybridization. For *Borrelia* genospecies identification by RLB, PCR products were hybridised to 15 different oligonucleotide probes (75 pmol and 100 pmol) as described in Gern et al. [1] for 9 different *Borrelia* genospecies (*B. burgdorferi* sl, *B. burgdorferi* ss, *B. garinii*, *B. afzelii*, *B. valaisiana*, *B. lusitaniae*, *B. spielmanii*, *B. bissettii* and *B. relapsing fever-like* spirochetes (*B. myamotoi*) [1] (Tab. 1 [1, 18, 19]). To avoid cross-reactivity more than one oligo probe for particular genospecies was used. For example, a GANE1 probe gives weak signal for some *B. burgdorferii ss*, LusiNE for *B. afzelii* or LusiNE1 for *B. garinii* [1]. All probes were blotted in lines on an activated Biodyne C membrane (Pall Europe Ltd., Portsmouth, UK) using a Miniblotter 45 (Immunetic, Cambridge, MA, USA). Hybridisation was visualized by incubating the membrane with enhanced chemiluminescence detection liquid (GE Healthcare, Otelfingen, Switzerland) and by exposing the membrane to X-ray film (Hyperfilm, GE Healthcare, Europe).

Table 1. RLB oligonucleotide sondes used in the research

Oligonucleotide sondes	Sequences (5'- 3')	*Borrelia burgdorferi* genospecies (target)	References
SL	CTTTGACCATATTTTTATCTTCCA	B. burgdorferi sensu lato	Rijpkema et al. (1995)[a]
SS	AACACCAATATTTAAAAAACATAA	B. burgdorferi sensu stricto	Rijpkema et al. (1995)[a]
GA	AACATGAACATCTAAAAACATAAA	B. garinii	Rijpkema et al. (1995)[a]
GANE	CAAAAACATAAATATCTAAAAACATAA	B. garinii	Poupon et al. (2006)[b]
AF	AACATTTAAAAAATAAATTCAAGG	B. afzelii	Rijpkema et al. (1995)[a]
VS4	TATATCTTTTGTTCAATCCATGT	B. valaisiana	Poupon et al. (2006)[b]
LusiNE (Lusi3)	TCAAGATTTGAAGTATAAAATAAAA	B. lusitaniae	Poupon et al. (2006)[b]
RFLNE or Miya2	CTATCCATTGATCAATGC	B. relapsing fever-like B. myamotoi-like	Gern et al. 2010[c]
SpiNE2	GAATGGTTTATTCAAATAACATA	B. spielmanii	Gern et al. 2010[c]
SpiNE3	GAATAAGCCATTTAAATAACATA	B. spielmanii	Gern et al. 2010[c]
LusiNE1	CATTCAAAAAAATAAACATTTAAAAACAT	B. lusitaniae	Gern et al. 2010[c]
LusiNE2	AAATCAAACATTCAAAAAAATAAAC	B. lusitaniae	Gern et al. 2010[c]
GANE1	AAAATCAATGTTTAAAGTATAAAAT	B. garinii	Gern et al. 2010[c]
BisNE1	AAACACTAACATTTAAAAAACAT	B. bissetti	Gern et al. 2010[c]
BisNE2	AACTAACAAACATTTAAAAAACAT	B. bissetti	Gern et al. 2010[c]

[a] [19]; [b] [18]; [c] [1]

RESULTS

A total of 205 ticks consisting of 74 nymphs, 91 males and 40 females were screened for *Borrelia* infection (Tab. 2). A total of 14 (6.83%) ticks were infected by *B. burgdorferi* sl using PCR followed by RLB. Among them, only 2 genospecies were identified: *B. garinii* was detected in 4/205 (1.95%) ticks and *B. afzelii* in 10/205 (4.88%) (Tab. 3, Fig. 2). No co-infection was observed. Half of the infections (n=7) were detected in nymphs, 6 were identified as *B. afzelii* and 1 as *B. garinii*, which gives an infection rate for nymphs of 9.45% (7/74). The 7 infections in adults were detected in 4 males and in 3 females, giving and infection rate of 4.39% (4/91) for males and 7.5% (3/40) for females.

Table 2. Detected pathogens samples including site, gender and stage of vector

Detected pathogens (gender, stage and agent of infection)		Areas of Podlasie (Sites of tick collection) Jakubin (53°10'N23°30'E)	Kolno (53°24'N 21°56'E)	Grajewo (53°39'N22°27'E)	Suwałki (54°07'N 22°56'E)	Siemiatycze (52°26'N 22°52'E)	Białowieża (52°42'N 23°52'E)	Total
[a]N	Total	15	15	14	15	0	15	74
	B. afzelii	1	1	1	2	0	1	6
	B. garinii	1	0	0	0	0	0	1
[b]M	Total	15	15	16	15	15	15	91
	B. afzelii	0	0	0	1	1	1	3
	B. garinii	1	0	0	0	0	0	1
ba [c]F	Total	20	0	0	0	0	20	40
	B. afzelii	0	0	0	0	0	1	1
	B. garinii	2	0	0	0	0	0	2
Total		5 of 50	1 of 30	1 of 30	3 of 30	1 of 15	3 of 50	**14 / 205**

[a] N – Nymph; [b] M – Male; [c] F – Female;

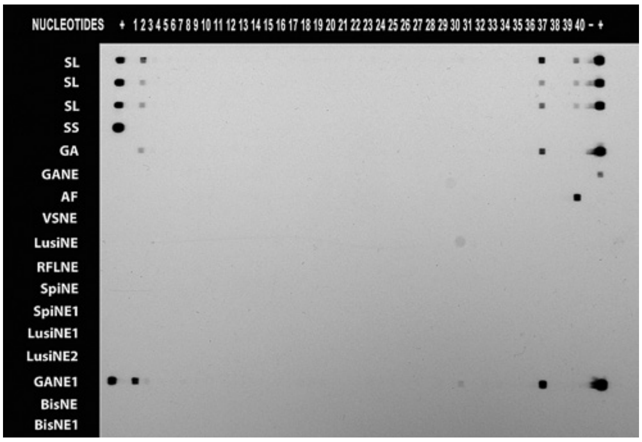

Figure 2. X-ray film illustrating RLB results from 40 DNA extraction samples. Oligonucleotides probes used: SL – *B. burgdorferi* sl; SS – *B. burgdorferi* ss; GA, GANE – *B. garinii*; AF – *B. afzelii*; VSNE – *B. valaisiana*; LusiNE – *B. lusitaniae* (+ AF – DNA of *B. afzelii*); RFLNE – B. relapsing fever-like; SpiNE, SpiNE1 – *B. spielmanii*; LusiNE1 (+ GA or GANE – DNA of *B. garinii*); LusiNE2- *B. lusitaniae*; GANE1 – *B. garinii* (+ SS – B. *burdgorferi* ss); BisNE, BisNE1 – *B. bissettii*.
+ – positive control; - – negative control; sample 2 (SL, GA, GANE1) – *B. garinii*; sample 30 (SL, GANE1) – *B. garinii*; sample 37 (SL, GA, GANE1) – *B. garinii*; sample 40 (SL, AF) – *B. afzelii*; samples 1–20: DNA extractions from females; samples 21–35: DNA extractions from males; samples 36-41: DNA extractions from nymphs

Table 3. *B. burgdorferi* sl infection in *I. ricinus* ticks

Gender and stage	No. of ticks	No. of infected ticks	No. of infected ticks with genospecies differentiation 1	2	3	4	5	6	7	8	9
Nymph	74	7	7	6	1	0	0	0	0	0	0
Male	91	4	4	3	1	0	0	0	0	0	0
Female	40	3	3	1	2	0	0	0	0	0	0
Total	205	14	14	10	4	0	0	0	0	0	0

1. *B. burgdorferi* sl, 2. *B. afzelii*, 3. *B. garinii*, 4. *B. burgdorferi ss*, 5. *B. valaisiana*, 6. *B.lusitaniae*, 7. *B. spielmanii*, 8. *B. bissettii*, 9. *B. relapsing fever-like (B. myamotoi-like)*

At 3 of the 6 sites in north-eastern Poland (Jakubin, Suwałki, Białowieża) the number of infected ticks was higher than at the other 3 (Fig. 1). The highest infection rate (5/14, 35.7%) was observed at Jakubin. All *B. garinii* (4/4, 100%) infections were detected in this area and observed in 1 nymph, 1 male and 2 females.

DISCUSSION

Lyme borreliosis is one of the most commonnly diagnosed infectious diseases transmitted to humans by ticks in Poland [24]. In 2010, the incidence of this disease was 9,159 cases, according to the Polish National Institute of Public Health [25]. During the last 13 years, a dramatically increasing tendency has been observed. Determination of the number of infected ticks in particular regions of the country provides important information about Lyme borreliosis epidemiology and risk areas. The use of the PCR and RLB techniques gives the possibility to detect and differentiate diverse genotypes belonging to the *B. burgdorferi* sl complex. Moreover, it is supposed that RLB will significantly facilitate epidemiological studies on tick-borne diseases by simultaneous detection of different types of tick-borne pathogens, such as other bacteria, e.g. *Anaplasma phagocytophilum*, or protozoa of the *Babesia* and *Theileria* species, simultanteously in one common reaction [26, 27]. Additionally, using species-specific oligonucleotide probes covalently linked to the membrane allows multiple uses of the same membrane, up to 15 times. It has not only clinical and epidemiological significance, but also an economic one.

The RLB technique combining hybridization with standard PCR increases the sensitivity of the method 1,000 fold in conventional amplification [28]. In PCR, highly conservative regions of 4 *B. burgdorferi* genes: outer surface protein A (*OspA* gene) [28], *fla* gene [30, 31] *16S rRNA* [20, 32] gene and used in this project, the intergenic spacer *5S 23S rDNA* [1, 19, 33], have been targeted. Another advantage of RLB hybridization in comparison with other molecular biology technique is that it is a more friendly method for the users. Chemiluminescence instead of radioactivity detection is safer and gives easy to read and store results.

In the presented study, a 6.83% prevalence of *B. burgdorferi* was observed in ticks collected in north-eastern Poland, and only 2 genospecies, *B. afzelii* and *B. garinii*, were identified. Similar results were obtained by Cisak et al. [10]. They reported that 11.6% of *I. ricinus* ticks (n=406) from the Roztocze National Park in South-Eastern Poland were infected by *B. burgdorferi* sl with 2 genospecies. The most frequent genospecies was *B. burgdorferi* ss (26/47, 55.3%) followed by *B. afzelii* (n=18, 38.3%). In 2 cases (4.3%), the genospecies was unidentified and 1 (2.1%) mixed infection with *B. burgdorferi ss* and *B. afzelii* was reported. Stańczak et al. [11] observed 13.8% (162/1172) infection prevalence of *B. burgdorferi* sl in *I. ricinus* ticks collected in forests from different Polish regions during a 3-year study (1996–1998). For genospecies identification, 153 positive tick samples were investigated by nested PCR using three species-specific primers. Single infections were in 54.2% cases (83/153), consisting in *B. afzelii* that was identified in 38/153 samples (24.9%), followed by *B. burgdorferi* ss in 23/153 (15%) and *B. garinii* in 22/153 (14.4%). In a group of 121 adult ticks, double infections with *B. burgdorferi* ss and *B. afzelii* were detected in 17 samples (14%), followed by both *B. burgdorferi* ss/*B. garinii* and *B. garinii*/*B. afzelii* co-infections observed in 11 cases (9.1%). Triple infections were observed twice (1.6%). In 2006, Strzelczyk et al. using a *fla* gene sequence as a target in PCR [31] reported an infection rate of 16.5% (14/85) among ticks from recreational areas of Silesia in South-Western Poland (11 mono-infections, 2 double infections and one undetermined infection). Three *Borrelia* species were detected with similar frequency: *B. burgdorferi* ss (n=6), *B. garinii* (n=5) and *B. afzelii* (n=4). The *B. burgdorferi* genospecies diversity depends on geographical distribution in Europe and on particular areas of country. In Eastern Europe, *B. afzelii* infections are observed more often than in Western part of the continent and in Scandinavian countries where *B. garinii* prevails [42]. Dominating in North America *B. burgdorferi* ss is rarely detected in Eurasia.

In the presented study, the dominant genospecies was *B. afzelii*, followed by *B. garinii*. Similar results were reported by Paulauskas et al. [34] in different regions of Lithuania by RLB – *B. afzelii* was identified in 73% (n=143) cases, whereas *B. garinni* in 10% (n=19) cases and *B. burgdorferi ss* only in 7% (n=14) of cases. *B. afzelii* dominant was defined by Stańczak et al. [11] in different regions of Poland, Derdáková et al. [33] in the southern part of the Czech Republic and Quessada et al. [35] in France (Lyon region). Other authors in Europe showed a dominance of *B. garinii*, for example, Missone et al. [21] in Belgium, Rijpkema et al. [19] in the Netherlands, as well as Escudero et al. [36] in Spain and Jouda et al. [37, 38] in Switzerland.

Scientists frequently emphasize the significant meaning of animal reservoirs in *B. burgdorferi* genospecies diversity and localization in the environment. Distinct *Borrelia* compatibility to vertebrate organism is caused by CRASPs proteins (Complement Regulator-Acquiring Surface Proteins) that deactivate the complement of the specific host species, and consequently, influence bacteria spreading out in the particular environment [39]. For example, in research by Kurtenbach et al. [40], small rodents were infected in 19% with *B. garinii*, but no transmission of this bacteria to ticks in the same area was observed (only about 1.3% of the xenodiagnostic ticks were infected with *B. garinii*). In comparison, ticks collected from the same area, derived from pheasants, were infected with *B. garinii* and *B. valaisiana* in more than 50% of cases. According to the review of Piesman and Gern [1, 41], small rodents are responsible for *B. afzelii* spreading and birds are regarded as main reservoirs for *B. garinii*.

Research on *B. burgdorferi* DNA detection among ticks collected from 6 different areas of North-Eastern Poland by using RLB method draw out some epidemiological important conclusions. Nymphs were more frequently infected (9.45%, 7/74) than adults (5.34%, 7/131). It is important to mention that contact with this immature stage of the vector might remain unnoticed by humans because of its small size. Distinct higher frequency of *B. burgdorferi* genospecies detection in eastern part of the studied region (Jakubin, Białowieża, Suwałki) indicated a higher prevalence of tick infections in areas of big forest complexes and primeval forests. According to the presented results, it appears that *B. afzelii* (10/14, 71.43%) dominates in the studied areas of North-Eastern Poland. All *B. garinii* infections (4/4, 100%) were detected among ticks collected at one site only – Jakubin. It might probably be connected with animal reservoir in this area.

CONCLUSIONS

To sum up, RLB used in genospecies-identification of different tick-borne pathogens, allowed identification of *B. afzelii* and *B. garinii* domination in ticks collected at various sites in North-Eastern Poland. Green, eastern, recreational regions with big forest complexes, such as: Białowieża, Suwałki and Jakubin, because of higher number of infected ticks seems to be potentially more dangerous for people. The most frequently infected stage of the tick were, usually hard to noticed, nymphs.

RLB is a safe, highly sensitive molecular-biology method that might be useful in genospecies differentiation of *Borrelia burgdorferi* and other tick-borne pathogens.

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