A comparative analysis of molecular markers for the detection and identification of *Borrelia* spirochaetes in *Ixodes ricinus*

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Borrelia burgdorferi sensu lato, carried by *Ixodes* ticks, is one of the most significant human pathogens, causing Lyme disease. As there is no standardized PCR method for detection and identification of spirochaete DNA, we carried out a comparative analysis using a set of complementary primers for three regions in the genomic DNA of these bacteria (genes *fla* and *rrs* and the non-coding *rrs-rrlA* region). DNA extracted from 579 *Ixodes ricinus* ticks was subjected to nested PCR. DNA of the examined spirochaetes was detected in 43 (7.4 %) lysates when the *fla* gene was used as a molecular marker, in 7 (1.2 %) lysates when using primers complementary to the *rrs* gene, and in 12 (2.1 %) lysates using primers complementary to the non-coding *rrs-rrlA* sequence. RFLP analysis based on the *fla* gene helped identify species from the *B. burgdorferi sensu lato* complex (*B. burgdorferi sensu stricto*, *Borrelia afzelii*, *Borrelia garinii*, *Borrelia valaisiana*), detect co-infections, and also identify *Borrelia miyamotoi*. Therefore, the *fla* gene is the most sensitive and specific molecular marker for the detection and identification of *Borrelia* spirochaetes in *I. ricinus*.

INTRODUCTION

Spirochaetes belonging to the *Borrelia burgdorferi sensu lato* complex, carried by *Ixodes* ticks, are one of the most significant human pathogens transmitted by ticks and cause a multi-system disease, borreliosis, known also as Lyme disease (Liveris *et al.*, 1999; Šitum *et al.*, 2000; Wang *et al.*, 1999). Since the bacterium was isolated from *Ixodes scapularis* ticks (Burgdorfer *et al.*, 1982) in the USA, 125 000 cases of this disease have been recorded, and in Europe 50 000 cases are recorded annually. In Poland, about 7000 cases are recorded annually, according to the Polish National Institute of Hygiene (<http://www.pzh.gov.pl>).

Difficulties with the extraction of *B. burgdorferi sensu lato*, ineffective culture, and the ambiguity of results in immunoserological analyses necessitated the search for new, quick, sensitive and specific diagnostic methods based on the detection and analysis of genetic material in these bacteria (Lin *et al.*, 2002; Sparagano *et al.*, 1999; Wang *et al.*, 1999). PCR enabled the detection of very small amounts of the bacteria, identifying DNA in human material, in animal reservoir hosts, and in vectors, i.e. *Ixodes* ticks. It was first

used for the detection of *B. burgdorferi sensu lato* DNA by Rosa & Schwan (1989), and since then it has been developed with regard to the selection of markers (Aguero-Rosenfeld, 2003; Picken, 1992; Picken *et al.*, 1996; Skotarczak *et al.*, 2002; Sparagano *et al.*, 1999; Wang *et al.*, 1998; Wodecka & Skotarczak, 2005; Zhang *et al.*, 1993). However, lack of standardization of PCR for the identification of *Borrelia* spirochaetes makes it impossible to compare the reports on this subject matter. PCR detection of *B. burgdorferi sensu lato* uses various molecular markers, i.e. DNA sequences located in the bacterial chromosome and in plasmids (Picken, 1992; Wodecka & Skotarczak, 2005). Usually, genes encoding antigens are used, e.g. the *fla* gene encoding the flagellum protein flagellin (Aguero-Rosenfeld, 2003; Michalik *et al.*, 2005; Picken *et al.*, 1996; Skotarczak *et al.*, 2008; Wodecka, 2007; Wodecka & Skotarczak, 2005), and *ospA*, *ospB* and *ospC*, encoding surface proteins (Aguero-Rosenfeld, 2003; Wang *et al.*, 1999, 2000). Additional significant genetic markers are genes encoding rRNA in both ribosomal subunits, especially *rrs* encoding 16S rRNA in the small ribosomal subunit (Burgdorfer *et al.*, 1982; Fraenkel *et al.*, 2002; Zhang *et al.*, 1993) and non-coding DNA within the operon that contains genes for rRNA (Kondrusik *et al.*, 2004; Liveris *et al.*, 1999; Ranka *et al.*, 2004). However, research on plasmid genes that encode surface proteins (*OspA*, *OspB* and *OspC*) has also shown a great variability of these sequences and therefore little usefulness for the identification of *Borrelia* spirochaetes (Lin *et al.*, 2002;

The GenBank/EMBL/DDBJ accession numbers for the *fla* gene sequence of *B. burgdorferi sensu stricto* G112-04, *B. afzelii* D131-07, *B. garinii* G23-07, *B. valaisiana* G43-0507 and *B. miyamotoi* D110-07 are FJ518808, FJ518805, FJ518806, FJ518807 and FJ518804, respectively.

Schwan *et al.*, 1993; Wang *et al.*, 1999). The *vlsE* gene, encoding a variable surface antigen, the *p66* gene, involved in the synthesis of the surface membrane protein, the *p13* gene, encoding P13 protein, and the *dbpA* and *dbpB* genes, encoding decorin-binding proteins, are even less frequently used, due to their high variability in individual species from the *B. burgdorferi sensu lato* complex (Iyer *et al.*, 2000; Pinne *et al.*, 2004; Roberts *et al.*, 1998). Genes of basic metabolism, i.e. encoding sigma factor B and D subunits (*rpoB* and *rpoD*), and the *hbb* gene encoding histone-like protein, are also rarely used for the identification of *Borrelia* species (Lee *et al.*, 2000).

Due to the lack of a standardized method for *B. burgdorferi sensu lato* detection, this study examined the usefulness of the *fla* gene, the *rrs* gene and the non-coding *rrs-rrlA* region as molecular markers for the detection of *B. burgdorferi sensu lato* in the common tick (*Ixodes ricinus*). Our analysis also shows the potential application of individual markers for the identification of species from the *B. burgdorferi sensu lato* complex.

METHODS

Tick collection. A total of 579 *I. ricinus* ticks were collected during the spring–summer seasons of 2007 in two endemic localities of recreation sites in the city of Szczecin (lake Głębokie surroundings and Dąbie Forest Park). Collections were performed by flagging flannel cloth over vegetation along forest paths. The mean temperature was 21 °C and mean air humidity was 70 %. Study sites were located in mixed forests where the highest occurrence of *Borrelia* species had previously been observed (Wodecka, 2003). The ticks were placed in 1.5 ml Eppendorf test tubes. After collection, ticks were identified according to a guidebook of invertebrates (Siuda, 1991) and stored at –20 °C until DNA extraction.

DNA extraction. DNA from questing ticks was extracted using a method previously described by Guy & Stanek (1991). Ticks were squashed one by one with a sterile pestle and then suspended in PBS buffer and placed in 1.5 ml Eppendorf test tubes. Then 100 µl 0.7 M

ammonium hydroxide was added and the mixture was boiled at 100 °C for 15 min. The content of tubes was vaporized to reduce the volume down to 50 µl. The extracted DNA was stored at –70 °C until further analysis.

Nested PCR assay. The PCR detection of *B. burgdorferi sensu lato* DNA in *I. ricinus* was carried out using three independent sets of primers, complementary for *fla*, *rrs* and the non-coding *rrs-rrlA* sequence. The sequence of individual primers and the length of amplification products are presented in Table 1.

The reactive mixture (10 µl) contained 0.5 U polymerase Allegro Taq DNA (Novazym), 70 mM Tris/HCl (pH 8.6 at 25 °C), 16.6 mM (NH₄)₂SO₄, 3.5 mM MgCl₂, 0.75 µM of each deoxyribonucleoside triphosphate, and 2 pmol of each primer from the pairs of outer and inner primers. The mixture used in the reaction with the outer primers was a 1 µl suspension of DNA extracted from the ticks, and in the reaction with the inner primers 1 µl of 10 times diluted postreactive mixture obtained with the inner primers was used (Wodecka, 2007). DNA from the *B. burgdorferi sensu stricto* IRS strain (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany) was used as the positive control, and sterile water was used as the negative control.

The initial denaturation took place at 94 °C for 10 min. It was followed by 40 cycles of amplification, including denaturation at 94 °C for 30 s, annealing for 45 s at a temperature dependent on the applied primers (*fla*, outer primers at 50 °C and inner primers at 54 °C; non-coding *rrs-rrlA* region, 50 °C and 55 °C; and *rrs*, both primers at 65 °C), the elongation of the DNA chain at 72 °C for 1 min and final elongation at 72 °C for 7 min.

The separation of the nested PCR products was carried out on a 2 % agarose gel (ICN) with the addition of ethidium bromide (Sigma–Aldrich) in the TBE buffer (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA).

The genomic species of *B. burgdorferi sensu lato* in *I. ricinus* were determined using PCR-RFLP analysis, which involved the positive DNA samples obtained with nested PCR based on primers complementary to the *fla* and *rrs* genes. The identification of species using the intergenic *rrs-rrlA* space was based on the analysis of nested PCR products, with different lengths for individual species.

DNA sequencing. Partial sequencing of the *fla* gene obtained with primers 220f and 823r was performed for positive samples giving a

Table 1. Primers used in this study

Marker	Primer	Size of the amplification product (bp)	Reference
<i>fla</i>	Outer	132f: 5'-TGGTATGGGAGTTTCTGG-3' 905r: 5'-TCTGTCATTGTAGCATCTTT-3'	Wodecka (2007)
	Inner	220f: 5'-CAGACAACAGAGGGAAAT-3' 823r: 5'-TCAAGTCTATTTTGGAAAGCACC-3'	
<i>rrs</i>	Outer	19f: 5'-AGAACTAACGCTGGCAGTG-3' 593r: 5'-TGCATAGACTTATATATCCGCC-3'	This study
	Inner	38f-rlb: 5'-CGTCTTAAGCATGCAAGTCAAACG-3' 472r-bor: 5'-CGTCATCACTTTGTCTATTCCTACA-3'	
<i>rrs-rrlA</i>	Outer	P95: 5'-GGTTAGAGCGCAGGTCTG-3' Pa: 5'-GGTATGTTTAGTGAGGG-3'	Ranka <i>et al.</i> (2004)
	Inner	P97: 5'-GATGTTCAACTCATCCTGGTCCC-3' Pb: 5'-CGTACTGGAAAGTGCGGCTG-3'	
		<i>B. afzelii</i> 435 <i>B. garinii</i> 532 <i>B. burgdorferi sensu stricto</i> 950	

restriction pattern characteristic for each *Borrelia* species detected in this study. The products were extracted with a Clean-Up extraction kit (A&A Biotechnology) according to the protocol of the manufacturer and DNA sequencing was performed by dye termination cycle sequencing. Each strand was analysed using ABI fluorescence automated sequencers by the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences, Warsaw, Poland.

The obtained sequences were compared to those of reference strains from GenBank. Sequences were aligned using the multisequence alignment program DNAMAN 5.2.9 (Lynnon Biosoft).

PCR–RFLP. Amplified DNA, obtained using primers complementary to *fla* and *rrs*, was subjected to restriction enzyme digestion with restriction enzymes selected according to the applied marker and the analysis of gene sequences obtained from GenBank, performed with DNAMAN 5.2.9 (Lynnon Biosoft).

Products obtained with primers 220f and 823r, complementary to *fla* gene, were digested with restriction enzyme *HpyF3I* (Fermentas), which recognizes the CTNAG sequence. This enzyme yielded seven different restriction patterns used for the identification of six species from the *B. burgdorferi sensu lato* complex, *B. burgdorferi sensu stricto*, *Borrelia afzelii*, *Borrelia garinii*, *Borrelia valaisiana*, *Borrelia lusitaniae* and *Borrelia bissetti*, and also *Borrelia miyamotoi*, a species from the relapsing fever borreliae (Fig. 1a).

DNA was amplified with primers 38f-rlb and 472r-bor (complementary to the *rrs* sequence), and digested with the restriction enzyme *FspBI* (Fermentas), which recognizes the CTAG sequence. The analysis of the *rrs* gene sequence yielded six restriction patterns for the identification of *B. burgdorferi sensu stricto*, *B. afzelii*, *B. garinii*, *B. valaisiana*, *B. lusitaniae* and *B. miyamotoi* (Fig. 2a). The restriction enzyme digestion products were separated electrophoretically on a 3 % agarose gel.

Statistical analysis. The obtained results were analysed using the chi-square test to determine whether the type of applied molecular

marker was significant for the frequency of detecting *B. burgdorferi sensu lato* in *I. ricinus*.

RESULTS AND DISCUSSION

Nested PCR results

Out of 579 ticks examined, 43 (7.4 %) were positive for spirochaete DNA by nested PCR with primers complementary to the *fla* gene. Using primers complementary to the non-coding region between the *rrs* and *rrlA* genes, *B. burgdorferi sensu lato* genetic material was detected in 12 (2.1 %) samples. Using primers complementary to *rrs*, DNA of the examined spirochaetes was found in only seven (1.2 %) lysates.

PCR–RFLP results

PCR–RFLP analysis concerned 43 positive DNA samples, obtained with nested PCR using the *fla* gene. Out of the examined DNA lysates, we identified four enzyme digestion patterns specific for the *B. burgdorferi sensu lato* complex, *B. garinii*, *B. valaisiana*, *B. burgdorferi sensu stricto* and *B. afzelii*, and one for *B. miyamotoi*, from the relapsing fever borreliae. We also identified co-infections: *B. afzelii*–*B. valaisiana*, *B. garinii*–*B. valaisiana*, *B. garinii*–*B. afzelii* and *B. burgdorferi sensu stricto*–*B. valaisiana* (Fig. 1b; Table 2).

By analysis of seven restriction patterns in PCR-positive DNA samples obtained with nested PCR (*rrs* gene), we

Fig. 1. (a) Restriction pattern obtained on the basis of the *fla* gene presented schematically. The resulting RFLP patterns were obtained after *HpyF3I* endonuclease treatment of specific amplified *B. burgdorferi sensu lato* flagellin sequence products. (b) Agarose gel electrophoresis. Lanes: M, molecular marker; 8 and 14, *B. burgdorferi sensu stricto*; 7 and 13, *B. afzelii*; 2, 3, 5, 9, 15, 17 and 18, *B. garinii*; 1, 6 and 16, *B. valaisiana*; 19, *B. miyamotoi*; 4, *B. afzelii* and *B. valaisiana* co-infection; 10, *B. garinii* and *B. valaisiana* co-infection; 11, *B. burgdorferi sensu stricto* and *B. valaisiana* co-infection; 12, *B. garinii* and *B. afzelii* co-infection.

Fig. 2. (a) Restriction pattern obtained on the basis of the *rrs* gene presented schematically. The resulting RFLP patterns were obtained after *Fsp*BI endonuclease treatment of specific amplified *B. burgdorferi sensu lato rrs* sequence products. (b) Agarose gel electrophoresis. Lanes: M, molecular marker; 1 and 2, *B. burgdorferi sensu stricto*; 3, *B. afzelii*; 4, *B. miyamotoi*; 5 and 6, *B. garinii*; 7, *B. valaisiana*. *Undigested PCR product.

identified five species of *Borrelia* spirochaetes, similarly to the analysis based on *fla*, but no co-infections were observed (Fig. 2b; Table 2). Two distinct restriction patterns were obtained for *B. garinii* which allowed the differentiation of genetic types of this species. However, because of the low efficacy of PCR amplification with primers for *rrs*, this method of differentiation of *B. garinii* types seems to be hardly effective.

In the analysis of nested PCR products based on the intergenic *rrs-rrlA* spacer, we observed only the presence of three species from *B. burgdorferi sensu lato*. We identified products with a length of 435 bp, characteristic for *B. afzelii*, 532 bp for *B. garinii* and 950 bp for *B. burgdorferi sensu stricto*. Similarly to the *rrs* gene, we did not identify any co-infections. Moreover, the analysis of this sequence did not allow the detection of *B. miyamotoi* (Table 2).

Results of *Borrelia* species identification obtained with each primer set (i.e. 220f and 823r for *fla*, 38f-rlb and 427r-bor for *rrs*, and P97 and Pb for *rrs-rrlA*) were compatible. The

results confirmed the sensitivity of the *fla* gene as a marker for detection of *Borrelia* species.

DNA sequences

fla gene sequences representative of five *Borrelia* species detected in this study were 100 % identical to the sequences of the *fla* gene obtained in other studies conducted earlier in north-western Poland (Wodecka & Skotarczak, 2005; Wodecka, 2007). Due to the small amount of PCR products obtained with primers for the *rrs* gene and the *rrs-rrlA* intergenic spacer, sequencing of these products was not done.

Statistical analysis of results

Statistical analysis of results obtained using nested PCR with primers complementary to *rrs* and the intergenic *rrs-rrlA* region (χ^2 test) showed statistically insignificant deviation ($P=0.250$). Statistically significant deviations

Table 2. *Borrelia* species detected in *I. ricinus* ticks (BB, *B. burgdorferi sensu stricto*; BG, *B. garinii*; BA, *B. afzelii*; BV, *B. valaisiana*; BM, *B. miyamotoi*; Co, co-infection)

Marker	Stage	n	%	BB (n/%)	BG (n/%)	BA (n/%)	BV (n/%)	BM (n/%)	Co (n/%)
<i>fla</i>	Adult	6/102	5.88	—	2/1.96	—	4/3.92	1/0.98	—
	Nymph	37/477	7.76	5/1.05	13/2.73	6/1.26	5/1.05	2/0.42	5/1.05
	Total	43/579	7.4	5/0.86	15/2.59	6/1.04	9/1.55	3/0.52	5/0.86
<i>rrs</i>	Adult	2/102	1.96	1/0.98	1/0.98	—	—	—	—
	Nymph	5/477	1.05	1/0.21	1/0.21	1/0.21	1/0.21	1/0.21	—
	Total	7/579	1.2	2/0.35	2/0.35	1/0.17	1/0.17	1/0.17	—
<i>rrs-rrlA</i>	Adult	1/102	0.98	—	1/0.98	—	—	—	—
	Nymph	11/477	2.31	1/0.21	8/1.68	2/0.42	—	—	—
	Total	12/579	2.1	1/0.17	9/1.55	2/0.35	—	—	—

were obtained for the comparison between the results for nested PCR with primers complementary to *fla* and *rrs* sequences ($P < 0.001$) and for *fla* and the non-coding *rrs-rrlA* region ($P < 0.001$).

The causative agent of Lyme disease, *B. burgdorferi sensu lato*, is genetically highly divergent. Sixteen species within the *B. burgdorferi sensu lato* complex have been described based on their phenotypic and genetic characteristics since the discovery of *B. burgdorferi* in 1982 and its subsequent description (Lünemann & Krause, 2003; Margos *et al.*, 2009). Three of them, *B. burgdorferi sensu stricto*, *B. garinii* and *B. afzelii*, are responsible for most cases of Lyme borreliosis worldwide.

Various phenotypic and genotypic techniques have been used to study variability and to identify species in the *B. burgdorferi sensu lato* complex. As our study shows, the selection of the DNA detection technique and appropriate molecular marker for *B. burgdorferi sensu lato* significantly affects the sensitivity and specificity of the reaction, and therefore is crucial for obtaining the best possible results (Basta *et al.*, 1999; Cinco *et al.*, 1998; Skotarczak *et al.*, 2002; Stańczak *et al.*, 1999).

In nested PCR used for the identification of *Borrelia* spirochaetes, many molecular markers may be used, sequences in both bacterial chromosomes and in plasmids. Until recently, many authors have most commonly applied the fragments of plasmid genes encoding surface proteins, mainly OspA and OspC, but further research has shown too high a variability in these sequences, and thus their limited usefulness in the identification of *Borrelia* spirochaetes. Nowadays, the detection and identification of *B. burgdorferi sensu lato* in ticks is carried out using more conservative genes in the bacterial chromosome. These are, for example, genes encoding antigens, such as *fla*, encoding flagellin, a flagellum protein (Aguero-Rosenfeld, 2003; Cisak *et al.*, 2005; Picken *et al.*, 1996), and DNA encoding rRNA in both ribosomal subunits. This type of DNA belongs to the class of particles with very high conservativeness, especially *rrs* encoding 16S rRNA in the small ribosomal subunit (Fraenkel *et al.*, 2002; Zhang *et al.*, 1993). Modern diagnostics also extensively uses non-coding DNA in the *rrn* operon that contains genes for rRNA and accumulates large differences in sequences between the species of *B. burgdorferi sensu lato* (Liveris *et al.*, 1999; Ranka *et al.*, 2004).

Results obtained in this study indicated that the *fla* gene is the most sensitive molecular marker for the detection of *Borrelia* spirochaetes in *I. ricinus* using the nested PCR technique. Moreover, PCR-RFLP analysis using this gene identified species from the *B. burgdorferi sensu lato* complex, detected co-infections, and identified all the *Borrelia* species carried by this tick species, including *B. miyamotoi* related to the group of relapsing fever borreliae.

rrs appeared much less useful in the detection of *Borrelia* spirochaetes in *I. ricinus*, and less effective in the identification of individual species. Moreover, the detec-

tion of co-infections was not possible when we used this gene. The non-coding region between *rrs* and *rrlA* was a slightly more sensitive molecular marker than *rrs*, but significantly less useful than *fla*. This sequence was the least effective when used for the identification of *Borrelia* spirochaetes. The large number of non-specific products obtained with nested PCR based on this sequence seriously hindered the identification of individual species.

The significantly higher sensitivity of the *fla* gene in the detection of *B. burgdorferi sensu lato* in *I. ricinus*, and more effective identification of individual species than by other DNA markers, is consistent with results obtained in Italy and the Czech Republic (Basta *et al.*, 1999; Cinco *et al.*, 1998). Also Šitum *et al.* (2000), in their analysis of *fla* and *rrs* genes with respect to the detection and identification of *B. burgdorferi sensu lato* species in patients with erythema chronicum migrans, obtained results showing that this flagellin-encoding gene is a more sensitive and specific marker than ribosomal genes. The *rrs* gene, in contrast to *fla*, showed lower sensitivity and specificity in the detection of *B. burgdorferi sensu lato* in ticks in this study, but higher sensitivity in the blood of patients examined in our previous study (Skotarczak *et al.*, 2002). The non-coding region between *rrs* and *rrlA* was a significantly less effective molecular marker for *Borrelia* detection and less useful in the identification of individual species in ticks examined in this study and also in human blood (Skotarczak *et al.*, 2002). However, other authors effectively detected and identified *B. burgdorferi sensu lato* DNA using primers complementary to this sequence, both in lysates from ticks (Ranka *et al.*, 2004) and in skin biopsies and blood from Lyme disease patients (Liveris *et al.*, 1999).

Our results show that the *fla* gene is more accessible than the sequence of the rRNA to primers. This is because within the sequences of rRNA genes there are regions that are mutually complementary, where the annealing of primers is hindered to a considerable extent (Basta *et al.*, 1999; Cinco *et al.*, 1998; Zhang *et al.*, 1993). There is also a hypothesis that the sequences in *rrs* genes and the intergenetic *rrs-rrlA* space are susceptible to damage during extraction. Fragmentation of the DNA chain in the *rrn* operon may lead to the lower detectability of *B. burgdorferi sensu lato* spirochaetes, especially in the analysis of *rrs* (Schwartz *et al.*, 1992). Therefore, the *fla* gene may be deemed an especially sensitive and specific marker for the detection of *B. burgdorferi sensu lato* in tick lysates.

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