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RIBOTYPING AND WHOLE-CELL PROTEIN ANALYSIS OF SPIROCHETES ISOLATED FROM ARTHROPODS IN THE CZECH REPUBLIC

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Abstract: In the presented work, six *Borrelia burgdorferi* sensu lato isolates were recovered from *Ixodes ricinus* ticks and one strain of spirochete with uncertain taxonomic position isolated from *Culex pipiens* mosquito collected in the Valtice area (South Moravia, Czech Republic), as well as representative type strains, were further characterized by ribotyping and whole-cell protein fingerprinting. The ribotype patterns obtained with HindIII and EcoRV ribotype patterns showed good correlations with the recently proposed genotypes and grouped each genospecies in a well-separated cluster. The whole-cell protein profiles of genospecies were more heterogenous than the ribotype patterns. The non-identified spirochetal strain was clearly separated from the *Borrelia* isolates in both methods thus prooving their good capacity to characterize *Borrelia burgdorferi* sensu lato strains.

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Key words: *Borrelia burgdorferi* sensu lato, Czech Republic, ribotyping, whole-cell protein analysis, taxonomy.

INTRODUCTION

Lyme borreliosis (LB) is currently the most common vector-borne disease in Eurasia and North America and represents a new global public health problem [2, 51]. The number of Lyme borreliosis patients is growing throughout the world and the widespread distribution of Lyme borreliosis has become the most prevalent tick-transmitted zoonosis [44]. The causative agents of this disease are several genospecies of *Borrelia burgdorferi* sensu lato (s.l.) [43].

This species complex comprises more than 12 pathogenic and nonpathogenic *Borrelia* species that can be determined on the basis of differences in nucleotide sequences, for example, in rrf-rrl intergenic spacer or in rrs loci [2, 28, 34, 38]. In Europe, seven genospecies have been documented: *B. burgdorferi* sensu stricto (s.s.), *B. garinii*, *B. afzelii*, *B. valaisiana*, *B. lusitaniae*, *B. bissettii* and *B. spielmanii* [20, 38, 46]. Investigations into the geographical distribution of *B. burgdorferi* s.l. in Europe have indicated that *B. garinii* is the most frequently isolated species, followed by *B. afzelii* and *B. burgdorferi* s.s., *B. valaisiana* and *B. lusitaniae* have been detected in *Ixodes ricinus* ticks in a few countries [20], *B. spielmanii* from human skin in several countries in Western and Central Europe [38] and *B. bissettii* in Slovenia [43, 46].

B. burgdorferi s.l. has a complicated life cycle, involving vectors (ticks of the *Ixodes ricinus* complex) and mammalian hosts or birds. The transmission and/or coinfection between borreliae (one or more genospecies) and their vectors occur during the tick attachment and feeding [1, 12, 13,

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14, 32, 41, 51]. In Central and Western Europe, the main vector for *B. burgdorferi* s.l. transmission is *I. ricinus* tick. Additional tick vectors may play a role in the maintenance of *B. burgdorferi* s.l. in enzootic cycles in nature [51]. In the Czech Republic, *B. garinii* and *B. afzelii* were the most frequently isolated genospecies from *I. ricinus* ticks [20]. In South Moravia and Eastern Bohemia, spirochetes and borreliae were isolated rarely also from mosquitoes [18, 19, 39, 53, 54].

Protein profiles of *B. burgdorferi* s.l. are heterogeneous containing more than 100 various proteins, which generate phenotypical variations among *B. burgdorferi* s.l. isolates [51]. *B. burgdorferi* has been shown to alter transcription and protein expression in response to the environment and conditions of cultivation, particularly pH and temperature [10, 37]. Outer surface proteins (Osp) A, B and C are major constituents of the *B. burgdorferi* outer membrane. One of the most immunodominant proteins, particularly in early infection, is 41-kDa flagellin protein [3].

Organization and transcription of rRNA genes have been highly conserved among prokaryotes during their evolution therefore enabling their use as a universal probe [5]. *B. burgdorferi* has a unique organization of rRNA genes. There is a single copy of rrs and two copies of rrl and rrf genes which are tandemly repeated in this spirochete [16, 42].

The southern Moravian region near the town Valtice represents a suitable habitat for ixodid ticks which are also frequently infected with borreliae [e.g. 21–24].

The aim of the present study was to characterize the Moravian isolates with regard to their phenotypic and genotypic characteristics by ribotyping and whole-cell protein analysis. Fatty acids profiles of the spirochetal strains isolated from arthropods collected in Moravia were published earlier [11]. Information about the diversity of *B. burgdorferi* s.l. and non-identified spirochetal isolates from arthropods collected in the environment of the Czech Republic can be important for the understanding of epidemiology and diagnostics of Lyme borreliosis.

MATERIAL AND METHODS

Bacterial strains and culture conditions. Spirochetal isolates from arthropods collected in South Moravia (Czech Republic) are listed in Table 1. Tick-isolates belonging to the *B. burgdorferi* s.l. group were confirmed as *Borrelia* species and identified to the species level according to Postic *et al.* [33]. Strain BR91 isolated from mosquito allowed no bands [33] characteristic for *Borrelia* spp. and was therefore assigned as up to now non-identified spirochete. *B. burgdorferi* s.l. type strains were chosen (Tab. 1) to compare the validity of analyses.

All spirochetal strains were grown in BSK-H medium (Sigma, USA) supplemented with 6% rabbit serum (Gibco, USA). The medium was also supplemented by rifampicin (50 μg/ml; Sigma) and phosphomycin (100 μg/ml; Sigma). Spirochetes were harvested by centrifugation at 8,500x g for 30 min when grown to a highly motile condition as determined by dark field microscopy. Bacterial cells were washed three times and finally resuspended in phosphate-buffered saline (PBS; pH 7.4; Oxoid, UK).

Table 1. Spirochetes used in this study.

Strain	Source[a] /country	Species designation
B31T	*Ixodes scapularis*/Shelter Island, NY, USA	*B. burgdorferi* sensu stricto
BR194	*Ixodes ricinus*, F/Valtice, Czech Republic	*B. burgdorferi* sensu stricto
20047T	*Ixodes ricinus*, N/Brittany, France	*B. garinii*
BR14	*Ixodes ricinus*, N/Valtice, Czech Republic	*B. garinii*
BR92	*Ixodes ricinus*, F/Valtice, Czech Republic	*B. garinii*
BR122	*Ixodes ricinus*, F/Valtice, Czech Republic	*B. garinii*
BR132	*Ixodes ricinus*, F/Valtice, Czech Republic	*B. garinii*
VS461T	*Ixodes ricinus*, F/Valais, Switzerland	*B. afzelii*
BR130	*Ixodes ricinus*, F/Valtice, Czech Republic	*B. afzelii*
VS116 T	*Ixodes ricinus*, F/Valais, Switzerland	*B. valaisiana*
BR91	*Culex* (*Culex*) *pipiens*, F/Valtice, Czech Republic	non-identified spirochete

[a] F – females, N – nymphs; T type strain.

DNA isolation and ribotyping. Bacterial cells were lysed and DNA was extracted by the method described by Le Febvre *et al.* [27] with a few modifications, briefly: Cell lysis solution was amended with 10 ng/ml of RNAase (Sigma) and proteinase K (5 ng/ml; Sigma) and the mixture incubated 30 min at 37°C. Proteins were removed by two subsequent phenol:chloroform:isoamylalcohol (25:24:1) extractions and obtained DNA precipitated by absolute ethanol and dissolved in TE buffer (10 mM Tris, Sigma; 1 mM EDTA, Sigma; pH 8.0). Ribotyping with a digoxigenin-labeled probe made complementary to *E. coli* 16S and 23S rRNA was carried out as described previously [47]. Briefly, all strains were separately digested with two restriction enzymes, EcoRV (TaKaRa, Japan) and HindIII (TaKaRa), according to the conditions recommended by the manufacturers. Horizontal electrophoresis was performed in 0.6% agarose gel in 1% TBE buffer. λDNA cleaved by EcoRI (TaKaRa) and HindIII used as a molecular weight marker. The DNA fragments were transferred to a membrane (BiodyneB 0.45 μ; Pall, USA) and hybridized at 56°C. Band-pattern analysis and consequent cluster analysis were carried out using GelCompar 4.1 software (Applied-Maths, Belgium). Dendrograms were calculated by the UPGMA clustering method using Dice coefficients.

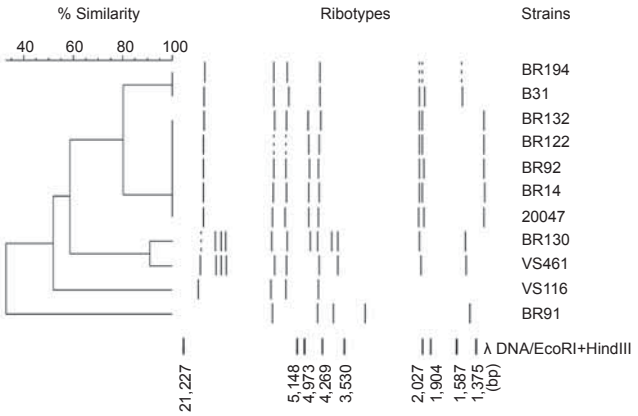

Figure 1. Ribotype patterns obtained with HindIII restriction enzyme. The dendrogram was calculated with Dice coefficient using UPGMA clustering method.

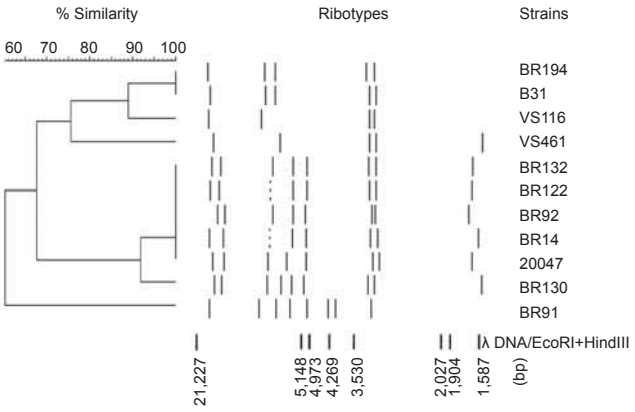

Figure 2. Ribotype patterns obtained with EcoRV restriction enzyme. The dendrogram was calculated with Dice coefficient using UPGMA clustering method.

Whole-cell protein fingerprinting. For protein analysis, whole-cellular debris of cultured spirochetes were used. Spirochetal cells were incubated and harvested as described above. Whole-cell samples of all strains were boiled for 10 min in sample buffer containing 2% SDS (Sigma) and 5% mercaptoethanol (Sigma). Electrophoresis of whole-cell lysates (1.25 μg of protein per lane) was performed in a discontinuous buffer system using 12% polyacrylamide gels, as described by Pot *et al.* [35], with the application of Maxigel System (Biometra, Germany). Weight Range SigmaMarker (Sigma), containing proteins of defined molecular mass (200, 116, 97, 66, 55, 45, 36, 29, 24, 20, 14.2 and 6.5 kDa), was used as a molecular weight marker. Protein fingerprints were visualized by staining with 0.1% silver nitrate (Sigma), as described by Kirkeby *et al.* [26]. The Bionumerics v. 4.601 software (Applied-Maths, Belgium) was used to analyze the protein banding patterns of the densitometric traces. Dendrogram was calculated with the Pearson product moment correlation coefficients (r) using the unweighted pair group method with arithmetic averages (UPGMA).

RESULTS

Ribotype patterns obtained with HindIII (Fig. 1) clustered all strains into a few well-separated groups. Ribotype patterns consisted of 4–12 bands ranging from 1,270–19,111 bp. *B. burgdorferi* s.s. as well as *B. garinii* strains revealed identical ribopatterns containing 7 and 8 bands, respectively. Hybridization profiles of two *B. afzelii* strains revealed differences in two fragments. *B. valaisiana* showed four bands. The non-identified strain BR91 was clearly separated from all *Borrelia* strains.

Ribotypes obtained with endonuclease EcoRV (Fig. 2) divided all strains into five clusters in accordance with their taxonomic position. Ribotype patterns comprised 4–9 bands ranging from 1,477–18,881 bp. Both *B. burgdorferi* s.s. strains produced identical patterns containing 5 fragments. All strains belonging to *B. garinii* generated the same pattern (8 fragments). *B. valaisiana* revealed four bands. Great differences were observed between *B. afzelii* strains. Hybridization profile of type strain VS 461 showed 5 bands, whereas strain BR130 showed 9 bands. The non-identified strain BR91 remained unclustered, similarly as with HindIII endonuclease.

Figure 3. Whole-cell protein profiles of analyzed strains. The dendrogram was calculated with Pearson correlation coefficient using UPGMA clustering method. Molecular weight standard: 116; 97; 66; 55; 45; 36; 29; 24; 20; 14.2 and 6.5 kDa.

Whole-cell protein fingerprints of analyzed strains obtained with SDS-PAGE separated analyzed strains into species-specific clusters (Fig. 3). The whole-cell protein profiles of all *Borrelia* isolates showed two basic patterns of low-molecular-mass major proteins (range 30–36 kDa): the outer surface protein bands at 34–36 kDa. A major protein at 31–32 kDa was not expressed by the non-identified strain BR91. The third major band was at 41 kDa (probably flagellin). A higher number of bands in the area between 30–55 kDa were observed in the profile of all tested *Borrelia* strains. Protein profiles revealed much higher heterogeneity in the area at and below 49 kDa and between 70–80 kDa, and for bands higher than 90 kDa. A certain degree of variability was evident in protein size and levels of expression for some isolates: B31 and 20047 differed in the relative migration of the protein band found at 30 instead of 32 kDa, and strain 20047 displayed higher expression of the 22 kDa protein. Non-identified strain BR91 did not display protein bands between 29–36 kDa.

DISCUSSION

The present study demonstrates some phenotypic and genotypic characteristics of *B. burgdorferi* s.l. strains isolated from arthropods collected in the Valtice region (South Moravia, Czech Republic). Identification of spirochetes which were isolated from ticks in this area revealed the presence of *B. burgdorferi* s.l., namely the genomic species *B. garinii* and *B. afzelii* [21]. Ribotyping was carried out to confirm identification of genospecies obtained by restriction fragment length polymorphism of rrf (5S)-rrl (23S) intergenic spacer amplicons, as well as to investigate intraspecies variability. Previously, various restriction endonucleases were successfully used for the identification of *B. burgdorferi* s.l. at the genospecies level, for example, EcoRI, EcoRV, HincII, HindIII, PstI [51]. Baranton *et al.* [4] recommended the use of EcoRV and HindIII, two enzymes which gave more reliable results for species identification than other enzymes. In our study, we confirmed these results, after digestion with HindIII, ribotypes of *Borrelia* type strains and strains isolated in South Moravia clustered in accordance with the type strains. Dendrogram based on HindIII ribotypes of *Borrelia* strains confirmed the distant relationship of *B. garinii* to *B. burgdorferi* s.s. strains. *B. afzelii* strains fell into a separate cluster, similarly as *B. valaisiana* strain. The non-identified spirochetal strain remained unclustered.

Ribotyping showed that all *Borrelia* strains formed 3.2-kb EcoRV and 2.1-kb HindIII restriction fragments. Similar data were previously reported by other authors [4, 16, 17, 29, 50]. In our study, we observed, similar to Wang *et al.* [50], species-specific fragments after HindIII cleavage: 1.45-kb for *B. burgdorferi* s.s., 1.2-kb for *B. garinii*, 4.0- and 1.45-kb for *B. afzelii*. Various ribotypes within *B. afzelii* strains were obtained by EcoRV digestion. *B. afzelii* strains differed for bands in the area between 3.2- to 3.4-kb and in patterns between 5.0–6.5-kb. Although no heterogeneity in the ribotype patterns of this genospecies has been previously reported in the literature, we found some variability. The strains of the same genospecies exhibited different ribotypes. The reason could be found in the different geographic areas where analyzed strains were isolated (strain BR130 from South Moravia, strain VS461 from Switzerland).

In some cases, ribotyping could represent a suitable and reliable method in epidemiological studies of *B. burgdorferi* s.l., but the selection of suitable restriction enzymes, as well as the use of an appropriate probe appears to be crucial. Probably, more restriction enzymes have to be used, as suggested in Baranton *et al.* [4] and Fukunaga *et al.* [15, 16] or ribotyping should be combined with some other method – for example, PCR-RFLP analysis as described by Postic *et al.* [33] to obtain reliable results for epidemiological studies.

The analysis of whole-cell protein patterns obtained with SDS-PAGE has proved to be reliable for comparing and grouping a large number of bacterial strains [35]. Obtained whole-cell protein fingerprints separated analyzed borreliae into individual species-specific clusters; however, they generally differ in the amount of the proteins and the molecular weight of the proteins they express. SDS-PAGE results demonstrated that strong 22- to 23 kDa bands probably represented OspC [49] and bands in the 30- to 35 kDa range OspA – OspB [7, 31]. In our study, a very strong band was found in the area between 22–23 kDa in *B. garinii* type strain 20047 and the same strong bands were also observed in *B. valaisiana* and *B. afzelii* strains. The heterogeneity of antigen expression might also be important in the virulence and pathogenesis of borrelial infections. Differences in the expression of proteins may indicate distinct organotropism of strains, but the exact role of these proteins in the virulence of the Lyme borreliosis causative agent remains to be determined.

Quantitative variations in the outer surface proteins during tick infections have been frequently reported [49]. Growth of borreliae *in vitro* along the growth curve or repeated passaging of a culture in BSK medium may cause changes in expression of certain proteins [8, 36, 40]. Different expression levels of some proteins may be partly due to the changes in temperature or in pH of their environment [9, 10, 30, 37, 45], but there are also some data available evidencing genetic and antigenic stability during a long-term cultivation, and probably also during a persistent infection [6]. These changes may be explained by the loss of plasmids, particularly the linear ones [48]. Variations between strains were also described earlier [25, 52]. In our study, quantitative variations among strains were observed, especially between *B. afzelii* isolates, as demonstrated by SDS-PAGE. The existence of local variations in the genomic species distribution in the Czech Republic given by the type of reservoirs is possible; thus, there may exist the dominant circulation of these genospecies in the

Valtice area. Such a result is in agreement with statements of other investigators [49]. In contrast, Janouškovcová *et al.*, [25] found higher variability in protein profiles among *B. garinii* strains.

CONCLUSION

In general, our analyses of borreliae isolated from haematophagous arthropods in South Moravia coincided with previously published data, and proved that ribotyping could be a reliable method in epidemiological studies of borreliae [4, 16]. Despite the detected protein pattern variability in strains representing three genomic groups, ribotyping clarified their taxonomic positions and confirmed their identification based on other genotypical and phenotypical data.

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