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MOLECULAR CHARACTERIZATION OF BORRELIA BURGDORFERI SENSU LATO STRAINS ISOLATED IN THE AREA OF BELGRADE, SERBIA

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ABSTRACT

This is the first report of the molecular characterization and identification of *Borrelia burgdorferi sensu lato* strains isolated in Serbia. Isolates A1, A2 and M1, from *Ixodes ricinus*, belong to *Borrelia burgdorferi sensu stricto*, while isolate K1 from *Apodemus flavicollis* is a mixture of *Borrelia afzelii* and *B. burgdorferi s.s.*

Key words*: Borrelia burgdorferi s.l., Ixodes ricinus*, Lyme borreliosis, Serbia

Lyme borreliosis is a multisystemic disorder caused by *Borrelia burgdorferi sensu lato* (*s.l.*) (2,14). *Borrelia burgdorferi s.l.* have been classified into at least 11 species (8) that are transmitted by the *Ixodes* species (*Ixodes ricinus, I. persulcatus, I. scapularis*, etc.) of hard ticks. Lyme borreliosis is a widely distributed zoonosis, and the *Ixodes ricinus* tick is the prominent vector for transmitting 5 species, *Borrelia burgdorferi sensu stricto* (*s.s.*), *Borrelia garinii*, *Borrelia afzelii*, *Borrelia lusitaniae* and *Borrelia valaisiana*, in Europe (15). Among these, *B. burgdorferi s.s.*, *B. afzelii* and *B. garinii* are pathogenic to humans. Concerning the incidence of Lyme borreliosis in the European countries neighboring Serbia, 120 cases/100.000 and 130/100.000 were reported in Slovenia and Austria, respectively (9). Similarly, Lyme borreliosis patients reported in 2003 numbered more than 800 in Serbia. Lyme borreliosis agents were isolated in most European countries included the neighboring countries, Croatia (12), Bulgaria (3), and Turkey (5). In Serbia, *B. burgdorferi s.l.* was first isolated in 1992 from the spleen of an *Apodemus flavicollis* mouse from the region of Belgrade's recreational park Kosutnjak, and was named K1 (4,13), but detailed identification of the isolate has not been performed. The aim of this study was to characterize the *B. burgdorferi s.l.* isolated from ticks and mammals in the area of Belgrade, the capital of Serbia.

Ticks were collected with 1 m^2 flannel flags from vegetation in Belgrade areas including recreational parks (Kosutnjak and Hyde Park), grass surfaces in Belgrade's settlements (Zvezdara, Banjica, and Miljakovac), and the mountain slopes of Avala. Ticks were dissected in biological safety cabinets, and the midgut tissues were inoculated into 5 ml of Barbour-Stonner-Kelly (BSK) II medium (1) and incubated at 33ºC for 3 months. The cultures were examined under dark-field microscopy. Three isolates from *I. ricinus* ticks from the regions of Avala (strains A1, A2) and Miljakovac (strain M1), and strain K1 previously isolated from *A. flavicollis* were used for further analyses.

Aliquots (0.5 ml) of cultures in the exponential growth phase were washed and the cells were resuspended in 100 µl of water. The resultant cell suspensions were boiled at 100ºC for 10 min in 1% Triton-X 100 solution. The supernatants were used as templates for subsequent PCR. PCR was performed by a previously described method (7). The intergenic spacer and flagellin gene (*flaB*) sequences of the isolates were amplified with primers corresponding to the 3' end of 5S rDNA (*rrf*) (5'-CTG CGA GTT CGC GGG AGA-3') and the 5' end of 23S rDNA (*rrl*) (5'-TCC TAG GCA TTC ACC ATA-3') (13), primer A corresponded to the 5' end of *fla* (5'-TCT GAT GAT GCT GCT GCT GGT ATG G-3') and primer D corresponded to the 3' end of *fla* (5'- TCC AAA AGT TAT CGT ATG AG-3') (6) , respectively.

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RFLP analysis of the intergenic spacer amplicon was accomplished by digestion of the PCR amplicon with *Mse*I and *Dra*I, as described previously (11). The PCR amplicon was cloned into the pGEM-T vector Easy vector system (Promega, Madison, WI, USA) according to the manufacturer's instructions and recombinant plasmid was transformed into *Escherichia coli* JM109. The recombinant plasmids from at least three *E. coli* clones were used for DNA sequencing with M31 (-29) and M13 reverse primers. A DNA cycle sequencing reaction was performed using a Thermo Sequenase pre-mixed cycle sequencing kit (Amersham, Piscataway, NJ) and a model SQ5500EL DNA-sequencer (Hitachi, Tokyo, Japan). Based on the determined DNA sequence of the amplicon, the RFLP pattern of the 5S-23S rDNA intergenic spacer was determined. CLUSTAL-X software was used to align the sequences, and the phylogenetic distance was calculated by the neighbor-joining method. A phylogenetic tree was drawn using N-J plot software (http://bips.u"strasbg.fr/fr/Documentation/ClustalX/).

B. burgdorferi s.l. was first isolated in Serbia in 1992 from an *A. flavicollis* mouse from the region of Kosutnjak recreational park, and was named K1 (4). In the same year, *B. burgdorferi s.l.* was isolated from an *I. ricinus* tick in the region of Kosutnjak and Zvezdara. However, the cultivation of these isolates was not successful. During 2002, *B. burgdorferi s.l.* was isolated from *I. ricinus* ticks in the regions of Avala (strains A1 and A2) and Miljakovac (strain M1). A specific amplicon was obtained from the cultures of all analyzed isolates after 5S-23S rDNA intergenic spacer specific-PCR. Based on 5S-23S rDNA intergenic spacer RFLP analysis, it was concluded that isolate K1 was a mixture of *B. burgdorferi s.s.* (sequence K1-1) and *B. afzelii* (sequence K1-2), while strains A1, A2, and M1 belonged to *B. burgdorferi s.s.* (Table 1). Sequencing analysis of 5S-23S rDNA intergenic spacer and *fla B* gene pointed to the same conclusions according to A1, A2, and M1 strains, but there were some dilemmas with K1 strain. On the phylogenetic tree constructed from 5S-23S rDNA intergenic spacer sequences, isolate K1 was clustered in *B. burgdorferi s.s.* (sequence K1-1) and *B. afzelii* (sequence K1-2), while on the phylogenetic tree constructed from *fla B* gene sequences, isolate K1 belonged to *B. afzelii* (Fig. 1). The problem with strain K1 could be explained by the fact that the wild mouse was infected with both *Borrelia* species and during *in vitro* cultivation *B. afzelii* became dominant. Physicians are faced to the significant problem that one tick can be coinfected with several *Borrelia* species, and with other microorganisms, which presents an additional problem for the differential diagnosis of Lyme borreliosis (9).

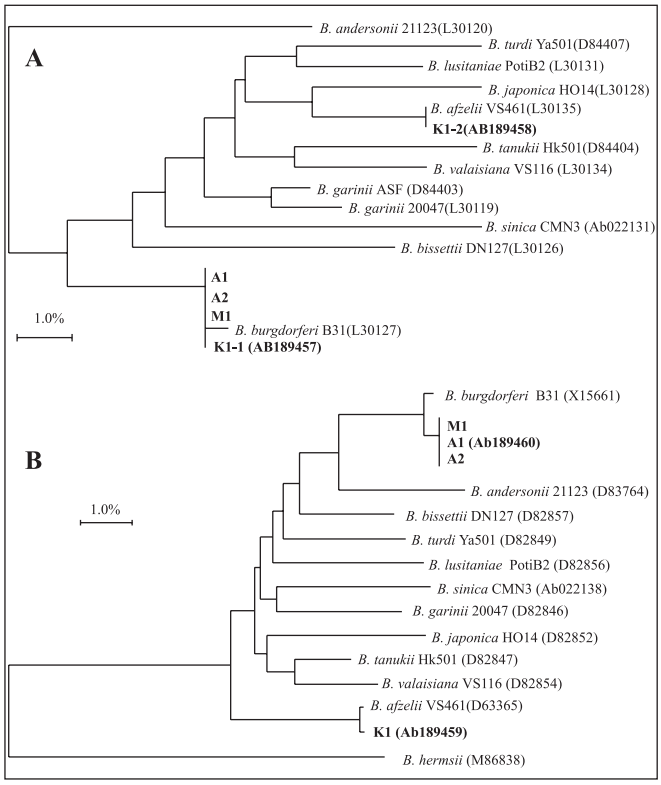

Figure 1. Phylogenetic tree based on the 5S-23S rDNA intergenic spacer sequence (A) and flagellin gene sequence (B). Sequence accession numbers are shown in parentheses. Bar, 1.0% sequence divergence. Isolates from Serbia are in bold.

Table 1. RFLP analysis of the 5S-23S rDNA intergenic spacer.

Strain	*Dra*I	*Mse*I	Amplicon (bp)	Species
K1-1	144, 53, 29, 28	107, 52, 38, 29, 28	254	*B. burgdorferi*
K1-2	173, 52, 20	106, 68, 51, 20	245	*B. afzelii*
A1	144, 53, 29, 28	107, 52, 38, 29, 28	254	*B. burgdorferi*
A2	144, 53, 29, 28	107, 52, 38, 29, 28	254	*B. burgdorferi*
M1	144, 53, 29, 28	107, 52, 38, 29, 28	254	*B. burgdorferi*
B31	144, 53, 29, 28	107, 52, 38, 29, 28	254	*B. burgdorferi*
VS461	174, 52, 20	107, 68, 51, 20	246	*B. afzelii*
20047	201, 52	107, 95, 51	253	*B. garinii*
VS116	203, 52	174, 51, 23, 7	255	*B. valaisiana*
PotiB2	145, 83, 29	107, 82, 39, 29	257	*B. lusitaniae*

Exact fragments sizes were determined based on the results of sequencing analysis.

Research carried out on Lyme disease in the territory of Serbia so far pointed to the species *Ixodes ricinus* L. (*Acari: Ixodidae*) as a source, vector and reservoir in the epizootic process of *Borrelia burgdorferi*. The species *Ixodes ricinus* is

predominant in material originating from Serbia. Moreover, this species is one of the most widely distributed in Serbia (10). On the basis of our findings we concluded that the *I. ricinus* ticks in our area are infected with *B. burgdorferi s.s.* The isolation of *B. afzelii* is strongly connected with the frequent appearance of the skin manifestation of Lyme borreliosis in our area. Although isolation of *B. garinii* has still not been achieved in our region, neuroborreliosis is not a rare manifestation of Lyme disease in Serbia. Further field surveying is needed for clarification, as it is clear that genotypic and phenotypic heterogeneity of *Borrelia burgdorferi* strains have ecological, epidemiological, clinical and microbiological significance.

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RESUMO

Caracterização molecular de cepas de *Borrelia burgdorferi sensu lato* isoladas na região de Belgrado, Sérvia

Trata-se do primeiro relato de identificação e caracterização molecular de cepas de *Borrelia burgdorferi sensu lato* isoladas na região de Belgrado, Sérvia. As cepas A1, A2 e M1, isoladas de *Ixodes ricinus*, pertencem à *Borrelia burgdorferi sensu stricto*, enquanto a cepa K1, isolada de *Apodemus flavocollis* é uma mistura de *Borrelia afzelii e B. burgdorferi s.s.*

Palavras-chave: *Borrelia burgdorferi s.l., Ixodes ricinus, Lyme borreliosis,* Sérvia

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