

Detection and Genotyping of *Borrelia burgdorferi sensu lato* by Polymerase Chain Reaction

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Aim. To isolate and genotype *Borrelia burgdorferi* genospecies in serum samples of Croatian patients with erythema migrans.

Methods. DNA isolates from sera of patients with erythema migrans were analyzed by nested polymerase chain reaction (PCR), amplifying a segment of flagellin gene with primers encompassing the conserved region of the gene. To screen PCR products for heterogeneity, we performed single-stranded conformation polymorphism (SSCP) analysis. The samples showing differences in SSCP patterns were sequenced, and the sequence compared in the GeneBank for sequence homology with known *Borrelia burgdorferi* genospecies. We also constructed phylogenetic tree of all known borrelial sequences.

Results. The nested PCR method using specially designed flagellin gene primers, achieved the sensitivity of 10 genome copies (0.01 pg of purified *Borrelia burgdorferi* DNA from culture) by dilution analysis. The assay specificity was confirmed by amplification of a part of the flagellin gene from different bacterial species. The primer pairs successfully amplified only *Borrelia burgdorferi* flagellin gene. The genome of *Borrelia burgdorferi sensu lato* was detected in the sera of all 10 tested patients with erythema migrans. Sequence data and phylogenetic analysis confirmed that all amplified samples belonged to *Borrelia afzelii* genospecies.

Conclusion. Phylogenetic tree analysis placed the borrelial isolates together with *Borrelia afzelii* sequences into a single group. This finding was additionally supported by sequence homology analysis, which produced a homology score of 99%. In patients with erythema migrans who come from the northwest Croatia, an endemic area for Lyme borreliosis, *Borrelia afzelii* was the cause of skin manifestations of Lyme borreliosis.

Key words: bacterial gene proteins; bacterial typing techniques; biotyping, bacterial; *Borrelia burgdorferi*; Croatia; erythema chronicum migrans; flagellin; gene products, bacterial; polymerase chain reaction; nested PCR

Borrelia burgdorferi (*B. burgdorferi*), a spirochete mostly transmitted by ticks of the Ixodidae family, causes Lyme borreliosis, a multisystemic disease that affects the skin, joints, central nervous system, and heart (1-3). Atypical and most common skin manifestation of Lyme borreliosis is erythema migrans, whereas neurologic symptoms, joint involvement, and chronic skin alterations can develop at later stages of the disease (4,5).

B. burgdorferi contains more than a hundred of various proteins, which generate phenotypical variations of *B. burgdorferi* isolates (6). The genetic variation of *B. burgdorferi* is reflected in the macrorestriction pattern of linear chromosomes (7), and polymorphism of the 16 S rRNA and 5S-23S rRNA genome regions (8). Analysis of the *B. burgdorferi* protein composition by two-dimensional electrophoresis, restriction of rRNA genes (9,10), and monoclonal antibody reactivity revealed 10 genomic groups to date (11-13). In Eurasia, they are *B. burgdorferi*

sensu stricto, *B. garinii* (group 20047) (9), *B. afzelii* (group VS461) (9,10), *B. valaisiana* (groups VS116 and M19) (8,14), *B. lusitaniae* (group PotiB2) (8,15), and *B. japonica* (group F63B) (8,16). In North America they encompass *B. burgdorferi sensu stricto* (7), *B. andersoni* (groups 21123 and 21038) (8,17-21), and *B. bissetti* (groups DN 127, CA55 and 25015) (17-22).

The classification of *B. burgdorferi sensu lato* into genomic groups has a clinical relevance for Lyme borreliosis in Europe. The association of *B. garinii* with neurologic symptoms, *B. afzelii* with skin manifestations, and *B. burgdorferi sensu stricto* with arthritis has been demonstrated in a number of studies (17,20,23). It seems that *B. japonica* causes no disease in humans (24), whereas the pathogenetic potential of *B. valaisiana*, *B. lusitaniae*, and *B. andersonii* has not yet been defined.

The genome of the genus *Borrelia* is organized in the linear central DNA and a linear plasmid (25). This is rarely seen among eubacteria, which are generally characterized by circular DNA (26). Genetic restriction maps have been designed and numerous genes isolated for most *Borrelia* strains. To date, 162 genes have been identified. All *B. burgdorferi sensu lato* species have two proteins of a constant molecular mass of about 40 kDa (p60). In 1988, Hansen et al (27) described protein p60 as an antigen of broad cross-reactivity. Protein p41 (p23) or flagellin, is one of the most numerous antigens and the main flagellum constituent localized in the periplasmic flagellum, below the external membrane (28).

Antibodies to flagellar antigen appear soon after infection, so that flagellin antigen is convenient for immunodiagnostic tests (29). Although variations have been found among different genomic groups, sequence variation of flagellin in *B. burgdorferi sensu lato* was 94-99% and in other *Borrelia* strains from 85 to 93% (30). Accordingly, flagellin appears to be genus specific.

In Croatia, *B. burgdorferi* was first isolated in 1991, at the Department of Dermatovenerology, Zagreb University Hospital Center, from the skin of a patient with erythema migrans, and was named P1 Zagreb. Electrophoretic analysis of the *B. burgdorferi* proteins showed six most important proteins of different molecular mass (OspA, OspB, OspC, p41, p60, and p100), classifying the isolate to *B. burgdorferi sensu lato* group (31).

In the northwest Croatia, an endemic area for Lyme borreliosis, four genomic *B. burgdorferi sensu lato* groups were identified in the *Ixodes ricinus* ticks: *B. afzelii*, *B. garinii*, *B. valaisiana* (group VS116), and *B. burgdorferi sensu stricto* (32).

Low number of *B. burgdorferi* in pathologic lesions and tissue fluids, low antigen level, and the ability of *B. burgdorferi* to escape the host's immune response result in slow, poor, and unreliable formation of specific antibodies (33). That is why the use of polymerase chain reaction (PCR) has proved very useful in the diagnosis of Lyme borreliosis (34-36).

In patients with Lyme borreliosis-associated skin alterations (erythema migrans and acrodermatitis chronica atrophicans), borrelia DNA was detected in 75-92% of skin biopsies (37) and in the serum (38). PCR is also used for *B. burgdorferi sensu lato* determination, together with addi-

tional methods such as hybridization, sequence specific primers (SSP) amplification, and DNA restriction fragment length polymorphism (RFLP) (20,39-41). Direct sequencing of particular genes is a highly precise and accurate technique of genotyping *B. burgdorferi sensu lato* isolates.

The aim of this study was to isolate and genotype *B. burgdorferi* genospecies in serum samples of patients with erythema migrans from the northwest Croatia, an endemic area for Lyme borreliosis.

Material and Methods

The study was carried out at the Department of Dermatovenerology, Zagreb University Hospital Center, and Croatian Institute of Transfusion Medicine in Zagreb. The study included analysis of sera from 40 patients with erythema migrans. Bacterial strains used for PCR specificity testing were obtained by the courtesy of Professor Z. Modrić from the Institute of Microbiology, Zagreb University School of Veterinary Medicine. *B. burgdorferi sensu lato* isolated and cultured at the Department of Dermatovenerology, Zagreb University Hospital Center, was used as a positive control.

DNA Isolation

One milliliter of serum was centrifuged for 10 min at 15,000 rpm at room temperature to concentrate the fraction with bacteria. The precipitate was washed with 1 ml PBS buffer, pH 7.0, and centrifuged at 12,000 rpm at room temperature for 5 min. To the precipitate 200 µL buffer II (100 µL NaCl 5 mol/L; 150 µL 10% sodium dodecyl sulfate; 2750 µL TE buffer, pH 8.0) and 10 µL proteinase K (10 mg/mL), were added and incubated for 2 h at 50 °C and for 10 min at 95 °C. Upon cooling down, 400 µL of a mixture of phenol, chloroform, and isoamyl alcohol were added and stirred for 5 min, then centrifuged for 10 min at 12000 rpm at room temperature. The aqueous phase was separated and DNA precipitated with a double volume of 100% ethanol at -20 °C overnight. DNA precipitate was washed with 70% ethanol, dried in a vacuum centrifuge for 5 min, dissolved in 30 µL water with 1 h incubation at 37 °C, and stored at 4 °C.

Primer Design

Two segments of the *Borrelia* species genome, one encoding ribosomal 16S RNA and chromosomal gene for flagellin protein, were chosen as targets of amplification.

The segment of ribosomal 16S RNA gene is highly preserved and broad specific (BS) for eubacteria, and the selected primers have been described in the literature (34).

The primers for BS 16S RNA gene amplified a region of 215 base pairs (Table 1). This amplification was used to control the yield and quality of DNA isolation (34).

The primers for flagellin gene were created on the basis of multiple comparison of the sequences obtained from GenomeNet-DNA Database of the National Institute of Genetics, Kyoto, Japan, using the BLAST software for an optimal comparison of two sequences and the ClustalW software for multiple sequence comparison, which also provides output data for phylogenetic analysis based on sequence variation (42).

In order to increase the method sensitivity, nested PCR, requiring the synthesis of two primer pairs, external and internal, was chosen. The external and internal primer sequences, shown in Table 1, generated a final PCR product of 535 bp.

DNA Amplification

The final components of the PCR included 50 mmol/L KCl; 10 µmol/L tris (hydroxymethyl) aminomethane pH 8.3; 1.5 µmol/L MgCl₂; 0.1% (wt/vol) gelatin; 100 µmol/L each dATP, dGTP, dCTP and dTTP; 2.5 U Amplitaq DNA Polymerase (Perkin-Elmer, Branchburg, USA), and 20 pmol of each primer.

The following conditions were used for amplification of ribosomal 16S RNA gene: initial denaturation at 94 °C for 3 min, denaturation at 94 °C for 30s, annealing at 42 °C for 30s and extension at 72 °C for 30s for 35 cycles, and final extension at 72 °C for 7 min, on the thermal cycler GeneAmp 2400 (Perkin-Elmer, Branchburg, NJ, USA).

The conditions for flagellin gene amplification were: initial denaturation at 94 °C for 3 min, denaturation was performed at 94 °C for

Table 1. Primers used in the study

Flagellin primers^a

BAF 51 5' – GCTGCTGGCATGGGGTTTCT -3'
(128-149)

BAF 31 5' – CAATAGCATACTCAGTACTATTCT -3'
(832-856)

BAF 52 5' – CAGACAACAGAAGGGAATTTAAATG -3'
(219-244)

BAF 32 5' – CAAGTGATGTATTAGCATCAACTG -3'
(729-754)

16 S rRNA primers^b

BS1 5' – GAGGAAGGTGGGGGATGA - 3'

BS2 5' – GCCCGGAACGGATTAC - 3'

^aThe size of the amplification product was 728 bp for the first amplification, and 535 bp for the second amplification.

^bThe size of the amplification product was 215 bp.

30s, annealing at 50 °C for 30s, extension at 72 °C for 30s, 30 cycles, and final extension at 72 °C for 7 min.

As the flagellin gene was submitted to double amplification, 5 µL of the first PCR products were used in another round of amplification with new internal primers, under the same amplification condition.

To test the specificity of PCR primers used in this study, DNA isolates from the following bacterial strains were tested: *B. burgdorferi sensu lato* and *B. afzelii*, *Staphylococcus aureus*, *Enterococcus* sp., *Staphylococcus epidermidis*, *Sarcina lutea*, *Klebsiella oxytoca*, *Escherichia coli*, *Proteus mirabilis*, *Yersinia* sp., *Salmonella enteritidis*, *Shigella sonnei*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Bacillus cereus*, *Treponema pallidum*, and *Leptospira interrogans*.

For PCR analysis, blank controls containing 10 µL of water substituted for DNA were used. As a positive control, *B. burgdorferi sensu lato* DNA isolated from culture of the control strain was run in parallel with each amplification assay.

Product Detection

For the detection of PCR products, 2.5% agarose gels in TBE buffer with ethidium bromide (2 µL/100 mL buffer) were used. The gel was photographed by a Polaroid camera.

Single-Stranded Conformation Polymorphism (SSCP) Analysis

SSCP analysis (43) was performed using a 10% MiniClean gel system (Pharmacia, Uppsala, Sweden) under conditions recommended by the manufacturer. Specific PCR product (4-6 µL) was mixed with 2 volumes of formamide-dye solution (88% formamide, 10 mmol/L EDTA, 0.01% xylene cyanol), heat-denatured at 95 °C for 5 min, and cooled rapidly in ice water (0 °C, 5 min). Eight microliters of the dena-

tured DNA solution was loaded onto a 10% polyacrylamide MiniClean gel. The electrophoresis conditions were 10 min at 200 V, 10 mA, 5 W and 2 h at 375 V, 15 mA, 10 W, all at 15 °C. The gels were silver stained (43,44).

Subcloning of PCR Products

PCR products were separated by agarose gel electrophoresis using low melting point agarose (Gibco-BRL, Eggenstein, Germany). Gel slices containing flagellin fragments of the expected size were excised and agarose pieces removed by microcentrifugation. The supernatant was used for subcloning with the aid of a Sure-Clone ligation kit (Pharmacia Biotech, Freiburg, Germany). Competent *Escherichia coli* XL1-Blue bacteria were used for transfection. Plasmid DNA was isolated using a Qiagen Plasmid isolation kit (Qiagen GmbH, Hilden, Germany).

Sequencing

PCR products of the flagellin gene were sequenced by the dideoxy chain-termination method (45) using an AutoRead sequencing kit (Pharmacia Biotech) and fluorescent universal and reverse primers annealing to the multiple cloning sites of the pUC18 vector. Sequencing was carried out by on a A.L.F. DNA sequencer (Pharmacia Biotech).

Computer Analysis

The data obtained by the reading of the sequence reaction were processed on the appropriate Apple Macintosh computer. The sequences were analyzed using the MacVector program (International Biotechnologies, Tecnomara, Fernwald, Germany) and the Genetics Computer Group sequence analysis software package (version 7.2, 1992; Genetics Computer Group, University Research Park, Madison, USA).

In the ClustalW program, we combined the sequences obtained with other flagellin gene sequences derived from the GeneBank.

The Drawtree program was transferred from the software base along with the complete PHYLIP software (Phylogeny Inference Package), available free over the Internet, distributed by the author J. Felsenstein, Department of Genetics, University of Washington, Seattle, USA, for the genome phylogenetic analysis. All sequences of the genus *Borrelia* were used (*B. afzelii*, *B. andersonii*, *B. anserina*, *B. barbouri*, *B. burgdorferi sensu stricto*, *B. parkerii*, *B. coriaceae*, *B. crocidurae*, *B. duttonii*, *B. hermsii*, *B. hispanica*, *B. lonestarii Texas*, *B. garinii*, *B. bisseti*, *B. lonestarii New Jersey*, *B. lusitaniae*, *B. miyamotoi*, *B. recurrentis*, *B. tanukii*, *B. theileri*, *B. turdae*, *B. turicatae*, *B. valaisiana*, as well as the sequences of *B. burgdorferi sensu lato* strains: *B. afzelii*, *B. japonica*, *B. andersonii*, *B. lusitaniae*, *B. bissetii*, *B. garinii*, *B. burgdorferi sensu stricto*, and *B. valaisiana*).

The phylogenetic trees were constructed by the neighbor-joining method of Saitou and Nei (46), using the PHYLIP package.

Results

PCR Reaction Sensitivity and Specificity

The sensitivity of the PCR assay was determined using *B. burgdorferi sensu lato* DNA samples isolated from culture (32) and serially diluted in water. Due to the scarcity of organisms in clinical samples, it was very important to develop a PCR method with a very high sensitivity. This was possible by using the nested PCR, which revealed a detection limit of 5 to 10 borrelial genomes per PCR reactions. The assay specificity was assessed by PCR amplification with different *B. burgdorferi* species and different bacterial strains. Under identical experimental conditions, specific amplification occurred only with *Borrelia burgdorferi* flagellin gene (Fig. 1). The efficacy of DNA isolation was controlled by amplification of 16S rRNA gene with primers conserved (BS1/BS2) among all eubacteria (Fig. 1).

Figure 2 shows successfully amplified flagellin gene fragment, isolated from sera of 5 patients with erythema migrans in comparison with a positive control. In another 5 tested patients we also detected borrelial flagellin gene (data not shown). A DNA isolate from *B. burgdorferi*

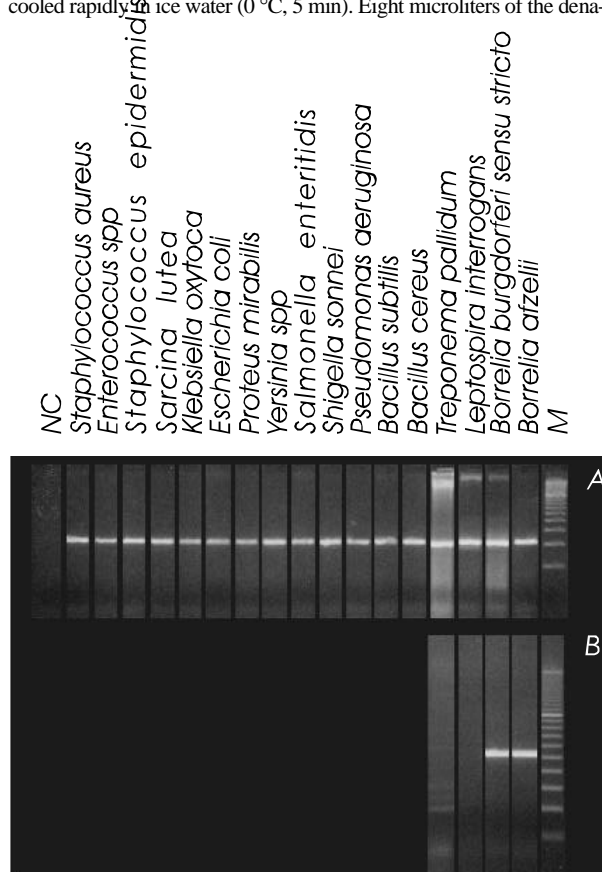


Figure 1. Specificity of primers amplifying the part of flagellin gene tested on DNA isolates from different bacterial strains. NC, negative control; M, molecular marker. (A) PCR products amplified with primers specific for highly preserved part of the 16S rRNA gene (product size: 215 bp). (B) PCR products amplified with primers specific for borrelial flagellin gene (product size: 535 bp).

sensu lato culture grown in 1991 at the Department was used as positive control (32).

Single-Stranded Conformation Polymorphism

The flagellin gene amplification products of positive samples were analyzed by SSCP (43), to determine the possible DNA chain heterogeneity, pointing to differences among PCR products. Two amplified DNA samples and the control sample revealed the presence of different chains within a single product, forming heteroduplexes, and all other samples showed an identical SSCP pattern (Fig. 3).

Sequencing and Phylogenetic Analysis

SSCP analysis was used for the selection of PCR products to be sequenced.

A total of 8 PCR samples were sequenced, among them samples with a mixed strand type on SSCP and samples with a single strand type. Using all sequences of the *Borrelia* species available, we constructed a phylogenetic tree depending on the flagellin gene similarity to the published sequences. Distances (percent of divergence) between all pairs of sequences from multiple alignments were calculated. Neighbor-joining method (46), was applied to the distance matrix giving an unrooted tree. In our case, branch length corresponding to exact genetic distances have been omitted and we constructed the tree in which particular sequence positions correspond to genetic distance in relative manner, producing the final tree with a maximum likelihood method which repetitively gave the tree with the same topology (Figs. 4 and 5).

Phylogenetic analysis revealed that *B. afzelii* was present in 10 patients samples. Comparison on the BLAST server computer yielded a 99-100% homology with *B. afzelii*, confirming the results of phylogenetic analysis. The homology with the species *B. tanukii* was 93%, and with *B. garinii*, *B. japonica*, *B. andersonii*, *B. burgdorferi sensu stricto*, *B. lusitaniae* and *B. valaisiana*

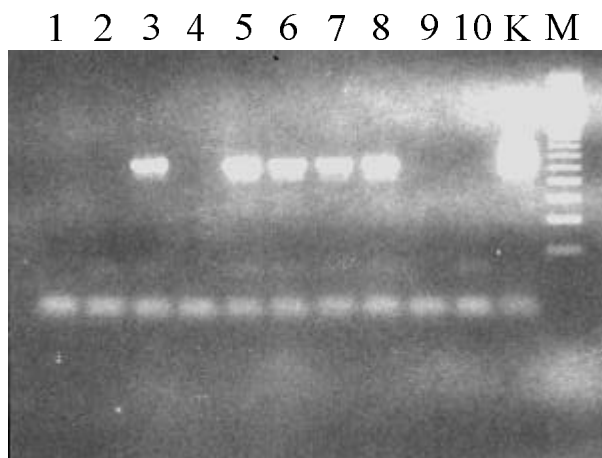


Figure 2. PCR results of the flagellin gene amplification in patient samples and controls. Lanes 1, 2, 4, and 9; negative controls. Lanes 3 and 5-8, amplification products with primers for flagellin gene (fragment size of 535 bp). K, positive control (culture of *B. burgdorferi sensu lato*); M, molecular marker.

it was 91-92%. The isolate of control strain *B. burgdorferi sensu lato* was also defined as *B. afzelii*.

Discussion

PCR methods for the detection of DNA from *B. burgdorferi sensu lato* were evaluated by analysis of sera from 40 patients with erythema migrans. The method using a combination of flagellin BAF51/BAF31 and BAF52/BAF32 primers as nested PCR achieved high sensitivity and specificity. Good sensitivity obtained by nested PCR of the borrelial flagellin gene in clinical samples has been reported (47,48), however, no data on the use of identical primer pairs could be found in available literature. Analysis of various bacterial strains was used to verify the method specificity. Our study demonstrated that the designed flagellin gene primers, being localized in the preserved gene regions, successfully amplified the target sequence of *B. afzelii* and *B. burgdorferi sensu stricto*, which differ in the flagellin gene sequence by 8% to 10%. The combination of BS1/BS2 primers for the preserved segment of 16S ribosomal gene proved sensitive enough for the control of DNA isolation. As they amplify the preserved region and yield products in the presence of DNA of other bacteria that may be found in the sample, they have been recommended as controls (34).

The technique of SSCP is suitable for rapid screening of sequence identity (43,44). In the present study, SSCP proved valuable in amplified DNA preselection for sequencing, as all samples with identical SSCP patterns also possessed identical sequences.

The phylogenetic tree revealed the clustering of the borrelial isolates with *B. afzelii* sequences into the same group, indicating that borrelial isolates from 10 patients with erythema chronicum migrans and from the *B. burgdorferi sensu lato* culture belong to the same genospecies. This finding is supported by sequence homology analysis that produced the homology score of

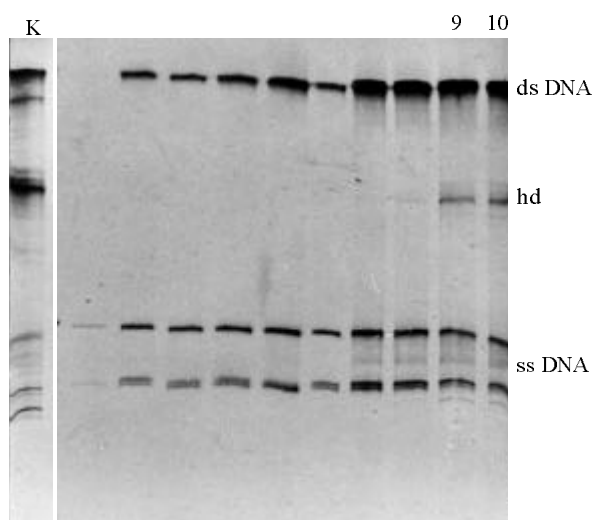


Figure 3. SSCP analysis of positive PCR products. Lane K (control, *B. burgdorferi sensu lato* culture) and 9 and 10 (2 out of 10 tested samples) contained heteroduplexes, whereas other samples showed identical strands. ds DNA, double-stranded DNA; hd, heteroduplexes; ss DNA, single-stranded DNA.

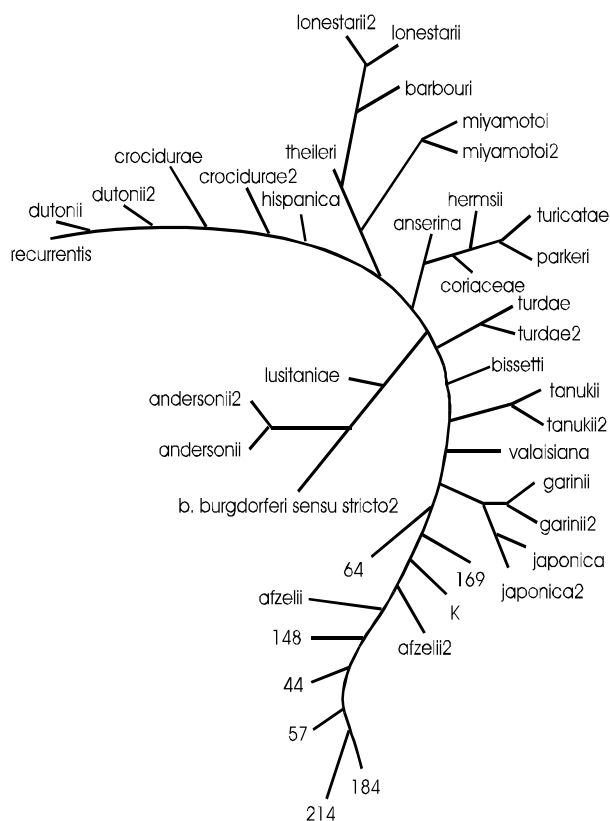


Figure 4. A phylogenetic tree designed on the basis of similarity of the flagellin gene sequences of the sequenced samples and published sequences of various borrelial species. For convenience, the length of particular branches is approximative and does not correspond to the respective phylogenetic distance. K, isolate from the culture *B. burgdorferi sensu lato*; 44, 57, 64, 148, 184, and 214, sequences of the tested samples.

99% between borrelial isolates and *B. afzelii* sequence data from the GeneBank.

B. afzelii was detected in all SSCP preselected and sequenced samples. As erythema migrans patients were from northwest Croatia, which is considered an endemic area for Lyme borreliosis, this finding was not unusual. Golubia et al (49) reported on the distinct predominance of *B. afzelii* in ticks from northwest Croatia, where it was identified in 37 out of 56 (66%) ticks. *B. afzelii* is the most common cause of skin manifestations of Lyme borreliosis, primarily erythema migrans (4,10).

Our study comprised specific endemic areas of Lyme disease in Croatia, and our results confirmed that *B. afzelii* is the main causative agent of erythema chronicum migrans in the studied group from that area. As similar studies have not been made in other parts of Croatia, there is a dilemma whether *B. afzelii* is the causative agent of the most frequent manifestation of the Lyme disease – *erythema chronicum migrans* in other parts of Croatia too. This research should be expanded by studies on the diseased from other clinical types of Lyme disease (lyme arthritis, neuroborreliosis), in order to determine whether other *Borrelia burgdorferi* genospecies are also included in the etiology of extracutaneous types of Lyme disease.

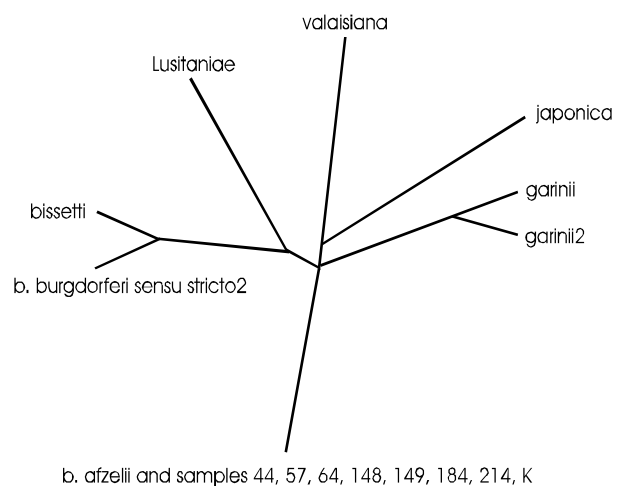


Figure 5. A phylogenetic tree designed on the basis of similarity of the flagellin gene sequences of the sequenced samples and published sequences of *B. burgdorferi sensu lato*. Contrary to Fig. 4, the length of particular branches corresponds algorithmically to sequence differences. For convenience, the illustration has been adjusted by PC Paintbrush software due to the great similarity of 10 sequences in a single branch. The length of lines is identical to those in the original Drawtree tree. K, isolate from the culture *B. burgdorferi sensu lato*; 44, 57, 64, 148, 184, and 214, sequences of the tested samples.

References

- 1 Šitum M. Lyme bolest. In: Lipozenèa J. editor. Dermatovenerologija. Zagreb: Naklada Zadro; 1999. p. 101-3.
- 2 Curl A. Bolesti prenesene i izazvane ubodom krpelja. Acta Derm Iug 1991;18:123-9.
- 3 Anderson JF. Epizootiology of Lyme borreliosis. Scand J Infect Dis 1991;77:23-4.
- 4 Steere AC. Lyme disease. N Engl J Med 1989;321:586-96.
- 5 Steere AC, Bartenhagen NH, Craft EI. Clinical manifestation of Lyme disease. Zbl Bakt Hyg A 1986;263:201-5.
- 6 Luft BJ, Jiang W, Munoz P, Dattwyler RJ, Gorevic PD. Biochemical and immunological characterization of the surface proteins of *Borrelia burgdorferi*. Infect Immun 1989;57:3637-45.
- 7 Casjens S, Delange M, Ley 3rd HL, Roisa P, Huang WM. Linear chromosomes of Lyme disease agent spirochetes: genetic diversity and conservation of gene order. J Bacteriol 1995;177:2769-80.
- 8 Postic D, Assous M, Grimont PAD, Baranton G. Diversity of *Borrelia burgdorferi sensu lato* evidenced by restriction fragment length polymorphysm of rrf(5S)-rrl(23S) intergenic spacer amplicons. Int J Syst Bacteriol 1994;44:743-52.
- 9 Baranton G, Postic D, Saint Girons I, Boerlin P, Piaffaretti JC, Assous M, et al. Delineation of *Borrelia burgdorferi sensu stricto*, *Borrelia garinii* sp. nov. and group VS461 associated with Lyme Borreliosis. Int J Syst Bacteriol 1992;42:378-83.
- 10 Canica MM, Nato F, du Merle L, Mazie JC, Baranton G, Postic D. Monoclonal antibodies for the identification of *Borrelia afzelii* sp. nov. associated with late cutaneous manifestations of Lyme Borreliosis. Scand J Infect Dis 1993;25:441-8.

- 11 Theisen M, Frederisen B, Lebech AM, Vuust J, Hansen K. Polymorphism in OspC gene of *Borrelia burgdorferi* and immunoreactivity of OspC as diagnostic antigen. *J Clin Microbiol* 1993;31:2570-6.
- 12 Wilske B, Preac-Muršič V, Jauris S, Hofmann A, Pradel I, Soutschek E, et al. Immunological and molecular polymorphism of OspC and immunodominant major outer surface protein of *Borrelia burgdorferi*. *Infect Immun* 1993;61:2182-91.
- 13 Wilske B, Preac-Muršič V, Schierz G, Gueye W, Herzer P, Weker K. Immunochemische Analyse der Immunantwort bei Spätmanifestationen der Lyme Borreliose. *Zbl Bakt Hyg A* 1988;267:549-58.
- 14 Wang G, Van Dam AP, Le Fleche AI, Postic D, Reter O, Baranton G, et al. Genetic and phenotypic analysis of *Borrelia valaisiana* sp. nov. (*Borrelia* genomic groups VS116 and M19). *Int J Syst Bacteriol* 1997;47:926-32.
- 15 Le Fleche A, Postic D, Girardet K, Peter O, Baranton G. Characterisation of *Borrelia lusitanae* sp. nov. by 16S ribosomal DNA sequence analysis. *Int J Syst Bacteriol* 1997;47:921-5.
- 16 Kawabata M, Masuzawa T, Yanagihara Y. Genomic analysis of *Borrelia japonica* sp. nov. isolated from *Ixodes ovatus* in Japan. *Microbiol Immunol* 1993; 37:843-8.
- 17 Assous MV, Postic D, Paul G, Nevot P, Baranton G. Western blot analysis of sera from Lyme Borreliosis patients according to the genomic species of the *Borrelia* strains used as antigens. *Eur J Clin Microbiol Infect Dis* 1993;124:261-8.
- 18 Assous MV, Postic D, Paul G, Nevot P, Baranton G. Individualization of two new genomic groups among America *Borrelia burgdorferi sensu lato* strains. *FEMS Microbiol Lett* 1994;121:93-8.
- 19 Magnarelli LA, Anderson JF, Johnson RC. Cross-reactivity in serological tests for Lyme disease and other spirochetal infections. *J Infect Dis* 1987; 156:183-8.
- 20 Eiffert H, Ohlenbuch A, Christen HJ, Thomssen R, Spielman A, Matuschka FR. Nondifferentiation between Lyme disease spirochetes from vector ticks and human cerebrospinal fluid. *J Infect Dis* 1995; 171:476-9.
- 21 Marconi RT, Liveris D, Schwartz I. Identification of novel insertion elements, restrictions fragment length polymorphism patterns, and discontinuous 23S rRNA in Lyme Disease Spirochetes: phylogenetic analyses of rRNA genes and their intergenic spacers in *Borrelia japonica* sp. nov. and genomic group 21038 (*Borrelia andersoni* sp. nov.) isolates. *J Clin Microbiol* 1995;33:2427-34.
- 22 Postic D, Marti Ras N, Lane RS, Henderson M, Baranton G. Expanded diversity among Californian *Borrelia* isolates and descriptions of *Borrelia bissetti* sp. nov. (formerly *Borrelia* group DN127). *J Clin Microbiol* 1998;36:3497-504.
- 23 Dreesler F, Ackerman R, Steere AC. Antibody responses to three genomic subgroups of *Borrelia burgdorferi* in European Lyme Borreliosis. *J Infect Dis* 1994;169:313-8.
- 24 Masuzawa T, Kurita T, Kawabata H, Muramatsu K, Uchikawa K, Yanagihara Y. Infectivity and early antibody response to *Borrelia burgdorferi sensu lato* isolated in Japan in outbred mice. *Microbiol Immunol* 1994;38:621-7.
- 25 Davidson BE, MacDougall GJ, Saint-Girons I. Physical map of linear chromosome of bacterium *Borrelia burgdorferi* 212, a causative agent of Lyme disease and localization of rRNA genes. *J Bacteriol* 1992; 174:3766-74.
- 26 Old IG, MacDougall GJ, Saint-Girons I, Davidson BE. Mapping of genes on the linear chromosome of the bacterium *Borrelia burgdorferi* possible location for its origin of replication. *FEMS Microbiol Lett* 1992; 99:245-50.
- 27 Hansen K, Bangsberg JM, Fjordvang H, Pederson NS, Hindersson P. Immunochemical characterization and isolation of the gene for a *Borrelia burgdorferi* immunodominant 60-kilodalton antigen common to a wide range of bacteria. *Infect Immun* 1988;56: 2047-53.
- 28 Barbour AG, Schrupf ME. Polymorphisms of major surface proteins of *Borrelia burgdorferi*. *Zbl Bakt Hyg A* 1986;263:83-91.
- 29 Hansen K, Hinderson P, Strandberg Pederson N. Measurement of antibodies to the *Borrelia burgdorferi* flagellum improves serodiagnosis in Lyme disease. *J Clin Microbiol* 1988;26:338-46.
- 30 Picken RN. Polymerase chain reaction primers and probes derived from flagellin gene sequences for specific detection of the agents of Lyme disease and North American relapsing fever. *J Clin Microbiol* 1992;30:99-114.
- 31 Bolanča-Bumber S, Šitum M, Balač-Winter A, Skerlev M, Lipozenič J, Dobrič I, et al. First isolation of *Borrelia burgdorferi sensu lato* in Croatia. *Acta Dermatovenerol Croat* 1997;5:95-9.
- 32 Golubič D. Epidemiološke, ekološke i kliničke karakteristike Lyme borelioze u sjeverozapadnoj Hrvatskoj. Dissertation. Zagreb: Sveučilište u Zagrebu; 1997.
- 33 Berg D, Abson KG, Prose NS. The laboratory diagnosis of Lyme disease. *Arch Dermatol* 1991;127:866-70.
- 34 Malloy DC, Nauman RK, Paxton H. Detection of *Borrelia burgdorferi* using the polymerase chain reaction. *J Clin Microbiol* 1990;28:1089-93.
- 35 Saiki RK, Gelfand DH, Stoffler S, Scharf SJ, Higuchi R, Horn GT, et al. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 1988;239:487-91.
- 36 Goodman JL, Bradley JF, Ross AE, Goellner P, Lagus A, Vitale B, et al. Bloodstream invasion in early Lyme disease: results from a prospective, controlled, blinded study using the polymerase chain reaction. *Am J Med* 1995;99:6-12.
- 37 Moter SE, Hofmann H, Wallich R, Simon MM, Kramer MD. Detection of *Borrelia burgdorferi sensu lato* in lesional skin of patients with erythema migrans and acrodermatitis chronica atrophicans by OspA-specific PCR. *J Clin Microbiol* 1994;32:2980-8.
- 38 Guy EC, Stanek G. Detection of *Borrelia burgdorferi* in patients with Lyme disease by the polymerase chain reaction. *J Clin Pathol* 1991;44:610-1.
- 39 Liveris D, Gazumyan A, Schwartz I. Molecular typing of *Borrelia burgdorferi sensu lato* by PCR-restriction fragment length polymorphism analysis. *J Clin Microbiol* 1995;33:589-95.
- 40 Demaerschalck I, Ben Massoud A, De Kesel M, Hoyois B, Lobet Y, Hoet P, et al. Simultaneous presence of different *Borrelia burgdorferi sensu lato* genospecies in biological fluids of Lyme disease patients. *J Clin Microbiol* 1995;33:602-9.
- 41 Rosa PA, Hogan D, Schwan TG. Polymerase chain reaction analyses identify two distinct classes of *Borrelia burgdorferi*. *J Clin Microbiol* 1991;29:524-32.
- 42 Thomson JD, Higgins DG, Gibson TJ. CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Research* 1993;22:4673-80.
- 43 Orita Miwahana H, Kanazawa H, Hayashi K, Sekiya T. Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphism. *Proc Natl Acad Sci USA* 1989;86:2766-70.

- 44 Mohabeer AJ, Hiti AL, Martin WJ. Non-radioactive single strand conformation polymorphism (SSCP) using the Pharmacia "PhastSystem". *Nucleic Acids Res* 1991;19:3154-7.
- 45 Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* 1977;74:5463-7.
- 46 Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 1987;4:406-25.
- 47 Picken RN. Polymerase chain reaction primers and probes derived from flagellin gene sequences for specific detection of the agents of Lyme disease and North American relapsing fever. *J Clin Microbiol* 1992;30:99-114.
- 48 Huppertz HI, Schmidt H, Karch H. Detection of *Borrelia burgdorferi* by nested polymerase chain reaction in cerebrospinal fluid and urine of children with neuroborreliosis. *Eur J Pediatr* 1993;152:414-7.
- 49 Golubiæ D, Rijpkema S, Tkalec-Makovec N, Ružić E. Epidemiologic, ecologic and clinical characteristics of Lyme Borreliosis in Northwest Croatia. *Acta Med Croatica* 1998;52:7-13.

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