Characterisation of Lyme Borreliosis Spirochetes

By Eva Ružić-Sabljić

Introduction

Lyme borreliosis is a tick-borne zoonosis caused by a spirochete of the genus *Borrelia*. The isolated spirochete was named *B. burgdorferi* in honour of Willy Burgdorfer, who first reported the spirochete aetiology of Lyme borreliosis in 1981. Later analysis of the Borrelia genome demonstrated that *Borreliae* are not homogeneous, and the former single species was re-classified into several species. Today, twelve different species have been identified, although at least three of them have been demonstrated to cause Lyme borreliosis in humans: *B. afzelii, B. garinii* and *B. burgdorferi sensu stricto*. Human pathogen strains that are not identified on the species level are reported as *B. burgdorferi sensu lato.*

Many wild and domestic animals and birds are natural reservoirs of different *Borrelia* species. Because *Borreliae* do not adversely affect their hosts, they can maintain spirochetes for a long time. Different species of blood-sucking arthropods can transmit *Borreliae* from one species to another. Hard ticks of the *Ixodes ricinus* complex are of the greatest significance as vectors. In enzootic foci, a high prevalence of *Borrelia* infection within the animal and tick population can be found. Human infection occurs only if *Borrelia* transmission results from the bite of an infected tick.

Assistant Professor Dr Eva Ružić-Sabljić.

Lyme borreliosis is present in the Northern hemisphere between 33 and 60 degrees latitude, which includes parts of Europe, Asia and America. The global distribution of Lyme borreliosis coincides with the geographic distribution of the main vector, Ixodes ticks, and is considered to be the most frequent tick-borne disease in these parts of the world. Lyme borreliosis is present all over Slovenia, where the incidence is among the highest in Europe.

The pathognomic manifestation of Lyme borreliosis is a skin disorder, erythema migrans, which develops on the skin at the site of a tick bite. *Borreliae* can disseminate from the primary lesion and different organs may be affected (skin, nervous system, joints, eyes, heart, etc.). Multiorgan infection can be indicated by a variety of clinical manifestations with signs and symptoms ranging from mild to severe. Many clinical manifestations are not pathognomic for Lyme borreliosis; in these patients, *Borrelia* infection must be confirmed by microbiological tests.

Description of *B. burgdorferi* sensu lato

Borreliae are typical, highly motile spirochetes 5 to 30 µm long and 0.2 to 0.3 µm wide. The main part of the cell consists of flagella that mediate spirochete motility. Flagella are rigid structures that insert bipolarly and subterminally and overlap in the middle of the cell. A protoplasmic cylinder wraps around the flagella, either left-handed or right-handed. Both structures, flagella and protoplasmic cylinders, are enveloped with a fluid, outer cell membrane containing proteins, lipids and carbohydrates. Although *Borreliae* are slow-growing spirochetes, they can be cultivated in complex rich media.

The *Borrelia* genome is composed of a linear chromosome and different numbers of linear and circular plasmids in the range of 8 to 65 kb. *Borreliae* are the only spirochetes and one of the rare bacteria species that contain a linear chromosome. Additionally, the *Borrelia* chromosome is characterised by its small size of about 1 Mb and by low G + C content of 27 to 32 mol%. *Borreliae* carefully maintain the plasmid copy number, and control plasmid replication, so *Borrelia* plasmids can be observed as minichromosomes. Comparing plasmid profiles of isolated strains, it is evident that the plasmid content varies significantly.

Analyses of whole cell lysates from *B. burgdorferi* sensu lato strains show numerous *Borrelia* proteins. Strains isolated from various biological sources in the same restricted geographical area can differ in terms of the proteins they express and the amount of expressed protein. The molecular weight of a particular protein can also vary among strains. Many *Borrelia* proteins share antigen characteristics on their surface, and in the great majority of hosts *Borrelia* antigens are capable of stimulating the immune system for specific antibody production. The common *Borrelia* antigen is flagellin, a genus-specific polypeptide of 41 kDa. Polypeptides of 100 kDa, 39 kDa and outer surface proteins (Osp) with variable low molecular weights (36 to 22 kDa) are considered to be species-specific antigens. OspA, OspB and OspC are of primary importance for *Borrelia* pathogenesis. The antigen heterogeneity of the major *Borrelia* proteins must be considered with respect to serodiagnosis, immune prophylaxis, taxonomy and pathogenesis.

Methods for *B. burgdorferi sensu lato* characterisation

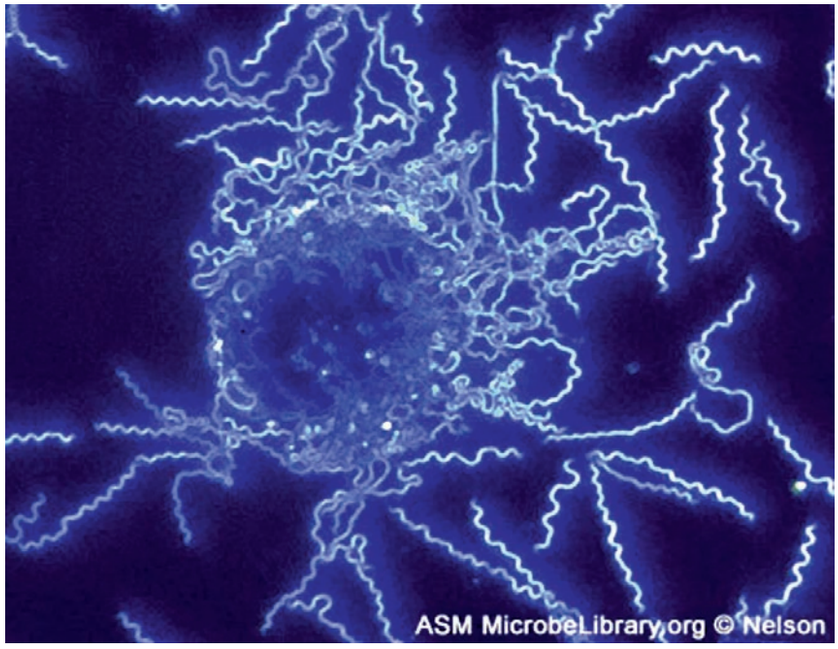
Thanks to their typical shape and motility, *Borrelia*e have always been delineated from other spirochetes and placed in a separate taxonomic branch. Twenty years ago, when Lyme disease was first described as a new clinical entity and *Borreliae* were discovered to be its etiological agent, great attention was paid to this spirochete. Over the years, a large number of *Borrelia* isolates have been provided from different human specimens, ticks and animal material. Requests for their full analysis were made. With the development of new microbiological, especially molecular methods, characterisation of *B. burgdorferi sensu lato* can now be provided in detail.

For a more systematic approach to *Borrelia* heterogeneity, a **serotyping system** has been developed. With a panel of monoclonal antibodies, *Borrelia*e were serotyped in relation to the antigenic characteristics of the OspA protein. At least seven different serotypes have been described. Later studies show a clear correlation between the particular OspA serotype and the species classification. The immunological diversity of the OspC protein, showing 13 different serotypes, appears to be significantly greater than that of OspA. Characterisation of *Borrelia* phenotypes is not so reliable, because *Borrelia*e may switch off the expression of a particular protein and change its protein profile and immunoreactivity.

Molecular typing based on the genetic characteristics of *B. burgdorferi sensu lato* can provide more precise information on the diversity of isolated strains. The *Borrelia* genome presents the main target for assessing the genetic relationships among the species, and a number of genotyping methods have been developed for identifying and differentiating particular strains.

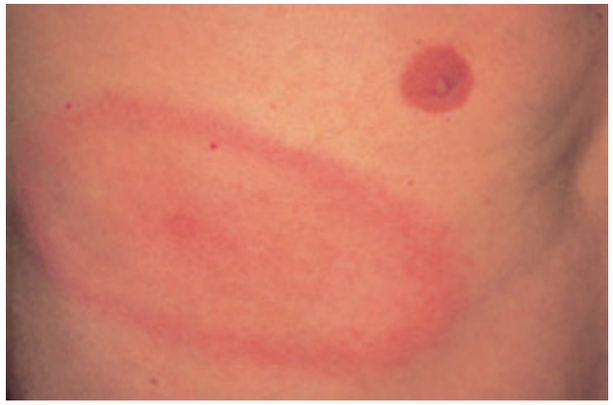
Chromosomal DNA restriction profiles can be shown by pulsed-field gel electrophoresis (PFGE). It is a highly effective molecular typing method for *B. burgdorferi sensu lato* species identification. The *Borrelia* genomic DNA is separated by PFGE after digestion with a restriction enzyme, and the discrimination of strains is based on the large restriction fragment length polymorphism (RFLP) of the chromosomal DNA. *Borrelia* DNA is digested by numerous restriction enzymes to construct physical maps of the genomes and to define different groups of strains. *Mlu*I is a restriction enzyme with relatively few recognition sites and PFGE patterns after *Mlu*I digestion demonstrates specific bands for each species. In addition to species identification, *Mlu*I digestion shows genetic diversity and permits discrimination between strains within each *Borrelia* species.

Plasmid fingerprint. *B. burgdorferi sensu lato* strains have an unusual plasmid content of linear and circular plasmids that may vary in number and size. Only linear plasmids can be well separated by pulsed-field gel electrophoresis. The plasmid fingerprint is the best method of comparing and differentiating strains within the same species. The overall plasmid profiles might correlate with the particular species of *B. burgdorferi sensu lato* but definitive species-specific plasmids could not be identified.



Ribotyping is based on the profiles obtained by restriction fragment patterns of chromosomal DNA digested by appropriate restriction enzymes (*Eco*RI, *Eco*RV, *Pst*I, *Hind*III etc.) and hybridised with a probe derived from highly conserved rRNA. *Borrelia* strains can be distinguished by ribotyping at both species and subspecies levels.

Numerous **polymerase chain reaction** (PCR)-based methods are used for *Borrelia* genotyping. The main principle of any PCR is to get numerous copies of the target DNA sequence. An amplified DNA sequence can be separated by electrophoresis and stained with ethidium bromide.



PCR amplification with species-specific primers, either for chromosomal or plasmid genes, permits species identification directly. The most frequently used primer pairs for species-specific identification of *Borrelia* strains are those created for conserved 16S rRNA genes. Cross-amplification between species does not occur, even when a large input of template DNA is used.

Arbitrary primed PCR, also referred to as random amplified polymorphic DNA, allows PCR amplification of discrete sequences in genomes without any previous knowledge about them. Methods are based on the use of short random sequence primers that amplify different DNA segments of various bacteria species. Following separation by gel electrophoresis, PCR replicons demonstrate a pattern of bands which is characteristic for the particular bacterial strain. Randomly amplified polymorphic DNA analysis of *B. burgdorferi sensu lato* isolates is a reliable technique for identifying

From left to right: Brigita Repše, Milena Rici, Frančiška Čurila-Mikuž and Tina Šivic. In the background: Urška Glinšek and Eva Ružić-Sabljić.

different *Borrelia* species, as well as for discriminating between strains within the same species.

If PCR amplicons are not specific enough to recognise species or heterogeneity among strains, they must be further processed. A simple and rapid method of identification is digestion of a PCR product with different restriction enzymes and visualisation of restricted patterns in polyacrylamide gel. Restriction fragment length polymorphism (RFLP) of the amplified *rrf* (5S)-*rrl* (23S) rRNA intergenic spacer region is a widely used typing method. The uniqueness of the rRNA gene organisation is tandemly repeated 23S - 5S rRNA genes. This specific organisation of the *Borrelia* genome, not present in other bacteria, is a suitable tool for species identification despite the lack of primer specificity. Digestion of amplicon with *Mse*I and *Dra*I enzymes results in different restriction fragments with species-differentiating characteristics.

In addition to digestion with restriction enzymes, PCR products can be analysed by DNA sequencing. PCR amplicons of 16S rRNA (*rrs*), *flagellin*, *OspA*, *OspC*, *p93*, *hsp60*, *p39* and other *B. burgdorferi sensu lato* genes have been sequenced and phylogenetic trees have been constructed from specific gene sequence analyses. Determination of the nucleotide sequence of multiple genes from numerous *Borrelia* strains is considered to be the gold standard for typing. The great advantage is a central database and the portability of sequence data. In 1997, the entire genome of *B. burgdorferi sensu stricto* strain B31 was reported.

Our results on Slovenian isolates

Lyme borreliosis is endemic to Slovenia. All major clinical manifestations of Lyme borreliosis have been found in Slovenian patients. The incidence of infection is among the highest in Europe. The first isolation of *Borrelia* strains from Slovenian patients was made in 1988. The great majority of Slovenian strains have been isolated from the skin of patients with early (erythema migrans) and chronic (acrodermatitis chronica atrophicans) infection, but also in patients with very rare (lymphocytoma, granuloma annulare) or

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nonspecific clinical manifestations, as well as from normal-looking skin at the site of a previous tick bite or previous skin disorder. Numerous isolates from cerebrospinal fluid have been isolated in patients with early and chronic infection of the central nervous system, but also in patients with skin manifestations without signs of central nervous system affection. *Borreliae* have also been isolated from the blood of Slovenian patients suffering from various clinical manifestations of Lyme borreliosis. In many cases, we have been able to confirm early dissemination of spirochetes and their presence in skin, blood or cerebrospinal fluid without any clinical or biochemical signs of their presence. Moreover, we have also reported on *Borrelia* persistence in skin and cerebrospinal fluid after appropriate antibiotic therapy, as well as re-infection in patients who have recovered from Lyme borreliosis, both confirmed by isolation.

We have set up a laboratory in our institute for *Borrelia* cultivation and identification, which is one of the very few centres to be involved in this kind of work and which has one of the best-know collection of *Borrelia* isolates.

The most frequently used method for *B. burgdorferi* sensu lato identification in our laboratory is a chromosomal DNA restriction profile followed by PFGE. Plasmid profiles are shown by PFGE, protein profiles by polyacrylamide gel electrophoresis. Identification of strains by different PCR methods has also been developed in our laboratory. The great majority of strains isolated from Slovenian patients have been identified as *B. afzelii* followed by *B. garinii*. *B. burgdorferi* sensu stricto strains are rarely isolated in our patients. The prevalence of particular *Borrelia* species depends on specimen origin: among skin and blood isolates *B. afzelii* is a widely prevalent species, while among isolates from cerebrospinal fluid, *B. garinii* is more frequently isolated. Although *B. burgdorferi sensu stricto* rarely causes infection in Slovenian patients, it has been isolated from skin as well as from cerebrospinal fluid of our patients. In several patients, isolated strains have not belonged to any of the three previously mentioned species, but are distinct new species. Some of these strains have been identified as *B. bissettii* and *B. spielamnii*, while other are still waiting to be identified.

Comparison of plasmid profiles is a very good tool for discrimination of strains within a species. In Slovenia, we have found numerous different plasmid profiles, suggesting great heterogeneity of strains in a relatively restricted area. We have also found unusual plasmid content, either plasmid dimer or multiple copies of large plasmid in nearly 13% of all Slovenian isolates.

Analysing the protein profiles of our strains, we have found a phenotypic heterogeneity that may affect pathogenesis, serological tests and vaccine development. We have found also a correlation between several Osp protein expressions and *Borrelia* species: *B. garinii* strains more often expressed the OspC protein than *B. afzelii* strains, while *B. afzelii* strains more often expressed OspB protein than *B. garinii*.

Tjaša Cerar, graduate microbiologist. Employed as young researcher in the Laboratory for microbiological diagnosis of borreliosis and leptospirosis.

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