Chapter 21
Borreliae

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21.1 Introduction

Borreliae are classified in the order Spirochaetales. All members of the genus *Borrelia* are highly motile, spiral-shaped organisms. The cells are comprised of a protoplasmic cylinder enclosed by an inner and outer membrane [1]. The periplasmic compartment contains a number of endoflagella which are responsible for both cellular motility and helical cell shape [2]. The outer membrane is devoid of lipopolysaccharides, but contains a large number of lipoproteins [3, 4]. Common features of members of this genus are (1) vertebrate host association, (2) transmission to new hosts by hematophagous arthropod vectors parasitized with borreliae, and (3) relatively low mol% G+C content (27–32%) in their DNA [1]. All borreliae cultured to date are microaerophilic, slow growing, and require complex culture media for propagation [1]. The genus *Borrelia* is divided into two major pathogenic groups—the Lyme disease (LD) borreliae and relapsing fever (RF) borreliae. The Lyme disease group of *Borrelia* consists of at least 14 different species and some members of this group are the agents of Lyme disease. The relapsing fever group includes more than 20 *Borrelia* species [5] that are agents of tick-borne (TBRF) or louse-borne (LBRF) relapsing fever (27–32%) [6]. LD borreliae in nature are maintained in enzootic cycles involving a variety of mammalian and avian hosts and hard ticks of the genus *Ixodes* as vectors. The primary bridging vectors to humans are *Ixodes scapularis and I. pacificus* in North America, *I. ricinus* in Europe, and *I. persulcatus* in Asia [7, 8]. Vectors of the RF *Borrelia* agents are soft-bodied ticks (family Argasidae), mainly of the genus *Ornithodoros*; the one notable exception is *Borrelia recurrentis,* which is transmitted by the human louse (*Pediculus humanus humanus*) [9].

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Many aspects of *Borrelia* spp. biology, epidemiology, and pathogenesis have been thoroughly covered in previous reviews [1, 9–11]. In this chapter, we focus on the different molecular methods employed in species identification, typing and subtyping of LD and RF borreliae. The contribution of these typing methods to taxonomy, epidemiology, and diagnostics will be discussed.

21.1.1 Molecular Typing of Borreliae

B. burgdorferi was identified as a new human pathogen in 1983 [12–14]. Since then, hundreds of isolates have been cultured worldwide from *Ixodes* ticks, small mammals and birds and from Lyme disease patients. Molecular characterization of these isolates has shown that they are genetically diverse [15–22]. Taxonomic classification of LD spirochetes has been revised in the last two decades based on information obtained from molecular typing methods with increasing discriminatory power [10, 23, 24]. Presently, 14 species comprise the cluster of genetically related isolates [10, 23, 25–27]. Five species (*B. burgdorferi*, *B. andersonii, B. bissettii, B. californiensis,* and *B. carolinensis*) have been detected in the United States and only *B. burgdorferi* has been associated with human disease. The remaining nine species (*B. afzelii, B. garinii, B. japonica, B. lusitaniae, B. sinica, B. spielmanii, B. tanukii, B. turdii,* and *B. valaisiana*) have been identified exclusively in Eurasia. In this latter group, only *B. afzelii and B. garinii* have been definitively associated with human Lyme disease, although limited human cases of infection with *B. bissettii*, *B. valaisiana,* and *B. spielmanii* have been reported [28–31]. Human infection by these *Borrelia* species usually results in a characteristic skin rash, erythema migrans (EM) [32, 33]. Dissemination of the spirochetes from the initial site of infection in skin can result in extracutaneous manifestations that may be dependent on the infecting species [15, 34]. *B. burgdorferi* infection more frequently causes Lyme arthritis, whereas infection with *B. garinii* and *B. afzelii* is more frequently associated with neuroborreliosis and a chronic skin condition (acrodermatitis chronica atrophicans), respectively.

Classification of RF borreliae has been traditionally based on geography, e.g., the Afro-tropical species of *B. duttonii* and *B. crocidurae* as Old-World TBRF and *B. hermsii* and *B. turicatae* found in the western and south-central US as New-World TBRF species [35]. The specific relationship between spirochetes and their arthropod vectors has been used as a means of speciation of RF borreliae [5, 36, 37]. Specifically, *B. hermsii* and *B. turicata* are exclusively transmitted by *O. hermsii* and *O. turicata* ticks [38]. Several new species of RF agents collectively named *B. miyamotoyi* sensu lato have been recently reported [9]. These include *B. miyamotoyi*, *B. miyamotoyi*-like, and *B. lonestari* transmitted by the hard ticks *I. persulcatus*, *I. scapularis,* and *Amblyomma americanum*, respectively [39–41]. The disease potential of these species is currently unknown. *B. miyamotoyi* has not been isolated from humans and only a single case of human infection with *B. lonestari* has been reported [42].

21.1.2 Phenotypic Typing

Conventional bacterial phenotyping approaches such as biotyping, antibiotic susceptibility profiling, and bacteriophage typing cannot be applied to the genus *Borrelia* due to the extreme fastidiousness of the organisms and the inability to form confluent lawns on solid media. Although a phage has been reported for *B. burgdorferi* [43–45], its utility for strain typing is unknown. Typing of *Borrelia* by fatty acid content and total protein profiling by SDS-PAGE are unreliable since both methods require defined growth media and specific metabolic states of the cells being typed [10]. Multi-locus enzyme electrophoresis (MLEE) typing, which involves comparison of the mobility of metabolic enzymes on gel electrophoresis, has been employed in a limited number of studies, but its utility for *B. burgdorferi* is limited because it is labor intensive and requires large amounts of cultured organism [10, 46, 47]. Serotyping of LD spirochetes based on two outer surface proteins, outer surface protein A (OspA) and outer surface protein C (OspC), has provided some important insights into vaccine development and clinical outcome, but this approach has been supplanted by genetic typing methods [48–51].

Phenotyping of RF borreliae has been hampered by the inability to cultivate many of these species and is not in common use. Biotyping has been largely based on vector specificity and geographic location. Biological assays, such as guinea pig resistance to *B. duttonii* [52], primate susceptibility to *B. recurrentis* [53], and bird susceptibility to *B. anserina* [54], have also been employed. In addition, for cultivable species, variations in sugar fermentation [55] and in structural traits, such as the number of flagella [56], have been reported. Serotyping of RF borreliae has been reported, but its applicability for typing is questionable since these species undergo continuous antigenic variation in mammals [35].

21.1.3 Genetic Typing

A variety of genetic typing methods, targeting both chromosomal and plasmid loci, have been applied to *Borrelia* species. These include DNA–DNA relatedness analysis, rRNA ribotyping, pulse-field gel electrophoresis (PFGE), plasmid fingerprinting, species-specific PCR, PCR-based restriction fragment length polymorphism (PCR-RFLP) analysis, single-strand conformational polymorphism (SSCP) typing, DNA sequence analysis of species-specific genes, variable-number tandem repeat (VNTR) analysis, and multi-locus sequence typing (MLST). In general, these approaches can be divided into those that require purified DNA (DNA–DNA hybridization, ribotyping, PFGE, plasmid fingerprinting) and those that can be accomplished by PCR with less purified material. The former methods are cumbersome and require in vitro cultivation of the spirochetes. Information gathered from these analyses is often used for bacterial species identification and taxonomy. In contrast, PCR-based typing schemes are relatively simple, rapid, and can be performed directly on large numbers of environmental or patient samples without the necessity of prior

cultivation. Results obtained from these latter analyses can be employed for both species/strain differentiation and elucidation of population dynamics of *Borrelia* species in nature. Furthermore, the potential problem of clonal selection of isolates by in vitro cultivation is avoided [21, 57]. In the following sections, each of these methods and their applications are discussed.

It should be noted, of course, that the ultimate typing approach is complete genome sequencing. The genome of *B. burgdorferi* strain B31 was among the first bacterial genomes to be completely sequenced. This revealed a unique genetic structure among prokaryotes, as the complete genome was comprised of a single, large linear molecule (referred to as the chromosome) of 910 kb and a collection of 12 linear and 9 circular plasmids with a combined size of approximately 600 kb [58, 59]. Although the genomic sequences of other *Borrelia* species are deposited in GenBank, that for strain B31 remains the only *fully* sequenced genome; the remaining genome sequences lack information on varying numbers of plasmids. The genomic features described for the *B. burgdorferi* B31 genome are also characteristic of other LD and RF *Borrelia* species sequenced to date in that they are comprised of a large linear chromosome and multiple linear and circular plasmids [60, 61]. The complete genomes of the RF borreliae, *B. reccurentis, and B. duttoni* have been reported [62] and those of *B. hermsii* and *B. turicatae* have been deposited in the NCBI genome database.

21.1.3.1 Genetic Typing Methods Employing Purified Genomic DNA

DNA–DNA Hybridization

The reference method for measurement of DNA relatedness between two organisms is DNA–DNA hybridization. This approach was employed for speciation of LD borreliae [63, 64]. The strength of this method is based on the fact that classification relies on comparison of total genomic sequences for the organisms under investigation. A DNA relatedness of 70% is the cut-off for defining members of a given species [65]. DNA homology among LD *Borrelia* species ranges from 48 to 70%, whereas that between LD and RF borreliae range from 30 to 44% [14, 63].

For North American RF spirochetes, *B. hermsii* shows 86 and 77% homology to *B. turicatae* and *B. parkeri*, respectively; DNA relatedness between *B. hermsii* and other RF agents is much lower (17–63%) [1]. A confounding factor is the variable plasmid content observed among *Borrelia* species and isolates. DNA–DNA hybridization of *B. turicatae* and *B. parkeri* suggests that they are members of a single species but the absence of circular plasmids in the latter and transmission of each species by a unique vector tick species suggests that they are distinct species [38].

Plasmid Typing

All members of the genus *Borrelia* contain multiple linear and circular plasmids. The number of plasmids and their molecular size vary among isolates of LD borre-

liae [66–69]. This variation has been the basis of plasmid fingerprinting as a typing method. For example, among 40 LD isolates from different sources and geographical locations, plasmid fingerprinting data correlated with *Borrelia* species designations by other typing methods, but no species-specific plasmids were identified [70]. The reported loss of both linear and circular plasmids during in vitro propagation and the possibility of plasmid recombination limit the usefulness of this method for LD agent typing [71–73]. This may be less of a problem for RF *Borrelia* species [74]. However, no comprehensive plasmid fingerprinting studies have been yet described for RF borreliae. The reported absence of circular plasmids from the genomes of *B. parkeri*, *B. anserina,* and *B. recurrentis* may be useful for differentiating these RF species from other RF borreliae [38].

Ribotyping

Ribosomal RNA genes are highly conserved and are universally present in bacteria. This facilitates the use of *E. coli*-based probes for identification of restriction fragments containing rRNA genes for many bacteria. The technique involves digestion of total genomic DNA with one or more restriction enzymes, electrophoretic separation of restriction fragments and Southern blotting with a probe for conserved regions of rRNA [75]. Individual species of *B. burgdorferi*, *B. garinii*, and *B. afzelii* can be identified by specific *Hin*dIII DNA fragments [15]. A study of 51 LD isolates demonstrated that all 18 *B. burgdorferi* strains belonged to a single ribotype, while 23 *B. garinii* and 10 *B. afzelii* isolates were distributed into nine and three ribotypes, respectively [76]. The method is relatively simple and highly reproducible, but has been superseded by PCR-based methods targeting rRNA genes (see below).

Pulsed-Field Gel Electrophoresis (PFGE)

A second typing method based on restriction enzyme digestion of total genomic DNA is pulsed-field gel electrophoresis. This approach employs restriction enzymes whose recognition sequences rarely appear in the *Borrelia* genome such that digestion of total genomic DNA produces a relatively small number of large restriction fragments. *Mlu*I has been the most useful enzyme for PFGE analysis of LD spirochetes. Most species can be identified by characteristic species-specific digestion fragments (e.g., 135 kb for *B. burgdorferi*, 220 kb and 80 kb for *B. garinii,* and 460, 320, and 90 kb for *B. afzelii*) [77, 78]. Strain differentiation within a species can also be accomplished by PFGE. Among 20 *B. burgdorferi* strains there were 10 *Mlu* I types and among 24 *B. garinii* strains there were 4 *Mlu* I types; interestingly, no variation was observed among 20 *B. afzelii* isolates [77]. *Mlu*I-based PFGE analysis of 186 North American *B. burgdorferi* isolates revealed 19 different patterns, although just a few of these predominated [18]. Six different PFGE types were identified in 48 *B. burgdorferi* clinical isolates from early Lyme disease patients [79]. In general, there is excellent correspondence between PFGE and other typing

methods [18, 79]. PFGE has also been beneficial for constructing physical maps of the *B. burgdorferi* chromosome [80, 81]. PFGE analysis remains a valuable tool for *B. burgdorferi* typing, but a drawback is the requirement for culture and large amounts of purified DNA.

21.1.3.2 PCR-Based Typing Methods

DNA Sequencing of PCR-Amplified Loci

Genetic typing methods involving PCR amplification of various genomic targets of LD borreliae are relatively simple, fast, and discriminative at the species and, in some instances, at the isolate level [17, 24, 82–84]. Analysis of PCR-amplified 16S rRNA products by either RFLP analysis [85] or by direct DNA sequencing results in species-level differentiation among LD borreliae [86]. DNA sequence homology among *B. burgdorferi*, *B. garinii*, *B. afzelii*, *B. lusitaniae,* and *B. valaisiana* at the *rrs* (16S rRNA) locus varies from 95.3 to 99.6% [86]. The GenBank database contains over 100 *rrs* sequences for LD *Borrelia* species. While these sequences are useful for species identification, they are less suitable in discriminating between strains of the same species. Numerous other genetic loci have been employed to identify LD *Borrelia* species and to investigate the taxonomic and evolutionary relationships among these spirochetes. These include the genes encoding flagellin (*flaB*) [87–89], outer surface protein A (*ospA*) [18, 87, 90], outer surface protein C (*ospC*) [88, 91], P66 (*p66*) [84, 92], histone-like protein (*hbb*) [93], P39 (*bmpA*) [94], and heat-shock proteins (*hsp60* and *hsp70*) [87]. Dykhuizen and co-workers have characterized over 20 different *ospC* genotypes in North America and Europe and *ospC* sequence analysis has become a primary tool for *B. burgdorferi* genotyping. Several studies have correlated *ospC* genotype with disseminated infection in LD patients [95–102]. Despite the substantial utility of *ospC* for genotyping, however, evidence of *osp*C intragenic recombination and lateral transfer between strains makes *ospC* unsuitable for phylogenetic studies [103–105].

DNA sequence variation at the *fla*B locus allow for discrimination between LD and RF *Borrelia* species [41, 89]. *glpQ,* a gene encoding glycerophosphodiester phosphokinase, is found in all RF borreliae, but not in LD species and thus can be employed for identification of RF agents [106]. Sequence variation in *glpQ* was used to differentiate between *B. lonestari* and *B. miyamotoi* [107]. Sequence variation at the *vtp* locus of *B. hermsii* (an ortholog of *ospC*) has been described; however, the utility of this target for RF species typing is unknown [108].

Ribosomal RNA Spacer RFLP Analysis

Genome analyses have shown that all LD *Borrelia* species possess a unique rRNA gene organization that is different from that of other prokaryotes, including RF *Borrelia* species [109]. The region consists of a single 16S rRNA gene (*rrs*) followed

by a large intergenic spacer (IGS); 3.2 kb in *B. burgdorferi* and 5.0 kb in *B. garinii* and *B. afzelii*. This is followed by a tandem repeat of 23S rRNA-5S rRNA genes (*rrlA-rrfA—rrlB-rrfB*); the tandem copies are separated by a short spacer of 225–266 bp [58, 110–112]. It is assumed that the rRNA IGS accumulate higher levels of sequence variation between related species than do coding regions because IGS regions do not encode functional gene products.

rRNA spacer restriction fragment length polymorphism (PCR-RFLP) typing has been extensively applied to study LD *Borrelia* species. PCR amplification of either *rrs-rrlA* or *rrfA-rrlB* intergenic spacers, followed by RFLP analysis with *Mse* I, allowed differentiation of LD species [16, 17, 113–116]. Postic et al. employed *Mse*I digestion of PCR amplified *rrfA-rrlB* IGS for differentiation of eight LD *Borrelia* species [16]. A modification of this typing method based on reverse line blotting of the *rrfA-rrlB* PCR product followed by hybridization with species-specific DNA probes enabled investigators to directly assess presence of different LD species in ticks [113]. Use of single strand conformational polymorphism (SSCP) in conjunction with PCR amplification of the *rrfA-rrlB* IGS has also been reported [117]. This facilitated identification of a new variant isolate of *B. burgdorferi* previously undetectable with RFLP analysis. The relatively small size of the *rrf*A–*rrl*B IGS (225–266 bp) limits the amount of information that can be obtained by RFLP analysis for typing of individual isolates.

PCR amplification of the 941 proximal base pairs of the *rrs-rrlA* spacer, encompassing the region immediately downstream of *rrs* and terminating at the conserved *ileT* locus, followed by RFLP analysis with either *Mse*I and *Hinf* I restriction enzymes, was able to distinguish LD borreliae at the species level and, more importantly, *B. burgdorferi* at the strain level [17, 21, 115, 116]. Isolates with different RFLP profiles were designated as ribosomal spacer types RST1, RST2, and RST3. Studies using this typing method with uncultivated *B. burgdorferi* in human tissues and field-collected ticks have shown that both LD patients and ticks may be simultaneously infected with one or more distinct genotypes of *B. burgdorferi* [21, 118]. Numerous studies have demonstrated that RST genotype correlates with pathogenic potential in humans and mice [17, 21, 102, 115, 116, 119–122]. A recent study of more than 400 *B. burgdorferi* clinical isolates showed that a combination of RST and *ospC* genotyping permitted the identification of *B. burgdorferi* genotypes that pose the greatest risk of hematogenous dissemination in humans [102].

Bunikis et al. modified this method by direct sequence analysis of the IGS PCR products [84]. They concluded that polymorphism in the first 250 nucleotides of the *rrs-rrlA* IGS was sufficient to discriminate between genotypes; 68 *B. burgdorferi* isolates could be classified into 10 distinct IGS genotypes and the 107 isolates of *B. afzelii* tested were classified into 11 IGS types [84]. By sequencing the entire IGS, Hanincova et al. were able to identify 16 IGS alleles among 127 *B. burgdorferi* clinical isolates [123]. The results of these studies also demonstrated linkage disequilibrium between the *rrs-rrlA* IGS and *ospC* loci [84, 102, 123]. A correlation between RFLP-generated RST types and sequence-generated IGS types concluded that IGS typing of the *rrs-rrlA* partial spacer was sufficient for strain typing of *B. burgdorferi* [84].

IGS typing has also been applied to New World and African RF *Borrelia* species [124, 125]. This facilitated differentiation of *B. hermsii*, *B. turicatae*, *B. miyamotoi,* and *B. lonestari* in North America [124]. *B. crocidurae* could be distinguished from *B. recurrentis* and *B. duttonii*, but this approach did not permit differentiation between the latter two African species [125].

Real-Time PCR

Differentiation of LD *Borrelia* species using real-time quantitative PCR targeting *p66* or *recA*, followed by melting curve analysis, has been described [126]. A simplified method of speciation and quantitation of *B. burgdorferi*, *B. garinii,* and *B. afzelii* has been developed and successfully applied to *I. ricinus* ticks in Germany. This typing method involved a single-run real-time PCR reaction targeting *ospA* and melting curve analysis of the amplified products to distinguish the *Borrelia* species [127]. Of the 1,055 *I. ricinus* ticks tested, 35% were infected; 53% with *B. afzelii*, 18% with *B. garinii* and 11% with *B. burgdorferi*, 0.8% could not be identified and 18% with mixed infections [127]. This approach has diagnostic value, but as yet has not been applied to strain typing.

Multi-Locus Sequence Typing

Differential distribution of 10 variable-number tandem repeat (VNTR) loci was utilized for both species and strain identification in a group of 41 globally diverse LD isolates [128]. This analysis was able to identify *B. burgdorferi*, *B. garinii,* and *B. afzelii*, and to discriminate between strains of *B. burgdorferi* [128]. However, the inability to detect VNTR loci in all *Borrelia* samples is problematic.

Multi-locus sequence typing (MLST) was proposed in 1998 as a highly discriminatory technique that generates accurate data for epidemiological, evolutionary, and population studies of bacterial pathogens [129]. This technique uses a concept similar to multi-locus enzyme electrophoresis, but instead of electrophoretic mobility of metabolic enzymes, the alleles at each locus are defined by nucleotide sequence. A number of studies employed a combination of DNA sequence analyses at multiple genetic loci to characterize the genus *Borrelia* and to elucidate its population structure [23, 26, 84, 130, 131]. These multi-locus sequence analysis methods, however, differ from classic MLST since the analyzed loci included not only housekeeping genes, but other non-coding and plasmid-borne polymorphic loci. In contrast, classic MLST schemes use only housekeeping genes that slowly diversify by random accumulation of nearly neutral mutations and retain signatures of longer term evolution. To ensure highly discriminatory power of relatively uniform housekeeping genes, the combined sequences of multiple housekeeping gene loci are analyzed [132].

An MLST scheme for *B. burgdorferi* has been described that is based on a set of eight different housekeeping loci. The method was employed to evaluate 64 *B. burgdorferi* cultured isolates from North America and Europe [24]. Results indicated that

the North American and European populations of *B. burgdorferi* are genetically distinct and further suggest that *B. burgdorferi* may likely have originated in Europe and not in North America as has been previously thought [133]. In addition, it was demonstrated that the previously used genetic markers of *B. burgdorferi* such as the *rrs-rrl*A intergenic spacer and the *ospC* locus evolve differently, as compared to the eight housekeeping genes [24]. In a separate study, the MLST scheme was applied to a phylogeographical study of 16 specimens of *B. lusitaniae* isolated from ticks in two climatically different regions of Portugal [134]. MLST analysis was able to demonstrate that the *B. lusitaniae* populations from these two regions constituted genetically distinct subpopulations, which appeared monophyletic based on *ospC* and *ospA* phylogenetic analyses [134]. More recently, this MLST approach has been employed to demonstrate that the distribution of *B. burgdorferi* genotypes in ticks varies between the Northeastern and Midwestern United States [135], to study the phylogeography of *B. burgdorferi* in the United States [136] and to define a new *Borrelia* species [137]. Based on these recent studies, it is reasonable to expect that MLST will gain wider use for typing and phylogenetic analyses of *Borrelia* species.

21.2 Conclusion

Application of any typing method to borreliae depends on the objectives of the particular study, the level of resolution desired (species vs. strain), and the laboratory conditions and technical expertise available. The most specific information is provided by complete genome sequencing and DNA–DNA hybridization, but these methods require cultivation of the species of interest. Similarly, PFGE has been very useful for strain typing, but relatively large amounts of DNA are required. Since most *Borrelia* species are difficult to cultivate and grow very slowly (especially newly isolated species), typing methods involving PCR amplification are currently most commonly used. Among these approaches, sequencing of specific genes following PCR amplification and PCR-RFLP or rRNA spacer regions has been most widely used. The recently developed MLST method is likely to become the method of choice in the future.

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