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Molecular Typing of *Borrelia burgdorferi* Sensu Lato: Taxonomic, Epidemiological, and Clinical Implications

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INTRODUCTION

Lyme borreliosis (LB), or Lyme disease, represents a new global public health problem (16). It is now the most common vector-borne disease in North America (41, 237) and Eurasia (172, 291). In the United States, more than 100,000 LB cases have been reported from 47 states in 1982 through 1996, with more than 16,000 cases in 1996 (41). It is estimated that annually about 50,000 cases occur in Europe (172).

In 1982, the bacterium that causes LB was first isolated by Willy Burgdorfer and colleagues from the hard tick *Ixodes dammini* (now *Ixodes scapularis* [175]) collected on Long Island, N.Y. (32). The isolate was subsequently identified as a new species of the genus *Borrelia* and was named *Borrelia burgdorferi* in 1984 (111). Since then, hundreds of *B. burgdorferi* isolates have been cultured worldwide from various geographic regions and biological sources, including *Ixodes* ticks, their reservoir hosts, and specimens from patients with different clinical syndromes. Molecular analysis has indicated that these *B. burgdorferi* isolates are genetically and phenotypically divergent. A closely related cluster containing several tick-borne *Borrelia* species and genomic groups associated with LB has been defined (14, 35, 68, 111, 116, 125, 143, 201, 267). The term

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TABLE 1. Major clinical manifestations of Lyme borreliosis in North America and Europe

Stage[a]	Clinical feature[b]	Incidence (reference) in:	
		North America[c]	Europe[d]
I	Early local infection		
	EM	Common (60–90%) (44, 79)	Common (~77%) (23)
	Tick bite recalled	25% (245)	64% (245)
	Central clearing of EM	35% (245)	68% (245)
	Systemic symptoms	50–69% (161, 245)	38–51% (245, 246)
II	Early disseminated infection		
	Multiple EM	Common (>18%) (161)[e]	Unusual (6%) (246)[e]
	Neuroborreliosis	Common (10–20%) (44, 79)	Common (16–80%) (23, 43)
	Meningoradiculitis	3–21% (44, 79)[f]	37–61% (92, 178)[f]
	Meningitis	2–17% (44, 79)[f]	4–27% (23, 43, 178)[f]
	Carditis	0.5–10% (44, 79)[f]	0.5–4% (46, 178)
	Borrelial lymphocytoma	Rare	Well documented (3%) (23)
III	Late LB		
	Lyme arthritis	Common (51–57%) (44, 242)[g]	Uncommon (~7%) (23)
	ACA	Rare (194)	Well documented (3%) (23)
	Peripheral neuropathy	30–70% late NB (44, 91)[f]	40–63% ACA patients (120)
	CNS involvement	Well documented (90)	<9% NB (178)[f]
	Encephalomyelitis	Rare (0.1%) (91)	4–6% (92, 178)[f]
	Meninggencephalitis	9% (44)[f]	0.5–4% (92, 178)[f]

[a] Stages of the clinical features are those of Steere (236).
[b] NB, neuroborreliosis; CNS, central nervous system.
[c] Data were based mainly on an earlier report by the Centers for Disease Control and Prevention on LB surveillance from 1984 to 1986 (44) and a population-based study in children in Southern Connecticut (79), except for those indicated specifically.
[d] Data were based mainly on a population-based study in Southern Sweden (23), except for those indicated specifically.
[e] Data were based on 76 American (161) and 231 European (246) culture-confirmed patients with EM.
[f] NB patients were used as denominator to calculate the relative prevalence. European data were based on data from 330 NB cases from Germany (178) and 176 NB cases from Denmark (92).
[g] A recent population-based study showed that only 7% of children with LB developed Lyme arthritis (79). About 10% of patients with Lyme arthritis may develop chronic antibiotic treatment-resistant arthritis (88).

"*B. burgdorferi* sensu lato" is now collectively used to refer to all *Borrelia* isolates within this cluster and to distinguish it from the species "*B. burgdorferi* sensu stricto" (strict sense of *B. burgdorferi*) (14).

Like other *Borrelia* species, *B. burgdorferi* sensu lato is a spiral-shaped, gram-negative bacterium with 7 to 11 periplasmic flagella. It varies from 10 to 30 μm in length and 0.2 to 0.5 μm in width (18). The genome of the type strain *B. burgdorferi* sensu stricto B31 contains a linear chromosome of 910,725 bp, with an average G+C content of 28.6%, and 21 plasmids (9 circular and 12 linear) with a combined size of more than 613,000 bp (38, 65). The G+C content of individual plasmids ranges from 23.1 to 32.3% (65).

Human infection due to *B. burgdorferi* sensu lato may involve multiple organs or tissues, resulting in skin, cardiac, neurological and musculoskeletal disorders. Lyme disease (236) was described as a new clinical entity in 1977 because of a geographic clustering of children with rheumatoid-like arthritis in Lyme, Conn. (239, 240, 241). Retrospective analysis revealed that many of the clinical manifestations of LB had been separately recorded by European clinicians since the end of 19th century (270). Table 1 lists the spectrum of the major clinical manifestations of human LB in North America and Europe. It has been shown that multiple erythema migrans (EM) and Lyme arthritis are more common in the United States than in Europe, whereas neuroborreliosis has more frequently occurred in European patients, especially in children with LB (23, 43, 44, 79, 178, 259). Borrelial lymphocytoma and acrodermatitis chronica atrophicans (ACA) are well documented in European LB patients but are rarely recognized among LB patients in the United States (194, 235). As a result of its protean clinical manifestations, LB was described as the new "great imitator" of various human diseases (179).

Numerous studies indicate that the *B. burgdorferi* sensu lato population is genetically highly divergent (1, 12, 14, 26, 71, 109, 130, 132, 144, 145, 147, 155, 168, 197, 259, 268). Different *Borrelia* species may be associated with distinct clinical manifestations of LB (9, 11, 14, 35, 56, 173, 186, 194, 259). In this paper, various molecular methods used for the identification and typing of *B. burgdorferi* sensu lato are reviewed. The current taxonomic status of *B. burgdorferi* sensu lato and the epidemiological and clinical implications of typing of *B. burgdorferi* sensu lato, especially the correlation between the various clinical presentations of LB and the infecting *Borrelia* species, are discussed.

PHENOTYPIC METHODS FOR TYPING OF *B. BURGDORFERI* SENSU LATO

Molecular techniques used for the identification and typing of microorganisms can be categorized as either phenotypic or genetic on the basis of the macromolecular targets used for analysis (249). For *B. burgdorferi* sensu lato, phenotypic typing systems such as biotyping, phage typing, and antibiotic susceptibility analysis, which are used for various bacterial species, are not feasible. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis protein (187, 257, 276) and fatty acid profile analysis (135, 136) have been used for typing of *B. burgdorferi* sensu lato, but the conclusions based on both of these phenotypic characteristics are not always reliable. Therefore, serotyping represents the most commonly used phenotypic method for *B. burgdorferi* sensu lato.

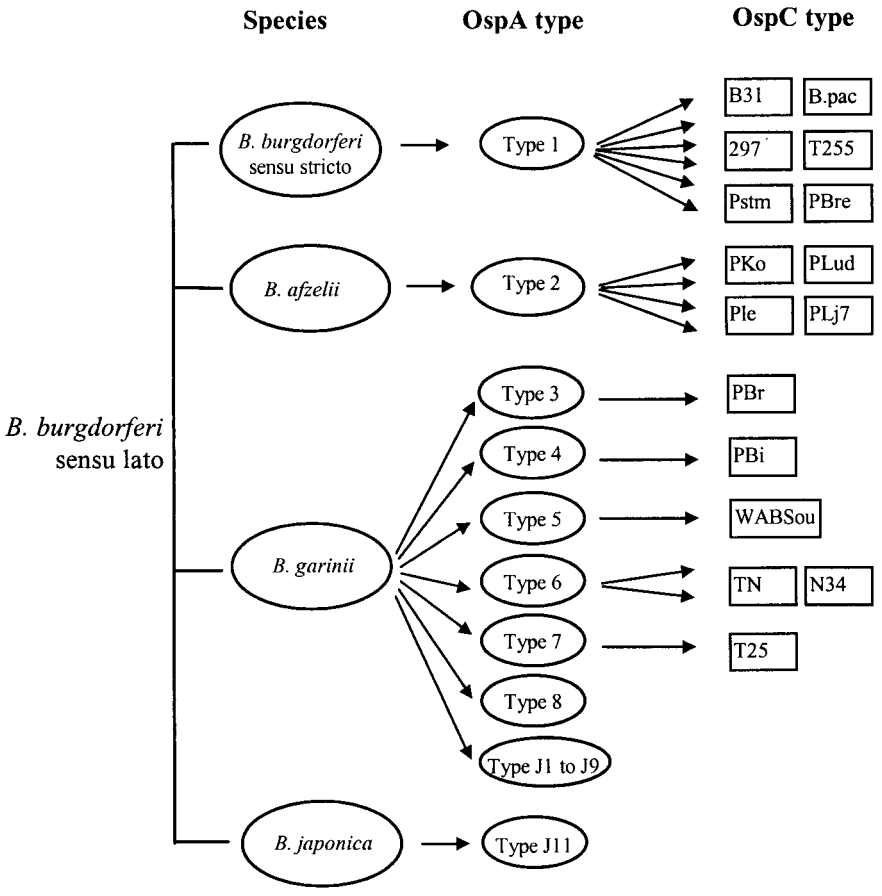

FIG. 1. Relationship between species and OspA and OspC serotypes of *B. burgdorferi* sensu lato. OspA serotype J10 of Japanese isolates corresponds to the European *B. afzelii* strains of serotype 2. Data from references 152 and 279 are included. Adapted from reference 278 with permission of the publisher.

Serotyping

The protein profiles of *B. burgdorferi* sensu lato isolates are heterogeneous (19, 276, 279, 280). Currently, two serotyping systems, based on the heterogeneity of outer surface protein A (OspA) and outer surface protein C (OspC) of *B. burgdorferi* sensu lato, are well established.

OspA serotyping. OspA is one of the major outer membrane lipoproteins of *B. burgdorferi* and has been used for serological diagnosis as well as for vaccine development (97, 119). The molecular mass of OspA ranges from 31 to 34 kDa among the different species of *B. burgdorferi* sensu lato (267, 280). Based on the differential reactivities of 112 European and 24 North American *B. burgdorferi* sensu lato isolates with eight OspA-specific monoclonal antibodies (MAbs), seven different OspA serotypes (serotype 1 to 7) were defined by Wilske et al. in 1993 (280). Later, an additional OspA serotype 8 was reported (277). Among Japanese *B. burgdorferi* sensu lato isolates, OspA serotypes J1 to J11 were recognized (152, 287).

Analysis of phenotypic characteristics in parallel with genetic markers showed that OspA serotypes correlated well with the current classification of *B. burgdorferi* sensu lato (280). As shown in Fig. 1, OspA serotypes 1, 2, and J11 correspond to *B. burgdorferi* sensu stricto, *B. afzelii*, and *B. japonica*, respectively, and serotypes 3 to 8 (types J1 to J9 in Japan) correspond to *B. garinii* (278, 287). OspA serotypes are likely to be differentially distributed among tick and human isolates and have been associated with distinct manifestations of LB (268, 277, 278, 280). In a report of 201 European *B. burgdorferi* sensu lato isolates, 53% of the 90 tick isolates were OspA serotype 6 but only 9% were OspA serotype 2. In contrast, 56% of the 111 isolates from patients with LB were OspA serotype 2 whereas only 13% were OspA serotype 6 (278). Although OspA serotype 4 has often been detected from cerebrospinal fluid CSF specimens of patients with Lyme neuroborreliosis (277), this serotype has not been observed among more than 90 tick isolates from various regions in Europe (278).

OspC serotyping. OspC is the predominant seroreactive antigen in the early stage of human infection due to *B. burgdorferi* sensu lato (2, 56, 74, 185). The molecular mass of OspC among the different species of *B. burgdorferi* sensu lato varies from 20 to 25 kDa (180, 279). Similar to OspA serotyping, analysis of the reactivity of *B. burgdorferi* sensu lato with a set of MAbs specific for OspC has led to the definition of 16 different OspC serotypes for European and North American isolates (278, 279). Comparison of OspA and OspC serotypes of *B. burgdorferi* sensu lato suggests that OspC is much more heterogeneous than OspA, especially for *B. burgdorferi* sensu stricto and *B. afzelii* strains. While only one OspA serotype for each of these species is known, six corresponding OspC serotypes for *B. burgdorferi* sensu stricto and four OspC serotypes for *B. afzelii* were identified (278). The relationships among OspA and OspC serotypes and the delineated *Borrelia* species are summarized in Fig. 1.

OspA and OspC serotyping provides a simple, straightfor-

TABLE 2. Reactivity of *B. burgdorferi* sensu lato species with different species-specific MAbs

Borrelia species	Reactivity with MAb[a]:						Reference(s)
	H9724	H5332	H3TS	D6	I17.3	O1141b	
B. burgdorferi sensu stricto	+	+	+	−	−	−	14, 148
B. garinii	+	+ or −	−	+	−	−	14, 148
B. afzelii	+	−	−	−	+	−	35, 148
B. japonica	+	+	−	−	−	+	148

[a] The reactivity is referred to as positive (+) and negative (−).

ward approach for the analysis of phenotypic characteristics among *B. burgdorferi* sensu lato at the species and subspecies level. Identification of the major *B. burgdorferi* sensu lato species can be made based on the reactivity of *Borrelia* isolates with the well-characterized species-specific MAbs (Table 2). However, the utility of both serotyping systems may be hampered due to the lack of OspA or OspC, the aberrant expression of these proteins, and possibly their variation during growth in vitro as well as in vivo (100, 244, 280).

Multilocus Enzyme Electrophoresis

Multilocus enzyme electrophoresis (MLEE) is a protein-based typing method whose results can be directly correlated with the genotype. MLEE is accepted as a promising method to elucidate the population genetics of bacteria (254). For this method, bacterial lysates are subjected to electrophoresis under nondenaturing conditions and the electrophoretic mobility of metabolic enzymes is determined after specific staining. Each electromorph is equated with an allele at the corresponding enzyme genetic locus. Thus, by associating each isolate with an electrophoretic type (ET), MLEE allows the differentiation of isolates by marking them with significant characteristics.

MLEE was used to analyze the population genetics of *B. burgdorferi* sensu lato in 1992 (26). In this study, 50 *B. burgdorferi* sensu lato isolates were grouped into 35 ETs, constituting three main divisions separated at a genetic distance greater than 0.75. Each of these three divisions corresponded to either *B. burgdorferi* sensu stricto, *B. garinii*, or *B. afzelii* (26). Later, this method was used by Balmelli and Piffaretti (12) to determine the overall genetic polymorphism of 54 *B. burgdorferi* sensu lato isolates from different regions of the world. A total of 12 genetic loci were characterized, and 50 ETs were distinguished. Cluster analysis of a matrix of genetic distances for pairs of ETs revealed 11 divisions separated from each other at a genetic distance greater than 0.65. Of these 11 divisions, 10 corresponded to *B. burgdorferi* sensu stricto (division I), *B. garinii* (divisions II and III), *B. afzelii* (division VII), *B. japonica* (division VIII), *B. valaisiana* (division V), *B. lusitaniae* (division IV), *B. andersonii* (division XI), and *B. bissettii* sp. nov. (divisions IX and X). Division VI contained only one isolate (CA2). Previously, this isolate had been placed into *B. bissettii* sp. nov., formerly genomic group DN127 (197, 201). Additional studies are needed to determine whether this strain constitutes a distinct species.

MLEE provides an estimate of the overall genetic relatedness and genetic diversity of *B. burgdorferi* sensu lato population. Almost all *Borrelia* isolates analyzed can be classified at the species level, and these assignments agree well with the results obtained from various genetic typing techniques (12, 26). However, this method is labor-intensive, mainly because large quantities of spirochetes have to be grown to obtain enough lysate for MLEE analysis. Therefore, MLEE is now used mainly to elucidate the population genetics of *B. burgdorferi* sensu lato.

GENOTYPIC METHODS FOR TYPING OF *B. BURGDORFERI* SENSU LATO

Molecular typing based on the genetic characteristics of microorganisms can provide more precise information on the diversity of pathogenic bacteria. During the past several years, a number of genotyping methods have been used to assess the genetic relationships among *B. burgdorferi* sensu lato species.

DNA-DNA Reassociation Analysis

DNA-DNA reassociation analysis is acknowledged to be a superior method for examining relationships between closely related taxa and represents the best applicable procedure for bacterial taxonomy at present (231). As recommended by the Ad Hoc Committee on reconciliation of approaches to bacterial systematics, the phylogenetic definition of a species generally would include strains with approximately 70% or more DNA-DNA relatedness and with a ΔT_m of 5°C or less (269).

The DNA reassociation value among strains in the genus *Borrelia* varies, ranging from 30 to 100% (18, 111). It is reported that the level of DNA homology between *Borrelia* species causing relapsing fever and those causing LB is about 30 to 44% (18). Among different *B. burgdorferi* sensu lato species, the level of DNA relatedness is about 48 to 70% (14, 111, 197). For example, the type strain *B. afzelii* VS461 has 48, 65, 54, 64, and 58% DNA homology to the type strains *B. burgdorferi* sensu stricto B31, *B. garinii* 20047 (14), *B. japonica* HO14 (116), *B. valaisiana* VS116, and *B. lusitaniae* PotiB2, respectively (197).

Because plasmid DNA may account for one third of the total genome of *B. burgdorferi* sensu lato, the results of DNA-DNA hybridization will also be affected by differences in the plasmid contents between strains. It is expected that plasmid loss occurring during in vitro cultivation (222) may result in a DNA-DNA relatedness below 100% between an isolate and its subcultured variants or among certain highly related strains. However, no data is currently available on this issue, and *Borrelia* isolates showing less than 70% homology to each other in DNA-DNA reassociation analysis do belong to different genospecies when other genetic techniques such as sequencing of the chromosomal genes are used for typing (14, 57, 127).

DNA hybridization is currently used as the reference method for species delineation of *B. burgdorferi* sensu lato (14, 116, 197). On the basis of DNA-DNA relatedness, several different *Borrelia* species or genomic groups associated with LB have been identified (see "Taxonomy of *B. burgdorferi* sensu lato," below, for details).

rRNA Restriction Analysis (Ribotyping)

Ribotyping has been frequently used for both taxonomic purposes and subgroup characterization of microorganisms belonging to different genera and species (195). Grouping of bacteria by ribotyping is based on the profiles obtained by restriction fragment patterns of chromosomal DNA digested with appropriate restriction enzymes and hybridized with a probe derived from a highly conserved rRNA. The restriction enzymes *Eco*RI, *Eco*RV, *Pst*I, *Hinc*II, *Hpa*I, and *Hin*dIII and probes from 16S + 23S rRNA of *Escherichia coli* (14, 267) or from 16S rRNA (144), 23S rRNA (71), or 5S rRNA (132, 225) of the LB spirochete have been successfully used to identify *B. burgdorferi* sensu lato strains at the species level. An example of the restriction patterns obtained after *Eco*RV digestion of genomic DNAs from isolates belonging to different *Borrelia* species is summarized by Postic et al. (196). It is noteworthy that almost all of the LB-related spirochetes from a 3.2-kb *Eco*RV (14, 71), a 3.2-kb *Hpa*I (132, 225), or a 2.2-kb *Hin*dIII (259) restriction fragment. Therefore, these bands can be used as genetic markers for the identification of *B. burgdorferi* sensu lato species. In addition, species-specific *Hin*dIII fragments were recognized by ribotyping for *B. burgdorferi* sensu stricto (1.45 kb), *B. garinii* (1.2 kb), and *B. afzelii* (4.0 and 1.45 kb) (259). These species-specific bands can be used to distinguish the three human pathogenic *B. burgdorferi* sensu lato species from each other.

A large number of *B. burgdorferi* sensu lato isolates from different sources have been analyzed by ribotyping (14, 71, 130, 164, 168, 259). In these studies, the restriction patterns of *B. burgdorferi* sensu stricto and *B. afzelii* appear very homogeneous whereas *B. garinii* is more heterogeneous. In a study including 51 *Borrelia* isolates (21 from Far Eastern Russia, Japan, and China; 20 from Europe; and 10 from North America), the 18 *B. burgdorferi* sensu stricto isolates belonged to only one ribotype while the 10 *B. afzelii* isolates showed three ribotypes and the 23 *B. garinii* isolates exhibited nine ribotypes (71). Thus, *Borrelia* strains can be distinguished by ribotyping at both species and subspecies levels. The result of ribotyping is rather reproducible. A relative drawback of this method is that more spirochetal DNAs are required than for other PCR-based typing approaches.

Pulsed-Field Gel Electrophoresis

Pulsed-field gel electrophoresis (PFGE) was first described in 1984 as a tool for examining the chromosomal DNA profiles of eukaryotic organisms (224). Subsequently, it has proven to be a highly effective molecular typing technique for many bacterial species (250). For this method, the bacterial genomic DNA is separated by PFGE after digestion with a restriction enzyme having relatively few recognition sites. Discrimination of the species and among strains is based on the large restriction fragment length polymorphism (LRFLP) of the chromosomal DNAs.

After digestion of the genome with *Mlu*I, the LRFLPs of *B. burgdorferi* sensu lato are both species and strain specific. As described by Belfaiza et al. (20) and confirmed by others, all three LB-causing *B. burgdorferi* sensu lato species are characterized by one to three species-specific fragments: a band at 135 kb for *B. burgdorferi* sensu stricto, two bands at 220 and 80 kb for *B. garinii*, and three bands at 460, 320, and 90 kb for *B. afzelii* isolates (33, 34, 72, 155, 191, 192). The PFGE method also allows the discrimination between strains within each *Borrelia* species. For example, 20 *B. burgdorferi* sensu stricto isolates were separated into 10 *Mlu*I LRFLPs, while 24 *B. garinii* and 6 *B. japonica* isolates exhibited 4 and 2 LRFLPs, respectively (20, 198, 199). Strikingly, only a single *Mlu*I pattern was observed among the 20 *B. afzelii* isolates (20, 198). In addition, PFGE can be used to construct physical maps of the genomes and to define different groups of *B. burgdorferi* sensu lato by combination of the chromosomal LRFLPs resulting from digestion of a set of restriction enzymes, e.g., *Mlu*I, *Sac*I, *Bss*HII, *Eag*I, *Sma*I, *Apa*I, *Csp*I, and *Sgra*AI (36, 37, 51, 174).

PFGE represents a reproducible and highly discriminative typing method for *B. burgdorferi* sensu lato. Characterization of isolates by PFGE usually correlates well with species designation of *B. burgdorferi* sensu lato by other methods (20, 72). The findings from studies using this method have both taxonomic and clinical implications (20, 155). Mathiesen et al. (155) reported that *B. burgdorferi* sensu lato isolates from patients in the United States, irrespective of their geographic region, belonged to a single rDNA cluster. In this study, results obtained from PFGE and *ospA* sequence analysis were in accordance.

Plasmid Fingerprinting

B. burgdorferi sensu lato strains have unusual double-stranded linear plasmids, in addition to the typical supercoiled circular plasmids (17, 65, 98, 107). Usually, plasmids are present at a low copy number of approximately one to two per chromosome equivalent (37, 98). There may be as many as 21 or more different plasmids within one spirochete cell (38, 65). Since both the number and the size of plasmids can vary among strains, plasmid profile analysis may be used for strain and species identification of *B. burgdorferi* sensu lato.

Xu and Johnson (285) studied 40 *B. burgdorferi* sensu lato isolates from diverse biological sources and geographical locations by plasmid fingerprinting. The number of plasmids in the *B. burgdorferi* sensu lato isolates ranged from 4 to 10, and the size of these plasmids ranged from 13.3 to 57.7 kb. The overall plasmid profiles might correlate with the species of *B. burgdorferi* sensu lato, although definite species-specific plasmids could not be identified in the three *Borrelia* species studied. Nevertheless, only linear plasmids, but not circular plasmids, are well separated in plasmid fingerprinting (285). The usefulness of plasmid fingerprinting for the classification of *B. burgdorferi* sensu lato seems to be limited since the results could be affected simply by the loss of plasmids during in vitro cultivation (15, 222, 286) or by inter- and intraplasmidic recombination (38, 212).

Randomly Amplified Polymorphic DNA

There is an increasing interest in the molecular typing of bacteria by using randomly amplified polymorphic DNA (RAPD) fingerprinting (275), or arbitrarily primed PCR (AP-PCR) (271). Both of these techniques use low-stringency PCR amplification with a single primer with an arbitrary sequence to generate strain-specific arrays of anonymous DNA fragments. In an early AP-PCR study, 29 *B. burgdorferi* sensu lato isolates were divided into three genospecies-specific groups (272). Later, AP-PCR was used to investigate the evolution of *B. burgdorferi* sensu stricto (64). More recently, this approach was evaluated by typing a large collection of *B. burgdorferi* sensu lato isolates from various biological and geographical sources (268). In this study, a total of 136 *B. burgdorferi* sensu lato strains were divided into seven genetic clusters, based on the DNA fingerprints generated with four arbitrary primers (Fig. 2). Six clusters contained 135 isolates corresponding to the well-defined species. One isolate did not belong to any of the known LB-related *Borrelia* species. Furthermore, pathogenic subgroups of *B. garinii* from LB patients with disseminated infections were identified by this analysis (268). Since RAPD is

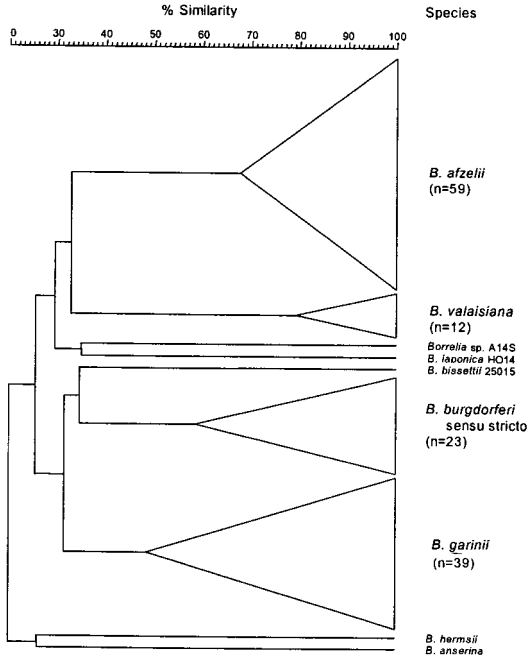

FIG. 2. Simplified dendrogram of 136 Lyme disease spirochete isolates from different *B. burgdorferi* sensu lato species based on their RAPD fingerprints. The numbers of isolates for each *B. burgdorferi* sensu lato species studied is indicated in parenthesis. The size of the vertical bars of the triangles for the four major *B. burgdorferi* sensu lato species represents the number of isolates studied, while the position of the left angle of these triangles is representative of the percent similarity within each of these species. Modified from reference 268.

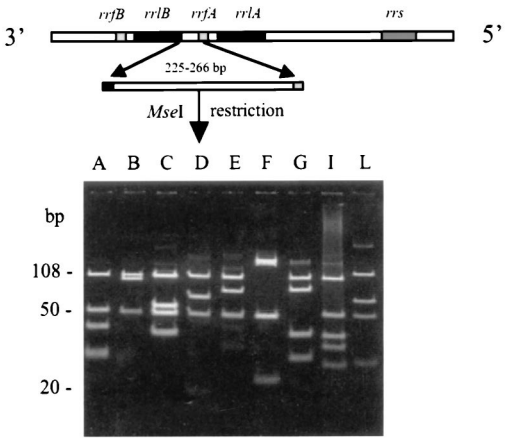

FIG. 3. Typing of *B. burgdorferi* sensu lato isolates by using the *rrfA-rrlB* intergenic spacer PCR-RFLP analysis. The *rrfA-rrlB* intergenic spacer was amplified by PCR, and this was followed by the analysis of *Mse*I restriction polymorphism of PCR products. The eight *B. burgdorferi* sensu lato species included are *B. burgdorferi* sensu stricto (pattern A), *B. garinii* (patterns B and C), *B. afzelii* (pattern D), *B. japonica* (pattern E), *B. valaisiana* (pattern F), *B. lusitaniae* (pattern G), *B. bissettii* sp. nov. (pattern I), and *B. andersonii* (pattern L). Modified from reference 196 with permission of the publisher.

a highly discriminatory method and is easy to perform, this method is now a powerful tool to distinguish the different *Borrelia* species from each other as well as to recognize *Borrelia* strains within each of the species (268).

PCR and PCR-Based Restriction Fragment Length Polymorphism Analysis

Species-specific PCR. PCR amplification with species-specific primers, in which the conserved 16S rRNA gene (131, 141) or species-specific plasmid gene loci (159, 160) are targeted, can be used directly for species identification of the LB spirochetes. The former approach has been used to differentiate the three human pathogenic species, *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* (121, 141), as well as *B. valaisiana* (131), from each other. However, cross-amplification between *Borrelia* species may occur when using primers derived from the species-specific plasmid sequences (159).

rDNA PCR-RFLP analysis. The *rrn* cluster of most *B. burgdorferi* sensu lato strains contains a single copy of 16S rRNA (*rrs*) and tandem repeated 23S rRNA (*rrlA* and *rrlB*) and 5S rRNA (*rrfA* and *rrfB*) (Fig. 3) (73, 77, 174, 225). The rDNA gene cluster is located at the center of the linear chromosome and is arranged in the following order: *rrs-rrlA-rrfA-rrlB-rrfB*. Three different PCR-RFLP approaches have been designed for *B. burgdorferi* sensu lato, based on the unique rRNA structure of these microorganisms, i.e., *rrs*, *rrfA-rrlB* spacer, and *rrs-rrlA* spacer.

DNA sequence analysis of *rrs* gene is able to discriminate various *B. burgdorferi* sensu lato species (see the following section). Several species of *B. burgdorferi* sensu lato can also be distinguished from each other on the basis of their *Bfa*I restriction patterns of PCR-amplified *rrs* (127, 203) or by single-strand conformational polymorphism–PCR analysis of the *rrs* (273). However, RFLP analysis of the PCR-amplified DNA from spacers between the ribosomal genes shows more discriminatory power.

rrfA-rrlB spacer PCR-RFLP analysis is a widely used typing system for *B. burgdorferi* sensu lato (197). Development and application of this method led to the description of eight different species or genomic groups within *B. burgdorferi* sensu lato in 1994 by Postic et al. (197). PCR amplification of the highly variable *rrfA-rrlB* intergenic spacer usually yields a 225- to 266-bp amplicon among strains from different species of *B. burgdorferi* sensu lato (149, 197, 265). No amplification occurred with the relapsing-fever borreliae. Digestion of amplicons with *Mse*I resulted in different restriction fragments with species-differentiating characteristics (Fig. 3) (197). Table 3 summarizes the *Mse*I restriction patterns of *B. burgdorferi* sensu lato identified to date. The results based on the *Mse*I restriction patterns are generally in accordance with those of DNA-DNA hybridization (197).

The *rrs-rrlA* intergenic spacer is about 3.2 kb in *B. burgdorferi* sensu stricto and 5 kb in *B. garinii* and *B. afzelii* (77, 174, 225). Liveris et al. reported that amplification of the partial *rrs-rrlA* spacer, followed by digestion with *Hin*fI and *Mse*I, produced both species- and subspecies-specific RFLP patterns (132, 134). This method has been applied to typing of *B. burgdorferi* directly in human tissue specimens and field-collected ticks, thus obviating the need for culture isolation (133, 134). These studies demonstrated that a significant proportion of human EM lesions contained mixtures of different *B. burgdorferi* genotypes. Furthermore, typing of *B. burgdorferi* isolates by this method may allow the differentiation of isolates with different degrees of pathogenic potential (134, 284).

Ribosomal spacer DNA RFLP analysis of *B. burgdorferi* sensu lato has a number of advantages. It is a rather simple and useful tool for rapid screening and species identification of large collections of *B. burgdorferi* sensu lato isolates. Furthermore, it can be used for typing uncultured samples collected from ticks and patients with LB. This facilitates epidemiological monitoring of the distribution of the different *B. burgdorferi* sensu lato species in *Ixodes* ticks and reservoir hosts. By

TABLE 3. *Mse*I restriction fragments of the 5S-23S rRNA (*rrfA-rrlB*) intergenic spacer of *B. burgdorferi* sensu lato

Species	Strain	Amplicon size (bp)	RFLP pattern	*Mse*I restriction fragment size (bp)	Reference
B. burgdorferi sensu stricto	B31	254	A	108, 51, 38, 29, 28	197
B. garinii	20047	253	B	108, 95, 50	197
	NT29	253	C	108, 57, 50, 38	197
B. afzelii	VS461	246	D	108, 68, 50, 20	197
B. japonica	HO14	236	E	108, 78, 50	197
B. vailaisiana	VS116	255	F	175, 50, 23, 7	267
	Am501	249	Q	169, 51, 23, 6	149
B. lusitaniae	PotiB2	257	G	108, 81, 39, 29	197
	PotiB3	255	H	108, 79, 52, 16	197
B. bissettii	DN127	257	I	108, 51, 38, 33, 27	197
	CA	226	J	108, 51, 38, 29	197
	25015	253	K	108, 51, 34, 27, 17, 12, 4	197
B. andersonii	19857	266	L	120, 67, 51, 28	197
	CA2	255	M	91, 50, 40, 28, 22, 17, 7	197
B. tanukii	Hk501	245	O	174, 51, 20	149
B. turdi	Ya501	248	P	107, 51, 38, 21, 16, 8, 7	149
Borrelia sp.	A14S	225	R	108, 66, 51	265

using reverse line blotting of the *rrfA-rrlB* spacer which was amplified by PCR and subsequently hybridized with species-specific oligonucleotide probes, Rijpkema et al. were able to identify four different *Borrelia* species in ticks collected in the Netherlands (207, 208). The prevalence of *B. burgdorferi* sensu lato infection in ticks and various reservoir hosts in several European countries was also investigated successfully by reverse line blotting (47, 118, 206).

DNA Sequence Analysis

Whole DNA-DNA reassociation analysis is the most robust approach by which *B. burgdorferi* sensu lato can be classified based on phylogenetic relationships since the results are ultimately based on the entire genome sequence of the organism. Generally, this method is useful for the study of bacterial genetics, evolution, taxonomy, and epidemiology (231). However, DNA sequence analysis of some highly conserved gene loci can be used as a suitable alternative method. For example, *rrs*, *fla*, and *ospA* have been used for this purpose with *B. burgdorferi* sensu lato.

Sequence analysis of bacterial *rrs* has been widely used for assessing the evolutionary history and species identification of *B. burgdorferi* sensu lato (68, 127, 142, 143, 201, 267). More than 200 *rrs* sequences from various *B. burgdorferi* sensu lato species have been determined (many of them partial sequences) and are now available from the GenBank database (22). The DNA sequences of *rrs* among strains from different *B. burgdorferi* sensu lato species are highly homologous, ranging from 95.3 to 99.6% (127). By using a 1,368-bp portion of the *rrs* gene from representative isolates of each *Borrelia* species obtained from diverse geographic regions and various biological sources (Table 4), a phylogenetic tree was constructed by the neighbour-joining method and is presented in Fig. 4. *Borrelia* isolates belonging to distinct species fall into individual clusters in the phylogenetic tree. Thus, sequence analysis of the 16S rRNA gene represents a reliable method for inferring the taxa of *B. burgdorferi* sensu lato (68, 127, 142, 201, 267).

The *fla* gene (76), now known as *flaB*, encodes a 41-kDa flagellin protein or FlaB (78) and is located on the linear chromosome (65). By phylogenetic analysis based on *fla* gene sequences, the relapsing-fever borreliae can be separated from the LB-related borreliae and different *B. burgdorferi* sensu lato species can also be distinguished from each other (69, 70).

The *ospA* gene, located on a 49- to 57-kb linear plasmid (24, 217), is present in almost all *B. burgdorferi* sensu lato isolates (266, 285). Sequence analysis of *ospA* genes showed homogeneity within *B. burgdorferi* sensu stricto and *B. afzelii* but revealed major subgroups within the *B. garinii* species (274, 280). Although lateral gene transfer and recombination of *ospA* between different *Borrelia* species may occur, the frequency of such events is rather low (57, 212). Thus, cluster analysis of the *ospA* gene could provide useful phylogenetic information. Usually, the clustering of *Borrelia* strains in the phylogenetic tree based on the sequence of *ospA* and its predicted amino acid sequence is in agreement with the classification based on sequence analysis of conserved chromosomal genes such as *rrs* (155), *p93* (57), and *fla* (69), as well as with data obtained by PFGE (33, 155) and RAPD fingerprinting (64, 268). However, a recent study of eight *B. valaisiana* isolates showed that *ospA* sequence analysis might not be appropriate for species identification of *B. burgdorferi* sensu lato (266).

Comparative analysis of the deduced amino acids sequences from *B. burgdorferi* sensu lato revealed a species-specific motif in the conserved amino-terminal of the OspC protein (66, 67, 109, 137). Although lateral transfer of *ospC* genes between species has been described (137, 205, 264), the species-specific motif region had not been transferred except for a few *B. valaisiana* isolates, which possibly obtained a complete *ospC* gene from *B. afzelii* or *B. garinii* (264). However, it is usually

TABLE 4. *B. burgdorferi* sensu lato strains used for phylogenetic analysis in Fig. 4

Species and strain	Biological source	Geographic region	Accession no. of *rrs*	Reference(s)
B. burgdorferi sensu stricto				
B31	*Ixodes scapularis*	United States	U03309	225
297	Human, CSF	United States	X85204	251
20004	*Ixodes ricinus*	France	M64310	141
DK7	Human skin, ACA	Denmark	X89195	251
B. garinii				
20047	*Ixodes ricinus*	France	D67018	67
PBi	Human, CSF	Germany	X85199	251
DK27	Human shin, EM	Denmark	X85193	251
JL20	Human, CSF	Sweden	X95198	251
ChY13p	*Ixodes persulatus*	China	AB007450	129
B. afzelii				
DK1	Human skin, EM	Denmark	X85109	251
DK2	Human skin, ACA	Denmark	X85198	251
R-IP3	*Ixodes persulatus*	Russia	L46697	141
HT61	*Ixodes persulatus*	Japan	D67019	67
17Y	*Ixodes persulatus*	Korea	U44939	GenBank
B. japonica				
HO14	*Ixodes ovatus*	Japan	L40597	67
IKA2	*Ixodes ovatus*	Japan	L40598	67
B. valaisiana				
VS116	*Ixodes ricinus*	Switzerland	X98232	265
UK	*Ixodes ricinus*	England	X98233	265
Am501	*Ixodes columnae*	Japan	D67021	67
B. lusitaniae				
PotiB2	*Ixodes ricinus*	Portugal	X98228	127
BR41	*Ixodes ricinus*	Czech Republic	X98231	127
B. andersonii				
19857	Rabbit	United States	L46688	67, 143
21038	*Ixodes dentanus*	United States	L46701	67, 143
B. tanukii				
Hk501	*Ixodes tanuki*	Japan	D67023	67, 68
OR2eL	*Ixodes tanuki*	Japan	D67020	67, 68
B. turdi				
Ya501	*Ixodes turdus*	Japan	D67022	67, 68
Ac502	*Ixodes turdus*	Japan	D67024	67, 68
B. bissettii sp. nov.				
DN127	*Ixodes pacificus*	United States	L40596	67, 197
B. hermsii				
HS1	*Ornithodoros hermsii*	United States	U42292	76

inconclusive for assignment of an isolate to a specific *Borrelia* species because of the overall high variability of *ospC* sequences (109, 252, 281) and lateral transfer of *ospC* gene between species (137, 205, 264).

Sequence analysis of other gene loci such as *p93* (57), *hbb* (258), *p39* (211), *hsp60* (263), and *hsp70* (263) can provide additional information for species identification of *B. burgdorferi* sensu lato.

Comparison and Selection of Typing Methods

Molecular typing systems can be characterized in terms of typeability, reproducibility, discriminatory power, ease of performance, and ease of interpretation (6, 249). The choice between various methods depends on a number of factors, such as the objectives of the study, the level of discriminatory power required, the kinds of DNA preparations and laboratory conditions available, and the technical skills of laboratory personnel. At this point, we recommend the ribosomal spacer PCR-RFLP as the method for *Borrelia* species identification during molecular epidemiological studies of *B. burgdorferi* sensu lato infection in *Ixodes* ticks, reservoir hosts, and patients. The discriminatory power of PFGE and RAPD is high, and both methods can be used to evaluate the genetic heterogeneity within *Borrelia* species. RAPD is easier to perform than PFGE and can be used to monitor large numbers of isolates. DNA-DNA reassociation analysis is currently the reference method for species delineation, although it is a time-consuming and

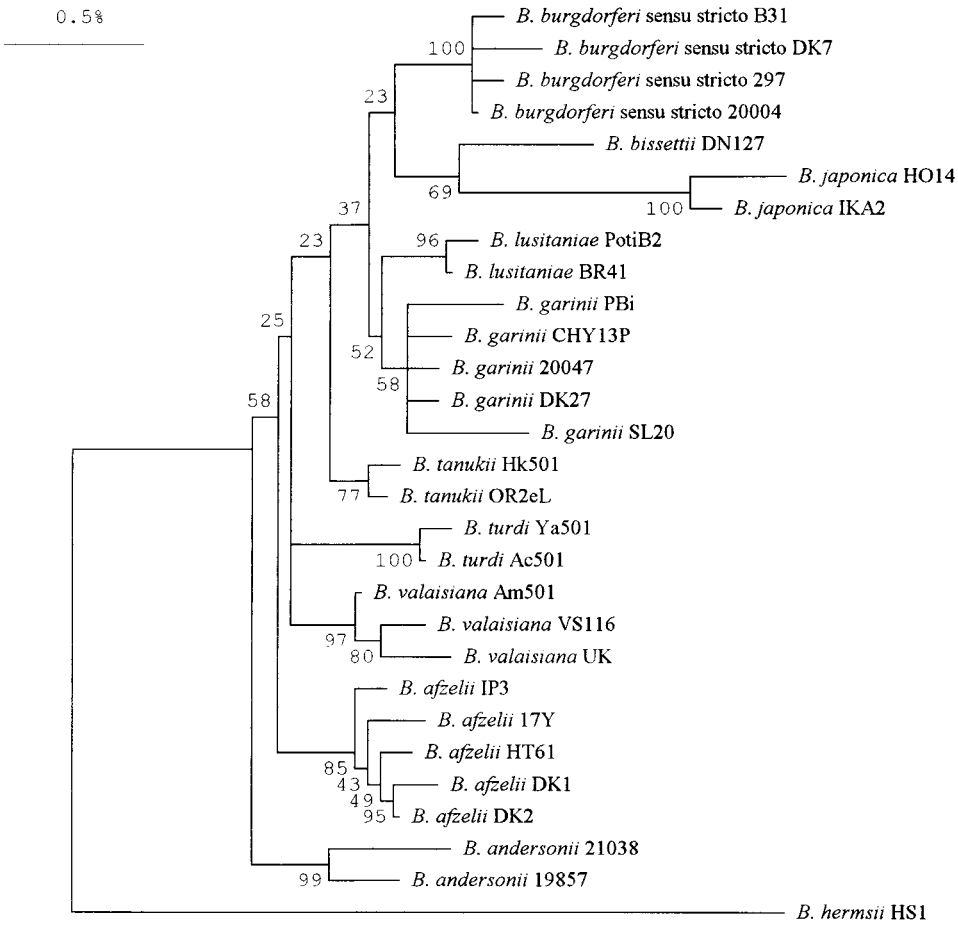

FIG. 4. Phylogeny of Lyme disease spirochete isolates as inferred from 16S rRNA gene sequence analysis. The phylogenetic tree was constructed by using the neighbor-joining method in the MEGA program as described in reference 267. A total of 28 *B. burgdorferi* sensu lato isolates representing 10 different *Borrelia* species were included in this analysis. *B. hermsii* HS1 was used as the outgroup. The sources of each isolate are given in Table 4. Numbers at the branch nodes indicate the results of bootstrap analysis. The bar represents 0.5% sequence divergence.

labor-intensive method. Therefore, this method is used mainly for confirmation of the taxonomic status of isolates that are not identifiable or typeable by ribosomal spacer RFLP and *rrs* sequence analysis, as well as for the identification of newly recognized *Borrelia* species or genomic groups.

TAXONOMY OF *B. BURGDORFERI* SENSU LATO

Taxonomy and Evolution of *B. burgdorferi* Sensu Lato

The spirochetes are one of the few major bacterial groups whose natural phylogenetic relationships are evident at the level of gross phenotypic characteristics. These organisms form a coherent taxon composed of six major groups as indicated by phylogenetic analysis (183, 282). The taxonomy of the order *Spirochetae* is out of the range of this review and can be found in two references (183, 233). Our interest focuses on the taxonomy of the genus *Borrelia*, especially the *Borrelia* species within the *B. burgdorferi* sensu lato complex.

The genus *Borrelia* represents a tight phylogenetic cluster, which is differentiated from other spirochetal phylogenetic groups by base signature analysis of *rrs* (183). More than 20 species have been identified within the genus so far (18, 223). These *Borrelia* species are usually categorized into two major categories, the relapsing-fever borreliae and the LB borreliae, on the basis of the differences between their ecological and genetic characteristics (18).

MLEE analysis has revealed a clonal population structure of *B. burgdorferi* sensu lato based upon the linkage disequilibrium of allele distributions (26), indicating that in the absence of recombination, all genes in strains belonging to the same species have a common evolutionary history. This conclusion has also been drawn from RAPD fingerprinting (13) and comparison of the sequences of the highly conserved chromosomal genes *fla* and *p93* (57). In addition, clonal populations were evidenced by the conserved chromosomal gene order in different *B. burgdorferi* sensu lato species (36, 174). It is not clear whether such populations are strictly clonal, since localized horizontal gene transfer of the outer membrane protein-encoding genes has been found in *B. burgdorferi* sensu lato (137, 145, 146, 205, 212, 264). For example, a close examination of the highly variable *ospC* gene indicated that lateral gene transfer might have occurred quite frequently, not only between members of the same species but also between strains from different species (137). As a result of such gene transfer and subsequent recombination, the linkage disequilibrium among different species might be disturbed. This would lead to a different topology of phylogenetic relationship based on *ospC* sequence analysis compared to that based on *rrs* gene analysis. Nevertheless, in general, *ospC* genes from strains of the same

TABLE 5. *Borrelia* species associated with LB, their ecological and pathogenic characteristics, and their geographic distribution

Taxon	Vector	Animal host	Human disease[a]	Distribution	Reference(s)
B. burgdorferi sensu lato	*I. scapularis* *I. pacificus*	Mammals, birds	EM, **arthritis,** carditis, neuroborreliosis	United States	14, 111
	I. ricinus			Europe	14
	I. persulatus?			Asia?	130
B. garinii	*I. ricinus* *I. persulatus*	Birds, small mammals	EM, arthritis, **neuroborreliosis**	Europe Asia	14
B. afzelii	*I. ricinus* *I. persulatus*	Small mammals	EM, arthritis, neuroborreliosis, **ACA**	Europe Asia	14, 35
B. japonica	*I. ovatus*	Small mammals	No	Japan	116
B. valaisiana	*I. ricinus* *I. granulatus* *I. columnae*	Birds	Unclear	Europe Asia	267
B. lusitaniae	*I. ricinus*	Unknown	No	Central Europe	127
B. andersonii	*I. dentatus*	Rabbit	No	United States	143
B. bissettii sp. nov.	*I. pacificus* *I. neotomae* *I. scapularis*	Rodents, birds	Unclear	United States	197, 201
	I. ricinus		EM, lymphocytoma	Slovenia	247
B. takunii	*I. takunus*	Small mammals	No	Japan	68
B. turdi[b]	*I. turdus*	Small mammals	No	Japan	68

[a] The clinical syndromes associated with distinct *B. burgdorferi* sensu lato species are in boldface type.
[b] The name has been corrected from *B. turdae* (68) by the International Union of Microbiological Societies (108).

species appear to be more closely related to each other than to *ospC* genes from different species. Furthermore, species-specific motifs recognized in the conserved amino terminal of the OspC proteins (67, 109, 137) indicate that *ospC* is still clonally inherited.

It is assumed that speciation among *B. burgdorferi* sensu lato is only a recent phenomenon, since divergence of the *rrs* gene sequences among representatives of different *Borrelia* species may not exceed 1% (127). Assuming a clonal evolution of *B. burgdorferi* sensu lato, one may predict that the greater the genetic diversity of the isolates within a given species, the longer the period available for evolution. Since the diversity of *B. burgdorferi* sensu lato in Eurasia is much greater than in North America, it is likely that this complex originated in Eurasia. Furthermore, *B. garinii*, which shows the greatest genetic heterogeneity by most analytical methods, is most likely the species which is closest to the common ancestor of *B. burgdorferi* sensu lato, as was recently proposed (64, 205).

Species within the *B. burgdorferi* Sensu Lato Complex

Different *Borrelia* species can be distinguished from each other by analysis of their phenotypic features, such as reactivity with species-specific MAbs (Table 2), or by genetic typing as outlined in the previous section. Since the discovery of *B. burgdorferi* in 1982, 10 different *Borrelia* species or genomic groups within the *B. burgdorferi* sensu lato complex have been identified. Nine are separated at the species level and were designated *B. burgdorferi* sensu stricto, *B. garinii* (14), *B. afzelii* (14, 35), *B. japonica* (116), *B. valaisiana* (267), *B. lusitaniae* (127), *B. andersonii* (143), *B. tanukii* (68), and *B. turdi* (68) (the name has been corrected from *B. turdae* [108]). More recently, the 10th genomic group, characterized by isolate DN127 (197), has been reevaluated and proposed as a new species, *B. bissettii* sp. nov. (201). The major ecological and human pathogenic characteristics of the LB-related *B. burgdorferi* sensu lato species, as well as their geographic distributions, are summarized in Table 5.

A number of atypical *Borrelia* isolates, not clearly belonging to one of the described species, have been cultured from North America (36, 155, 191, 201), Europe (193, 268), and China (130, 291). One of these was cultured from a patient with LB in Europe (265, 268). It is reasonable to expect that more *Borrelia* species will be recognized as more isolates are recovered from divergent biological and geographic sources and are studied by various molecular typing methods.

Borrelia Species Pathogenic to Humans

Not all strains from the described *Borrelia* species or genomic groups are pathogenic for humans. Currently, only *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* have been cultured frequently from patients with LB (33, 134, 155, 161, 246, 259, 280). These three major species appear to be responsible for causing different clinical syndromes of human LB (11, 35, 56, 173, 259). A recent study of nine *B. burgdorferi* sensu lato isolates recovered from patients with LB in Slovenia revealed that they were most closely related to the North American isolate 25015, which belongs to *B. bissettii* sp. nov. (193, 247). Thus, *B. bissettii* sp. nov. isolates may represent the fourth *Borrelia* species that could cause human LB. Interestingly, *B. bissettii* sp. nov. is the second reported *B. burgdorferi* sensu lato species (*B. burgdorferi* sensu stricto is the other) to be present in both the Old World and New World (193, 247). *B. valaisiana* is widely distributed in European countries as well as in Asia

(267). It might be pathogenic for humans, since DNA specific for this species has been detected by PCR from patients with LB (209).

Although it is uncertain whether the other LB-related *Borrelia* species cause human disease, their pathogenic role is far less important than that of the three major *Borrelia* species. For example, *B. japonica* was only weakly pathogenic in mice (114). Although *I. ovatus* ticks, the specific vector for *B. japonica*, are distributed widely in Japan and frequently bite humans, no LB patients with a confirmed *I. ovatus* tick bite have been reported, except for one case suspected on the basis of the serological findings (153).

EPIDEMIOLOGICAL IMPLICATIONS

Geographic Distribution of Different *Borrelia* Species

LB is a globally distributed zoonosis (21, 221). Human cases occur predominantly in the northern hemisphere (162, 221). Three LB-associated *Borrelia* species, *B. burgdorferi* sensu stricto (111), *B. andersonii* (143), and *B. bissettii* sp. nov. (201), are recognized in North America. *B. burgdorferi* sensu stricto is widely distributed in the northeast, midwest, and western regions of the United States (41). It is the only *Borrelia* species cultured from patients with LB in North America. *B. andersonii* and *B. bissettii* sp. nov. were cultured mainly from ticks and small mammals from New York and California, respectively (4, 28, 201).

B. burgdorferi sensu stricto, *B. garinii*, *B. afzelii*, *B. valaisiana*, *B. lusitaniae*, and *B. bissettii* sp. nov. have been documented in Europe. The geographic distribution of these species on the European continent has been reviewed recently based on a total of 1,263 records (738 isolates and 525 molecular DNA detections) from 26 countries (101). *B. garinii* and *B. afzelii* are the most frequently cultured species in Europe. *B. burgdorferi* sensu stricto is distributed mainly in western European countries but is rarely recognized in eastern areas of Europe. *B. valaisiana* has been cultured or detected in ticks or avian reservoirs from at least eight European countries, including the United Kingdom (123), Ireland (117, 118), The Netherlands (207, 267), Switzerland (105, 187, 188), Germany (131, 216), Italy (48), Croatia (86, 206), and the Czech Republic (200). This species seems abundantly present in ticks in Ireland, Germany, and Croatia (118, 131, 206). Only a few *B. lusitaniae* strains have been isolated in Portugal (197), the Czech Republic, Moldova, and Ukraine (200). Nine isolates with genotypic and phenotypic similarity to *B. bissettii* sp. nov. have been cultured in Slovenia (193, 247).

Six *B. burgdorferi* sensu lato species have been reported in Far Eastern Russia and Asian countries. *B. garinii* and *B. afzelii* are largely found throughout the range of *I. persulcatus* ticks in China (129, 248, 290), Japan (72, 164), Korea (181), and Far Eastern Russia (147, 200, 218). *B. japonica*, *B. tanukii*, and *B. turdi* are limited to Japan (68, 116, 149). One isolate from an *I. columnae* tick in Japan was classified as *B. valaisiana* (267). The presence of *B. burgdorferi* sensu stricto in Asia is controversial. Although most reports suggest that *B. burgdorferi* sensu stricto is absent (129, 147, 164, 200, 248), two recent studies indicated the presence of this *Borrelia* species in the mainland of China (130) and in Taiwan (226).

LB cases in the southern hemisphere including South America (10), Africa (158, 219), and Australia (157) have been reported. However, these cases were based on serology only. *B. burgdorferi* sensu lato has not been isolated from local *Ixodes* ticks or any other suspected vectors or patients (31, 213). Recently, *B. garinii* was recovered from a patient with LB in Australia; however, the infection may have been acquired in Europe (103).

Vectors and *Borrelia* Species Specificity

The principal vectors of *B. burgdorferi* sensu lato are ticks of the *I. ricinus* complex (31). These include *I. scapularis* (*I. dammini*) and *I. pacificus* in the United States, *I. ricinus* in Europe, and *I. persulcatus* in Asian Russia, China and Japan (3, 31, 53, 164, 290). These vectors for *B. burgdorferi* sensu lato are not strictly species specific, and the specificity displayed by many of the relapsing-fever borreliae for a single tick species is probably not applicable to the LB spirochetes. For example, the European sheep tick *I. ricinus* has been recognized as a vector of all three human pathogenic *Borrelia* species, *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* (80, 189). *I. scapularis* and *I. pacificus* in the United States and *I. ricinus* in Europe are vectors of *B. burgdorferi* sensu stricto (31, 208, 236).

On the other hand, *B. japonica* is found mainly in *I. ovatus* in Japan, and *B. andersonii* appears to be restricted to *I. dentatus* in the United States. Furthermore, *B. burgdorferi* sensu stricto, the most widely distributed *Borrelia* species in *I. scapularis* in North America and *I. ricinus* in Europe, is rarely found in the *I. persulcatus* regions in Far Eastern Asia (129, 164, 200). These findings suggest that some particular vector species will harbor and transmit only one specific *Borrelia* species. A recent comparative study of the three main clusters of European *I. ricinus* indicated significant differences in their susceptibility to *B. afzelii* (60). This finding may explain, in part, the uneven distributions of *Borrelia* species in Europe.

Both *I. ricinus* and *I. persulatus* can be infected with *B. garinii* and *B. afzelii* (102, 164, 200, 291). Other *Borrelia* species may also be transmitted by several vector species of the *I. ricinus* group (e.g., *B. bissettii* sp. nov. strains have been isolated from *I. neotomae* [now *I. spinipalpis*] [171], *I. scapularis*, and *I. pacificus* in the United States [197], as well as from *I. ricinus* in Europe [247]). Although *B. valaisiana* and *B. lusitaniae* are cultured mainly from European *I. ricinus* ticks, the former species has also been cultured from *I. columnae* ticks in Japan (67, 127, 267).

Additional tick vectors as well as hematophagous insects may play a role in maintenance of *B. burgdorferi* sensu lato in enzootic cycles in nature and occasionally in transmitting *B. burgdorferi* sensu lato to humans (31). These include *I. uriae*, *Dermacentor variabilis*, *D. parumapertus*, *Amblyomma americanum*, *Rhipicephalus sanguineus*, and *Haemaphysalis leporislustris* in North America (31); *I. hexagonus*, *I. uriae*, *I. trianguliceps*, *I. acuminatus*, *I. frontalis*, *D. reticulatus*, and *H. punctata* in Europe (80, 229); *I. tanuki* and *I. turdus* in Japan (67); and *I. granulatus*, *H. bispinosa*, *H. concinna*, and *H. longicornis* in southern China (290, 291). The relationship between these possible tick vectors and various *B. burgdorferi* sensu lato species remains unknown.

Mixed infections of multiple *B. burgdorferi* sensu lato species have been found in ticks (131, 160, 164, 190, 200, 206), reservoir hosts (123, 163, 164, 200), and in patients with LB (52, 133, 209). The prevalence of mixed infections in ticks varies from 5 to 40% in different geographic regions (131, 160, 190, 200). Such mixed infections may result directly from feeding on a host infected with multiple *Borrelia* species or through cofeeding transmission, by which various spirochetes may be exchanged among ticks infesting a single host (84, 165, 184, 204). Alternatively, ticks may acquire different *Borrelia* species from different individual hosts during their three life cycle stages, since the spirochetes can survive through the molts and are present in all subsequent stages of the vectors (3, 31).

TABLE 6. Distribution of *B. burgdorferi* sensu lato species in 472 isolates cultured from LB patients with different clinical syndromes[a]

Specimen	Total no.	*B. burgdorferi* sensu stricto	*B. garinii*	*B. afzelii*	Other[f]
		No. (%)	No. (%)	No. (%)	No. (%)
Skin, EM[b]	308	96 (31.2)	50 (16.2)	156 (50.7)	6 (1.9)
CSF[c]	68	9 (13.2)	46 (67.7)	10 (14.7)	3 (4.4)
Skin, ACA[d]	74	3 (3.0)	5 (6.8)	66 (89.2)	0 (0)
Others[e]	22	16 (72.8)	3 (13.6)	2 (9.1)	1 (4.5)
Total	472	124 (26.3)	104 (22.0)	234 (49.6)	10 (2.1)

[a] Only isolates with indication of clinical source and belonging to *Borrelia* species in published papers were included for analysis. These isolates were recovered from patients from 18 countries including Germany ($n = 123$), United States ($n = 92$), The Netherlands ($n = 74$), Slovenia ($n = 63$), Austria ($n = 45$), Denmark ($n = 21$), Japan ($n = 12$), Sweden ($n = 10$), Russia ($n = 7$), Switzerland ($n = 6$), Czech Republic ($n = 6$), China ($n = 4$), France ($n = 3$), Italy ($n = 2$), Poland ($n = 1$), Croatia ($n = 1$), Lithuania ($n = 1$), and Australia ($n = 1$). Isolates with mixed infections of two species were excluded. A detailed list of *Borrelia* isolates included in our analysis is available upon request.

[b] Isolates are from references 1, 20, 34, 64, 70, 85, 132, 137, 155, 168, 194, 197, 200, 251, 259, 268, 273, 281, and 285.

[c] Isolates are from references 1, 14, 33, 85, 132, 137, 141, 251, and 259.

[d] Isolates are from references 20, 85, 132, 137, 194, 229, 259, 268, and 273.

[e] This includes 17 isolates from human blood, 3 from synovial fluid, 1 from myocardial samples, and 1 from the skin of a patient with lymphocytoma (12, 216, 234).

[f] This includes 9 Slovenian isolates genetically similar to North America isolate 25015, which belonged to *B. bissettii* (201), and 1 isolate from a patient with EM in The Netherlands (265).

Hosts and *Borrelia* Species Association

B. burgdorferi sensu stricto in the United States and *B. garinii* in Eurasia have been isolated from a large diversity of mammalian hosts and birds (3, 81, 166, 176). In contrast, *B. afzelii* in Eurasia and *B. burgdorferi* sensu stricto in Europe were isolated mainly from rodents (81, 83). Many species of mammals are hosts of *B. japonica* in Japan (150, 151). However, birds are assumed to be the only hosts for *B. valaisiana* in Europe, since no *B. valaisiana* isolate has been cultured or detected from mammals or rodents to date (105, 123, 267). Only a few *B. lusitaniae* strains were isolated from *I. ricinus* in southern, central, and eastern Europe (127, 200). Data for hosts of this species are currently not available. *B. andersonii* and *B. bissettii* sp. nov. in North America seem to involve particular and narrow host spectra, i.e., cottontail rabbits (*Sylvilagus floridanus*) (4) and wood mice (*Neotomae fuscipes*) (28), respectively, although further studies are necessary to clarify these unique relationships.

Transmission Cycles of Different *Borrelia* Species in Nature

Increasing data indicate that two specific enzootic transmission cycles (rodent-tick and bird-tick) are involved in maintaining different *B. burgdorferi* sensu lato species in nature, as suggested by Nakao et al. (164) and Humair et al. (104). Reservoir hosts infected by various *B. burgdorferi* sensu lato species may not transmit them to ticks with equal efficiency (55, 122, 123, 154, 214). Hosts acting as filters seem to be predisposed to mainly transmitting one species to ticks, to the detriment of other genomic groups (123). The mechanisms underlying the apparent differential transmission of *B. burgdorferi* sensu lato by various mammalian and avian hosts remain to be determined, although a recent study revealed that differences in serum complement sensitivity among *Borrelia* species could be a key factor in LB ecology (124).

Different *B. burgdorferi* sensu lato species may be maintained through distinct transmission cycles in natural foci (83, 104, 105, 106, 123, 164). In North America, *B. burgdorferi* sensu stricto is maintained mainly by the rodent (white-foot mice, *Peromyscus leucopus*)-tick cycle, while the tick-infested resident and migrant birds are potentially important in regional spread and in dissemination of the spirochetes (3, 166, 230, 232). *B. andersonii* and *B. bissettii* sp. nov. have their own distinct enzootic cycles involving other tick vectors and vertebrate hosts (4, 28, 201). In Eurasia, the major species, *B. afzelii*, is circulated mainly between the reservoirs, particular *Apodemus* mice and *Clethrionomys* voles, and *Ixodes* ticks (81, 104, 106, 164) whereas *B. garinii* may be maintained predominantly through the bird-tick transmission cycle, especially in northern Europe (176, 177). *B. valaisiana* has been detected in various avian reservoirs but never in rodent hosts (105, 123, 267). Therefore, this species may not survive and persist in small mammals and most probably circulates between its avian reservoirs and ticks in natural foci (123).

IMPLICATIONS FOR CLINICAL DISEASE, DIAGNOSIS, AND VACCINE DEVELOPMENT

Given the genetic diversity of *B. burgdorferi* sensu lato and the protean disease manifestations of LB, the use of molecular typing provides valuable data for possible elucidation of the association of particular *Borrelia* species with the distinct clinical manifestations of LB (259). Molecular typing may also contribute to establishing a correlation between the spirochetal subtypes and the occurrence and severity of particular clinical manifestations of LB (133, 268, 284). Such data are important for the clinical and laboratory diagnosis of LB as well as for vaccine development.

Association of Clinical Disease and Infecting *Borrelia* Species

The difficulty in culturing *B. burgdorferi* sensu lato from clinical specimens, other than skin, has hampered assessments of the association between *Borrelia* species and distinct clinical entities. However, there is increasing evidence supporting such an association. First, numerous *Borrelia* isolates recovered worldwide from LB patients with different clinical syndromes have been subjected to molecular typing analysis. We reviewed these reports on isolates from LB patients present in the Medline database from 1992 through October 1998. Only cultured isolates that had been subjected to molecular typing analysis and assigned properly to their taxon, and whose clinical source was indicated, were included in the analysis. The distribution of *Borrelia* species of 472 *B. burgdorferi* sensu lato isolates recovered from patients is summarized in Table 6. Different species of *B. burgdorferi* sensu lato are associated with distinct clinical manifestations of LB: Lyme arthritis is associated with *B. burgdorferi* sensu stricto infection, neuroborreliosis is asso-

ciated with *B. garinii* infection, and ACA is associated with *B. afzelii* infection (11, 14, 35, 155, 173, 186, 194, 197, 259, 273, 280). In a recent study, Wormser et al. demonstrated a significant association between the infecting genotype of *B. burgdorferi* in an EM lesion and the presence of spirochetemia or multiple EM lesions, suggesting that hematogenous dissemination is related to the *B. burgdorferi* genotype (284). A limitation of this analysis may be the differences in culture recovery for different *B. burgdorferi* sensu lato species or genotypes. Two reports suggest that the distribution of genotypes in tick or human tissue specimens, as measured by sequencing or PCR-RFLP analysis, is different from that obtained by culture (133, 170). Another confounding factor may be publication bias (87). Nevertheless, molecular analysis clearly points to a correlation between genotype and clinical manifestation. Second, a number of European studies showed that the antibody responses in LB patients to the three *B. burgdorferi* sensu lato pathogens varied with disease manifestation (5, 8, 9, 56, 169). For example, Assous et al. studied sera from 67 LB patients and found that sera from 55% of the 20 patients with Lyme arthritis had preferential reactivity (a serum sample showed preferential reactivity when it detected two or more protein bands with a bacterial suspension of a given species compared to suspensions of the other *Borrelia* species) with a *B. burgdorferi* sensu stricto isolate, sera from 47% of the 23 patients with neuroborreliosis had more reactivity with a *B. garinii* strain, and sera from 100% of the 8 patients with ACA had greater reactivity with a *B. afzelii* strain in Western blot analysis (8, 9). Third, in vitro experiments in which the tissue tropism of *Borrelia* species was evaluated by incubation of various cultured *Borrelia* species with human brain tissue have provided evidence that *B. garinii* invaded brain tissue more efficiently than did *B. burgdorferi* sensu stricto and *B. afzelii* (289).

The mechanisms by which infection with different *Borrelia* species may lead to distinct clinical syndromes of LB are unclear. Different serum susceptibility (27, 260) and tissue tropism among *Borrelia* species may in part account for such differences (49, 128, 182, 253, 289). Although each of the three human pathogenic *B. burgdorferi* sensu lato species has been associated with different clinical syndromes of LB, the clinical spectrum caused by these species can largely overlap.

Erythema migrans. EM is an expanding red or bluish-red rash, often with central clearing, which occurs in up to 90% of patients with objective evidence of LB (162). Usually, EM appears at the early stage of *B. burgdorferi* sensu lato infection. All three species of *B. burgdorferi* sensu lato pathogenic to humans have been repeatedly cultured from skin biopsy specimens from patients with EM (Table 6). No significant difference between the occurrence of EM and infection with a particular *Borrelia* species has been reported. This is to be expected since all infections with *B. burgdorferi* causing LB result from an initial tick bite. However, it was noted that EM patients infected with *B. burgdorferi* sensu stricto in North America complained more frequently of systemic symptoms than did EM patients in Eurasia (161, 245, 246), where EM is caused predominantly by infection due to *B. afzelii* (216, 259).

Lyme carditis. Lyme carditis is a well-known clinical manifestation in both North American and European patients with LB (46, 238, 261). Early studies showed that the frequency of this syndrome was considerably higher in North America, where about 4 to 10% of untreated LB patients presented with cardiac abnormalities (44, 236), than in Europe, where only 0.5 to 4% of untreated LB patients presented with similar abnormalities (46, 162, 178). However, the incidence has been lower in two recent population-based studies in the United States and in Europe (23, 79). The only *Borrelia* isolate recovered from a myocardial specimen of an Austrian patient in Europe to date was typed as *B. burgdorferi* sensu stricto (216, 234).

Lyme arthritis and *B. burgdorferi* sensu stricto infection. Lyme arthritis is the most common musculoskeletal symptom resulting from *B. burgdorferi* sensu stricto infection in North America. It occurs either at the early disseminated stage or as a more chronic manifestation. About 60% of untreated patients with EM experienced brief attacks of monoarticular or oligoarticular arthritis in the United States (44, 236, 242). In contrast, only 3 to 15% of patients suffered from arthritis in Europe (23, 45, 178), where *B. garinii* and *B. afzelii* are more frequently recovered than *B. burgdorferi* sensu stricto (101, 216). This unequal distribution of *Borrelia* species may directly be related to the disparity in the reported incidence of Lyme arthritis between North America and European countries. Although different *B. burgdorferi* sensu lato species pathogenic to humans can be detected or recovered from the joint or synovial tissue in animal experimental studies (220, 288) and in patients with Lyme arthritis (59, 167, 262), the only three *Borrelia* isolates recovered from the synovial fluids of patients with Lyme arthritis were typed as *B. burgdorferi* sensu stricto (12, 216).

Neuroborreliosis and *B. garinii* infection. Neuroborreliosis is the most frequent manifestation of disseminated infection in Europe (43, 75, 113) and is a common symptom in North American LB patients as well (44, 91). Both the central and peripheral nervous system can be involved (90, 120). All three species, *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii*, are known to cause Lyme neuroborreliosis. In European patients, *B. garinii* constituted up to 72% ($n = 55$) of the 76 *Borrelia* isolates or DNAs detected in human CSF samples, whereas 8% ($n = 6$) and 20% ($n = 15$) of the these specimens were identified as *B. burgdorferi* sensu stricto and *B. afzelii*, respectively (101). Of 36 CSF isolates mainly recovered from patients with neuroborreliosis in Southern Germany, *B. garinii*, *B. afzelii*, and *B. burgdorferi* sensu stricto accounted for 58, 28, and 11%, respectively (33). These findings in European patients suggest an association between infection of *B. garinii* and the development of Lyme neuroborreliosis (11, 125, 186, 259, 277). This is not strictly the case, since obviously, *B. burgdorferi* sensu stricto is responsible for human neuroborreliosis in the United States. It may be noteworthy that the incidence of lymphocytic meningoradiculitis (Bannwarth syndrome) in LB patients is relatively lower in the United States (75, 89) than in European countries. This also suggests differences in pathogenicity between *B. burgdorferi* sensu stricto and *B. garinii*.

Acrodermatitis chronica atrophicans and *B. afzelii* infection. ACA is a late cutaneous manifestation of LB characterized by chronic and long-lasting progressive red and bluish-red lesions, usually on the extensor surface of the extremities (235). It was described as early as 1883 (270) and is widely encountered throughout Europe (7, 45, 235); there have been few reports from the United States (58, 115, 155). Molecular studies of ACA isolates from patients in several European countries have provided evidence suggesting an exclusive association of ACA and *B. afzelii* infection (9, 11, 259, 273). However, a Danish isolate, DK7, derived from an ACA lesion was classified as *B. burgdorferi* sensu stricto by OspA serotyping (280) as well as by 16S rRNA and *ospC* gene sequence analysis (251). In a recent study, 22 *B. burgdorferi* sensu lato isolates from Slovenian patients with ACA were subjected to PFGE and species-specific PCR analysis (194). Results showed that infection was due to *B. afzelii* in 17 cases, to *B. garinii* in 4 cases, to *B. burgdorferi* sensu stricto in 1 case. One American isolate from a patient with ACA was also typed as *B. burgdorferi* sensu stricto (155). These studies indicate that *B. afzelii* is the predominant but not the exclusive etiologic agent of ACA.

Implication for Serological Diagnosis

The diagnosis of LB is primarily clinical, but serological tests can provide very useful supporting evidence, especially in patients with disseminated or chronic infection. The serological tests usually used to assess *B. burgdorferi* antibodies include enzyme-linked immunosorbent assay (ELISA), immunofluorescence assays, and Western blotting. ELISA and immunofluorescence assays are the most widely used but have low specificity (99, 256). Cross-reactive antibodies can produce a false-positive test in patients with other illnesses, e.g., other spirochetal infections, nonspirochetal bacterial endocarditis, Epstein-Barr virus infection, rheumatoid arthritis, and systemic lupus erythematosus (139, 140). In the United States, a two-step protocol for the evaluation of the *B. burgdorferi* antibodies in sera was recommended by the Centers for Disease Control and Prevention (40). The performance of this protocol, as well as its simplified approach (255), may improve the specificity of serodiagnosis for LB (50, 110, 126), but the high percentage of seronegativity in 20 to 50% of patients, probably dependent on the stage of LB, remains a problem and limits the value of serological tests (2, 95, 99, 246). In addition, the antigenic heterogeneity of *B. burgdorferi* sensu lato may influence the sensitivity and specificity of serological tests for LB, especially on the European continent, where three pathogenic species of *B. burgdorferi* sensu lato and at least eight OspA serotypes are well documented (277, 280). Differences in the regional distributions of borrelial species may also affect preferential reactivities of sera from patients with LB (30, 169). Several studies have shown differences in the reactivity patterns in Western blot analysis, depending on the species, strain, or serotype used as the source of antigen (56, 94, 95, 156, 169). This issue should be readdressed, since molecular typing results based on hundreds of isolates have indicated that the genetic diversity of *B. burgdorferi* sensu lato is much greater than was previously thought, even in North America (134, 155, 197, 287). In a recent study, the influence of interspecies variability of *B. burgdorferi* sensu lato on the serodiagnosis of LB was evaluated by using immunoglobulin M and immunoglobulin G ELISA with antigens prepared from representative isolates of *B. burgdorferi* sensu stricto (isolates B31 and PKa2), *B. garinii* (PBi), and *B. afzelii* (PKo). Variations resulting from the use of different strains for antigenic preparations were noted in sera from 222 patients with clinically defined LB of all stages, 133 blood donors, and 458 forest workers (94). Such differences were also noted in ELISA or Western blotting by using whole-cell antigenic preparation (95), recombinant OspC (156), internal flagellin fragment (96), and P39 protein (211) from different species of *B. burgdorferi* sensu lato. Therefore, serological tests involving a combination of antigens derived from two or more strains (from different species in Europe) might be most informative as a response to the impact of strain heterogeneity, especially in Eurasia.

Implication for Vaccine Development

In the past years, different types of vaccines, including a whole-cell vaccine (112), live attenuated vaccine from an aflagellar mutant (215), recombinant OspA (61), OspC subunit vaccines (292), and OspA-based DNA vaccines (138, 228), have been developed (see also reference 283). Recombinant OspA vaccine has been highly successful in protecting laboratory animals against challenge by homologous strains of *B. burgdorferi* sensu lato (61). Recently, the efficacy of this recombinant OspA vaccine was evaluated in two large clinical trials involving more than 21,000 people from areas of the United States where LB is endemic (227, 243). The efficacy of these OspA vaccines was 76 to 92% in the second year after a booster dose (227, 243). Vaccine failure appears to be due to a low OspA antibody level. In December 1998, one of the OspA-based vaccine was approved for human use by the Food and Drug Administration in the United States (42). A recent study might raise questions about the safety of these OspA vaccines, since it seems theoretically possible that the OspA protein could provoke autoimmunity in susceptible individuals (88). The efficacy of this OspA-based vaccine in Europe may be considerably lower, given the substantial variations displayed in OspA proteins of *B. burgdorferi* sensu lato, as revealed by molecular typing analysis. Immunization with recombinant OspA derived from any one isolate may fail to protect against the heterogeneous population of organisms present in the natural focus. OspA immunization may protect mice from tick-borne infection with heterogeneous *B. burgdorferi* sensu lato from different geographic regions by blocking spirochetal transmission from the vector to the host (54, 63). However, it is not clear whether the specific antibody in persons who are immunized with an OspA vaccine derived from one isolate can also provide coverage against a wide range of strains. Passive and active immunization against OspC can serve in protective immunity. Recently, passive immunization with a high dose of anti-OspC immune sera appeared to be therapeutic for chronic LB in mice (292). However, the OspC-mediated protective immunity is probably strain specific (25). Thus, the general applicability of this approach may be limited by the well-documented heterogeneity of OspC among members of the species *B. burgdorferi* sensu lato (137, 279). One strategy to circumvent OspA and OspC heterogeneity is the use of vaccines made of a cocktail of various recombinant OspA and/or OspC gene products derived from different strains. In Europe, a multiple-subunit vaccine is expected to yield protective immunity against infections of different species of *B. burgdorferi* sensu lato. Gern et al. (82) reported that a polyvalent OspA vaccine prepared from three isolates of different species efficiently protected mice against infection due to either *B. burgdorferi* sensu stricto, *B. garinii*, or *B. afzelii*.

Other potential candidates for vaccines include decorin binding protein A (DbpA) (39, 93, 210), P35 (62), and P66 (29, 202). Further information on the distribution of various *Borrelia* species in their natural reservoir hosts and vectors and knowledge of the *Borrelia* antigenic compositions is needed to find representative immunogenic antigens for vaccine development.

CONCLUSIONS

Since different *Borrelia* strains may intrinsically be associated with distinct pathogenic properties, application of molecular typing methods to the classification of *B. burgdorferi* sensu lato will provide the framework for a systematic approach to characterize differences in infectivity as well as in pathogenicity between strains. Among the typing methods available for *B. burgdorferi* sensu lato, OspA serotyping can be used to analyze the phenotypic characteristics. Both PFGE and RAPD fingerprinting analysis are powerful tools in elucidating the genetic relationship between closely related *Borrelia* isolates within a species. rDNA spacer RFLP analysis is simple and can be applied to uncultured specimens; it is therefore ideally suited for molecular epidemiological and population genetic studies of *B. burgdorferi* sensu lato. Phylogenetic analysis based on 16S rRNA gene sequence may represent a potential method for inferring the taxa of *B. burgdorferi* sensu lato (68, 127). However, DNA-DNA reassociation data are generally required for designation of a new species because of the high degree of

sequence similarity of the 16S rRNA genes between closely related *Borrelia* species (127, 267). In general, there is excellent concordance in the typing results obtained by different molecular approaches at the species level, with the exception of DNA sequence analysis of *ospC*.

Ten *B. burgdorferi* sensu lato species have been identified. The pathogenicity of three of these species is well documented in humans. Distribution of *Borrelia* species and clinical syndromes of LB are different in North America and Eurasia, possibly due to the different pathogenicities of *Borrelia* species. Current data indicate that *B. burgdorferi* sensu stricto infection is associated with articular disorders, *B. garinii* infection is associated with neuroborreliosis, and *B. afzelii* infection is associated with ACA. Since the recovery of *Borrelia* from clinical specimens of patients with LB by culture is low, in particular in patients with Lyme arthritis and carditis, assessment of the correlation between infecting species and clinical manifestations will require other approaches. Appropriate serological tests capable of differentiating the antibody response of patients infected with different *Borrelia* species may be helpful in this regard. In addition, sensitive molecular typing techniques which do not require large amounts of patient material or cultivation of the spirochete will play an increasingly important role in elucidation of the pathogenic potential of different *B. burgdorferi* genotypes. Ultimately, the development of suitable animal models for investigation of the tissue tropisms of different *B. burgdorferi* sensu lato species will provide more direct evidence for the correlation between *Borrelia* species and clinical symptoms of LB.

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