

Three Species of *Borrelia burgdorferi* Sensu Lato (*B. burgdorferi* Sensu Stricto, *B. afzelii*, and *B. garinii*) Identified from Cerebrospinal Fluid Isolates by Pulsed-Field Gel Electrophoresis and PCR

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A total of 36 European *Borrelia burgdorferi* sensu lato cerebrospinal fluid isolates (mainly from southern Germany) were analyzed by pulsed-field gel electrophoresis (PFGE) for large restriction fragment pattern (LRFP) and linear plasmid profiles. Analyzing this large panel of isolates, we detected all three species of *B. burgdorferi* sensu lato pathogenic for humans in cerebrospinal fluid from patients with Lyme neuroborreliosis by PFGE typing after *Mlu*I digestion: 21 *B. garinii* (58%), 10 *B. afzelii* (28%), and 4 *B. burgdorferi* sensu stricto (11%) strains as well as 1 isolate with bands characteristic of both *B. afzelii* and *B. garinii*. Species classification by PFGE typing was confirmed by 16S rRNA-specific PCR. Eighteen isolates (11 *B. garinii*, 6 *B. afzelii*, and 1 *B. burgdorferi* sensu stricto isolate) were further characterized by LRFP with four different restriction enzymes (*Apa*I, *Ksp*I, *Sma*I, and *Xho*I). All *B. afzelii* isolates showed identical patterns for each restriction enzyme group. Considerable heterogeneity was demonstrated within the *B. garinii* group. Subsequent analysis of plasmid profiles revealed only marginal differences for *B. afzelii* strains but different patterns for *B. garinii* isolates. In one *B. afzelii* strain we found a linear plasmid of about 110 kbp not described before. LRFP analysis by PFGE is a suitable tool for the molecular characterization of *B. burgdorferi* sensu lato strains and allows determination not only of the species but also of the subtypes within *B. garinii*.

The spirochete *Borrelia burgdorferi* sensu lato is the causative agent of Lyme borreliosis and is transmitted to humans primarily by ticks of the genus *Ixodes* (13). Initially identified as one species (27a), *B. burgdorferi* sensu lato has recently been delineated into three pathogenic species: *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* (6, 15).

Lyme borreliosis exhibits a broad array of clinical manifestations, e.g., skin disorders like erythema migrans and acrodermatitis chronica atrophicans, carditis, arthritis, and neurological symptoms (e.g., lymphocytic meningoradiculitis [Bannwarth's syndrome] and meningoencephalitis) (38, 39, 49, 52). Lyme neuroborreliosis (LNB) may occur with or without antecedent erythema migrans or other symptoms (26, 29, 38, 50). In Germany, Bannwarth's syndrome is the most common presentation of LNB, whereas subacute basilar meningitis, with or without unilateral or bilateral facial palsy, is most common in the United States (37, 38). *B. burgdorferi* sensu lato was first cultured from cerebrospinal fluid (CSF) in the United States by Steere et al. (50) and in Europe by Preac-Mursic et al. (42).

B. burgdorferi sensu lato has been only rarely isolated from patients with neuroborreliosis (28, 43–45, 50). Thus, culture isolation has not become clinical practice. However, *B. burgdorferi* sensu lato has also been isolated from the CSF of seronegative patients (41, 43, 44). Presentations of Lyme disease vary in different geographic regions; e.g., mild LNB is more common in the United States, whereas severe neurolog-

ical and late skin manifestations like acrodermatitis chronica atrophicans occur more often in Europe (1, 40, 42, 49, 53). These observations suggest that clinical outcome might depend on infection with strains of different species and pathogenic potentials.

In order to evaluate this aspect, we characterized a large panel of 36 isolates from the CSF of patients with neuroborreliosis. We were mainly interested in seeing which species were involved in this disorder. To characterize these isolates, pulsed-field gel electrophoresis (PFGE) typing was used for designation of the isolates and to place them into the respective species as described by Belfaiza et al. (8). For 18 strains, we compared the species differentiation obtained by PFGE with those obtained by an established PCR based upon 16S rRNA sequence described by Marconi and Garon (33). We found predominantly *B. garinii* species and, less frequently, *B. afzelii* and *B. burgdorferi* sensu stricto species.

MATERIALS AND METHODS

Bacterial strains. *B. burgdorferi* strains (see Table 1) were isolated between 1984 and 1993 in the Max von Pettenkofer-Institut from the CSF of patients (mainly from southern Germany) with symptoms and diagnoses of LNB (e.g., lymphocytic meningoradiculitis [Bannwarth's syndrome] and lymphocytic meningitis). All CSF samples were clear upon receipt in the laboratory. For the present study the strains were grown in modified Kelly medium for 4 to 5 days at 33°C (44) to a cell density of 10⁸ cells per ml. Only strains subcultured less than 11 times were used. Cells were harvested by centrifugation and were washed three times in TN buffer (10 mM Tris-OH/HCl [pH 7.6], 1 M NaCl).

Restriction analysis of the whole genome and plasmid separation. Borrelial strains were embedded in agarose blocks, lysed with lysozyme, and digested with proteinase K as described previously (14). For plasmid profile analysis, agarose sheets were used without further treatment. For species differentiation, the DNAs of all strains were digested with *Mlu*I. Additionally, to compare hetero- or homogeneity within one species, the DNAs of 18 strains were digested with *Apa*I,

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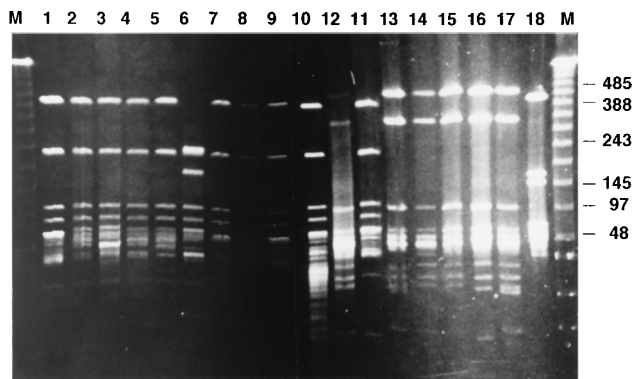


FIG. 1. LRFPs after *MluI* digestion. Lanes 1 to 11, *B. garinii* PBi, PBr, PHei, PMek, PHe, PRef, PWa, PFe, PMue, PFlk, and PSh, respectively, identified by two specific bands of 220 and 80 kbp; lanes 12 to 17, *B. afzelii* PLap, PMel, PAlt, PStb, PSpe, and PBoj, respectively, characterized by three bands of 460, 320, and 90 kbp; lane 18, a *B. burgdorferi* sensu stricto strain (strain PKa1) characterized by one specific band of 145 kbp; lanes M, marker (lambda concatemer ladder; monomer size, 48.5 kbp). Numbers on the right are in kilobase pairs.

KspI, *SmaI*, and *XhoI* restriction enzymes under the conditions recommended by the supplier (Boehringer Mannheim, Mannheim, Germany). PFGE was done with a CHEF (contour-clamped homogeneous electric field electrophoresis) DR (dynamic regulated) III apparatus (Bio-Rad, Munich, Germany). The DNA fragments obtained after *ApaI* digestion were separated for 30 h with pulse times of 1 to 20 s (*SmaI* and *XhoI*, 1 to 30 s; *KspI* and *MluI*, 1 to 40 s). The plasmid analysis was done with pulse times of 0.9 to 2.5 s for 30 h. Lambda concatemers with a monomer size of 48.5 kbp and Marker II (Boehringer Mannheim) were used as length markers.

16S rRNA-specific PCR. DNA isolation was done as described by Luft et al. (32). As a target for PCR amplification, the gene coding for 16S rRNA was selected. We applied the *B. burgdorferi* sensu lato-specific primers and species-specific primers for *B. burgdorferi* sensu stricto, *B. afzelii*, and *B. garinii* as published by Marconi and Garon (33). PCR was performed under the conditions described by Marconi and colleagues (33, 34) with the Hybaid OmniGene thermocycler (MWG-Biotech, Ebersberg, Germany), with 1 min of denaturation at 94°C, 1 min of annealing at 47°C, and 1.5 min of extension at 72°C for 25 cycles. The amplified DNA was separated by electrophoresis with a 2% agarose gel containing ethidium bromide to stain the bands.

OspA serotyping. Strains were analyzed for their OspA serotypes as described previously (56). Whole-cell lysates of borreliae (7.5 mg of protein per lane) were separated in sodium dodecyl sulfate (SDS)-15% polyacrylamide gels and were transferred to nitrocellulose by the semidry technique. After blocking, the blots were reacted with a panel of eight OspA-specific monoclonal antibodies. Binding of antibodies was detected with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins diluted 1:1,000 (Dakopatts, Copenhagen, Denmark).

RESULTS

Genome analysis by PFGE typing (LRFP pattern after *MluI* digestion). The CSF isolates were grouped into the different species according to their typical large restriction fragment patterns (LRFPs) described by Belfaiza et al. (8), with one minor exception that in our experimental setup the specific band identifying *B. burgdorferi* was 145 kbp instead of the previously described band of 135 kbp.

LRFP after *MluI* digestion showed two characteristic bands of 220 and 80 kbp for the 21 *B. garinii* strains (PBi, PBr, PHei, PMek, PHe, PRef, PWa, PFe, PMue, PFlk, and PSh [Fig. 1, lanes 1 to 11, respectively] and PLi, PKi, PSeS, PScf, PBaEII, PFin, POhm, PSoR, PFei, and PLa [data not shown]). Three characteristic bands of 460, 320, and 90 kbp were observed for the 10 *B. afzelii* strains (PLap, PMel, PAlt, PStb, PSpe, and PBoj [Fig. 1, lanes 12 to 17, respectively] and PBo, PHo, PKr, and PHa [data not shown]). One band of 145 kbp was typical for the *B. burgdorferi* sensu stricto strains PKa1 (Fig. 1, lane 18) and PFra, PHas, and PStm (data not shown). Remarkably, one strain (strain PFCa) showed the three bands characteristic for *B. afzelii* and the two specific bands characteristic for *B. garinii*.

This patient seems to have had a mixed infection (Table 1). The *SmaI* LRFP revealed the specific bands for *B. afzelii* and *B. garinii* for strain PFCa.

LRFP after *ApaI*, *KspI*, *SmaI*, and *XhoI* digestions. We randomly selected 11 *B. garinii* and 6 *B. afzelii* strains and analyzed them with four different restriction enzymes to examine the strains for hetero- or homogeneity within the species. Also, one isolate of *B. burgdorferi* sensu stricto was examined. To obtain a small number of fragments, restriction enzymes which recognize GC-rich DNA sequences were chosen. Only bands larger than 70 kbp were considered for analysis because smaller bands could be plasmid bands.

B. garinii strains were heterogeneous. Strains PBi, PWa, PMue, PFlk, and PSh showed an identical pattern after digestion with *SmaI* (fragments of 260, 135, 125, 105, and 90 kbp; Fig. 2, lanes 1, 7, 9, 10, and 11), *KspI* (band sizes of 475 and 425 kbp; see Fig. 4, lanes 1, 7, 9, 10, and 11), *ApaI* (band sizes of 115, 105, 85, and 70 kbp; data not shown), and *XhoI* (band sizes of 170, 160, 95, 90, 85, 80, and 70 kbp; Fig. 3, lanes 1, 7, 9, 10, and 11). These strains were grouped together in group LRFP 1; they all shared OspA serotype 4. Three other *B. garinii* strains, strains PBr, PMek, and PFe, revealed five bands with sizes of 260, 240, 110, 80, and 75 kbp after *SmaI* digestion (Fig. 2, lanes 2, 4, and 8), three bands with sizes of 415, 290, and 175 kbp after *KspI* digestion (Fig. 4, lanes 2, 4, 8), four bands of 160, 150, 90, and 80 kbp after *XhoI* digestion (Fig. 3, lanes 2, 4, 8), and six bands of 120, 105, 90, 85, 80, and 75 kbp after *ApaI* digestion (data not shown). These three strains were grouped together in LRFP 2 and shared OspA serotype 3.

Strains PHei, PHe, and PRef showed different patterns after digestion with the four different restriction enzymes. These strains belonged to OspA serotypes 5, 6, and 7, respectively, and were grouped together in LRFP 3 (Fig. 2 to 4, lanes 3, 5, and 6).

B. afzelii PLap, PMel, PAlt, PStb, PSpe, and PBoj were homogeneous after digestion with the four different restriction enzymes. The strains showed identical patterns after *SmaI* digestion (six bands of 145, 135, 125, 115, 85, and 80 kbp; Fig. 2, lanes 12 to 17), after *KspI* digestion (475, 385, and 110 kbp; Fig. 4, lanes 12 to 17), after *XhoI* digestion (170, 125, 110, 80, and 70 kbp; Fig. 3, lanes 12 to 17), and after *ApaI* digestion (160, 155, and 75 kbp bands; data not shown). One strain, strain PMel, showed an additional band after digestion with *SmaI* (110 kbp), *KspI* (120 kbp), and *ApaI* (110 kbp). This band was identified as a plasmid band (Fig. 5).

Plasmid analysis. The 11 *B. garinii* strains revealed six to eight linear plasmid bands of between 15 and 70 kbp. Five of the *B. garinii* strains (strains PBi, PWa, PMue, PFlk, and PSh; group LRFP 1) showed a unique plasmid pattern: three plasmids larger than 50 kbp and three to four plasmids of between 20 and 30 kbp. Three of the *B. garinii* strains (strains PBr, PMek, and PFe; group LRFP 2) showed very similar plasmid patterns, with a characteristic four- to five-band pattern of between 30 and 40 kbp (data not shown). The remaining three strains showed different patterns. The six *B. afzelii* strains showed similar plasmid patterns, with six to eight bands. All strains exhibited four plasmids of approximately 65, 40, 30, and 27 kbp. Strain PMel showed a unique plasmid of 110 kbp; this plasmid has not been described before for *B. afzelii* (Fig. 5, lane 13). *B. burgdorferi* sensu stricto PKa1 showed four plasmids of approximately 60, 43, 40, and 30 kbp.

16S rRNA-specific PCR. To confirm the validity of our species determination by PFGE typing, a species-specific PCR of 16S rRNA was performed for 18 strains. With the *B. burgdorferi* sensu lato primers, a specific 350-bp amplicon was generated from all isolates (expected band size, 357 bp) (Fig. 6a).

TABLE 1. CSF strains used in the study

No.	Strain	Yr of isolation	Place of residence	Species	OspA serotype ^a
1	PBi ^b	1984	Southern Germany	<i>B. garinii</i>	4
2	PBr	1984	Southern Germany	<i>B. garinii</i>	3
3	PHei	1987	Northern Germany	<i>B. garinii</i>	5
4	PMek	1992	Southern Germany	<i>B. garinii</i>	3
5	PHe	1993	Southern Germany	<i>B. garinii</i>	6
6	PRef	1988	Southern Germany	<i>B. garinii</i>	7
7	PWa ^b	1990	Southern Germany	<i>B. garinii</i>	4
8	PFe ^b	1992	Southern Germany	<i>B. garinii</i>	3
9	PMue	1992	Southern Germany	<i>B. garinii</i>	4
10	PFlk	1990	Southern Germany	<i>B. garinii</i>	4
11	PSh	1987	Southern Germany	<i>B. garinii</i>	4
12	PLap	1988	Southern Germany	<i>B. afzelii</i>	0
13	PMel	1990	Southern Germany	<i>B. afzelii</i>	X
14	PAlt ^b	1988	Southern Germany	<i>B. afzelii</i>	2
15	PSib ^b	1987	Southern Germany	<i>B. afzelii</i>	0
16	PSpe ^b	1986	Southern Germany	<i>B. afzelii</i>	2
17	PBoj ^b	1989	Poland	<i>B. afzelii</i>	0
18	PKa1 ^b	1983	Southern Germany	<i>B. burgdorferi sensu stricto</i>	1
19	PLi	1988	Southern Germany	<i>B. garinii</i>	5
20	PKi	1992	Southern Germany	<i>B. garinii</i>	X
21	PSeS	1990	Southern Germany	<i>B. garinii</i>	6
22	PScf	1992	Southern Germany	<i>B. garinii</i>	4
23	PBaEII	1990	Southern Germany	<i>B. garinii</i>	4
24	PFin	1991	Southern Germany	<i>B. garinii</i>	4
25	POhm	1991	Southern Germany	<i>B. garinii</i>	6
26	PSoR	1989	Southern Germany	<i>B. garinii</i>	6
27	PFei ^b	1986	Southern Germany	<i>B. garinii</i>	4
28	PLa	1988	Northern Germany	<i>B. garinii</i>	3
29	PBo	1987	Southern Germany	<i>B. afzelii</i>	0
30	PHo	1992	Southern Germany	<i>B. afzelii</i>	0
31	PKr	1992	Southern Germany	<i>B. afzelii</i>	2
32	PHa	1992	Northern Germany	<i>B. afzelii</i>	2
33	PFra	1991	Southern Germany	<i>B. burgdorferi sensu stricto</i>	1
34	PHas ^b	1992	Southern Germany	<i>B. burgdorferi sensu stricto</i>	1
35	PStm ^b	1992	Northern Germany	<i>B. burgdorferi sensu stricto</i>	1
36	PFCa	1992	Southern Germany	<i>B. afzelii/B. garinii</i>	214

^a Serotypes according to Wilske et al. (56).

^b Isolates from patients with Bannwarth's syndrome.

Eleven strains revealed an amplicon of approximately 570 bp (expected band size, 574 bp) only with the *B. garinii*-specific primers (Fig. 6b, lanes 1 to 11). These strains were identified as *B. garinii*. Six strains were identified as *B. afzelii* by an amplicon of approximately 590 bp (expected band size, 591 bp) only with a specific primer pair for *B. afzelii* (Fig. 6c, lanes 12 to 17). With the specific primers for *B. burgdorferi sensu stricto*, only one strain showed an amplicon of approximately 570 bp (expected band size, 574 bp) and was identified as *B. burgdorferi sensu stricto* (Fig. 6d, lane 18). The PCR findings corresponded completely to the results obtained by PFGE typing.

DISCUSSION

We set out to examine 36 isolates from the CSF of patients with neuroborreliosis by using PFGE typing. This is the largest number of CSF isolates analyzed until now. Among these CSF isolates, we found 21 *B. garinii* isolates (58%), 10 *B. afzelii* isolates (28%), 4 *B. burgdorferi sensu stricto* strains (11%), and one isolate with a mixed pattern. To confirm these findings, we examined 18 randomly selected strains by 16S rRNA-specific PCR (33). Both methods revealed identical results. This is the first report on the differentiation of isolates from the CSF of patients with neuroborreliosis by both methods.

The degree of heterogeneity or homogeneity within the species of *B. garinii* (11 strains) and *B. afzelii* (6 strains) was ad-

ditionally examined by LRFP after digestion of genomic DNA with four different restriction enzymes (*ApaI*, *KspI*, *SmaI*, and *XhoI*). This method has been successfully used to analyze isolates from ticks for heterogeneity or homogeneity within the species without subsequent hybridization with specific probes (14). Our six *B. afzelii* isolates from CSF showed identical patterns after digestion with each of four different restriction

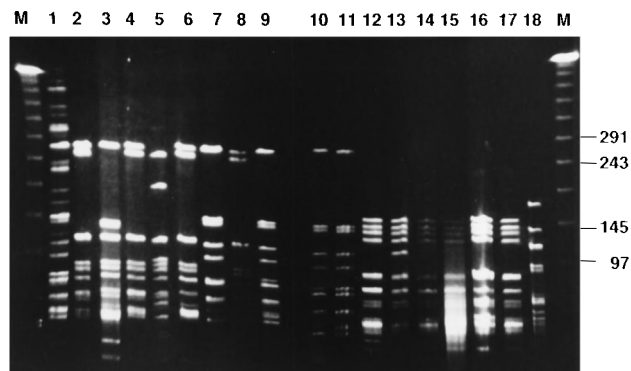


FIG. 2. LRFPs after *SmaI* digestion. The lanes are the same as those described in the legend to Fig. 1.

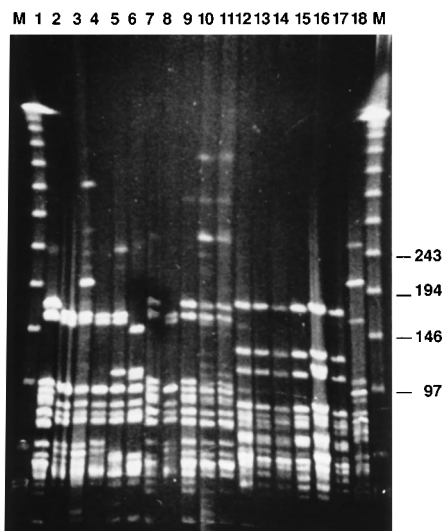


FIG. 3. LRFPs after *Xho*I digestion. The lanes are the same as those described in the legend to Fig. 1.

enzymes, thus reflecting homogeneity within the isolates of the *B. afzelii* group investigated. Among 20 *B. afzelii* isolates from different origins, Belfaiza et al. (8) found indistinguishable patterns after *Mlu*I digestion; 15 of 20 isolates had identical *Sma*I patterns, but 5 of 20 isolates revealed different *Sma*I patterns because of one missing or additional DNA fragment. Characterization of strains from skin and CSF by Boehmer et al. (11) revealed marginal differences within the *B. afzelii* species after hybridization with different probes. By contrast, Chetcuti et al. (17) observed major variations in the *B. afzelii* group when they investigated strains isolated from ticks and skin biopsy specimens. Our *B. afzelii* isolates from skin also showed a heterogeneous pattern (unpublished data). These differences are possibly explained by the different biological sources (CSF, skin, and ticks) of the *B. afzelii* strains investigated. While other investigators found at least marginal differences, our investigation with only *B. afzelii* strains from CSF showed homogeneity within this group. Homogeneity within *B. afzelii* strains isolated from CSF but heterogeneity within

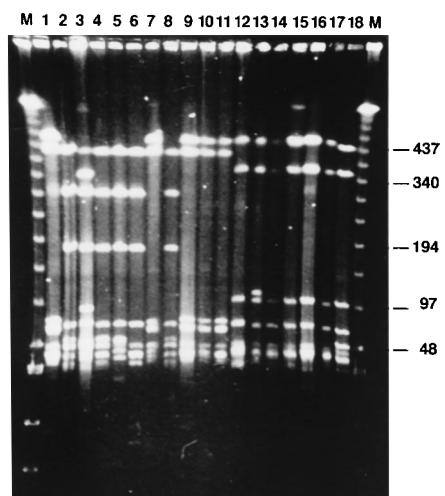


FIG. 4. LRFPs after *Ksp*I digestion. The lanes are the same as those described in the legend to Fig. 1.

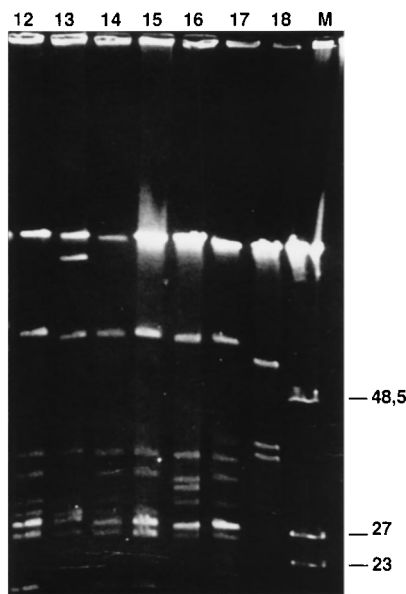


FIG. 5. Plasmid profiles of *B. afzelii* PLap (lane 12), PMel (lane 13), PAlt (lane 14), PStb (lane 15), PSpe (lane 16), and PBoj (lane 17) and the *B. burgdorferi* sensu stricto strain (lane 18); lane M, marker (lambda DNA digested with *Hind*III).

B. afzelii strains from different sources suggests that subgroups may exist within the *B. afzelii* species. These subgroups may exhibit different pathogenic potentials and/or different affinities to various tissues. Members of these subgroups may preferentially penetrate into the CSF, while others may remain localized in the skin. More strains must be examined to validate these findings.

These closely related strains of *B. afzelii* were further characterized by plasmid analysis since this technique has been successfully used for strain differentiation (7, 48, 59). The plasmid pattern analysis revealed no major differences, another strong indication for homogeneity within the *B. afzelii* isolates from CSF. Xu and Johnson (59) also found only minor differences within the *B. afzelii* strains by analyzing the plasmid patterns of strains from different sources. However, one interesting finding was the existence of a 110-kbp plasmid (approximate size) in one of our *B. afzelii* isolates (strain PMel). Plasmids of this size have, to our knowledge, not been described for the three species of *B. burgdorferi* sensu lato pathogenic for humans but have been described for *B. japonica*, group 21038, and relapsing fever borreliae (10, 16, 47).

In contrast to *B. afzelii*, *B. garinii* isolates from CSF show heterogeneity in their LRFPs, representing at least three groups (LRFPs 1, 2, and 3). Comparison of the LRFPs with OspA serotypes (56) revealed that LRFP 1 correlates with OspA serotype 3 and that LRFP 2 correlates with OspA serotype 4. The group LRFP 3 includes only a single isolate of OspA serotypes 5, 6, and 7. This heterogeneity could also be detected by plasmid analysis. When comparing the three different methods, there was a clear correlation between the plasmid pattern, LRFPs 1 and 2, as well as OspA serotype 3 and 4, respectively. However, a larger number of strains needs to be analyzed to confirm these results. Our findings of heterogeneity within the *B. garinii* species ties in with the observations made by using other methods (5, 11, 12, 18, 23, 30, 46, 55, 56, 60).

It has been speculated that the different clinical manifestations of Lyme borreliosis might be specifically correlated with

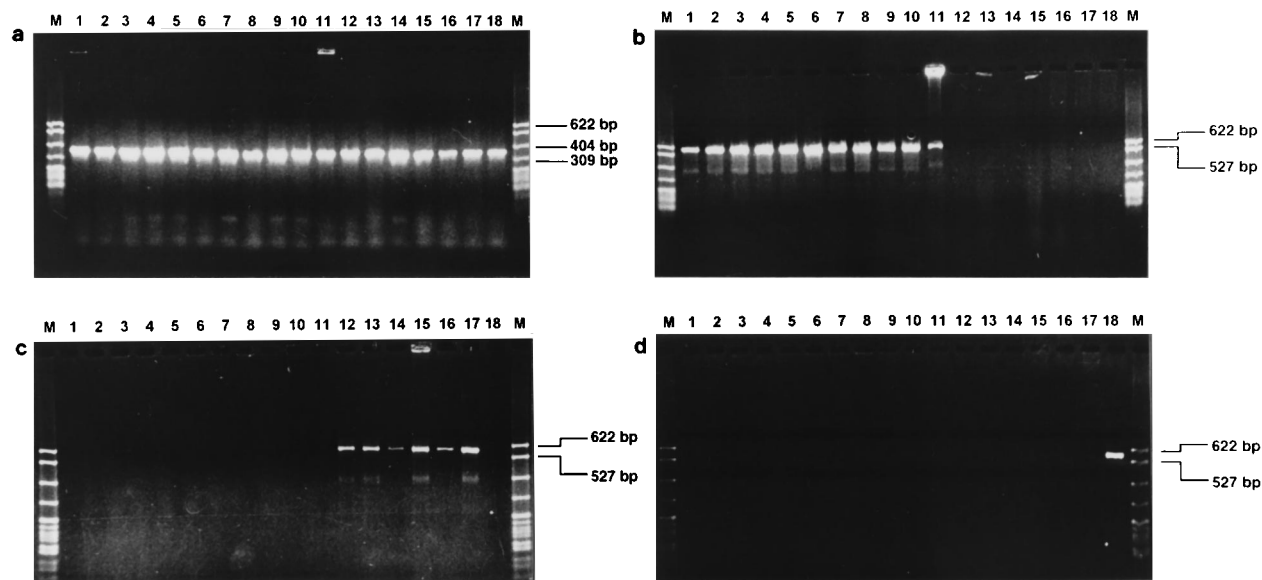


FIG. 6. PCR amplification with 16S rRNA-specific primer. Lanes 1 to 11, *B. garinii* strains (PBi, PBr, PHei, PMek, PHe, PRef, PWA, PFe, PMue, PFlk, and PSH, respectively); lanes 12 to 17, *B. afzelii* PLap, PMel, PAlt, PStb, PSpe, and PBoj, respectively; lane 18, *B. burgdorferi* sensu stricto strain (strain PKa1); lane M, marker DNA (pBR322 DNA digested with *MspI*). (a) PCR amplification with *B. burgdorferi* sensu lato-specific primer. (b) PCR amplification with *B. garinii*-specific primer. (c) PCR amplification with *B. afzelii*-specific primer. (d) PCR amplification with *B. burgdorferi* sensu stricto-specific primer.

certain subtypes of *B. burgdorferi* sensu lato (2, 3, 12, 51, 56, 58). Many investigators have observed *B. afzelii* to be prevalent among skin isolates from Europe (6, 15, 51, 54, 56, 58). In contrast, it has been discussed and is controversial whether a similar association of certain subtypes of *B. burgdorferi* sensu lato with isolates from CSF can be established. Since it is difficult to isolate borreliae from the CSF, only a few isolates have been investigated. Whereas only *B. garinii* has been isolated from the CSF of five patients from Scandinavia (30), analysis of 11 CSF isolates from patients in Germany demonstrated the presence of all three species (56). Van Dam et al. (51) found by rRNA gene restriction analysis of 10 isolates from patients with extracutaneous symptoms 9 *B. garinii* and 1 *B. burgdorferi* sensu stricto strains. Those investigators speculated that *B. garinii* might be associated with extracutaneous symptoms and that *B. afzelii* might be associated with cutaneous manifestations of Lyme disease. Using PCR, Eiffert et al. (22) found five different *ospA* sequences in isolates from the CSF of 12 pediatric patients with LNB and thus confirmed the heterogeneity of CSF isolates for *OspA* serotypes and sequences as well as the prevalence of *B. garinii*-associated *OspA* types described by Wilske et al. (56) for 18 strains from Europe. Demaerschalck et al. (21) demonstrated *ospA* DNA from all three species in one CSF sample and *B. burgdorferi* sensu stricto and *B. garinii* *ospA* DNA in another CSF sample.

Our results demonstrate considerable heterogeneity among CSF isolates from Europe. All three species have been isolated from patients presenting with classical Bannwarth's syndrome. Thus, this type of neurological disease is not associated with a certain species. One patient was even shown to have a mixed infection of *B. afzelii* and *B. garinii* by PFGE typing and *OspA* serotyping (55a). This observation was verified by the *SmaI* LRFP, which revealed the bands specific for *B. afzelii* and *B. garinii*. This is the first description of isolates from human CSF consisting of two different strains and confirms infection with more than one strain, as suggested by PCR (21, 36), in humans.

Halperin (25) discussed the fact that a milder form of neuroborreliosis is more frequent in the United States than in

Europe. It appears that the clinical outcome depends not only on infection with strains of different species. Certain types of outer surface proteins (*OspA*, *OspC*, and *OspD*) (9, 35, 57) are associated with certain species (27, 34, 50a, 51a, 55b, 56) and may play a role in pathogenesis (20, 24). In addition, platelet-binding activity (19), expression of lectin activity that promotes bacterial attachment to glycosaminoglycans (31), binding to glycosphingolipids (4), and other as yet unknown factors may be involved in the pathogenesis of the disease.

In conclusion, we found a considerable degree of heterogeneity among 36 isolates from CSF. This could explain the contradictory results obtained previously by analysis of small numbers of isolates from CSF. This heterogeneity has important implications for the microbiological diagnosis (serodiagnosis and PCR) as well as the development of a borrelia vaccine.

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