Isolation and Characterization of *Borrelia burgdorferi* Sensu Lato Strains in an Area of Italy Where Lyme Borreliosis Is Endemic

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Between 1993 and 1998, we isolated *Borrelia burgdorferi* sensu lato from 55 of the 119 patients with clinically diagnosed Lyme borreliosis who were admitted to “San Martino” Hospital in Belluno, Veneto, an Adriatic region in northeastern Italy where Lyme borreliosis is endemic. Upon hospitalization, all patients presented erythema migrans. Isolates were typed using ribosomal DNA PCR-restriction fragment length polymorphism (RFLP) analysis of the *rrfA-rrlB* intergenic spacer. Of the 41 isolates typed, 37 belonged to *Borrelia afzelii*, 2 to *Borrelia garinii*, and 2 to *B. burgdorferi* sensu stricto. Pulsed-field gel electrophoresis, performed on 21 strains (13 new isolates and 8 controls), revealed different RFLP patterns within the *B. garinii* and *B. afzelii* strains; among the five *B. garinii* strains and the 12 *B. afzelii* strains, three or two different RFLP patterns were identified, according to the restriction enzyme used. The protein patterns of the new isolates confirmed their genotypic classification and revealed the level of expression of some immunodominant proteins like OspA and other characteristic Osps. These findings constitute the first report of such a high recovery rate of *B. burgdorferi* from patients in a very restricted area in Italy; they also indicate the predominance of the genospecies *B. afzelii* in the study area and the heterogeneity of the circulating strains.

Lyme borreliosis (LB) is an emerging tick-borne zoonosis caused by spirochetes of the *Borrelia burgdorferi* sensu lato complex, which are usually transmitted to the host by the bite of infected hard ticks of the genus *Ixodes*. Nucleic acid and protein studies have revealed great genetic and phenotypic differences among the borrelial strains associated with LB. Based on these differences, *B. burgdorferi* was recently divided into a number of genomic species (3, 6, 20, 24, 28, 30, 35, 36, 37, 45). To date, four of these species have been found to be associated with LB: *B. burgdorferi*, *Borrelia garinii*, *Borrelia afzelii*, and *Borrelia bissettii* (6, 42). Three of these species are known to be present in Italy (11, 14, 15). In addition another species was found in Italian ticks, *Borrelia valaisiana*, whose pathogenicity for humans is still questionable (16).

Several studies have indicated that the clinical manifestations of LB may vary according to the specific infecting genomic species of *B. burgdorferi* sensu lato: from patients affected by LB arthritis, the most commonly isolated strains belong to *B. burgdorferi* sensu stricto, whereas *B. garinii* seems to be involved in cases showing prevalent neurological manifestations. Strains of *B. afzelii* have been found prevalent in skin forms of the disease, erythema migrans (EM) and especially acrodermatitis chronica atrophicans. (2, 5, 12, 44). However, the distribution and the prevalence of the different species within various geographic regions, which could provide information on the epidemiology of LB, have still not been clearly defined.

Molecular techniques used for identifying and typing micro-

organisms can be categorized as either phenotypic or genetic on the basis of the macromolecular targets used for analysis. For *B. burgdorferi* sensu lato, the phenotypic typing systems include sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of proteins (33), profile analysis of fatty acids (30), and serotyping (47, 48); the genetic typing systems, which can provide more precise information on the diversity of *B. burgdorferi*, include restriction enzyme analysis (1, 27), DNA-DNA hybridization (27, 38), PCR-restriction fragment length polymorphism (RFLP) analysis of the *rrfA-rrlB* intergenic spacer (36), plasmid profile analysis (7), PCR (31), and arbitrarily primed PCR (46). Another molecular typing method, the pulsed-field gel electrophoresis (PFGE) of large DNA fragments produced by rare-cutting restriction enzymes, has provided results that are consistent with the division of *B. burgdorferi* sensu lato into the genomic species and has been successfully used to differentiate closely related borrelial isolates (9, 14, 21, 22, 34).

Between 1993 and 1998, 119 persons with an illness consistent with LB were admitted to “San Martino” Hospital in the city of Belluno, which is situated in Veneto, an Adriatic region in northeastern Italy where LB is endemic. Various cases of LB have been reported in the Belluno area in recent years, and a recent tick-spirochete survey identified *Borrelia* infection in 0.8% of the nymphs and 3.1% of the adult *Ixodes ricinus* ticks collected, in addition to isolating two genomic species (i.e., *B. burgdorferi* sensu stricto and *B. garinii*) (L. Ciceroni, S. Ciarrochi, B. Carnielli, and M. Romano, Abstr. 8th Int. Conf. Lyme Borrel. Other Emerg. Tick-Borne Dis. 1999, abstr. P-318, p. 85, 1999).

The aim of the present study, which was performed as part of an LB survey in the Veneto region, was to identify the

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TABLE 1. PFGE restriction patterns obtained after *Mlu*I digestion from *B. garinii* and *B. afzelii* isolates

Genospecies	Pattern	Strains	Bands after digestion with <i>Mlu</i> I (kb)
<i>B. garinii</i>	A	N34, G25, BL30	418, 220, 100, 80, 63
	B	BL21	418, 220, 100, 80
	C	BZ5	460, 220, 100, 80, 63
<i>B. afzelii</i>	A	VS461, BL11, BL12, BL25, BL29, BL31, BL33, BL34, BL37, BL41, BL48	460, 320, 90
	B	BL43	460, 320, 90, 60

genospecies of the borreliae isolated from these hospital patients: PCR-RFLP analysis of the *rrfA-rrlB* intergenic spacer was directly applied to the fresh culture, first passage, to identify the genospecies; PFGE was subsequently applied to culture-adapted, cloned strains to further assess their intraspecies heterogeneity. The results of genetic typing were compared with the protein profiles of the isolates.

MATERIALS AND METHODS

Clinical samples and isolation of spirochetes. The 119 persons were hospitalized between October 1993 and October 1998 and were clinically diagnosed with LB on the basis of EM and other clinical symptoms (13). Skin biopsies, performed for all patients prior to antibiotic therapy, were taken near the margin of the EM and inoculated in 5 ml of modified Barbour-Stoenner-Kelly (BSK) II medium containing 5% normal rabbit serum. All specimens were incubated at 32°C for at least 2 months, and samples of each culture were examined weekly for spirochetes by dark-field microscopy.

Borrelial strains and growth conditions. As controls, strains of six different genospecies of *B. burgdorferi* sensu lato were used: *B. burgdorferi* sensu stricto (B31, IRS), *B. garinii* (N34, G25), *B. afzelii* (VS461), *B. valaisiana* (VS116), *Borrelia lusitaniae* (Poti B1), and *B. bissettii* (DN127). We also used two local strains as controls: *B. garinii* BZ5, isolated from *I. ricinus* ticks collected in the bordering area of Alto Adige-South Tyrol, and *B. garinii* BITS, isolated from the nearby Karst region (11, 17). The North American type strain *B. burgdorferi* sensu stricto B31 (from *Ixodes dammini* ticks; Shelter Island, N.Y.) and the two *I. ricinus* strains IRS (from Switzerland) and G25 (from Sweden) were kindly provided by R. C. Johnson (University of Minnesota, Minneapolis). As for the other reference strains, *B. afzelii* VS461, *B. valaisiana* VS116, *B. lusitaniae* Poti B1, and *B. bissettii* DN127 were obtained from D. Postic (Unité de Bactériologie Moléculaire et Médicale, Institut Pasteur, Paris, France) and N34 was kindly supplied by R. Ackermann (Department of Virology, University of Cologne, Cologne, Germany). The *B. garinii* BZ5 strain was taken from our collection (Istituto Superiore di Sanità). All spirochetes were grown in BSK II medium at 32°C for 7 to 10 days (8).

PCR-RFLP analysis of the *rrfA-rrlB* intergenic spacer. PCR-RFLP analysis was performed on the first passage of clinical samples showing borrelial growth. For the analysis, 2 ml of culture was washed twice in phosphate-buffered saline and resuspended in 50 µl of distilled water. The preparation was boiled at 100°C for 10 min, and this suspension was used for PCR. Positive controls included strains B31, BITS, VS461, VS116, Poti B1, and DN127. Positive control DNA was extracted as previously described (19); 1 ng of isolate DNA was used for each positive control. PCR amplification of the 5S-23S intergenic spacer DNA was performed as described by Postic et al. (36). This was followed by endonuclease *Mse*I (Boehringer Mannheim, Mannheim, Germany) digestion, performed according to the manufacturer's instructions. The restriction fragments were sub-

sequently subjected to electrophoresis on a 16% acrylamide–0.8% bisacrylamide gel for 3 h at 100 V.

Chromosomal analysis by PFGE. Genomic DNAs were prepared using a slightly modified version of the procedure described by Taylor et al. (43). Exponential-phase borreliae were washed with 50 mM Tris containing 150 mM NaCl buffer, pH 8.0 (TN), at 20°C and were adjusted to an optical density of 1.5 at 600 nm in the same buffer. A portion of this suspension was mixed with an equal volume of molten 1.5% low-melting-temperature agarose (Bio-Rad) in TN buffer, pipetted into 90-µl rectangular plug molds (Bio-Rad, Richmond, Calif.), and then allowed to harden at 4°C for 15 min. The solidified agarose plugs were immersed in a digestion solution of 50 mM Tris–50 mM EDTA–1% SDS (pH 8.0) and 1 µg of proteinase K (Boehringer Mannheim) per ml and were incubated at 50°C for 16 to 24 h. The plugs were then washed four times for 1 h with Tris-EDTA (10 mM Tris–1.0 mM EDTA, pH 8.0) and stored at 4°C in the same buffer. Two restriction endonucleases, *Mlu*I and *Sma*I, were used to compare the different strains (Tables 1 and 2). For digestion, DNA corresponding to half a plug was digested by incubation with 15 U of the restriction enzymes, according to the manufacturer's instructions. The plugs were then loaded onto 1% Pulsed Field Certified Agarose Gels (Bio-Rad) in 0.25× Tris-borate-EDTA (TBE) (89 mM Tris–20 mM EDTA–89 mM boric acid, pH 8.3). PFGE was performed with a contour-clamped homogeneous electric field apparatus (CHEF Mapper; Bio-Rad) at 14°C, with buffer circulation and a constant voltage of 200 V. Runs were carried out with increasing pulse times (from 5 s to 15 s for 15 h and from 20 s to 25 s for 9 h). Gels were then stained with ethidium bromide, destained in water, and photographed under UV illumination.

SDS-PAGE. Whole-cell lysates were prepared as described elsewhere (11). Proteins were separated by SDS-PAGE using Laemmli's buffer system and polyacrylamide gels (26); molecular standards were run in each gel (SDS-PAGE Molecular Weight Standards; Bio-Rad). After electrophoresis, gels were stained with Coomassie brilliant blue.

RESULTS

Patients. Of the 119 patients, 52 were female and 67 were male; they ranged in age from 31 to 60 years. They all lived in or near Belluno. All of them met the case definition for the public health surveillance of LB of the U.S. Centers for Disease Control and Prevention. All of the patients had an erythematous skin lesion, whereas 19 of 119 (16%) of them had symptoms and/or signs suggestive of systematic infection. None of the patients had arthritis. None of them reported having been bitten by a tick bite outside the area of Belluno, and none had received antibiotic treatment prior to clinical diagnosis. Of the 119 skin specimens cultured for *Borrelia*, 55 (46%) were

TABLE 2. PFGE restriction patterns obtained after *Sma*I digestion from *B. garinii* and *B. afzelii* isolates

Genospecies	Pattern	Strains	Bands after digestion with <i>Sma</i> I (kb)
<i>B. garinii</i>	A	G25, N34, BL21, BL30	230, 180, 105, 70
	B	BZ5	260, 230, 105, 70
<i>B. afzelii</i>	A	VS461, BL11, BL25, BL29, BL31, BL33, BL34, BL41, BL43, BL48	250, 150, 140, 130, 120, 80
	B	BL12	194, 150, 130, 120, 80
	C	BL37	250, 150, 140, 130, 97, 80

FIG. 1. Typing of *B. burgdorferi* sensu lato isolates by using *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. DNA was electrophoresed on a 16% acrylamide gel, stained with ethidium bromide, and UV illuminated. The names of strains in each lane are indicated on the top of each well in the photos. The molecular sizes of DNA fragments (in base pairs) are shown on the right of the gels. Expected fragment sizes for *B. burgdorferi* sensu stricto strains were 108, 50, 38, and 29 to 28 bp. Expected fragment sizes for *B. garinii* strains were 108, 95, and 50 bp. Expected fragment sizes for *B. afzelii* strains were 108, 68, 50, and 20 bp. The 20-bp expected size was not easily visible.

positive. Following diagnosis, all patients were successfully treated with antibiotics.

Genetic typing of the isolates: PCR-RFLP analysis of the *rrfA-rrlB* intergenic spacer. Based on the analysis of patterns obtained after restriction by *Mse*I, we were able to classify 41 of the 55 culture-positive samples. PCR amplification of the spacer region ranged from 216 to 226 bp. The patterns obtained after restriction by *Mse*I were compared with the patterns reported by Postic et al. (36) for each of the genospecies: the analysis of the amplicon patterns, reported in Fig. 1 for 30 of the 41 strains, revealed that the BL45 and BL47 strains belonged to *B. burgdorferi* sensu stricto and that BL21 and

BL30 belonged to *B. garinii*. BL44 showed a pattern with bands of both *B. burgdorferi* sensu stricto and *B. afzelii*, and consequently the isolate contained two distinct genospecies. The other 36 samples revealed a pattern identical to that of the VS461 strain and were thus assigned to *B. afzelii*. Of these strains, four (BL53, BL54, BL49, and BL34) showed an additional band of about 45 bp.

PFGE analysis. Of the 41 isolates typed by PCR-RFLP, which were maintained for more than three passages in BSK medium, 13 were further characterized by PFGE (i.e., BL11, BL12, BL21, BL25, BL29, BL30, BL31, BL33, BL34, BL37, BL41, BL43, and BL48); the isolates were cloned by limiting dilution method.

The genomic DNAs analyzed by PFGE after cleavage with the enzymes *Mlu*I and *Sma*I are shown in Fig. 2 and 3. Additional strains, belonging to *B. garinii* and *B. burgdorferi* sensu stricto, *B. afzelii*, *B. valaisiana*, and *B. lusitanae*, were used for comparison purposes. Since the bands smaller than 58 kb could be the result of uncut or cut plasmids, greater fragments were used to define restriction patterns. As expected, the strains analyzed using *Mlu*I showed five different restriction patterns characteristic of the five species (9, 14), as shown in Fig. 2.

The restriction patterns of *B. burgdorferi* sensu stricto B31 showed three bands, at 418, 150, and 135 kb. These three bands were also found in the pattern of *B. burgdorferi* IRS sensu stricto, which showed an additional band at 101 kb. The isolates BL21 and BL30 (Fig. 2B), as well as strains N34 and G25, showed patterns typical of the genospecies *B. garinii*, consisting of two bands at 220 and 80 kb and three additional bands at 418, 100, and 63 kb, although the 63-kb band was not visible in strain BL21. A band at 460 kb, instead of 418 kb, was detected in strain BZ5. Thus, when *Mlu*I was used, three patterns were observed for the *B. garinii* strains: one included strains N34, G25, and the new isolate BL30; a second pattern included the isolate BL21; and a third pattern included the strain BZ5. The remaining 11 isolates (i.e., BL11, BL12, BL25, BL29, BL31, BL33, BL34, BL37, BL41, BL43, and BL48) exhibited restriction patterns identical to that of strain VS461, consisting of three bands at 460, 320, and 90 kb, confirming that they belonged to *B. afzelii*. Isolate BL43 showed an additional band at 60 kb. The type strains of *B. valaisiana* and *B. lusitanae* each yielded a unique *Mlu*I restriction pattern.

Like the patterns obtained with PFGE after *Mlu*I digestion, those obtained after *Sma*I digestion (Fig. 3) allowed us to differentiate among the five *B. burgdorferi* sensu lato species on the basis of characteristic bands and to identify BL21 and BL30 as *B. garinii* and the remaining 11 isolates as *B. afzelii*. For fragments greater than 58 kb, no differences in restriction patterns were observed in comparisons of BL21 and BL30. Isolates BL21 and BL30 yielded a *Sma*I restriction pattern that was indistinguishable from that of G25 and N34 but distinct from that of BZ5. Isolates BL11, BL25, BL29, BL31, BL33, BL34, BL41, BL43, and BL48 had restriction patterns indistinguishable from that of VS461, the type strain of *B. afzelii* (Fig. 3A). The remaining two isolates identified as *B. afzelii* (BL12 and BL37) yielded distinct *Sma*I restriction patterns, which were easily distinguishable from that of VS461, as indicated by arrows in Fig. 3A.

Protein profiles by SDS-PAGE. The protein patterns of the 13 new isolates kept in culture were compared with those of

FIG. 2. PFGE of *Mlu*I-cleaved genomic DNAs from *B. burgdorferi* sensu lato strains. The digestion products were separated at 200 V for 24 h in 1% agarose–0.25× TBE with two increasing pulse times, from 5 to 15 s (15 h) and from 20 to 25 s (9 h). The names of strains are indicated on the top of each lane in the photos. M, size marker (108 bp). The molecular sizes of DNA fragments (in kilobases) are shown on the left of the gels. Reference strains and genospecies used were B31 and IRS, type strains of *B. burgdorferi* sensu stricto; G25, type strain of *B. garinii*; VS461, type strain of *B. afzelii*; VS116, type strain of *B. valaisiana*; and Poti B1, type strain of *B. lusitaniae* (A); and G25 and N34, type strains of *B. garinii* (B). An additional band for BL43 is indicated by a small arrowhead.

the strains used as controls. The overall protein patterns of BL21 and BL30 (Fig. 4A) were consistent with those for *B. garinii* and were thus similar to those for strains N34 and G25; strain BZ5 differed from the other *B. garinii* strains in that it had a higher molecular-mass-protein, OspA (33 kDa). The profiles of the isolates classified as *B. afzelii* were, in general, similar to that of the *B. afzelii* strain VS461 (Fig. 4B). A certain degree of variability was evident in the protein size and level of expression of some *B. afzelii* isolates: BL12 and BL37 differed in the relative migration of the protein band at 37 instead of 35 kDa, and strain BL12 displayed a higher expression of the 23-kDa protein.

DISCUSSION

LB was first recognized in Italy in 1985 (18) and is endemic in several regions. Most cases have been reported in northern Italy, and in the period from 1985 to 1996, only 6.1% of the cases were reported in central and southern Italy and the

Islands. The review of the national literature shows that, in the same 13-year period, at least 368 cases were recorded in the region of Veneto (14), representing 27.8% of the cases reported nationwide during this period.

Since the first LB case was described in 1985, the number of clinically diagnosed cases in the Belluno Hospital has progressively increased: 15 cases in 1995, 45 in 1996, 85 in 1997, 75 in 1998, and 102 in 1999.

With regard to the isolation rate of *B. burgdorferi*, the 46% found in our study is quite high, although rates of *B. burgdorferi* in skin biopsy specimens have been reported to be as high as 100% in Europe (10, 25, 32, 41).

The ribosomal DNA (rDNA) PCR-RFLP analysis showed that the majority of the isolates belonged to *B. afzelii*; two isolates were classified as *B. garinii* and two as *B. burgdorferi* sensu stricto. Since the amplification was performed on the first culture inoculated with the skin sample, we were able to type all of the isolates, including those that did not subse-

FIG. 3. PFGE of *Sma*I-cleaved genomic DNAs from *B. burgdorferi sensu lato* strains. The digestion products were separated at 200 V for 24 h in 1% agarose–0.25× TBE with two increasing pulse times as follows: from 5 to 15 s (15 h) and from 20 to 25 s (9 h). The names of strains are indicated on the top of each lane in the photos. M, size marker (λ). The molecular sizes of DNA fragments (in kilobases) are shown on the left of the gels. Reference strains and genospecies used were B31 and IRS, type strains of *B. burgdorferi sensu stricto*; G25, type strain of *B. garinii*; VS461, type strain of *B. afzelii*; VS116, type strain of *B. valaisiana*; Poti B1, type strain of *B. lusitaniae* (A); and G25 and N34, type strains of *B. garinii* (B). Additional bands for BL12 and BL37 are indicated by small arrowheads.

quently adapt to the BSK medium. In the case of isolate BL44, a hybrid profile was obtained, which was interpreted as coinfection with *B. afzelii* and *B. burgdorferi sensu stricto*; this phenomenon is quite common in the first isolation. The results of the PFGE analysis of the 13 strains that were kept in culture, compared to the strains of the five genospecies of *B. burgdorferi sensu lato* and to the local tick strain BZ5, were consistent with the results obtained with rDNA PCR-RFLP, yet they revealed heterogeneity within the strains. In fact, we obtained three patterns within *B. garinii* strains by *Mlu*I digestion and two patterns by *Sma*I digestion; even BL21 and BL30, though isolated in the same restricted area of Belluno, differed for one band after digestion with *Mlu*I. However, the BL30 strain showed a close relationship with G25, a Swedish tick isolate and N34. Strain BZ5 was always shown to have a unique pattern. This heterogeneity within the *B. garinii* genospecies was previously reported by other authors; in fact, Postic et al. (35, 39) described up to four RFLPs in 20 *B. garinii* isolates.

Although no heterogeneity in the RFLP of *B. afzelii* strains has been reported in the literature, we found two different patterns after *Mlu*I digestion and three patterns after *Sma*I digestion. These results demonstrate that strains that belong to the same genomic species yet are characterized by different PFGE restriction patterns may be present in a small geographic area. By contrast, strains indistinguishable by their PFGE restriction patterns can be found in geographically distant areas.

Our results show that in the small geographic area served by the Belluno Hospital, the three main pathogenic genospecies of *B. burgdorferi sensu lato* are all present and that the dominant genospecies is *B. afzelii*; an analogous prevalence of this genospecies has been reported in humans in Slovenia, which borders northeastern Italy (34). The net prevalence of *B. afzelii* in human isolates in Belluno is quite unexpected if compared to the genospecies prevalence rates in *I. ricinus* ticks in the nearby region of Friuli Venezia Giulia (16). In this region, the rate of infected ticks was 28% for *B. burgdorferi sensu stricto*,

FIG. 4. Coomassie brilliant blue-stained proteins in whole-cell lysates of *B. burgdorferi* sensu lato strains. The names of strains are indicated on the top of each lane in the photos. LMW, low-molecular-weight standards; HMW, high-molecular-weight standards. The molecular sizes of protein standards (in kilodaltons) are shown on the right of the gels. Components were separated by SDS-PAGE

13.2% for *B. garinii*, and only 1.1% for *B. afzelii*. The majority of the ticks were coinfecting: 25.5% with *B. burgdorferi* sensu stricto and *B. garinii*; 6.6% with *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii*; and only 1.1% with *B. burgdorferi* sensu stricto and *B. afzelii*. There was thus a very low rate of tick infection by *B. afzelii* alone or associated with other *Borrelia* genospecies. In the rest of Europe, *B. garinii* appears to be prevalent, and *B. afzelii* constitutes approximately 37% of *B. burgdorferi* sensu lato isolates (23). The reasons for these discrepancies are unknown, though the following hypotheses can be proposed: (i) There exist local variations in the distribution of genomic species in Italy due to the type of reservoirs; thus, there exists a true dominant circulation of this genospecies in the Belluno area. (ii) All of our isolates were from patients manifesting EM. Even though EM is the first lesion common to all LB manifestations, there is general agreement that there is a preferential association of *B. afzelii* with cutaneous mani-

festations and that the majority of isolates from EM and acrodermatitis chronica atrophicans are *B. afzelii* (4, 5, 40, 44).

In conclusion, our data contribute to the knowledge of the repartition of the *B. burgdorferi* species in northern Italy, which is important for the better use of antigens in serology and for defining future vaccination policy.

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