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Characterization of Lyme Borreliosis Isolates from Patients with Erythema Migrans and Neuroborreliosis in Southern Sweden

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Southern Sweden is an area of Lyme borreliosis (LB) endemicity, with an incidence of 69 cases per 100,000 inhabitants. The most frequent clinical manifestations are erythema migrans (77%) and neuroborreliosis (16%). There was no record of human *Borrelia* strains being isolated from patients in this region before the prospective study reported here. *Borrelia* spirochetes were isolated from skin and cerebrospinal fluid (CSF) from LB patients living in the region. A total of 39 strains were characterized by OspA serotype analysis, species-specific PCR, and signature nucleotide analysis of the 16S rRNA gene. Of 33 skin isolates, 31 (93.9%) were *Borrelia afzelii* strains and 2 (6.1%) were *Borrelia garinii* strains. Of six CSF isolates, five (83.3%) were *B. garinii* and one (16.7%) was *B. afzelii*. Neither *Borrelia burgdorferi* sensu stricto strains nor multiple infections were observed. The *B. afzelii* isolates were of OspA serotype 2. Three *B. garinii* strains were of OspA serotype 5, and the remaining four strains were of OspA serotype 6. All of the *B. garinii* strains belonged to the same 16S ribosomal DNA ribotype class. Our findings agree with earlier findings from other geographic regions in Europe where *B. afzelii* and *B. garinii* have been recovered predominately from skin and CSF cultures, respectively. To further study the possible presence in Sweden of the genotype *B. burgdorferi* sensu stricto, which is known to be present in Europe and to occur predominately in patients with Lyme arthritis, molecular detection of *Borrelia*-specific DNA in synovial samples from Lyme arthritis patients should be performed.

Lyme borreliosis (LB) or Lyme disease (15) is probably the most common tick-borne human disease in Europe and the United States. The disease is a multisystemic infection caused by the tick-borne spirochete *Borrelia burgdorferi* sensu lato. Cases of LB and isolates of spirochetes have been identified in most countries in the Northern Hemisphere (1, 6, 12, 17, 20). The distribution of the disease is associated with the distribution of the *Ixodes* tick complex and, in Europe, *Ixodes ricinus* (26). This tick is known to be abundant especially in the southern coastal areas and central regions of Sweden, with a 5 to 26% prevalence of borrelia-infected ticks (11, 12, 23). Typical borrelial infections primarily involve the skin, nervous system, and joints. Involvement of the nervous system neuroborreliosis is the predominant result of morbidity caused by the *Borrelia* bacteria in Europe, and *Borrelia* bacteria are also the most common bacterial pathogen of the nervous system in Sweden (43).

Borrelia isolates that cause LB are divided into three different genospecies of the *B. burgdorferi* sensu lato group, *B. burgdorferi* sensu stricto, *Borrelia afzelii*, and *Borellia garinii*, on the basis of DNA sequence identity (6, 18, 46). However, human pathogenic *Borrelia* species that cannot be classified into any of these groups have recently been reported (45). The three *B. burgdorferi* sensu lato genospecies have been isolated from human skin, cerebrospinal fluid (CSF), and synovial fluid as well as from ticks in Europe (16, 17, 22, 25), whereas only the *B. burgdorferi* sensu stricto genotype has been found in humans in North America. The agents of LB are divided into eight serotypes based on their reactivity to OspA monoclonal antibodies (MAbs) (47, 48). Furthermore, clinical data suggest an association between infecting *Borrelia* species and clinical manifestations in Europe (4, 5, 16, 17, 21, 22, 27, 39, 40, 44). *B. burgdorferi* sensu stricto is the most common spirochete species associated with Lyme arthritis, and *B. afzelii* predominates in the skin, especially in cases of acrodermatitis chronica atrophicans (ACA) (39). *B. garinii* is most often associated with neuroborreliosis (5, 44). Most European skin isolates belong to OspA serotype 2 of *B. afzelii*, whereas a similar but less-stringent association has emerged between CSF isolates and *B. garinii* (47).

Southern Sweden has been identified as an area where LB is highly endemic, with an incidence of 69 cases per 100,000 inhabitants (10). The most frequent clinical manifestations are erythema migrans (EM) (77%), neuroborreliosis (16%), and arthritis (7%). No LB isolates had been recovered in this area of endemicity before our present study.

The aim of this study was to isolate and characterize the *Borrelia* species that cause LB in southern Sweden. LB isolates were recovered from skin and CSF. The isolated strains were characterized by species-specific PCR, signature nucleotide analysis by partial 16S rRNA gene sequencing (14, 34), and tests of immune reactivity to a panel of MAbs directed against OspA (47, 48).

MATERIALS AND METHODS

Study population. This prospective multicenter study was carried out at eight different locations in southern Sweden, an area where LB is endemic, from 1994 to 1997. Patients suspected of having EM and neuroborreliosis were studied. Only adults were included in the group that had skin biopsies. The ethics

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TABLE 1. Immunoreactivity of *B. burgdorferi* sensu lato strains with a panel of OspA-specific MAbs

Borrelia isolates	L32-1F11	L32-1C8	H5332	H3TS	L32-14G7	L32-1F7	L32-1G3	L32-1D11	OspA serotype
B31[a]	+	+	+	+	−	−	−	−	1
ACA1[a], LU54, LU58, LU68, LU71, LU72, LU81, LU82, LU85, LU156, LU157, LU165, LU169, LU171, LU188, LU192, LU203, LU207, LU209, LU212, LU215, LU216, LU218, LU235, LU253, LU254, LU256, LU9038	+	−	+/−	−	+	−	−	−	2
IP90[a], LU59, LU116, LU170	+	+	−	−	−	−	−	−	5
LU118, LU185, LU190, LU222	+	+	+	−	−	−	−	−	6

[a] Reference strains: B31, *B. burgdorferi* sensu stricto; ACA1, *B. afzelii*; and IP90, *B. garinii*. +, positive; −, negative; +/−, variable.

committee of Lund University, Lund, Sweden, approved the study. The departments involved in this study were the departments of infectious diseases, pediatrics, and dermatology at local hospitals in southern Sweden in the provinces of Skåne, Blekinge, Halland, and Kalmar, and an outpatient primary care center in the province of Blekinge.

Culture of *Borrelia* spp. *Borrelia* spirochetes were cultured from 4-mm skin biopsy specimens and 1-ml CSF samples in Barbour-Stoenner-Kelly medium II (7), with 10% rabbit sera (Sigma Chemical Co., St. Louis, Mo.) and 1.3% Bacto gelatin (Difco Laboratories, Detroit, Mich.). The medium used for skin biopsy specimens contained 100 μg of fosfomycin (Sigma) per ml and 50 μg of rifampicin (Sigma) per ml to inhibit the growth of contaminating microorganisms. Cultures were incubated at 32°C, and growth was recorded weekly using darkfield microscopy. The reference strains used were *B. burgdorferi* sensu stricto B31 (ATCC 35210) (15), a North American tick isolate; ACA1 *B. afzelii*, isolated from a Swedish ACA patient (2); and an IP90 *B. garinii* tick isolate from Russia (29).

OspA serotyping. All of the local isolates used for protein analysis were low-passage strains (<10 passages), and the cells were grown in Barbour-Stoenner-Kelly medium II until middle to late log phase. Sonicated whole-cell lysates prepared from *Borrelia* cells were separated on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis 4 to 15% gradient gel (Bio-Rad, Richmond, Calif.), and the proteins were transferred to polyvinylidene difluoride membranes (0.45-μm pore size; MSI Inc., Westborough, Mass.), as previously described (37). These membranes were probed with a panel of eight OspA MAbs (47, 48), including L32-1F11, L32-1C8, L32-14G7, L32-1G3, L32-1F7, and L32-1D11, which were provided by B. Wilske, and H5332 and H3TS (8), which were obtained from A. G. Barbour. The H3TS is type-specific for *B. burgdorferi* sensu stricto serotype 1, and L32-14G7 is type-specific for *B. afzelii* serotype 2. *B. garinii* shows a more divergent pattern that is covered by serotypes 3 to 8.

Genotyping: 16S rRNA gene (16S rDNA) analysis. DNA was isolated according to the method of Bunikis et al. (14). Standard PCR routines were used to prevent contamination (30). Briefly, filter tips (ART, San Diego, Calif.) were used, and amplified *Borrelia* DNA was handled in a different location. Contamination controls included extraction controls and negative buffer controls in parallel with strain samples. Genospecies-specific identification of LB isolates was determined by 16S rRNA gene analysis using PCR and primers described by Marconi and Garon (34): a (LD) primer set specific for *B. burgdorferi* sensu lato, a (BB) primer set genospecific for *B. burgdorferi* sensu stricto, a (BA) primer set genospecific for *B. afzelii*, and a (BG) primer set genospecific for *B. garinii*. Primers were synthesized by Interactiva Biotechnologie GmBh, Ulm, Germany. PCR mixtures contained a 0.2 μM concentration of each primer, and the PCR buffer solution contained 10 mM Tris-HCl, 1.5 mM MgCl, 50 mM KCl (pH 8.3), 0.2 mM concentrations of each deoxynucleoside triphosphate, and 1 U of *Taq* DNA polymerase (Boehringer Mannheim, Mannheim, Germany). PCRs were performed in an MJ PTC-100 thermocycler (MJ Research Inc., Waltham, Mass.), as previously described (34). The amplified DNA was separated by agarose gel electrophoresis, stained by ethidium bromide, and visualized using a standard UV transilluminator.

The PCR products were purified in MicroSpin Columns S-400 (Pharmacia Biotech, Uppsala, Sweden) prior to sequencing. Cycle sequencing was carried out using the PCR species-specific primers in an ABI PRISM rhodamine dye terminator cycle sequencing core kit with Ampli*Taq* DNA Polymerase FS (PE Applied Biosystems, Foster City, Calif.). The cycle-sequencing products were purified in MicroSpin S-200 Columns (Pharmacia Biotech) before being analyzed on an automatic sequencer (ABI PRISM 373A; PE Applied Biosystems). Sequences used for comparison were *B. burgdorferi* sensu stricto strain B31, *B. afzelii* strain DK4, and *B. garinii* strain IP90 (accession numbers UO3396, X85194, and M89937, respectively). Analysis of signature nucleotides was performed as described earlier (34), using the software BioEdit version 4.8.6 (Tom Hall, Department of Microbiology, North Carolina State University, Raleigh, N.C.)

RESULTS

Isolation of local human pathogen spirochetes. Seventy-nine skin biopsy specimens and 207 CSF cultures from suspected LB cases were included in the study. Seventy-five patients with EM fulfilled the Centers for Disease Control and Prevention (CDC) criteria for Lyme disease (19), with the exception that a skin lesion less than 5 cm in size was accepted. Four skin biopsy specimens were taken from patients who were considered not to have EM. Thirty-three strains were collected from the 75 skin biopsy specimens, which was a success rate of 42%. The skin strains were recovered from biopsy specimens from individuals whose ages ranged from 19 to 69 years. The female/male distribution was 16:17. Twelve patients with EM had general symptoms of disseminated LB with low-grade fever, headache, arthralgia, and myalgia, and one patient had concomitant meningitis (LU253).

Eighty of the 207 patients included in the study fulfilled the CDC criteria for neuroborreliosis (19). Of these patients, 10 did not have meningitis. Six CSF isolates were recovered from the 70 patients with meningitis who also fulfilled the CDC criteria for neuroborreliosis, which results in an 8.6% culture success rate. The CSF strains were all obtained from children with meningitis who ranged from 4 to 7 years of age. In addition, two of six children had meningitis and facial palsy. Three of the six children presented an EM or had a history of EM in the head region.

When contamination and the number of patients that were treated with an antibiotic before sampling were considered, the success rates for culturing EM skin biopsy specimens and CSF were 50% and 10.2%, respectively.

The 33 isolates originating from skin biopsy specimens from patients with EM were identified as LU30, LU34, LU35, LU54, LU58, LU65, LU68, LU71, LU72, LU81, LU82, LU85, LU116, LU118, LU156, LU157, LU165, LU169, LU171, LU182, LU188, LU192, LU203, LU207, LU209, LU212, LU215, LU218, LU235, LU253, LU254, LU256, and LU9038. The six CSF isolates are identified as LU59, LU170, LU185, LU190, LU216, and LU222.

TABLE 2. Summary of species-specific PCR

Borrelia isolate(s)	Amplification of *Borrelia* DNA with specific primer[a]			
	LD	BB	BA	BG
B31[a]	+	+	−	−
ACA1[a], LU30, LU34, LU35, LU54, LU58, LU65, LU68, LU71, LU72, LU81, LU82, LU85, LU156, LU157, LU165, LU169, LU171, LU182, LU188, LU192, LU203, LU207, LU209, LU212, LU215, LU216, LU218, LU235, LU253, LU254, LU256, LU9038	+	−	+	−
IP90[b], LU59, LU116, LU118, LU170, LU185, LU190, LU222	+	−	−	+

[a] LD, primer specific for *B. burgdorferi* sensu lato; BB, primer genospecific for *B. burgdorferi* sensu stricto; BA, primer genospecific for *B. afzelii*; and BG, primer genospecific for *B. garinii*. +, positive; −, negative.
[b] Reference strains: B31, *B. burgdorferi* sensu stricto; ACA1, *B. afzelii*; and IP90, *B. garinii*.

OspA serotyping. The results of the OspA serotyping are shown in Table 1. Three isolates were contaminated (LU34, LU35, and LU182) and therefore could not be used for specific protein analysis. Two strains could not be subcultured (LU30 and LU65), and only enough cells for DNA analysis were obtained. None of the strains showed any reactivity to the MAb H3TS that is type specific for *B. burgdorferi* sensu stricto and represents the OspA serotype 1. Twenty-six of the 28 strains (92.9%) isolated from skin and 1 of the 6 strains (16.7%) isolated from CSF reacted with the MAb L32-14G7 that is type specific for *B. afzelii* and represents OspA serotype 2. One of the skin strains (LU116) and two of the six strains isolated from CSF (LU59 and LU170) were classified as *B. garinii* OspA serotype 5. One of the skin strains (LU118) and three of the six strains isolated from CSF (LU185, LU190, and LU222) reacted as *B. garinii* OspA serotype 6.

16S rDNA PCR and 16S rDNA signature nucleotide analysis. The results of the species-specific PCRs are summarized in Table 2. All of the locally isolated strains could be amplified using the *B. burgdorferi* sensu lato-specific primer set LD, whereas none of the local strains could be amplified by the *B. burgdorferi* sensu stricto primer set BB. The DNA from 31 of 33 strains isolated from skin (93.9%) and 1 of 6 strains from CSF (16.7%) was amplified by the species-specific primer pair for *B. afzelii* (BA). Two of 33 strains originating from skin (6.1%) and 5 of 6 strains isolated from CSF (83.3%) were amplified with the species-specific primer pair for *B. garinii* (BG). None of the local isolates were amplified by both BA and BG primer sets.

The 16S rDNA signature nucleotide analysis was performed by partial sequencing of the 16S rRNA gene using the subspecies-specific primer pairs used in the PCR. The results of the signature nucleotide analysis are shown in Table 3. Thirty-one of the 33 skin strains and 1 of the 6 CSF strains amplified and sequenced with the BA species-specific primer pair demonstrated the same signature nucleotide pattern as *B. afzelii* reference strain DK4. All of the *B. garinii* strains isolated, two from skin and five from CSF, that were amplified and sequenced with the BG species-specific primer pair showed the same pattern as reference strain IP90, a *B. garinii* strain ribotype 1.

DISCUSSION

In this prospective study we have for the first time recovered and characterized *Borrelia* strains from humans with LB in southern Sweden, an area where the high endemicity of LB is well defined (10).

The genospecies that we found to predominate in early skin borreliosis was *B. afzelii* (93.9%), while *B. garinii* predominated in neuroborreliosis (16.7%). Few *B. garinii* strains were recovered from skin. Occasionally, the *B. afzelii* strain was recovered from CSF, and one patient with EM and concomitant meningitis had a *B. afzelii*-positive skin culture. The three different methods used for genospecies determination, 16S rDNA PCR, signal nucleotide analysis, and OspA serotyping, all showed the same results. Our findings are in concordance with earlier clinical studies in Europe (4, 5, 16, 21, 22, 39, 44). *B. garinii* and *B. afzelii* have also previously been recovered from skin and CSF in other geographic regions in Sweden (2, 3, 6, 16, 28, 31).

B. garinii has previously been shown to form three patterns of variable signal nucleotide positions in the partial 16S rDNA sequence (14). All of the *B. garinii* isolates in this study fell into the 16S rDNA signal nucleotide pattern seen in the Russian tick isolate *B. garinii* IP90 ribotype 1. The *B. garinii* isolates diverged into the two OspA serotypes 5 and 6. OspA serotype 6 has been shown to predominate in CSF isolates from children in Germany (47). The diversity of *B. garinii* OspA serotypes

TABLE 3. Analysis of signature nucleotide positions of the partial 16S rDNA in studied *Borrelia* strains

Primer[a] or accession number	*Borrelia* isolate(s)[b]	Nucleotide identity at 16S rRNA position[c]								
		126	170	174	175	189	252	273	376	451
UO3396	*B. burgdorferi* sensu stricto B31	T	G	A	G	T	A	A	G	G
X85194	*B. afzelii* DK4	T	G	A	A	T	G	G	A	G
M89937	*B. garinii* IP90	C	A	A	A	T	A	G	A	G
BA	LU30, LU34, LU35, LU54, LU58, LU65, LU68, LU71, LU72, LU81, LU82, LU85, LU156, LU157, LU165, LU169, LU171, LU182, LU188, LU192, LU203, LU207, LU209, LU212, LU215, LU216, LU218, LU235, LU253, LU254, LU256, LU9038	T	G	A	A	T	G	G	A	G
BG	LU59, LU116, LU118, LU170, LU185, LU190, LU222	C	A	A	A	T	A	G	A	G

[a] BA, primer genospecific for *B. afzelii*; BG, primer specific for *B. garinii*.
[b] Nucleotides of *B. burgdorferi* sensu stricto B31, *B. afzelii* DK4, and *B. garinii* IP90 are included for comparison purposes.
[c] *B. burgdorferi* B31 numbering; insertion of C in position 213 has been ignored. T, threonine; G, glycine; A, alanine; C, cysteine.

depends on the geographical distribution of LB strains (16, 17, 47, 48). Comparison of *B. garinii* OspA serotypes from tick isolates in Japan with *B. garinii* serotypes isolated in Europe shows that different OspA serotypes exist on these two continents as well (35).

The *B. burgdorferi* sensu stricto genotype has yet to be isolated in Sweden from humans, nor have *B. burgdorferi* sensu stricto genotypes been found among isolates from our patients. This genotype was isolated from a lesion from a patient with ACA (31) in neighboring Denmark and was also identified by DNA analysis in *I. ricinus* ticks that were collected from migrating birds in southern Sweden (38). *B. burgdorferi* sensu stricto is known to be present in European LB, and it has been recovered from both skin and CSF but has been found more often than *B. afzelii* and *B. garinii* in synovial samples when molecular analytical methods are used (22, 25, 27). Detection of *Borrelia*-specific DNA in synovial samples from Lyme arthritis patients should be performed in Sweden to examine the possible presence of the genotype *B. burgdorferi* sensu stricto in LB.

We did not identify any multiple infections of the various genospecies in our human LB isolates. Mixed infections and genetic diversity of *B. burgdorferi* sensu lato, as determined by culture and/or PCR, have been observed both in Europe and the United States (20, 32, 33). The possibility of identifying multiple infections probably depends on both variation in growth requirements and factors such as the origin of the spirochetes and the technique and method being used.

Isolation of *B. burgdorferi* by culture is the best diagnostic evidence of LB. The bacteria are not easily recovered from clinical specimens other than biopsy samples from EM lesions. A success rate of more than 80% with skin biopsy specimens from EM patients has been reported (9). Our success rate was lower, partly due to specimen contamination and antibiotic treatment of patients prior to sampling. All of the CSF isolates were recovered from children who contracted neuroborreliosis less than 3 weeks after subclinical symptoms of meningitis or an acute facial palsy were diagnosed. Adults seem to have a tendency to neglect their symptoms, as many delayed seeing a doctor for more than 3 weeks after onset of symptoms, which may have decreased the possibility of recovering *Borrelia* spp. from CSF. The *Borrelia* medium used to culture skin biopsy specimens was supplemented with antibiotics, while the medium for CSF was antibiotic-free, which may have influenced the growth of *B. garinii* in CSF.

Culture is generally not useful for detection of the *Borrelia* bacteria or confirmation of the LB diagnosis. For laboratory diagnosis of LB, the enzyme-linked immunosorbent assay and Western blot usually are used. The importance of different *B. burgdorferi* species used in the antigenic preparations for these tests has been studied in LB patients in Germany (24). Results from the present study indicate a variation in test results that favors a locally recovered *Borrelia* strain due to the different levels of expression of immunodominant proteins. Use of local LB strains, together with information on the seroreactivity in the population to be tested, should be taken into account when developing diagnostic serological assays for clinical diagnosis (13, 36).

Two *B. burgdorferi* sensu stricto OspA-based *Borrelia* vaccines have been developed (41, 42). The geographic diversity and the wide range of clinical manifestations found in European *B. burgdorferi* sensu lato strains present a great challenge for vaccine development. A prerequisite for developing an LB vaccine is access to a variety of microbiologically and clinically well-characterized *Borrelia* strains.

Our results confirm the presence of *B. afzelii* and *B. garinii* in LB in Sweden. Additional studies on synovial samples from Lyme arthritis with molecular methods would be interesting in order to explore the possible presence of the genotype *B. burgdorferi* sensu stricto in Sweden. An effective *Borrelia* vaccine for Sweden at the minimum should protect against infection with the two genospecies *B. afzelii* and *B. garinii*.

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