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First Isolation of *Borrelia lusitaniae* from a Human Patient

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The first human isolate of *Borrelia lusitaniae* recovered from a Portuguese patient with suspected Lyme borreliosis is described. This isolate, from a chronic skin lesion, is also the first human isolate of *Borrelia* in Portugal. Different phenotypic and molecular methods are used to characterize it.

In Portugal, Lyme borreliosis has been known since 1989 (9) and became a notifiable disease in 1999. Although the causative agent has not been isolated from humans, several genospecies of *Borrelia burgdorferi* sensu lato have been detected in *Ixodes ricinus* ticks. They are *B. garinii*, *B. afzelii*, *B. valaisiana*, and *B. lusitaniae* (1, 6, 7). The last, which was isolated from ticks for the first time in 1993 (in Portugal) (10), appears to circulate mainly around the western Mediterranean basin (1, 3, 6, 7, 14). To date, the reservoir of *B. lusitaniae* has not been defined, and little is known about the ecology of this genospecies. *B. lusitaniae* is known to cause experimental disease in the C3H/HeN mouse model (13), suggesting that some strains of this genospecies could also be associated with human Lyme borreliosis.

The present study describes and characterizes the first human isolate of *B. lusitaniae* and the first human isolate of *Borrelia* in Portugal. A 46-year-old woman from the Lisbon area in Portugal presented with skin lesions on her left thigh that had persisted for approximately 10 years. These chronic skin lesions were characterized by two ill-defined erythematous macules associated with a local diffuse infiltration of the subcutaneous tissues.

Punch biopsy samples (5 mm^3) were taken from the margin of the skin lesion and divided into two parts. One part was inoculated immediately into Barbour-Stoenner-Kelly H medium (Sigma) supplemented with gelatin, pH 7.6, and incubated at 32°C for 4 weeks. Cultures were examined weekly by dark-field microscopy for motile spirochetes. The second half of the skin biopsy sample was washed in physiological saline and then transferred to a dry tube and stored at −30°C until PCR was performed. The patient's blood was collected on the day of the biopsy and again 4 months later, and prepared sera were stored at −20°C.

For comparative phenotypic and genetic purposes, an additional 14 strains were evaluated (Table 1). The human isolate PoHL1 and the six Portuguese tick-derived strains PoTiBG4, PoTiBV6, PoTiBG20, PoTiBL37, PoTiBG86, and PoTiBG163 (1) were from low-passage cultures when analyzed. Whole-cell lysates of the spirochetes were centrifuged at 10,000 × *g* at 4°C for 30 min, washed twice in phosphate-buffered saline, resuspended in Tris-hydrochloride buffer, and stored at −20°C.

Genomic DNA from all strains and from the skin biopsy sample was extracted with the QIAamp DNA minikit (Qiagen) in accordance with the manufacturer's instructions. The tissue fragments were incubated overnight at 56°C in lysis buffer, concentrated by evaporation, and analyzed by PCR. A nested PCR targeting the 5S (*rrf*)-23S (*rrl*) intergenic spacer and the gene (*ospA*) encoding the outer surface protein A of *B. burgdorferi* sensu lato was performed as described previously (4, 5, 11).

Intergenic spacer amplicons were digested with *Mse*I at 37°C for 2 h. Electrophoresis was carried out on a 16% acrylamide-bisacrylamide gel for 1 h at 120 V, the gels were stained with ethidium bromide, and the amplicons were visualized with UV transillumination.

The *ospA* gene and the intergenic spacer of PoHL1, the human isolate, were sequenced (3). In addition, spacer amplicons were subjected to DNA-DNA hybridization by the reverse-line blot assay (5, 11).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (8). The patient's antibodies were detected in a two-step approach: an in-house indirect immunofluorescence assay with a polyvalent conjugate (2), followed by an in-house Western blot (12). Immunoblots were calibrated with the following *B. burgdorferi* monoclonal antibodies: anti-OspA (O31a), anti-OspB (H6831), antiflagellin (H9724), anti-OspC (CB625), anti-p72 (CB312), and anti-Hsp60 (O62a).

Borrelia was successfully isolated from the skin lesion of the human patient. The sequence of the intergenic spacer sequence of the *B. lusitaniae* human isolate (strain PoHL1) is shown in Fig. 1.

DNA-DNA hybridization by the reverse line blot assay showed that only the DNA probe specific for *B. burgdorferi* sensu lato hybridized to the human skin isolate, since no DNA probe specific for *B. lusitaniae* was available. Phylogenetic analysis of the partial *ospA* sequence revealed that the patient isolate clustered with *B. lusitaniae* (Fig. 2). The human isolate had an *ospA* allele that was not identical to those of previously described tick isolates of *B. lusitaniae*. A nucleotide similarity

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TABLE 1. Strains and GenBank *rrf-rrl* nucleotide sequences used in this study

Species	Strain or isolate	Origin	*rrf-rrl* accession no.
B. afzelii	PGau[a]	Human, Germany	NA[b]
	PKo	Human, Germany	NA
B. burgdorferi sensu stricto	B31	*I. scapularis*, United States	L30127
	VS219	Human, France	AY032919
	IP3[a]	Human, France	NA
B. garinii	Pbi[a]	Human, Austria	Z77175
	PoTiBG4	*I. ricinus*, Portugal	AY463164
	PoTiBG20	*I. ricinus*, Portugal	AY463166
	PoTiBG86	*I. ricinus*, Portugal	AY463168
	PoTiBG163	*I. ricinus*, Portugal	AY463169
B. lusitaniae	PoHL1	Human, Portugal	AY209179
	PoTiBL37[c]	*I. ricinus*, Portugal	AY463167
B. valaisiana	PoTiBV6	*I. ricinus*, Portugal	AY463165
B. japonica	HO14	*I. ovatus*, Japan	L30125

[a] Strain cultured and used as a positive control.
[b] NA, not applicable.
[c] Sequence identical to that derived from GT058.

of 84.2% to alleles of tick isolates PotiB1, PotiB2, and PotiB3 was observed. At the *rrf-rrl* locus, the alleles of the human isolate and the *B. lusitaniae* tick isolate PoTiBL37 differed by only 1 nucleotide, leading to a sequence identity of 99.5%.

The PCR-restriction fragment length polymorphism profile resulting from the *Mse*I-digested 230-bp 5S-23S intergenic spacer showed four fragments (78, 69, 39, and 29 bp) for both the human and the tick *B. lusitaniae* isolates. The observed restriction patterns were indistinguishable from each other but different from those of the other members of the *B. burgdorferi* sensu lato complex (Fig. 3).

Analysis of the whole-cell lysate protein profile of the human *B. lusitaniae* isolate with monoclonal antibodies revealed the presence of OspA, OspB, OspC, flagellin, p72, and Hsp60 proteins. The patient's serum specimens were analyzed for antibodies reactive with *B. lusitaniae*, *B. garinii*, and *B. afzelii* by immunoblotting. No immunoglobulin M (IgM) response was detected. However, a weak IgG reaction with proteins p72 and Hsp60 and a protein with a molecular size of approximately 14 kb was observed with the three studied strains. The immunofluorescence assay only showed a slight reactivity (1:32) in the second serum sample.

The results of the molecular analyses of the patient isolate allows the assignment of this strain to *B. lusitaniae*, a genospecies previously considered to be nonpathogenic in humans. This finding supports our recent genotyping of *B. lusitaniae* from directly amplified spirochetal DNA from skin biopsy samples from Portuguese patients with suspected Lyme disease. Besides being prevalent in Tunisia, *B. lusitaniae* is considered to be predominant in the south of Portugal (Grândola region), where it is the unique strain isolated so far from *I. ricinus* ticks or detected by DNA amplification in hard-tick species other than *I. ricinus* (1, 3). This predominance of *B. lusitaniae* in southern habitats, as well as its first isolation in a patient living near Lisbon, suggests an important transmission risk for this genospecies in these areas.

In conclusion, for this human case, the described weak serological response, which is present in a high percentage of our patients with unspecific and long-lasting skin manifestations, suggests a clinical pattern for *B. lusitaniae* different from those

TACTTTGACCATATTTTTATCTTCCATCTCTACTTTGCCAA
TTTGTTTATGCAACATAGAATAATATATATCTTTGTTTAATC
CATGTCAATATCTATTTTATTTTTTATATTTTTTTAAATCAA
ACATTCAAAAAAATGAACATTTAAAAAACATAAAAAATAAA
TTCAAGATTTGAAGTATAAAATAAAAAACCCTGGCAATAA
CCTACTCTCCCGCGAACTCGCAGTACCATCAGCGAATAA
GAGCTTAACTTCTGTGTTCGGAATGTTAACAGGTGTTTCC
TCTTTTCTTTAACCACCAGGGTTTTTATAAGGAAGACAAAA
ATATGGCCAAAGATACGGGTAA

FIG. 1. Sequence identification of the *B. lusitaniae* human isolate (PoHL1). GenBank accession number, AY209179.

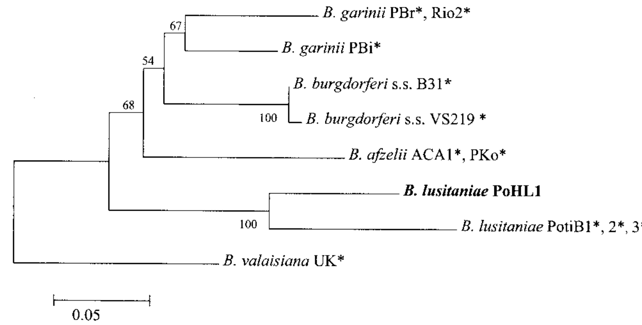

FIG. 2. Midpoint-rooted neighbor-joining tree of partial *ospA* sequences from the isolate obtained in this study (boldface) and GenBank downloads (*). The Kimura two-parameter model was used to estimate the pairwise distances of the aligned nucleotide sequences (MEGA program, version 2.1). Bootstrap values (500 replicates) are given next to the nodes. The scale indicates the estimated number of nucleotide substitutions per sequence position. GenBank accession numbers: A24014 (ACA1), X14407 (B31), X80257 (PBi), X80256 (PBr), A33374 (PKo), Y10837 (PotiB1), Y10838 (PotiB2), Y10839 (PotiB3), AF227319 (Rio2), AF095941 (UK), and AF369948 (VS219).

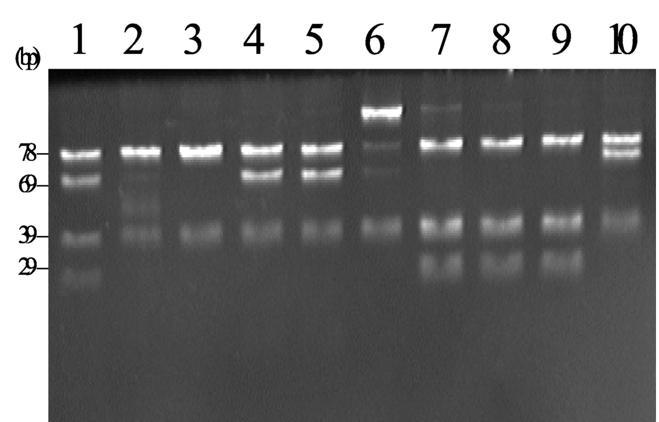

FIG. 3. *Mse*I restriction patterns of *B. burgdorferi* sensu lato *rrf-rrl* spacer nested-PCR products amplified from cultured strains. Lanes: 1, PoHL1, human isolate; 2, PoTiBG163, tick isolate, *B. garinii*; 3, PBi, *B. garinii*; 4 and 5, PKo and PGau, respectively, *B. afzelii*; 6, PoTiBV6, tick isolate, *B. valaisiana*; 7 to 9, B31, IP3, and Vs219, respectively, *B. burgdorferi* sensu stricto; 10, HO14, *B. japonica*.

for other *Borrelia* spp. in the Portuguese population examined so far.

Nucleotide sequence accession number. The intergenic spacer sequence of the *B. lusitaniae* human isolate (strain PoHL1) has been assigned GenBank accession number AY209179.

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