VECTOR-BORNE AND ZOONOTIC DISEASES
Volume 0, Number 00, 2009

DOI: 10.1089/vbz.2008.0210

SHORT COMMUNICATION

Molecular Characterization of a New Isolate of *Borrelia lusitaniae* Derived from *Apodemus sylvaticus* in Portugal

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Abstract

A total of 196 small mammals were collected in Portugal and tested for *Borrelia burgdorferi* sensu lato. Tissue samples were taken from each animal and cultured in Barbour-Stoenner-Kelly (BSK)-II medium. The single strain of spirochete isolated was confirmed as *Borrelia lusitaniae* by genetic analyses. This is the first report of *B. lusitaniae* isolated from *Apodemus sylvaticus*.

Key Words: *Apodemus sylvaticus*—*Borrelia lusitaniae*—Portugal—Reservoir.

Introduction

Lyme borreliosis is an emerging tick-borne disease with public health implications in countries such as Portugal (Lopes de Carvalho and Núncio 2006). The disease is caused by bacteria belonging to the *Borrelia burgdorferi* sensu lato (s.l.) complex, and is transmitted by *Ixodes* spp. ticks. *Borrelia lusitaniae* is the most prevalent *B. burgdorferi* s.l. species in Portugal (Baptista et al. 2004, Lopes de Carvalho et al. 2008b) and was first isolated from ticks collected in the southern regions of the country (Núncio et al. 1993). In 2004 and in 2008, two strains of *B. lusitaniae* were isolated from human patients, indicating its pathogenicity in humans (Collares-Pereira et al. 2004, Lopes de Carvalho et al. 2008a). Recent studies pointed out that regional strains of *B. lusitaniae* isolated in Portugal constitute genetically distinct populations (Vitorino et al. 2008). Other species of *B. burgdorferi* s.l., such as *B. garinii*, *B. afzelii*, *B. burgdorferi* s.s. and *B. valaisiana*, had already been detected in ticks in mainland Portugal as well as in Madeira Island (Baptista et al. 2004, Lopes de Carvalho et al. 2008b). To our knowledge, these other *Borrelia* genospecies have not been detected in human samples from Portugal.

Different *Borrelia* genospecies are associated with distinct ecologies and enzootic cycles, specific pathogenicity, and clinical symptomatology in patients. The list of reservoirs for *B. burgdorferi* s.l. in endemic areas of Europe is extensive. Some associations, however, between *Borrelia* genospecies and vertebrate hosts have been identified, such as *B. garinii* and *B. valaisiana* in birds, *B. afzelii* within small rodents, and *B. burgdorferi* s.s. in red squirrels and hedgehogs (Humair and Gern 1998); lizards may be the principal reservoir hosts of *B. lusitaniae* (Richter and Matuschka 2006, Amore et al. 2007). Although *B. lusitaniae* circulates between a range of ticks and host vertebrate species such as lizards, birds (Poupon et al. 2006), and small mammals, the vertebrate reservoir of *B. lusitaniae* had yet to be identified. In a study performed in the Grandola region, *B. lusitaniae* DNA was detected by polymerase chain reaction in all small mammal species captured (*Mus spretus*, *Apodemus sylvaticus*, *Rattus norvegicus*, and *Crocidura russula*), but it was not possible to isolate spirochetes. These observations indicate that there may exist a sylvatic cycle specific to this *Borrelia* genospecies, maintained by a variety of hosts and reservoirs (Baptista 2006). In Portugal, to address this question we initiated a study to determine whether small mammals can contribute to the maintenance of *B. lusitaniae* in natural foci in Portugal.

Materials and Methods

One hundred ninety-six small mammals (22 *A. sylvaticus*, 160 *M. spretus*, and 14 *Rattus rattus*) were captured using Sherman traps, between July 2002 and October 2004 in three different National Parks of Portugal: Vale Guadiana (W 8°7′19″, N 41°47′19″), Peneda Gerês (W 9°2′13″, N 38°27′28″), and Arrábida (W 7°39′50″, N 37°41′21″).

The rodents were brought to the laboratory and identified by external morphology and skull features. Animals were then anesthetized with ketamine hydrochloride (Imalgene; Merial,

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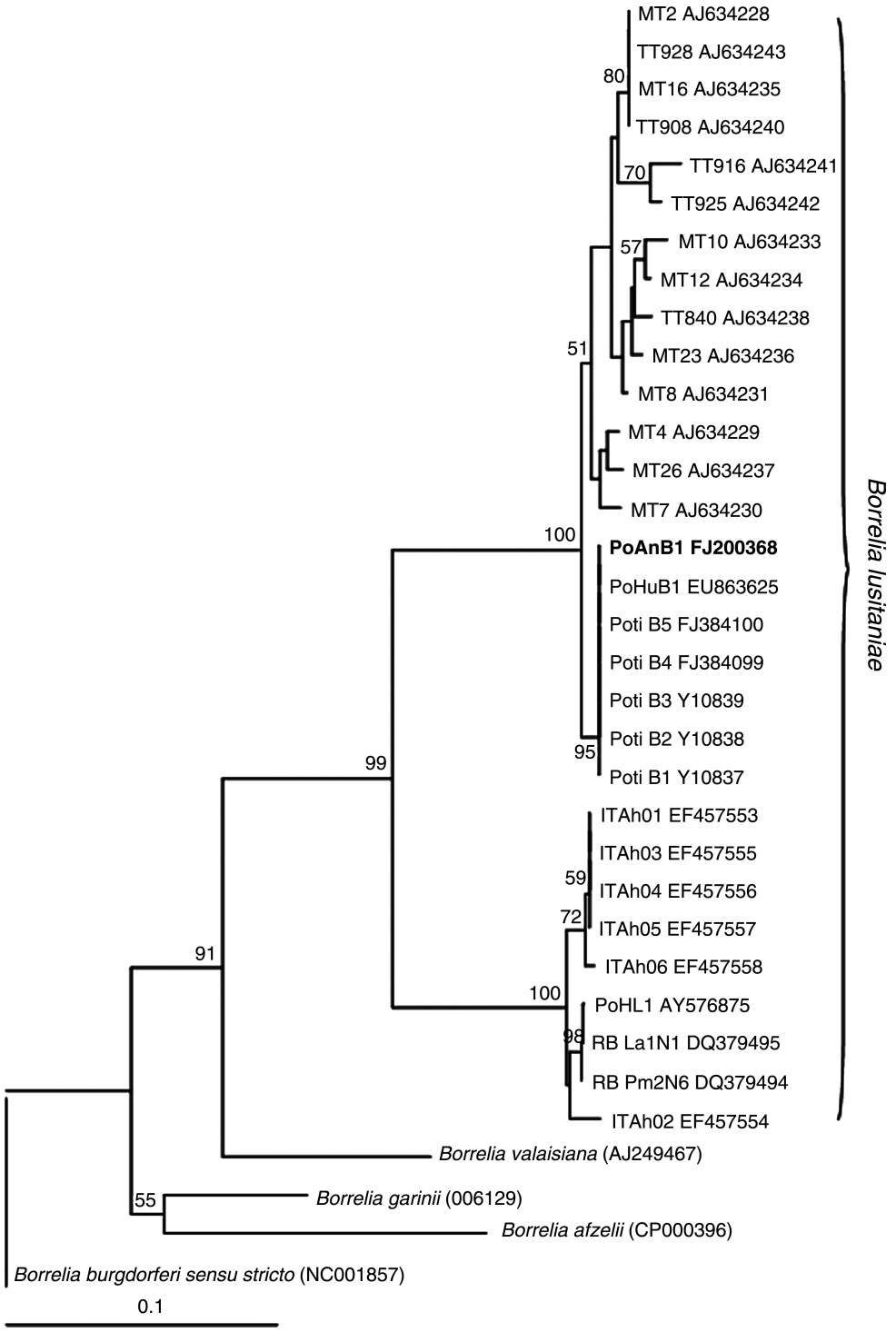

FIG. 1. Neighbor-joining tree inferred from partial *OspA* gene sequences from the isolate obtained in this study (bold) compared to GenBank sequence data. Distance matrices were calculated using the Kimura 2-parameter model to correct for multiple substitutions. Bootstrap values were obtained from 1000 replicate trees and are indicated at the nodes (>50%). Po, Portuguese strains; ITAh, Italian strains; MT, Morocco strains; TT, Tunisia strains; RB, German strains.

Lyon, France) at a dose of 10 mg/kg, bled by cardiac puncture for serodiagnostics, and euthanized by exposure to CO_2 gas. Serum samples were then tested for the presence of immunoglobulin G antibodies against *Borrelia* by indirect immunofluorescence assay (Doby et al. 1991), using a strain of *B. garinii* as antigen. Positive serology indicated by a titer of 32.

Target organs (skin, bladder, and heart) were aseptically harvested for culture.

The organs were first disinfected by successive immersion in iodine, 70% ethanol, and phosphate-buffered saline; tissues were then minced and placed directly into 8 mL of Barbour-Stoenner-Kelly (BSK)-II medium. All cultures were main-

tained at 34°C for 3 months and examined weekly by dark-field microscopy to monitor the presence of live spirochetes. Heart tissue from a single *A. sylvaticus* mouse captured in Vale Guadiana demonstrated viable spirochetes 8 weeks after culture in BSK-II medium; 1 mL of culture was centrifuged for 5 min at 7000 *g*, and the sediment washed twice with sterile phosphate-buffered saline and frozen at −20°C until DNA extraction. Total DNA was extracted using the QIAamp tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Three genes, *flaB* (9), *rrf (5S)-rrl* and *(23S)* intergenic spacer region of *B. burgdorferi* s.l. (Johnson et al. 1992, Rijpkema et al. 1995), and the outer surface protein A (*OspA*) gene, were used to characterize this isolate (Lopes de Carvalho et al. 2008a). The sequences were assembled by combining the sequences generated by each primer, using the BioEdit software. For phylogenetic inference the alignments were made using amino acid sequences and converted to DNA sequences using BioEdit software. All alignments were made using ClustalX program (Thompson et al. 1997) and manually inspected for misalignments. Primer sequences except for the last two (5′) to six (3′) nucleotides were removed from the alignment before phylogenetic analyses, regarding the polymorphisms observed in the remaining sequences. Neighbor-joining tree of DNA sequence alignment was conducted in PAUP* 4.0b10 software. Distance matrices were calculated using the Kimura two-parameter model to correct for multiple substitutions. Bootstrap analysis was obtained with 1000 replicates.

Results and Discussion

Of the 169 small mammals captured, 17 (10.6%) *M. spretus* and 1 (5.6%) *R. rattus* showed antibodies to *B. lusitaniae*; however, no positive cultures were obtained from these host species. Despite isolation of spirochetes from one *A. sylvaticus* captured in the Vale Guadiana National Park, no seropositive samples were obtained from this species. The *flaB* gene, the *rrf* (5S)–*rrl* (23S) intergenic spacer region of *B. burgdorferi* s.l., and the *OspA* gene were amplified from DNA extracted from the *A. sylvaticus* isolate, and further analysis of this isolate identified it as *B. lusitaniae*. Phylogenetic analyses based on the *OspA* gene grouped this new isolate near other *B. lusitaniae* isolated in Portugal and most closely aligned to the North African clade (Grego et al. 2007) (Fig. 1). OspA clustering via the Neighbor-joining algorithm was confirmed by Maximum Likelihood (ML) and MrBayes analysis (data not shown). Intergenic Spacer (IGS) sequences also confirmed the PoAnB1 strain as *B. lusitaniae* as the mean intra–*B. lusitaniae* group sequence identities vary between 95.8% and 91.6% and are below 90% with other *Borrelia* genospecies (89.8% with *B. garinii*, 86.9% with *B. afzelii*, 86.5% with *B. valaisiana*, and 85.1% with *B. burgdorferi* s.s.). The eco-epidemiological studies performed to date demonstrate that *B. lusitaniae* presents different characteristics to existing *B. burgdorferi* genospecies in other European countries (Zeidner et al. 2002, Grego et al. 2007). Previous serology results (Núncio 2002) as well as the DNA detection (Baptista 2006) and the isolation and molecular identification of *B. lusitaniae* indicate that small mammals, particularly, *A. sylvaticus*, may play a role in the maintenance of *B. lusitaniae* in Portugal. To our knowledge, this is the first report of live *B. lusitaniae* spirochetes isolated from *A. sylvaticus*, an indication that this mammal is a competent reservoir of *B. lusitaniae*.

Previous studies have demonstrated that *B. lusitaniae* is not as immunogenic as *B. burgdorferi* s.s. (Zeidner et al. 2002). Even in human cases, isolates derived by culture were achieved in a seronegative and in a borderline seropositive patient (Collares-Pereira et al. 2004, Lopes de Carvalho et al. 2008a). Thus, the low titer (Ig = 16) antibody detected in the *A. sylvaticus* mouse from which the borrelia was isolated seems to confirm a pattern regarding *B. lusitaniae*. Future studies, using an autochthonous borrelia strain as antigen, should improve the sensitivity of our serological testing.

Despite evidence in several other studies indicating lizards as main reservoir host of *B. lusitanie* in some geographical areas (Richter and Matuschka 2006, Amore et al. 2007), our findings demonstrate that more attention be given to small mammals as potential reservoirs. In addition, parallel studies in birds and lizards are currently ongoing to gain a more detailed assessment of both the ecology and pathogenicity of borrelia strains circulating in Portugal.

Nucleotide sequence accession number: The *rrf* (5S)-*rrl* (23S) intergenic spacer region of *B. burgdorferi* s.l., *flaB*, and *OspA* gene from PoAnB1 have been deposited in GenBank with accession numbers EU647595, EU122385, and FJ200368, respectively.

Acknowledgments

The authors wish to thank Mary Crabtree, Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, CO, for the help with the phylogenetic analysis. Teresa Luz, Centro de Estudos de Vectores e Doenças Infecciosas, is also acknowledged for her expert technical assistance. This study was partially supported by FLAD with a fellowship and FCT project (POCTI/ESP/39549/2001).

Disclosure Statement

No competing financial interests exist.

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