VECTOR-BORNE AND ZOONOTIC DISEASES
Volume 8, Number 4, 2008

DOI: 10.1089/vbz.2007.0245

Detection of *Borrelia lusitaniae*, *Rickettsia* sp. IRS3, *Rickettsia monacensis*, and *Anaplasma phagocytophilum* in *Ixodes ricinus* Collected in Madeira Island, Portugal

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ABSTRACT

A total of 300 *Ixodes ricinus* ticks were tested by polymerase chain reaction (PCR) for the presence of *Borrelia* spp., *Rickettsia* spp., and *Anaplasma phagocytophilum*. Sequence analysis demonstrated 8 (2.7%) ticks infected with *B. lusitaniae*, 60 (20%) with *Rickettsia* spp., and 1 (0.3%) with *A. phagocytophilum*. Seven (2.3%) ticks were coinfected with *B. lusitaniae* and *Rickettsia* spp., 2 (0.6%) with *R. monacensis*, and 5 (1.7%) with *Rickettsia* sp. IRS3. The results of this study suggest simultaneous transmission of multiple tick-borne agents on Madeira Island, Portugal.
Key Words: Madeira—*Ixodes ricinus*—*B. lusitaniae*—*Rickettsia*—*Anaplasma phagocytophilum*.

INTRODUCTION

MADEIRA, THE MAIN ISLAND of the Madeira archipelago, Portugal, is located in the north Atlantic Ocean about 1000 km from the European Coast and 800 km west of Africa. Climatic conditions make this island an ideal setting for *Ixodes ricinus* ticks, the most widely distributed tick species, colonizing various habitats and parasitizing several vertebrate hosts. Human parasitism by this species is a common occurrence in Portugal. In other parts of Europe, *I. ricinus* ticks play an important role in the transmission of *Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum*, and *Rickettsia* spp. to domestic animals and humans. In Portugal, *B. lusitaniae* is the most prevalent *Borrelia* spp. and was first isolated from ticks in southern Portugal (Núncio et al. 1993). In 2004, *B. lusitaniae* was isolated from a human patient presenting with erythemic skin lesions, indicating its pathogenicity in humans (Collares-Pereira et al. 2004). Subsequent reports from mainland Portugal indicate an incidence rate for Lyme borreliosis of 0.04/100,000 inhabitants. To date, only 2 clinical cases have been confirmed by laboratory testing on Madeira Island (Lopes de Carvalho and Núncio 2006).

Multiple *Borrelia* spp., including *B. afzelii*, *B. valaisiana*, and *B. burgdorferi* sensu stricto, have been detected in *I. ricinus* ticks collected on Madeira Island (Matuschka et al. 1998, Núncio et al. 2001). *A. phagocytophilum* has also been detected in 4% of actively questing *I. ricinus* collected from vegetation on this island (Santos et al. 2004). To our knowledge, no studies of *Rick-*

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TABLE 1. TICKS DETECTED WITH *B. LUSITANIAE*, *RICKETTSIA* SP. IRS3, *R. MONAENSIS*, AND *A. PHAGOCYTOPHILUM*

Agents found in ticks	*Positive ticks (%)*	*Stage/gender*[a]	*Studied genes*	*Accession no.*
B. lusitaniae (PoTiB6)	8 (2.7)	2F; 6M	*fla; ITS*	EF501757; EU078961
Rickettsia sp. IRS3 (PoTiR5dt)	21 (7)	7F; 13M; 1N	*opmA; gltA*	EF501755; EU078962
R. monaensis (PoTiR6dt)	39 (13)	12F; 16M; 11N	*opmA; gltA*	EF501756; EU078963
A. phagocytophilum (PoTiA1dt)	1 (0.3)	1N	*groESL*	EU004826[b]

[a]F, female; M, male; N, nymph.
[b]Accession number of partial sequence of *groESL* from Madeira Island *A. phagoytophilum* prototype, previously described in a work now in submission (Santos et al. in submission).

ettsia spp. detection in ticks have been undertaken in Madeira.

The aim of this study was to assess the potential acquisition of tick-borne infections on Madeira Island. For this purpose, we determined the prevalence of tick-borne agents and also determined coinfection rates of *Borrelia* spp. with other agents, namely, *A. phagocytophilum* and *Rickettsia* spp.

MATERIALS AND METHODS

A total of 300 *I. ricinus* (70 females, 68 males, and 162 nymphs), identified by one of the authors (M.S.N.), were randomly selected from an archival sample of ticks collected from 4 communities of Madeira, namely, Funchal, Calheta, Caniçal, and Porto Moniz, by flagging the vegetation. The number of ticks used in this study

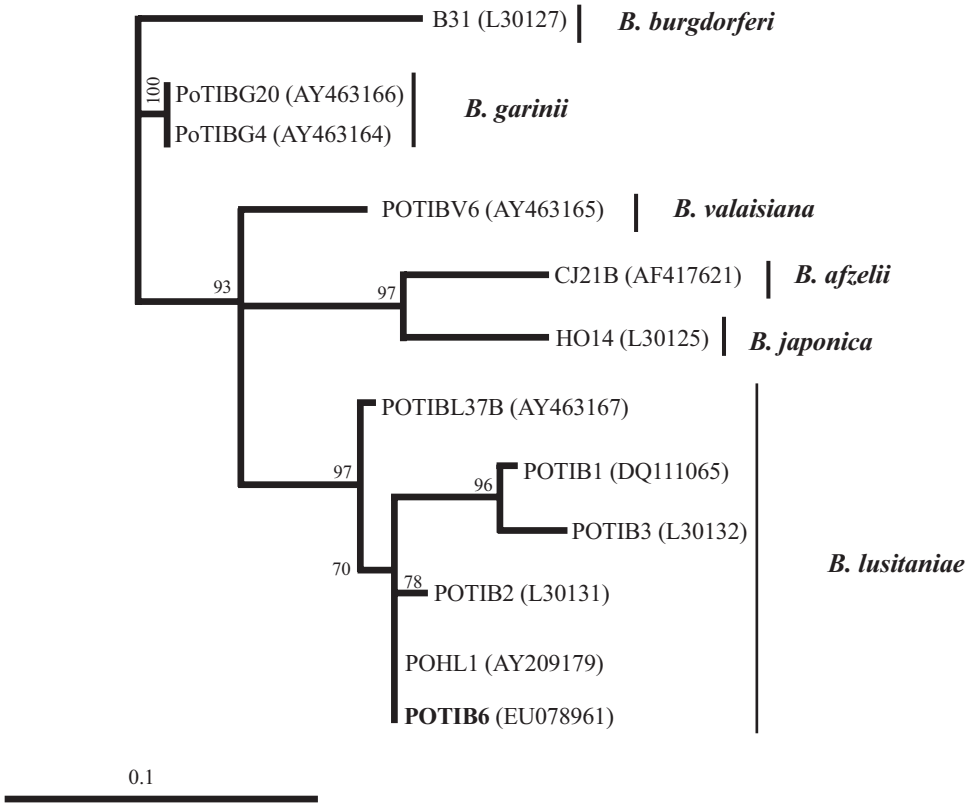

FIG. 1. Phylogenetic analysis based on the *rrf-rrl* gene nucleotide sequences. Maximum likelihood tree was performed by the PUZZLE program, with the HKY-85 model generated using a transition/transversion of 2.25, a nucleotide frequency of A = 0.403; C = 0.051; G = 0.13; T = 0.415. Log likelihood = −614.18. Branch lengths represent genetic distances between sequences. The branch values represent the support based on quartet puzzling steps of 1000 replicates. Only a single representative of 100% identical isolates obtained in this study was included in the tree (boldface).

was based on statistical analysis of the prevalence of tick infection with *Borrelia* spp. found previously (Núncio et al. 2001). DNA was extracted from ticks as described (Schouls et al. 1999). Polymerase chain reaction (PCR) assays were performed targeting 2 *B. burgdorferi* sensu lato genes: *fla* (Johnson et al. 1992), using outer 1 and 2 primers for the first reaction and inner 1 and 2 for the nested reaction, and the intergenic spacer region (*rrf-rrl*) (Rijpkema et al. 1995), using primer pairs 23SN1/23SC1 and 5SCB/23SN2 for the nested reaction. *Rickettsia* DNA was detected by amplification of citrate synthase gene *gltA* using RpCs415/RpCs1220 primers (Sousa et al. 2006), and the outer membrane protein A gene *ompA* with the primer pair Rr190.70p/Rr190.602n (Regnery et al. 1991). A single target gene of *A. phagocytophilum* was amplified, the heat shock operon (*groESL*) (Sumner et al. 1997), in a nested PCR, using primer pairs HS1/HS6 and HS43/HS45, respectively (Santos et al. unpublished data).

The resulting amplicons were sequenced and compared with published sequences of representative *A. phagocytophilum*, *Borrelia*, and *Rickettsia* species. Multiple alignments of the nucleotide sequences were generated by the ClustalW program, version 1.6 (Thompson et al. 1994), and phylogenetic analysis was carried out by maximum likelihood analysis in the TREE-PUZZLE program, version 5.1 (Strimmer and von Haeseler 1997), using a quartet puzzling algorithm to generate the tree. The analysis was run with the Hasegawa-Kishino-Yano (HKY-85) model of substitution (Hasegawa et al. 1985), and quartet puzzling support values based on 1000 puzzling steps were calculated.

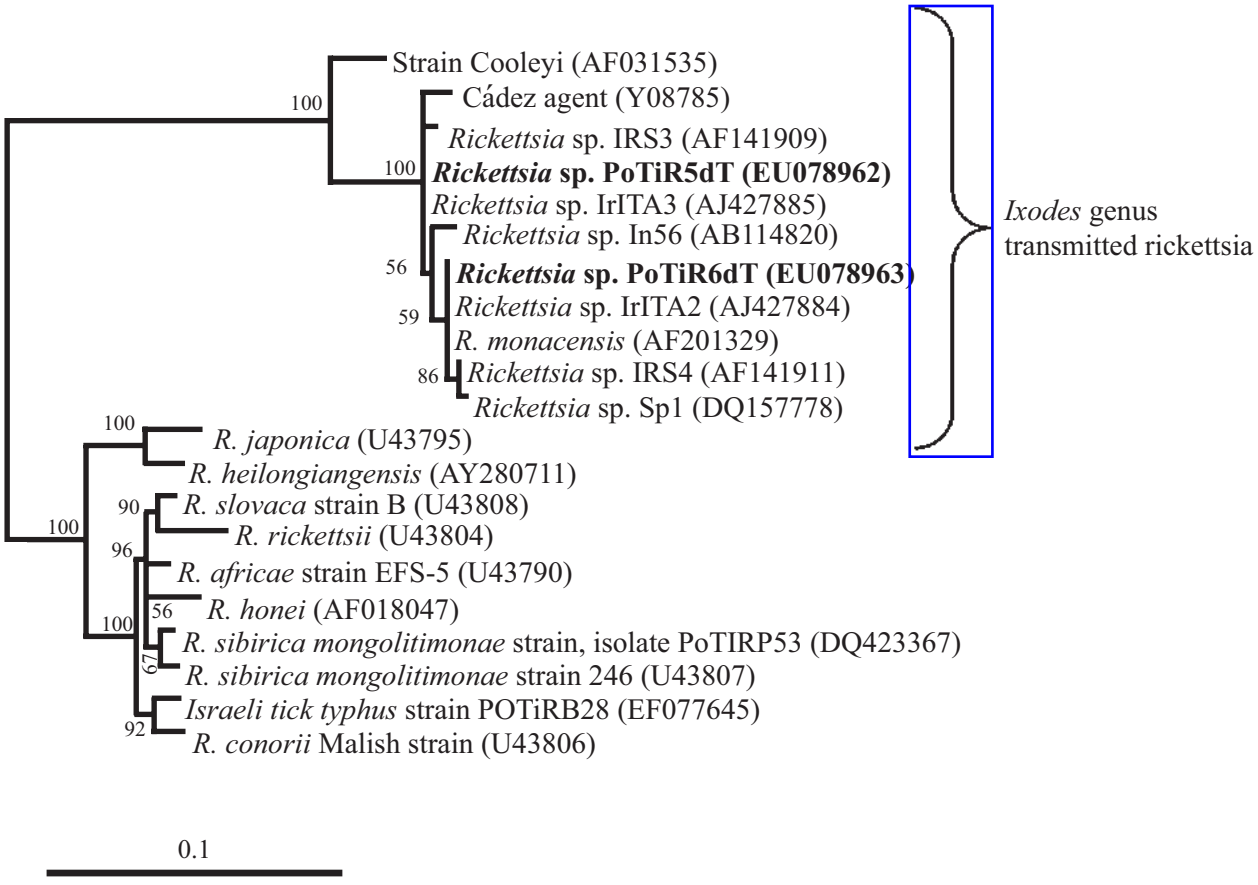

FIG. 2. Phylogenetic analysis based on the *ompA* gene nucleotide sequences. Maximum likelihood tree was performed by the PUZZLE program, with the HKY-85 model generated using a transition/transversion of 1.75, a nucleotide frequency of A = 0.271; C = 0.182; G = 0.228; T = 0.32. Log likelihood = −17914.85. Branch lengths represent genetic distances between sequences. The branch values represent the support based on quartet puzzling steps of 1000 replicates. Only a single representative of 100% identical isolates obtained in this study was included in the tree (boldface).

RESULTS AND DISCUSSION

In this study, *B. lusitaniae*, *Rickettsia* sp. IRS3, *R. monacensis*, and *A. phagocytophilum* were detected in *I. ricinus* collected from Madeira Island. The percentage of positives for each agent is shown in Table 1. Strains PoTiB6, PoTiR5dt, and PoTiR6dt are designations for *B. lusitaniae*, *Rickettsia* sp. IRS3, and *R. monacensis,* respectively.

Seven (2.3%) ticks infected with *B. lusitaniae* were also infected with *Rickettsia* spp., 2 (0.6%) with *R. monacensis*, and 5 (1.7%) with *Rickettsia* sp. IRS3. One (0.3%) tick was infected with *A. phagocytophilum*. Sequence analysis indicated 100% similarity to the *groESL* partial sequence, previously described in ticks from Madeira. The phylogenetic analysis of the intergenic spacer region (*rrf-rrl*) gene of *Borrelia* shows PoTiB6 clustered with the other *B. lusitaniae* strains isolated in Portugal from ticks, such as PoTiB2, and humans, PoHL1, with a nucleotide sequence identity of 99% (Fig. 1).

Concerning the phylogenetic analysis of the *Rickettsia ompA* gene, PoTiR5dt clustered with *Rickettsia* sp. IRS3 and *Rickettsia* sp. IrITA3, and PoTiR6dt clustered with *R. monacensis* and *Rickettsia* sp. IrITA2, both with a nucleotide sequence identity of 100% (Fig. 2).

The overall tree topology for *Borrelia* and *Rickettsia* was identical when phylogenetic analyses were performed for the *fla* and *gltA* genes, respectively (data not shown).

The prevalence of *Borrelia* spp. in *I. ricinus* found in this report, 2.7%, was lower compared with the 31.2% rate demonstrated in a previous study in Madeira (Núncio et al. 2001). This might be due to a difference in the sensitivity of assays used in these studies. However, the rate is similar to the prevalence found in a previous study (Matuschka et al. 1998), even though the authors detected *B. afzelii*, *B. garinii*, and *B. burgdorferi* sensu stricto, but not *B. lusitaniae*, as was the case in the study by Núncio et al. (2001).

A. phagocytophilum was also detected in fewer ticks (0.3%) compared with an earlier study performed on Madeira Island (4%) (Santos et al. 2004). This may be attributed to seasonal and geographical changes in this agent's prevalence or to differences in the detection methods used. Furthermore, some archival samples may have been degraded, resulting in false negative amplification.

An outbreak of murine typhus, caused by *R. typhi*, in Porto Santo in 1996 alerted clinicians to the occurrence of this disease in the Madeira archipelago. This points to the possibility of other pathogenic Rickettsiae circulating in Madeira Island. The detection of *Rickettsia* sp. IRS3 and *R. monacensis* in the current study, the latter species having recently been shown to be pathogenic in Spain (Jado et al. 2007), should therefore make competent authorities vigilant toward these potentially pathogenic agents.

Our report is the first documentation of *Rickettsia* sp. IRS3 and *R. monacensis* in *I. ricinus* ticks and their coinfection with *B. lusitaniae*. These findings raise a few questions concerning whether these agents' life cycle is altered by their coexistence in the same vector, for which further studies would be needed, to determine if this would result in coinfection with less typical Lyme borreliosis clinical presentation or disease severity.

As tourism becomes a larger portion of the economy of this island, other studies should be undertaken, looking at a broad range of tick species and agents, as well as specimens from multiple municipalities. In this way, clinical awareness will be enhanced and prevention methods can be readily established.

ACKNOWLEDGMENTS

The authors thank Dr. Nordin Zeidner, Centers for Disease Control and Prevention, for invaluable comments and revision of the manuscript, and Dr. Paulo Nogueira, National Institute of Health, for his help in the calculation of the number of samples used for this study.

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