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First isolation of human *Leptospira* strains, Azores, Portugal

Ana T. Gonçalves [a], Clara Paiva [b], Francisco Melo-Mota [c], Maria Luísa Vieira [a,g], Teresa Carreira [a,g], Mónica S. Nunes [a,g], Luísa Mota-Vieira [d], Ahmed Ahmed [e], Rudy A. Harstkeerl [e], Karyne Hyde [f], Margarida Collares-Pereira [a,g,*]

[a] *Unidade de Leptospirose e Borreliose de Lyme, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Rua da Junqueira 100, 1349-008 Lisboa, Portugal*
[b] *Serviço de Medicina Interna, Hospital do Divino Espírito Santo de Ponta Delgada (HDESPD), São Miguel, Açores, Portugal*
[c] *Serviço de Doenças Infecciosas, Hospital do Divino Espírito Santo de Ponta Delgada (HDESPD), São Miguel, Açores, Portugal*
[d] *Unidade de Genética e Patologia Moleculares, Hospital do Divino Espírito Santo de Ponta Delgada (HDESPD), São Miguel, Açores, Portugal*
[e] *Leptospira Reference Lab, Royal Tropical Institute (KIT), Amsterdam, the Netherlands*
[f] *Sector de Serologia do Serviço de Patologia Clínica, Hospital do Divino Espírito Santo de Ponta Delgada (HDESPD), São Miguel, Açores, Portugal*
[g] *Centro de Recursos Microbiológicos (CREM), Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Caparica, Portugal*

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SUMMARY

Objectives: The aim of this study was the first identification of *Leptospira* isolates from Azorean inpatients.
Methods: Whole blood samples from 68 inpatients attending the São Miguel Hospital between 2006 and 2008, with a clinical and epidemiological suspicion of leptospirosis, were inoculated in a transport medium broth at the patient's bedside and further processed using a serial dilution technique prior to culture. At admission, 62 (91%) patients were also analyzed for the presence of leptospiral DNA by a nested PCR and 40 (59%) for specific agglutinins by microscopic agglutination test (MAT). The isolates obtained were first assigned at the serogroup level by both MAT reactivity with hyperimmune rabbit antisera and a PCR-based assay with the single primer iRep1. The species identification was performed by DNA sequencing. The use of monoclonal antibodies allowed intraspecific discrimination at the serovar level.
Results: Of the 10 (14.7%) human *Leptospira* isolates, seven were identified as *Leptospira interrogans* serovar Copenhageni and three as *Leptospira borgpetersenii* serovar Arborea, which is in agreement with previous data from the Azorean rodent population.
Conclusions: This study represents a great step towards the definitive identification of the pathogenic leptospires in Azorean patients and confirms the bacteriological human–rodent connection for the first time.

Introduction

Leptospirosis is a bacterial zoonosis that affects human populations worldwide, mainly in tropical and temperate areas.[1] The Azores, a group of nine inhabited Portuguese islands in the North Atlantic Ocean, have been known for the endemicity of leptospirosis since 1993; it was recognized as a major health problem soon thereafter.[2] Human infection usually occurs after accidental contact with the urine of infected animals. Symptoms range from mild and flu-like manifestations to severe, potentially fatal septicemic complications.[3]

In recent years, the increasing number of fatal human cases in São Miguel and Terceira islands[4] has led to growing concern about the risk factors and potential infection sources,[5–7] reinforcing the need for bacteriological identification of the human infective leptospires through culture. This method, although with a prolonged incubation time and low diagnostic sensitivity in the clinical setting, has an important role in the study of outbreaks and global epidemiology, and provides a crucial pool of clinical strains for studies of pathogenesis. So far, human infections in the Azores have only been confirmed by the standard microscopic agglutination test (MAT), which only enables the presumptive serologic identification of the infective pathogen.[3]

The traditional identification of *Leptospira* isolates at both the serogroup and serovar levels uses group-specific rabbit antisera and cross-agglutinin absorption with homologous antigens, respectively.[8] However, this approach is very complicated and restricts its use to only a very few specialized laboratories. The method has been improved on with the introduction of panels of monoclonal antibodies (mAbs), which allow a more rapid serotyping.[7,9,10] In recent years, the identification of leptospiral isolates has also become faster with molecular techniques, which

* Corresponding author. Tel.: +351 21 3652600; fax: +351 21 3632105.
E-mail address: mcp@ihmt.unl.pt (M. Collares-Pereira).

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provide a complementary approach to the conventional antigenic identification.[11–15] Besides DNA sequencing, the use of PCR-based assays with single primers, namely iRep1, which hybridize with repetitive DNA elements within the *Leptospira* genome,[12,15] have demonstrated a great utility for rapid typing at the serogroup level.

The present work characterizes the first clinical Azorean *Leptospira* isolates obtained from patients admitted to the Hospital of Ponta Delgada (HDESPD), on São Miguel Island, and describes the results achieved by phenotypic and molecular techniques.

Materials and methods

Blood samples and reference/field strains: Leptospira detection

Between February 2006 and December 2008, blood samples from 68 inpatients attending the São Miguel Hospital with a clinical diagnosis of acute febrile illness and an epidemiology compatible with leptospirosis, were collected at the patient bedside, before antibiotic treatment. Whole blood (10% v/v) was immediately inoculated in a transport medium broth and processed as soon as possible, using a serial dilution technique in liquid EMJH (Difco, Detroit, MI, USA) medium prior to culture into semisolid EMJH, as previously described.[2] Briefly, two 10-fold serial dilutions of the inoculated transport medium were prepared in liquid EMJH. Eight and 15 drops of the highest dilution were inoculated into each of two 5-ml tubes of 0.1% semisolid EMJH supplemented with 200 μg/ml of 5-fluorouracil and 5% rabbit serum. Blood cultures were sent to the Instituto de Higiene e Medicina Tropical (Lisboa), where they were incubated at 30 °C for up to 20 weeks and checked for the presence of live leptospires.[3] Reference *Leptospira* strains representing six pathogenic serogroups and rodent field isolates from São Miguel ($n = 21$) and Terceira ($n = 19$)[16] were also analyzed to make fingerprint comparisons and phylogenetic analyses. In addition, 62 (91%) inpatients were examined at admission for the presence of *Leptospira* DNA in serum and urine samples through a nested-PCR, with minor modifications.[17] Briefly, the second primer set internal to primers A and B of the *rrs* (16SrRNA) gene were synthesized with the following sequences: A, nest 5′-TGCAAGTCAAGCGGAGTAGC-3′ and B, nest 5′-TTCTTAACTGCTGCCTCCCG-3′, resulting in a 292-bp fragment. The detection of anti-*Leptospira* MAT antibodies in sera from 40 (59%) inpatients was also carried out at admission.[5]

Isolates: phenotypic and molecular evaluations

Firstly, to differentiate *Leptospira interrogans* sensu lato from *Leptospira biflexa* sensu lato, isolate growth rates at both 13 °C and 30 °C, in the presence or absence of 8-azaguanine (225 μg/ml) and with or without NaCl (1 M), were evaluated.[18–20] In these studies, a pathogenic (*L. interrogans*, M20), a saprophytic (*L. biflexa*, Patoc 1), and an intermediate (*Leptospira inadai*, Portuguese human isolate) strain were included as internal controls.

The phenotypic identification of isolates was based on MAT reactivity with hyperimmune rabbit antisera (at the serogroup level), and panels of monoclonal antibodies (mAbs) (at the serovar level)[3] according to the most reactive presumptive serogroups known in the Azores population (Icterohaemorrhagiae, Ballum, and Sejroe).[4]

In the molecular approach, the genomic DNA of isolated strains and reference spirochetes was firstly extracted with a commercial kit (Puregene -Gentra®, QIAGEN Inc.Valencia, CA, USA). To differentiate *Leptospira* spp, a PCR-based assay was performed using the single primer iRep1 (5′GCG GAC TCA TAC CCG CT3′) according to Barochii et al.[12] The PCR mix included 1–2 μg of DNA from each strain, 250 pmol of primer, 5 mM $MgCl_2$, 1× PCR buffer, 250 μM of each deoxynucleotide triphosphate, and 1 U of Taq polymerase (Bioline, London, UK) in a final volume of 50 μl. After an initial denaturation at 94 °C for 5 min, the amplification program consisted of 35 cycles at 94 °C for 30 s, 50 °C for 90 s, and 72 °C for 4 min, with a final extension of 7 min at 72 °C. Amplified fragments were visually inspected on 2% agarose gel (Bioline, London, UK) stained with ethidium bromide (0.5 μg/ml; Bio-Rad, Hercules, CA, USA). Negative and positive controls were used in each PCR reaction. Primer specificity was tested by employing 1–2 μg of DNA (Puregene-Gentra®, QIAGEN Inc.Valencia, CA, USA DNA purification kit) from the related spirochete *Borrelia burgdorferi* sensu stricto. Blank samples lacking DNA were used as negative controls.

For speciation of the isolates, from both patients and rodents of São Miguel and Terceira,[16] the 245-bp fragment of the *secY* gene flanked by primers G1 and G2 was amplified and subsequently sequenced at the Royal Tropical Institute, Amsterdam, as described by Victoria et al.[21] DNA sequence clustal alignments were done using Vector NTI 10 software (Invitrogen, Carlsbad, CA, USA). Phylogenetic analysis was conducted using MEGA4.[22] One thousand bootstrap replications were used to provide confidence

Table 1
Agglutination titers (reciprocals) of monoclonal antibodies from serogroup Icterohaemorrhagiae with reference serovars/strains and Azorean human *Leptospira* isolates

Icterohaemorrhagiae		Reference serovars/strains		Portuguese patients	
mAb	Code	Icterohaemorrhagiae/RGA	Copenhageni/Dutch patient[a]	AzHuL01	AzHuL02
1	F12C3-11	10240	20480	20480	10240
2	F20C3-1	10240	10240	5120	5120
3	F20C4-1	20480	20480	20480	20480
4	F52C1-4	1280	80	80	80
5	F52C2-2	1280	10	10	10
6	F70C4-5	2560	1280	320	320
7	F70C7-11	20480	160	320	80
8	F70C13-5	160	640	640	1280
9	F70C14-10	20480	10	10	10
10	F70C20-3	10240	5120	5120	5120
11	F70C24-20	160	2560	1280	1280
12	F70C26-3	160	1280	1280	640
13	F82C1-3	10	10	10	10
14	F82C2-3	10	10	10	10
15	F82C7-3	10	10	10	10
16	F82C8-4	10	10	10	10
17	F89C3-3	10	10	10	10
18	F89C12-4	10	640	160	320

mAb, monoclonal antibody.
[a] Typed at KIT (Amsterdam) as serovar Copenhageni and confirmed by cross-agglutinin absorption test (CAAT).

Table 2
Agglutination titers (reciprocals) of monoclonal antibodies from serogroup Ballum with reference serovars/strains and Azorean human *Leptospira* isolates

Ballum		Reference serovars/strains		Portuguese patients		
mAb	Code	Arborea/Arborea	Ballum/M127	AzHuL04	AzHuL06	AzHuL09
1	F74C1	1280	640	640	640	2560
2	F74C4	10	5120	10	10	10
3	F74C7	320	640	640	640	1280

mAb, monoclonal antibody.

in the nodes. The tree was constructed by the neighbor-joining method using the Jukes–Cantor model.[22]

Results

Ten (14.7%) out of 68 examined patients with a clinical and epidemiological suspicion of leptospirosis showed a positive culture for *Leptospira* spp in the selective EMJH medium. Clinical isolates were obtained as follows: three in 2006 (AzHuL01 to AzHuL03), six in 2007 (AzHuL04 to AzHuL09), and one in 2008 (AzHuL10). The age of the culture-positive patients, all males, ranged from 19 to 56 years (mean 32 years, standard deviation (SD) ±11). Six out of the 10 patients had an occupation that involved contact with infected sources (dairy farmer, gardener, and mason). Their major clinical manifestations at admission (mean of 5 days after the onset of symptoms, SD ±2) included fever, chills, headache, and myalgia. Jaundice was only present in one case with acute renal failure (AzHuL05), and pneumonia was also diagnosed with anicteric leptospirosis (AzHuL02). The mean number of days after which cultures were scored positive was 33 (range 15–76, SD ±19). The presence of *Leptospira* DNA was confirmed in 19 (31%) out of 62 patients by blood and/or urine analysis. Patients with a positive culture who were studied by PCR (all but AzHuL01, not examined) showed DNA amplification in both the blood and urine samples collected at admission. In addition, five culture-positive patients also had a positive MAT serology at admission, whilst three showed an inconclusive titer and two were negative in the first serum sample.

All isolates showed the typical morphology and characteristic motility of the genus *Leptospira* under dark-field microscopy. The absence of leptospire growth at the differential temperature of 13 °C and in the presence of 8-azaguanine, as well as the morphologic change with NaCl (1 M), suggested the pathogenic status of all isolates and their assignment as *L. interrogans* sensu lato strains.[18–20] After a first typing of isolates according to serogroup affinities, Icterohaemorrhagiae (70%) and Ballum (30%), panels of respectively 18 and three specific monoclonal antibodies for each group were used (Tables 1 and 2). Agglutination profiles of all isolates within the serogroup Icterohaemorrhagiae were similar and consistent with that of serovar Copenhageni. The three Ballum profiles were most similar to the profile of serovar Arborea. Tables 1 and 2 show representative agglutination titers for isolates of both serogroups.

When analyzing the isolates and the reference strains through the iRep1-PCR fingerprints (Figure 1 and data not shown), it was possible to discriminate the same serological serogroups, i.e., the seven Icterohaemorrhagiae and the three Ballum isolates shared a

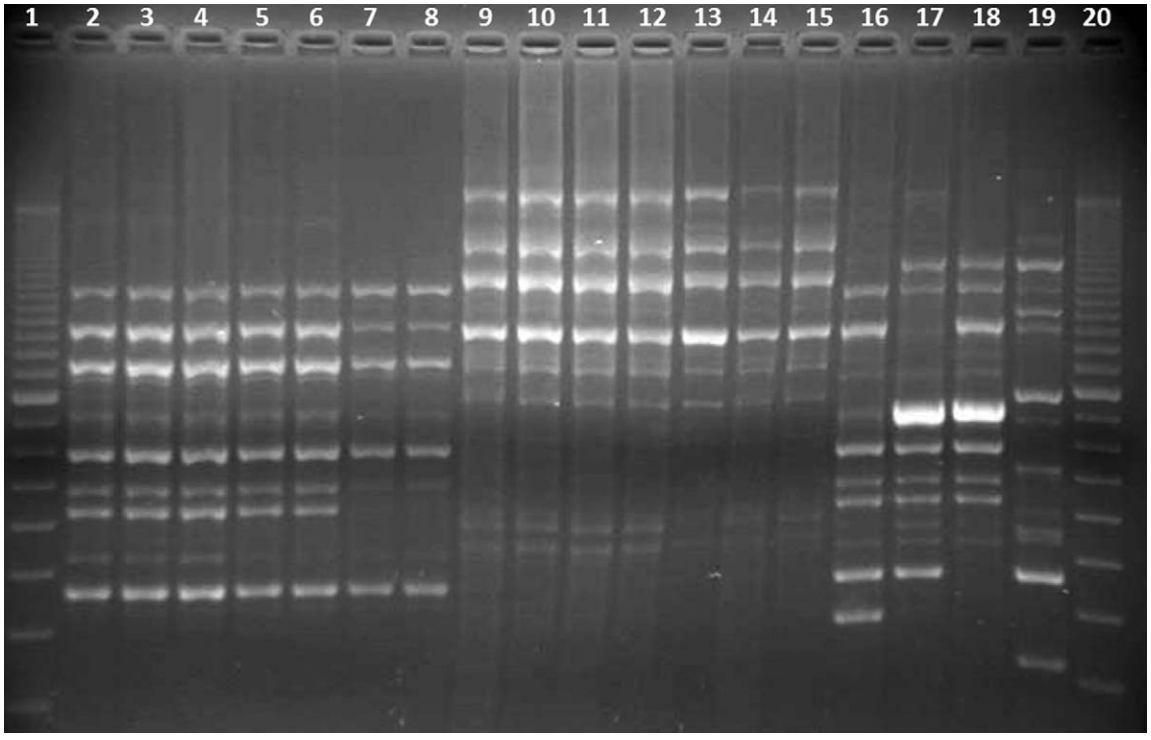

Figure 1. iRep1-PCR fingerprints of human and rodent Azorean isolates and reference strains from genus *Leptospira*. Lanes 2–4: Icterohaemorrhagiae human isolates (AzHuL01, AzHuL05, AzHuL08); lanes 5 and 6: Icterohaemorrhagiae reference strains (RGA serovar Icterohaemorrhagiae, M20 serovar Copenhageni); lanes 7 and 8: Terceira rodent isolates (AzTRoPu74, AzTRoH91); lanes 9–11: Ballum human isolates (AzHuL04, AzHuL06, AzHuL09); lanes 12 and 13: Ballum reference strains (Mus 127 serovar Ballum, Arborea serovar Arborea); lanes 14 and 15: São Miguel rodent isolates (AzSMRoS130, AzSMRoS184); lane 16: serogroup Sejroe (strain Hardjo-bovis serovar Hardjo); lane 17: serogroup Canicola (strain Hond Utrecht IV serovar Canicola); lane 18: serogroup Australis (strain Jêz Bratislava serovar Bratislava); lane 19: serogroup Pomona (strain Mozdok serovar Pomona); lanes 1 and 20: molecular size markers 3000 bp.

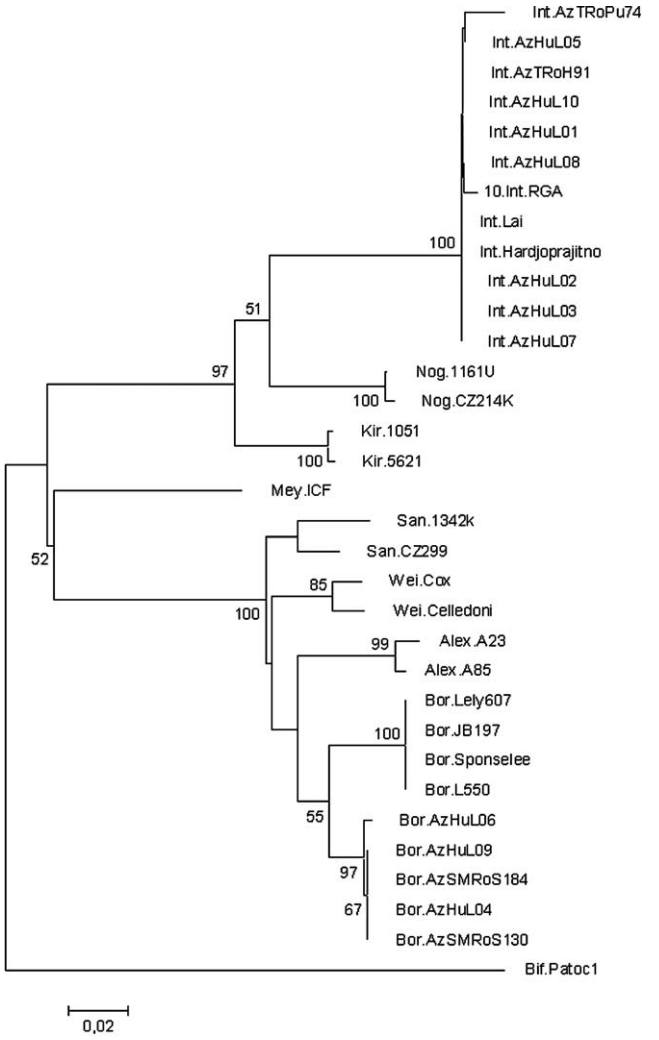

Figure 2. Phylogenetic tree based on Tamura-Nei distances and elaborated using the neighbor-joining method. Distances were calculated from G1–G2 restricted sequences and are based on 32 *Leptospira* strains (*L. interrogans* (Int.) isolates from rodents (AzTRoPu74, AzTRoH91) and humans (AzHuL01-03, AzHuL05, AzHuL07-08, AzHuL10); *L. borgpetersenii* (Bor.) isolates from rodents (AzSMRoS184, AzSMRoS130) and humans (AzHuL04, AzHuL06, AzHuL09); reference strains (the remaining sequences)). Numbers above branches represent the percentage of bootstrapping results (1000 replicates). Only bootstrap values above 50% are shown. *L. biflexa*, Patoc 1 was used as the outgroup. GenBank accession numbers of rodent and human isolates: **GQ144958**–**GQ144961** and **FJ911689**–**FJ911698**, respectively.

similar molecular pattern with the respective reference strains (RGA/M20 and Mus 127/Arborea), which are not differentiated by this molecular analysis (Figure 1).[15]

Sequencing and subsequent phylogenetic studies based on G1–G2 restricted sequences of *secY* allowed the identification of seven human isolates as belonging to species *L. interrogans* sensu stricto and the remaining to *Leptospira borgpetersenii* (Figure 2). Human isolates were assigned the GenBank accession numbers **FJ911689**–**FJ911698**.

To investigate the molecular relatedness among strains, a selection of São Miguel and Terceira rodent isolates was also analyzed by iRep1-PCR (Figure 1) and DNA sequencing (Figure 2 and data not shown). This additional research revealed the occurrence of similar DNA fingerprints and sequences between patients and rodents with similar *L. borgpetersenii* and *L. interrogans* profiles. The four rodent isolates were assigned GenBank accession numbers **GQ144958**–**GQ144961**.

As expected, the serological identification of the 10 isolates was in agreement with the detection of significant titers anti-Icterohaemorrhagiae and anti-Ballum in serial samples (Table 3), with the following geometric mean titers: $GMT_{Ictero.} = 2276$ and $GMT_{Bal.} = 1000$.

Discussion

This study was performed to isolate and identify for the first time the leptospires causing disease in Azorean inpatients. Leptospirosis has been considered a public health problem of increasing importance in the Azores, particularly in São Miguel and Terceira islands, due to both a high annual incidence rate (11.1 per 100 000 population, 1992–2003) and the occurrence of fatal cases among local inpatients, with rodents being the most incriminated sources.[4] However, in order to set up control and prevention measures, infection sources have to be identified based on the evidence of the same leptospires in both patients and suspected hosts.

In a recent epidemiological approach on these two islands,[16] determination of rodent distribution and relative abundance patterns as the major wild *Leptospira* reservoirs, revealed the presence of rodent-borne transmission foci (unpublished data). It also confirmed a wide dispersion and a high abundance of the three murine species *Rattus rattus*, *Rattus norvegicus*, and *Mus musculus*, together with a wide dispersion of two infectious spirochetes: *L. interrogans* sensu stricto serovar Copenhageni by the black rat (*R. rattus*) and brown rat (*R. norvegicus*), and *L. borgpetersenii* serovar Arborea by the house mouse (*M. musculus*) and the two rat species.[7] The overall *Leptospira* isolation rates were 61% (347 positive out of 567 necropsied animals) in São Miguel and 54% (246 positive out of 456) in Terceira, in 2006 and 2005, respectively (unpublished data).

So far, knowledge of the causative serovars in the Azores population has been presumptive, because it has been based on the highest titers in the MAT, which is a poor indicator of the infecting serovar.[23] Isolation is required for a definite identification. However, an attempt to isolate human *Leptospira* strains had never taken place on the islands, due to both local difficulties in the isolation procedure for these fastidious bacteria and the low contribution of culture to an early diagnosis, when antibiotic treatment is most effective.[1,3] This study aimed to provide a definitive bacteriological identification of the infecting human spirochetes in order to confirm rodents as the main natural infection source.

The relatively low success rate of leptospiral isolation by culture compared to the higher sensitivity of DNA detection by PCR is well recognized.[9] We applied an adopted blood culturing procedure at the patient bedside that enabled the isolation of leptospires from 14.7% of the patients suspected of having leptospirosis. This is a satisfying percentage considering that PCR confirmed 30.6% of the patients. This might partly be due to the use of blood instead of plasma or serum in the adopted procedure, ensuring a higher concentration of leptospires in the initial sample, whereas the inclusion of a dilution step reduces the occurrence of contaminants and growth inhibitors.

The isolates obtained in this research were assigned to species *L. interrogans* sensu stricto and *L. borgpetersenii*, showing a complete agreement between the Icterohaemorrhagiae and Ballum agglutination profiles developed at the serogroup level and the iRep1-PCR fingerprints. These results were also consistent with the predominant serological pattern found in the majority of hospitalized patients from São Miguel and Terceira since 1992.[5,16] Three presumptive major serogroups have been detected, with Icterohaemorrhagiae and Ballum as the dominant ones (approximately 86%), followed by serogroup Sejroe to a much lesser extent (10%) (unpublished data). Moreover, two different serovars, namely Copenhageni (of serogroup Icterohaemorrhagiae) and Arborea (of

Table 3
Serological and molecular characterization of *Leptospira* human isolates and microscopic agglutination test serodiagnosis of the culture-positive patients, Azores, 2006–2008

Human Leptospira isolate/year	Hyperimmune rabbit antisera Serogroup (titer)	iRep1-PCR Serogroup	Sequencing Species (GenBank accession No.)	MAT diagnosis (No. serum sample)
AzHuL01/2006	Icterohaemorrhagiae (1/8000)	Icterohaemorrhagiae	*L. interrogans* (**FJ911689**)	Icterohaemorrhagiae 1/10,240 (3rd; 46 days)
AzHuL02/2006	Icterohaemorrhagiae (1/8000)	Icterohaemorrhagiae	*L. interrogans* (**FJ911690**)	Icterohaemorrhagiae 1/2560 (2nd; 37 days)
AzHuL03/2006	Icterohaemorrhagiae (1/8000)	Icterohaemorrhagiae	*L. interrogans* (**FJ911691**)	Icterohaemorrhagiae 1/640 (3rd; 60 days)
AzHuL04/2007	Ballum (1/4000)	Ballum	*L. borgpetersenii* (**FJ911692**)	Ballum 1/1280 (3rd; 74 days)
AzHuL05/2007	Icterohaemorrhagiae (1/16000)	Icterohaemorrhagiae	*L. interrogans* (**FJ911693**)	Icterohaemorrhagiae 1/2560 (2nd; 16 days)
AzHuL06/2007	Ballum (1/4000)	Ballum	*L. borgpetersenii* (**FJ911694**)	Ballum 1/320 (3rd; 50 days)
AzHuL07/2007	Icterohaemorrhagiae (1/8000)	Icterohaemorrhagiae	*L. interrogans* (**FJ911695**)	Icterohaemorrhagiae 1/2560 (2nd; 25 days)
AzHuL08/2007	Icterohaemorrhagiae (1/16000)	Icterohaemorrhagiae	*L. interrogans* (**FJ911696**)	Icterohaemorrhagiae 1/1280 (3rd; 61 days)
AzHuL09/2007	Ballum (1/4000)	Ballum	*L. borgpetersenii* (**FJ911697**)	Ballum 1/2560 (2nd; 27 days)
AzHuL10/2008	Icterohaemorrhagiae (1/8000)	Icterohaemorrhagiae	*L. interrogans* (**FJ911698**)	Icterohaemorrhagiae 1/2560 (3rd; 62 days)

MAT, microscopic agglutination test.

serogroup Ballum), were identified by mAbs in seven and three isolates, respectively. This was in agreement with specific MAT titers in late-phase convalescent samples of the culture-positive patients.

According to the epidemiological information collected in the present research, occupational (six cases) and accidental (four cases) exposures favored a direct contact with animals (especially rodents and cattle) and infected soil or vegetation, respectively, as the major risk factors for leptospiral transmission. The important environmental risk exposure to the Copenhageni serovar through highly infected rat species and endemic Arborea infection in mice is also a significant transmission risk for humans.[6,16] At present, dairy cattle in the Azores have only been confirmed as Hardjo (serogroup Sejroe) renal chronic carriers.[16] Thus, the identification of similar iRep1-PCR fingerprints and DNA sequences in endemic strains from rodents and Azorean inpatients confirmed the expected correlation between these major reservoirs and human leptospirosis cases.

In conclusion, this study has characterized the first human *Leptospira* isolates in the Azores and shows the bacteriological human–rodent connection for the first time. In the future, the molecular study of these human isolates will also contribute to an understanding of the pathogenesis of the disease in the Azorean population.

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Conflict of interest: No conflict of interest to declare.

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