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Altodouro, a new *Leptospira* serovar of the Pomona serogroup isolated from rodents in northern Portugal

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ABSTRACT

A *Leptospira* strain (designated RIM 139) was isolated from the kidney of a house-mouse, *Mus musculus*, in Trás-os-Montes e Alto Douro region of northern Portugal. The isolate showed typical leptospiral motility and morphology under dark-field microscopy and was pathogenic for hamsters.

Species determination was carried out on basis of PCR products generated by species-specific primers and by sequencing of the *secY* gene. Putative serogroup typing was performed using the microscopic agglutination test (MAT) with a panel of rabbit anti-*Leptospira* sera representative of the major pathogenic serogroups. Serovar identification was carried out by a combination of monoclonal antibodies and cross-agglutinin absorption test (CAAT). The novel nature of the strain was confirmed by restriction endonuclease analysis (REA). Results showed that RIM 139 represents a new serovar. The name Altodouro is proposed for this new serovar. Speciation findings – PCR analysis of the *ompL1* gene and sequencing of the *secY* gene – indicated that it belonged to *Leptospira kirschneri* as did amplification with G1/G2 and B64-I/B64-II the primer sets.

1. Introduction

Leptospirosis is a zoonosis of worldwide distribution, caused by infection with pathogenic spirochetes of the genus *Leptospira*. The disease is maintained in nature by numerous reservoirs, free-living and domestic mammals, constituting a potential source of infection for humans, and other susceptible hosts.

There are 20 genomically distinct *Leptospira* species as delineated by DNA-DNA hybridisation (Levett et al., 2006; Adler and de la Penã-Moctezuma, 2010), From them 9 are pathogenic, 6 saprophytic and 5 intermediate species. Members of the genus *Leptospira* are also divided serologically based on similarity of surface-exposed epitopes as determined by using the cross-agglutinin absorption test (CAAT) and over 260 pathogenic serovars have been identified (Adler and de la Penã-Moctezuma, 2010) and the list is still updated (Corney et al., 2008; Sedigheh et al., 2010; Valverde et al., 2008). Since there is a poor correlation between the serological and genotypic classification systems, serologically similar strains may be genetically indistinguishable (LeFebvre and Thiermann, 1986; LeFebvre et al., 1987) and genetically similar strains may be serologically distinct (Thiermann et al., 1986). A definitive identification of the isolates would require the use of serological and molecular techniques (Brenner et al., 1999).

This study reports the isolation and identification of a new *Leptospira* serovar. Serological typing was based on the microscopic agglutination test (MAT) performed with rabbit hyper-immune sera, monoclonal antibodies (mAbs), and CAAT. Molecular typing involved species-specific polymerase chain reaction (PCR) and restriction endonuclease analysis (REA).

2. Materials and methods

2.1. Bacterial culture

Isolate RIM 139 was obtained from the kidney of a house-mouse *Mus musculus* in Trás-os-Montes e Alto Douro region of northern Portugal by Paiva-Cardoso (2009) using the following method. Approximately 1 g of pooled kidney tissue was macerated and homogenized aseptically in 1% (W/V) Bovine Serum Albumin (BSA) diluent (Ellinghausen, 1973), to produce a 10% suspension of the tissue. This suspension was diluted ten times and 2 to 5

Abbreviations: CAAT, cross-agglutinin absorption test; EMJH, Ellinghausen-McCullough-Johnson-Harris; MAT, microscopic agglutination test; MAb's, monoclonal antibodies; PCR, polymerase chain reaction; REA, restriction endonuclease analysis.

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Table 1
Primer sets used in this study.

Primer	Primer sequences (5′–3′)	References
LipL32 fwd	cgctgaaatgggagttcgtatgatt	Levett et al. (2005)
LipL32 rev	ccaacagatgcaacgaaagatccttt	Levett et al. (2005)
PFA	tgagtaacacgtgggtaatcttcc	Fearnley et al. (2008)
PRA	caggtaccatcatcacatygctgc	Fearnley et al. (2008)
G1	ctgaatcgctgtataaaagt	Gravekamp et al., 1993
G2	ggaaaacaaatggtcggaag	Gravekamp et al. (1993)
B64-I	ctgaattctcatctcaactc	Gravekamp et al. (1993)
B64-II	gcagaaatcagatggacgat	Gravekamp et al. (1993)
Intergroup A fwd	ctactggcggcttgatcaac	Reitstetter (2006)
Intergroup A rev	ctggatctgttccgtctgcgatc	Reitstetter (2006)
Intergroup B fwd	cttgatagaaccactggtggtgcc	Reitstetter (2006)
Intergroup B rev	ctggatcggttccatcgctcag	Reitstetter (2006)
Borgpeter fwd	cttgatagaacaacaggcggcatcatc	Reitstetter (2006)
Borgpeter rev	gctaataagtttgcaatgctcgtaac	Reitstetter (2006)
Kirschner fwd	cggtttgatcaatgcgagaagcacc	Reitstetter (2006)
Kirschner rev	ttggatccgttccgtctgcgatt	Reitstetter (2006)
Santarosai fwd	cttatcaatgcaagatctaccaaaggt	Reitstetter (2006)
Santarosai rev	gcggatatgttcccgagtagtaatc	Reitstetter (2006)
Noguchii fwd	gcggatttatcaatgcaagaagtaca	Reitstetter (2006)
Noguchii rev	ccggatcggttccgtctgcgatcag	Reitstetter (2006)
Weilii fwd	aggctgatattgcaggcttc	Reitstetter (2006)
Weilii rev	cggaatcgaatatgttcacgagtg	Reitstetter (2006)
SecY II	gaatttctcttttgatcttcg	Victoria et al. (2008)
SecY IV	gagttagagctcaaatctaag	Victoria et al. (2008)

Key: Y = C + T.

drops inoculated into tissue culture tubes containing the 7 ml of semi-solid media with 0.4% rabbit serum as described by Ellis et al. (1986). Cultures were incubated at 28–30 °C and examined weekly by dark-field microscopy for leptospiral growth (OIE, 2008). Positive cultures were transferred to fresh medium and adapted to liquid EMJH (Ellinghausen-McCullough-Johnson-Harris) culture medium (Johnson and Harris, 1967).

2.2. Animal testing

Initial pathogenicity studies were carried out in hamsters. The hamster model, including housing and handling of hamsters, intraperitoneal injection and monitoring response to challenge was carried out according to procedures described by Haake (2006), with some modification. Briefly, two Golden Syrian hamsters (Harlan Laboratories, UK) after a 7 day quarantine period were inoculated intraperitoneally with 1 ml of study strain RIM 139 (1×10^8 cells/ml), and subsequently the animals were examined twice daily. At the onset of symptoms, the hamsters were euthanized and during the course of a *post mortem* examination blood and kidney were collected for serology and culture, respectively. All operators involved in protocols and premises were licensed under the Animals (Scientific Procedures) Act (1986).

2.3. Serological typing: MAT with group sera and monoclonal antibodies

Serological identification of RIM 139 strain was initially performed by cross-agglutination. In this procedure the MAT was carried out using a panel of 17 *Leptospira* antisera against the 15 major pathogenic serogroups (Wolff, 1954; Dikken and Kmety, 1978; WHO, 2003). The *Leptospira* serogroups tested included Australis (serovars Australis and Bratislava), Autumnalis, Ballum, Canicola, Cynopteri, Grippotyphosa, Hebdomadis, Icterohaemorrhagiae, Javanica, Louisiana, Mini, Pomona, Pyrogenes, Sejroe (serovars Saxkoebing and Hardjo) and Tarassovi. Rabbit sera were prepared as described by WHO (2003).

Monoclonal antibody typing was done using a panel of mAbs (F43C9-5, F46C1-1, F46C2-4, F46C4-1, F46C5-1, F46C9-1, F46C10-1, F48C1-3, F48C3-3, F48C6-5, F58C1-2, F58C2-3, F61C7-1, F71C2-4, F71C3-5, F71C9-4, F71C13-4, F71C16-6, F71C17-5, F164C1-1, F165C1-4, F165C2-1, F165C3-4, F165C7-5, F165C8-3, F165C12-4) that characteristically agglutinate serovars belonging to serogroup Pomona (Terpstra et al., 1987). Two serogroup Pomona strains – serovars Kunming (strain K5) and Mozdok (strain 5621) were included in this test for comparison of their agglutination patterns with that of RIM 139.

2.4. Cross-agglutinin absorption test

The CAAT was performed using RIM 139 and *Leptospira kirschneri* serovar Kunming (strain K5) antigens (OIE Leptospirosis Reference Laboratory's collection, Belfast, UK) and antisera according to Dikken and Kmety (1978). This was done in duplicate and independently by two operatives to guarantee reproducibility.

2.5. Genetic characterization

Cultures of *Leptospira* were prepared for DNA extraction by centrifugation of 1.5 ml aliquots in a micro-centrifuge at 3500*g* for 15 min. The supernatant was removed and the pellet was used for DNA extraction with a QIAamp DNA Mini Kit (Qiagen).

To confirm the pathogenic status of the *Leptospira* isolate two polymerase chain reaction (PCR) analyses were performed using pathogen-specific primer pairs which amplify a 423 bp target on the gene encoding lipoprotein LipL32 (Levett et al., 2005) and PFA/PRA primers for 369 bp target of the 16S rRNA (Fearnley et al., 2008) (Table 1). Amplification was performed in a final volume of 25 µl containing 12.5 µl JumpStart™ REDTaq® ReadyMix™ for High Throughput PCR (Sigma), 100nM forward LipL32, 300 nM reverse LipL32 primers and 120 nM for PFA and PRA primers, 2.0 µl DNA extract and nuclease-free water to make up the final volume. The amplification profile was the same as that described by the authors.

PCR analyses were also applied with the seven species-specific primers pairs as described by Reitstetter (2006). Primer sets

Table 2
List of *Leptospira* reference strains of serogroup Pomona used in the restriction endonuclease analysis.

Species	Serogroup	Serovar	Strain	Country	Source	Year	Reference
L. interrogans	Pomona	Pomona	Pomona	Australia	Human	1937	Clayton et al. (1937)
L. interrogans	Pomona	Kennewicki	LT 1026	USA	Bovine	1967	Anonymus (1967)
L. interrogans	Pomona	Monjakov	Monjakov	Russia	Human	1936	Chernukha (1966)
L. kirschneri	Pomona	Mozdok	5621	Russia	Field vole	1965	Semenova (1965)
L. kirschneri	Pomona	Tsaratsovo	B81/7	Bulgaria	Harvest mouse	1976	Manev (1976)
L. santarosai	Pomona	Dania	K1	Denmark	Bovine	1967	Chernuka and Isaeva (1967)
L. noguchii	Pomona	Proechimys	1161 U	Panama	Spiny rat	1982	Sulzer et al. (1982)
L. santarosai	Pomona	Tropica	CZ 299	Panama	Spiny rat	1966	Gale et al. (1966)
L. kirschneri	Pomona	Kunming	K5	China	Apodemus chevrieri	1984	Zhang et al. (1987)