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Isolation and clinical sample typing of human leptospirosis cases in Argentina

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ABSTRACT

Leptospira typing is carried out using isolated strains. Because of difficulties in obtaining them, direct identification of infective *Leptospira* in clinical samples is a high priority. Multilocus sequence typing (MLST) proved highly discriminatory for seven pathogenic species of *Leptospira*, allowing isolate characterization and robust assignment to species, in addition to phylogenetic evidence for the relatedness between species. In this study we characterized *Leptospira* strains circulating in Argentina, using typing methods applied to human clinical samples and isolates. Phylogenetic studies based on 16S ribosomal RNA gene sequences enabled typing of 8 isolates (6 *Leptospira interrogans*, one *Leptospira wolffii* and one *Leptospira broomii*) and 58 out of 85 (68.2%) clinical samples (55 *L. interrogans*, 2 *Leptospira meyeri*, and one *Leptospira kirschneri*). MLST results for the *L. interrogans* isolates indicated that five were probably Canicola serogroup (ST37) and one was probably Icterohaemorrhagiae serogroup (ST17). Eleven clinical samples (21.6%), provided MLST interpretable data: five were probably Pyrogenes serogroup (ST13), four Sejroe (ST20), one Autumnalis (ST22) and one Canicola (ST37). To the best of our knowledge this study is the first report of the use of an MLST typing scheme with seven loci to identify *Leptospira* directly from clinical samples in Argentina. The use of clinical samples presents the advantage of the possibility of knowing the infecting strain without resorting to isolates. This study also allowed, for the first time, the characterization of isolates of intermediate pathogenicity species (*L. wolffii* and *L. broomii*) from symptomatic patients.

1. Introduction

Leptospirosis is a zoonotic disease of global distribution caused by spirochetes of the genus *Leptospira* spp. (Levett, 2007). It is maintained by chronic carrier hosts that excrete the organism into the environment, and human infection results from direct contact with infected animals or with a contaminated environment (Levett, 2007; Ko et al., 2009). Leptospirosis in humans can vary in severity according to the infecting *Leptospira* serovar, inoculum dosage and the patient's age, health and immunological competence. Clinical manifestations, when present, vary from a mild flu-like febrile illness to a severe disease with symptoms that may include jaundice, renal failure and pulmonary hemorrhage (Adler and de la Peña Moctezuma, 2010).

In recent decades typing and detection of *Leptospira* spp. based on molecular techniques has been introduced and widely applied to the field of leptospirosis study. Moreover, molecular methods have been exploited as an alternative or supplementary approach to the currently existing serological methods. The cross-agglutination absorption test (CAAT) analysis that led to the definition of serovar, is today considered to be the basic systematic unit for *Leptospira* spp. typing (Adler and de la Peña Moctezuma, 2010). However, CAAT is cumbersome and time-consuming for routine typing, mostly due to the time required for the preparation of rabbit immune sera. Therefore, few laboratories are able to perform CAAT (Terpstra et al., 1985). For this reason, most isolates are identified at serogroup level, using the Microscopic agglutination test (MAT) (Cerqueira and Picardeau, 2009).

Since the 1990s speciation of leptospires based on genomic DNA homology has been available. Molecular approaches such as heterologous DNA hybridization (Brenner et al., 1999; Levett, 2006; Ahmed et al., 2012) and more recently, average nucleotide identity and genome-to-genome distances (Bourhy et al., 2014), have led to the identification of 22 *Leptospira* species, ten pathogenic: *Leptospira interrogans*, *Leptospira borgpetersenii*, *Leptospira santarosai*, *Leptospira noguchii*, *Leptospira weilii*, *Leptospira kirschneri*, *Leptospira alexanderi*, *Leptospira alstonii*, *Leptospira kmetyi* and *Leptospira mayottensis*; five intermediate: *Leptospira inadai*, *Leptospira broomii*, *Leptospira fainei*, *Leptospira wolffii*, *Leptospira licerasiae*; and seven non-pathogenic: *Leptospira biflexa*,

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Leptospira wolbachii, *Leptospira vanthielii*, *Leptospira terpstrae*, *Leptospira meyeri*, *Leptospira idonii*, and *Leptospira yanagawae*. The intermediate group produces mild clinical symptoms or no symptoms at all. The pathogenic implication of this group is yet unknown.

DNA hybridization is a complicated method that requires the use of considerable amounts of isotope-labeled DNA of high quality. Other techniques based on PCR amplification, such as multilocus sequence analysis, are gaining importance as molecular tools for the speciation of *Leptospira* (Ahmed et al., 2012). The 16S ribosomal RNA (rRNA) gene allows identification due to inter-species differences and low intra-species variability. The comparison of 16S rRNA sequences is a powerful tool for deducing phylogenetic and evolutionary relationships among bacteria (Backstedt et al., 2015). Due to multiple benefits, Multilocus Sequence Typing (MLST), based on the generation of sequences from different genes and allowing a high-throughput scale, has become the method of choice. MLST is unambiguous and suitable for epidemiological research, population studies and the investigation of maintenance hosts during outbreaks (Ahmed et al., 2006, 2011; Maiden, 2006; Thaipadungpanit et al., 2007; Leon et al., 2010; Boonsilp et al., 2013). MLST has proved highly discriminatory for seven pathogenic species of *Leptospira*, providing both isolate characterization and robust species assignment in addition to phylogenetic evidence for relatedness between species. Crucially, this scheme is also supported by a public website (http://Leptospira.mlst.net/) (Boonsilp et al., 2013).

Leptospira typing is carried out using isolated strains. Although culture is rarely performed in routine clinical practice and is only positive in a minor number of cases, it continues to have an important role in defining the global epidemiology of infection (Thaipadungpanit et al., 2007; Slack et al., 2007; Adler and de la Peña Moctezuma, 2010; Thaipadunpanit et al., 2011). Identification of the infecting serovar can provide clues as to the chronic carrier host, since certain serovars may be associated with either a single or small number of mammalian or other species (Levett, 2001; Bharti et al., 2003). Such information makes an important contribution to the development of prevention strategies (Levett, 2001; Bharti et al., 2003; Ko et al., 2009). Nonetheless, because of difficulties in obtaining isolates, direct identification of infective *Leptospira* in clinical samples is a high priority for clinical, epidemiological, and basic scientific purposes (Agampodi et al., 2013).

In Argentina, there have been a few studies concerning species prevalence and many of these studies reported a number of cases with agglutination titers against multiple serogroups. In a study by Vanasco et al. (2008), Icterohaemorrhagiae and Pomona were identified as the major serogroups involved in human leptospirosis. Recently, Caimi et al. (2012) demonstrated the presence of serogroup Canicola and Icterohaemorrhagiae, using variable number tandem repeat and MLST characterization in bovine and porcine isolates.

In this context, the aim of this study was to characterize *Leptospira* strains circulating within Argentina. This was performed by the use of molecular typing methods applied to human clinical samples and isolates.

2. Materials and methods

2.1. Bacterial isolates and clinical samples

Human isolates (culture) and samples obtained between January 2004 and March 2014 were selected from a collection at the Leptospirosis laboratory of the National Respiratory Disease Institute (Instituto Nacional de Enfermedades Respiratorias, INER), Santa Fe, Argentina. Characteristics of participants are shown in Table 1. Eight isolates, and eighty five samples (78 serum samples and 7 whole blood) diagnosed as positive by real-time quantitative polymerase chain reaction (qPCR), were selected from the INER collection. The qPCR assay was performed according to Stoddard et al. (2009) using a TaqMan probe targeting *LipL32*.

Eight isolates were obtained from 0.2 ml of human whole blood cultured into Ellinghausen–McCullough–Johnson–Harris semisolid medium (EMJH). The cultures were incubated at 28 °C and examined weekly for 4 months (World Health Organization and International Leptospirosis Society, 2003).

Leptospires in serum samples were quantified using the threshold cycle (CT) of the qPCR reaction. Leptospiral culture dilutions from 10^8 to 1 *Leptospira*/ml were prepared and the number of cells in culture was determined as described by Smythe et al. (2002), except for the use of a Neubauer bacterial counting chamber. The CT of each culture was obtained by qPCR as described above, and the calibration curves were constructed.

2.2. Serological characterization of isolates

Serological characterization of isolates into serogroups was carried out by MAT using rabbit antisera against reference serovars (Faine, 1999), representing a standard battery of 23 serogroups: Grippotyphosa, Pomona, Ballum, Pyrogenes, Icterohaemorrhagiae, Sejroe, Tarassovi, Canicola, Bataviae, Semaranga, Autumnalis, Australis, Cynopteri, Javanica, Panama, Hebdomadis, Sarmin, Ranarum, Louisiana, Mini, Celledoni, Djasiman, Shermani. Each isolate was assigned to the serogroup of the group serum with the highest titer.

2.3. DNA extraction

Genomic DNA was extracted from serum, whole blood and EMJH cultures using QIAamp DNA Blood Mini Kits (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions.

2.4. 16S rRNA

Determination of species was performed as a first step, using 16S rRNA as the amplification target (Mérien et al., 1992). For each reaction, 1 U of GoTaq DNA polymerase (Promega, Madison, WI, USA), 200 μM deoxynucleoside triphosphates (dNTPs), and 1 μM of primers, were added to a total volume of 50 μl. Amplification was carried out using a Veriti Thermal Cycler (Applied Biosystems, Foster City, CA, USA) and the PCR products were analyzed on 2% agarose gels.

2.5. MLST

A previously published MLST scheme based on the amplification of seven housekeeping genes (*mreA*, *pfkB*, *pntA*, *sucA*, *tpiA*, *glmU* and *caiB*) was used (Boonsilp et al., 2013). Reaction mixtures were prepared using 1.25 U GoTaq DNA polymerase (Promega, Madison, WI, USA) with 5 pmol of each primer and 5 μl of DNA in a total volume of 50 μl. Amplification was carried out using a Veriti Thermal Cycler. The PCR products were analyzed on 2% agarose gels.

2.6. Sequencing and sequence analysis

PCR amplification products of 16S rRNA and MLST genes were purified using GeneJET PCR Purification Kit (Thermo Scientific, Waltham, MA, USA) prior to DNA sequencing. PCR products were then sequenced by Macrogen Inc. (Seoul, Korea). The sequences were edited using Chromas Lite 2.1.1 (Technelysium Pty Ltd., Australia). The contigs were assembled using the Staden Package software (MRC-LMB, UK) and the alignment and construction of phylogenetic trees was performed using MEGA 5 (Tamura et al., 2011). To obtain the *Leptospira* species, the assembled sequences of 16S rRNA were analyzed using the Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp).

Phylogenetic relationship among concatenated sequences of 16S rRNA was inferred using the maximum-likelihood (ML) method and the Tamura–Nei parameter, using MEGA 5.0 (Tamura et al., 2011).