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Leptospira wolffii, a potential new pathogenic *Leptospira* species detected in human, sheep and dog☆

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ABSTRACT

Leptospirosis is the most common zoonotic disease, which is transmitted to humans through contaminated water or direct exposure to the urine of infected animals. In this study, the presence and prevalence of *Leptospira* species in the infected samples of human (n = 369) and sheep (n = 75) sera and also dogs' urine (n = 150), collected from four provinces of Iran, were investigated by using nested-PCR/RFLP assay followed by sequencing analysis. Nested-PCR assay detected that 98/369 (26.5%) human, 13/75 (17.33%) of sheep's sera and 33/150 (22%) dogs' urine samples were positive for *Leptospira* DNA. RFLP assay detected that all positive cases had either pathogenic or intermediate *Leptospira* species. By sequence analysis, *Leptospira interrogans* was the most prevalent species among the examined samples of human (53/82, 64.6%) and sheep (11/13, 84.6%). However, in dog samples, *Leptospira wolffii* (27/29, 93.1%) was detected for the first time and was the dominant species. The presence of *L. wolffii* with 100% identity in clinical human samples and animals suspected with *Leptospira* may provide evidence for circulation of *L. wolffii* and its role in transmission cycle within human and animal hosts. In addition, this species can be potentially pathogenic to human and probably animal hosts. A large epidemiology survey would be needed to define the presence and the prevalence of this species in global endemic regions.

1. Introduction

Leptospirosis is a worldwide zoonotic disease which affects wild and domestic animals and humans. Infected animals excrete the organisms in their urine and contaminate the environment and human infection occurs through contaminated water or direct exposure to the urine of infected animals (Bharti et al., 2003; Ko et al., 1999). Early diagnosis of leptospirosis is critical because of the risk of severe complications (Ko et al., 1999; Laras et al., 2002).

Before 1989, the genus *Leptospira* was conventionally classified into two species, *L. interrogans* sensu lato and *L. biflexa* sensu lato, which comprised pathogenic and non-pathogenic strains, respectively (Faine and Stallman, 1982; Johnson and Faine, 1984). *L. interrogans* is the only pathogenic species that is distributed in approximately 160 mammalian species worldwide. Although classification based on serotyping remained gold standard according to the molecular taxonomy system developed by Yasuda et al. (1987), nine species were described, including *L. interrogans*, *L. biflexa*, *L. borgpetersenii*, *L. inadai*, *L. noguchii*, *L. santarosai*, *L. weillii*, *L. meyeri* and *L. wolbachii*. In 1992, 1998 and 1999, three species: *L. kirschneri*, *L. fainei* and *L. genome species 1–5* were described, respectively (Ramadass et al., 1992; Perolat et al., 1998; Brenner et al., 1999), and recently, two other species: *L. broomii* (Levett et al., 2006) and *L. wolffii* (Slack et al., 2008) have introduced. Phylogenetic analysis of these species using the 16S rRNA gene has resulted in a broad classification of the species (pathogenic, saprophytic and intermediates). In 2007, the Committee on the Taxonomy of Leptospiraceae decided to revise the nomenclature for genomospecies 1 and 3 as well as 4 and 5. The genomospecies 1 and 3 were designated *L. alstonii* and *L. terpstrae*, respectively as pathogenic and the genomospecies 4 and 5 were designated *L. vanthielii* and *L. yanagawae*, respectively as non-pathogenic *Leptospira* species (Adler and de la Pena Moctezuma, in press; Cerqueira and Picardeau, 2009). As antigenic relatedness, there are currently over 250 recognized pathogenic serovars (Cerqueira and Picardeau, 2009) that a few of them are found in

☆ *Note*: Nucleotide sequence data reported in this paper are available in the European Molecular Biology Laboratory (EMBL), GenBank and DNA Data Bank of Japan (DDJB) databases under the accession number(s) EU497658–EU497661 and GQ501005–GQ501010 (Human), FJ812167–FJ812170 (Sheep and Dog).

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more than one species of *Leptospira*. Hence, there is a poor correlation between the serological and genotypic classification systems (Brenner et al., 1999). It has been also observed that the strains of the same serovars are associated with different clinical syndromes.

Leptospirosis has emerged as a major public health problem in much of developing world. It also affects livestock animals worldwide, and is therefore of economic importance. Because, the control tools which are used today are ineffective, development of novel techniques that could detect the causative agent at early stage of infection is highly needed for treatment and prevention of this neglected disease. Recently, we developed a nested-PCR/RFLP assay using 16S rRNA, which is a rapid, specific technique that can differentiate pathogenic and non-pathogenic *Leptospira* species in early stage of infection (Djadid et al., 2009). Therefore, the objective of the current study was to detect *Leptospira* DNA and also frequency of the main *Leptospira* species in the suspected human, sheep and dogs using recently developed nested-PCR/RFLP methods (Djadid et al., 2009) in both sera and urine samples in four provinces of Iran. Furthermore, all positive samples were analyzed by sequencing method that surprisingly identified *L. wolffii* species in infected human, and for the first time in sheep and dogs.

2. Materials and methods

2.1. Human sample collection

Blood samples ($n = 369$) were collected from human with suspected leptospira infection (based on physician diagnosis and WHO Guidance for Diagnosis, Surveillance and Control of the Disease) during the transmission season (April–October) in 2005–2007. The patients had history or clinical manifestations of leptospirosis such as presence of fever with headache or body aches associated with jaundice 2–3 days prior to sampling. They were admitted to general hospitals in different parts of Guilan and Mazandaran provinces in northern part of Iran, located on the southern coast of the Caspian Sea (Fig. 1). The median age of participants with suspected leptospirosis was 40 years (ranged from 13 to 78 years) that 296/369 (80%) were men. In Caspian provinces of Iran, rice farming, agriculture, fishing and cattle husbandry are the main activities.

Approximately, 2–5 ml of venues blood was collected in a sterile tube and the serum was separated from the blood samples,

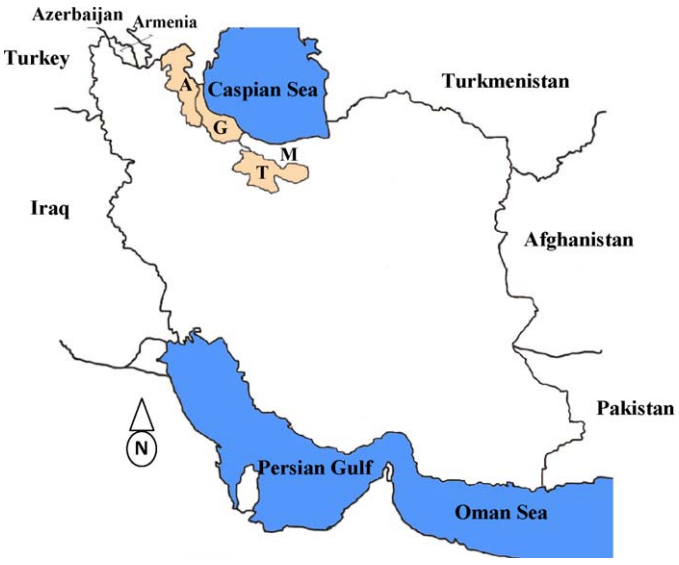

Fig. 1. Map of Iran showing study sites. M: Mazandaran province, G: Guilan province, A: Ardebil province, T: Tehran province.

followed by storage at −20 °C, and transferred to the main laboratory in Tehran. Prior to sample collection, written informed consent was obtained from the patients. This study was approved by the Ethical Review Committee of Research in Pasteur Institute of Iran.

2.2. Sheep and dog sample collection

A number of 75 and 150 sheep and dogs were screened for leptospirosis, respectively. Dogs' urine sample (10 ml) was collected by cystocentesis or catheterization and transferred to a 15-ml sterile plastic tube containing 2 ml of 0.1 M EDTA (pH 8) and 0.5% formaldehyde (Gerritsen et al., 1991). The blood samples were collected from sheep flocks from Ardebil province located in north-western of Iran (Fig. 1). Then, the serum was separated from the blood samples, followed by storage at −20 °C, and transferred to the main laboratory in Tehran.

2.3. DNA extraction

The leptospiral genomic DNA was extracted from suspected human and sheep sera and also from dogs' urine samples using the method previously described (Djadid et al., 2009; Cheng and Jiang, 2006). The DNA was air-dried, re-dissolved in TE buffer (10 mM Tris-HCL, pH 8.0, 0.1 mM EDTA) and kept at −20 °C until use. The DNA was quantified by both gel electrophoresis and spectrophotometrically by calculating the A260/A280 ratios and the A260 values to determine protein impurities and DNA concentrations.

2.4. Nested-PCR and restriction fragment length polymorphism (PCR-RFLP) for 16S rRNA gene amplification

Primer selection and PCR assay were performed as previously described (Djadid et al., 2009). Briefly, *Leptospira* DNA was amplified using primers 5′-GGCGGCGCGTCTTAAACATG-3′ and 5′-GTCCGCCTACGCACCCTTTACG-3′ (nest-1) and the second amplification (nest-2) was performed with primers 5′-CAAGT-CAAGCGGAGTAGCAA-3′ and 5′-CTTAACCTGCTGCCTCCCGTA-3′. These primers were designed based on particular region of 16S rRNA gene of *Leptospira* species that were described earlier (Djadid et al., 2009). PCR was performed in a total volume of 25 μl reaction mixtures containing 2 mM $MgCl_2$, 200 μM dNTP mixture (Invitrogen, Carlsbad, CA, USA), 1 unit of Taq polymerase (Invitrogen, Carlsbad, CA, USA), and a pair of primers (10 pmol each) for both nested-PCR reactions. The negative control containing all the components of the reaction mixture without DNA sample was used as control in all nested PCR. The second amplified DNA fragments were separated on 2% agarose gels by electrophoresis and visualized on an ultraviolet transilluminator following ethidium bromide staining.

RFLP was used to distinguish all pathogenic and non-pathogenic species (Djadid et al., 2009). Therefore, all second PCR products (nest-2) were separately digested with *ApoI* (Invitrogen, Carlsbad, CA, USA) according to the supplier's instructions.

2.5. Sequencing 16S rRNA PCR product

Amplified fragments were gel-purified using the QIAGEN DNA purification kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Direct sequencing of the DNA fragments was performed in both directions for each PCR product using the dideoxy chain termination procedure (Chemistry V3.1, Applied Biosystems) and the 3730XL DNA analyzer (Applied Biosystems) by MilleGen sequencing service (Labege, France). Nucleotide sequences were aligned with the corresponding *L. interrogans*