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# Leptospirosis in Caspian Sea littoral, Gilan Province, Iran

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ABSTRACT

In Iran, leptospirosis is endemic to Caspian Sea littoral. The disease appears as a seasonal infection mostly affecting people in rural areas involved in farming. We investigated the prevalence of this infection among suspected patients in Gilan Province by an indirect immunofluorescent assay (IFA), and two PCR protocols, a nested-PCR and a real-time PCR (qPCR), targeting *rrs* and *lipL32* genes, respectively. We also identified the common *Leptospira* species by sequencing a partial sequence of *rrs* gene. Out of the 128 sera examined by IFA, 25.78% were positive with the antibody titers ≥1/80. The antibody titer in 39.06% of sera ranged from 1/10 to 1/140, and 35. 16% showed no antibodies, all considered negative. Nested PCR and qPCR detected *Leptospira* DNA in 20.31% and 18.75% of the sera, respectively. The two PCR assays had 98.43% agreement ($K = 0.93$) and showed an inverse correlation with the IFA titers. Also, three pathogenic *Leptospira* species, *L. kirschneri* (n = 10), *L. interogans* (n = 8), and *L. borgpetersenii* (n = 2) were identified from the clinical specimens in the study area. In our hands both PCR assays proved very efficient for early diagnosis of illness and could be used in combination with IFA for both diagnosis and epidemiological studies, but nested PCR was cheaper and appeared more appropriate for our laboratories in rural settings.

## 1. Introduction

Leptospirosis is a global zoonotic disease caused by the pathogenic members of the spirochetes belonging to the genus *Leptospira*. Humans infection is through direct contact with infected animals or indirect contact with infected urine of animal hosts (Bharti et al., 2003; Levett, 2001; WHO, 2006). The clinical manifestations of human leptospirosis are very diverse ranging from mild, flu-like illness to life-threatening manifestations such as severe pulmonary hemorrhage syndrome and Weil's disease (McBride et al., 2005). The annual worldwide incidence of leptospirosis is estimated to be around 1.03 million cases with 58,900 deaths (Costa et al., 2015). The burden of endemic leptospirosis is thought to be very significant for people living in rural areas involved in farming mainly rice and sugarcane cultivations, and urban settings with inadequate sanitary system commonly defined as slums (Costa et al., 2015; WHO, 2006). In Iran, the infection is endemic to Caspian Sea littoral covering Golestan, Mazandaran, and Gilan provinces (Djadid et al., 2009; Ghasemian et al., 2016; Honarmand and Eshraghi, 2011; Zakeri et al., 2010b). In this area, the disease appears as a seasonal infection mostly affecting people in the countrysides involved in farming. Moreover, anti-*Leptospira* antibodies have been detected in humans residing in other parts of Iran. The disease has been reported as an occupational hazard for the people working in rice paddies (Alavi and Khoshkho, 2014) or those in close contact with farm animals or the carcasses in slaughterhouses (Esmaeili et al., 2016).

The accuracy of clinical diagnosis of leptospirosis is not very reliable as there can overlap with the other prevalent infectious diseases including rickettsial and hantavirus infections, Crimean-Congo hemorrhagic fever (CCHF), dengue, and malaria or fevers of unknown origin (Izurieta et al., 2008). In Thailand, a hospital-based study revealed the accuracy of the clinical diagnosis of leptospirosis to be only 20% in suspected cases (Wuthiekanun et al., 2007). In the Caspian Sea littoral, malaria has long been eradicated, but CCHF is occasionally reported, and due to the apparent seasonal pattern of the disease the physicians often rely on clinical features to make a diagnosis. For the past decade, a homemade indirect immunofluorescence assay (IFA) developed by Pasteur Institute of Iran has been in use for diagnosis and surveillance of the disease in the Caspian Seal littoral (Zakeri et al., 2010b).

In this study, we investigated the prevalence of leptospirosis infection among suspected patients in Gilan Province of Iran by the same

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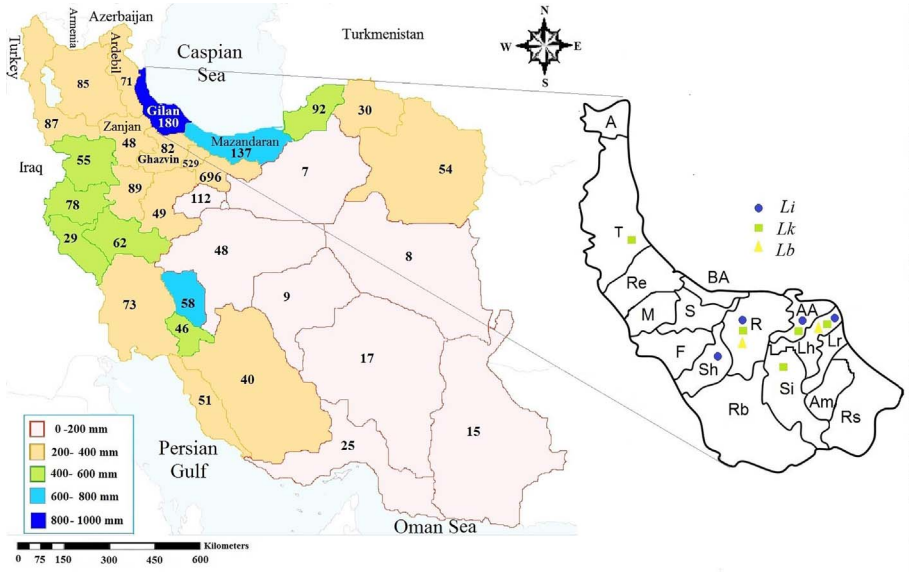


| Abbs. in Map | Counties | No. of Samples | Species |
|---|---|---|---|
| A | Astara | - | - |
| AA | Astaneh Ashrafiyyh | $16^{(6)}$ | $Lk^{(3)}$, $Li^{(3)}$ |
| Am | Amlash | - | - |
| BA | Bandar Anzali | $4^{(0)}$ | - |
| F | Fuman | $7^{(0)}$ | - |
| Lh | Lahijan | $11^{(6)}$ | $Lk^{(2)}$, $Li^{(2)}$ $Lb^{(1)}$, $NS^{(1)}$ |
| Lr | Langrud | - | - |
| M | Masal | $1^{(0)}$ | - |
| R | Rasht | $37^{(6)}$ | $Lk^{(1)}$, $Li^{(1)}$, $Lb^{(1)}$, $NS^{(3)}$ |
| Re | Rezvan Shahr | $6^{(0)}$ | - |
| Rs | Rudsar | $1^{(0)}$ | - |
| Rb | Rudbar | $1^{(0)}$ | - |
| S | Soumehe sara | $7^{(0)}$ | - |
| Sh | Shaft | $4^{(1)}$ | $Li^{(1)}$ |
| Si | Siahkal | $3^{(1)}$ | $Lk^{(1)}$ |
| T | Talesh | $5^{(1)}$ | $Lk^{(1)}$ |
| | Not defined | $25^{(5)}$ | $Lk^{(2)}$, $Li^{(1)}$, $NS^{(2)}$ |
| | Total | $128^{(26)}$ | $Lk^{(10)}$, $Li^{(8)}$, $Lb^{(2)}$, $NS^{(6)}$ |

**Fig. 1.** Map of Iran, the data of the clinical samples identified and sequenced based on the *rrs* fragment in this study. The values in the superscript parentheses in columns 3 and 4 represent the number of specimens sequenced and identified, respectively. Abbs. Abbreviations, *LK. Leptospira kirschneri*, *Li. Leptospira interrogans*, and *Lb. Leptospira borgpetersenii*. NS, Not Specified. The value in each province shows the density of population/km$^2$. The colors demonstrate the long-term average rainfall in different areas of Iran.

IFA, and two published DNA-based assays, a nested PCR and a real-time PCR (qPCR) targeting *rrs* (Merien et al., 1992) and *lipL32* genes (Bourhy et al., 2011; Stoddard et al., 2009), respectively. Our results also report the identification of the common *Leptospira* species in the area by sequencing a partial sequence of *rrs* gene.

## 2. Material and methods

### 2.1. Study areas

Gilan Province is in the north of Iran, lying along the south and west of the Caspian Sea. It borders Mazandaran Province in the east, Ardabil in the west, and Zanjan and Qazvin in the south. It also shares a small border with the Republic of Azerbaijan in the north, as well as Russia across the Caspian Sea (Fig. 1). Iran is in the dry zone of the world with the average rainfall of 250 mm. However, the Gilan climate is humid subtropical and this province, by a large margin, has the heaviest rainfall in the country reaching as high as 1000 mm (Iran Meteorological Organization, 2014) (Fig. 1). It is one of the most densely populated regions (Fig. 1) inhabited by 2,530,696 people according to 2016 census (Statistical Centre of Iran, 2016). The coastal plain along the Caspian Sea in this province is mainly used for rice paddies, where rodents and food-producing animals like cattle are abundantly seen. Hence, climate, environmental, and socioeconomic conditions are favorable for transmission of leptospirosis in this area.

### 2.2. Serum samples

Sera were obtained from 128 patients suspected with leptospirosis from rural areas of Gilan Province in the Caspian Sea littoral (Fig. 1) during late April to mid-June 2015. The patients referred to public health centers with fever and one or more other symptoms including chills, headache, and muscular pain. Volumes of 5 ml blood were obtained from the patients, and after incubation at 4 °C for 24 h, the sera were separated and transported to the Department of Parasitology, Pasteur Institute of Iran within 7–10 days in sealed styrofoam boxes with ice cubes. Informed consent was obtained from all the adult participants, the parents or legal guardians of the children. This study was reviewed and approved by the Ethical Committee of Research, Pasteur Institute of Iran (project No. 626).

### 2.3. Indirect immunofluorescent antibody assay (IFA)

Regarding the occurrence of CCHF in Caspian Sea littorals, all the sera were initially checked for anti-CCHF antibodies by Department of Arboviruses and Viral Hemorrhagic Fevers Laboratory (National Reference Laboratory), Pasteur Institute of Iran as previously described (Chinikar et al., 2012). Then, the serum samples were screened by a homemade IFA using conjugated antibodies. Simply, the stationary phase *Leptospira interrogans* spirochetes with the density $10^9$/ml were inactivated with formaldehyde 0.25% for 10 min, centrifuged at 1712 × *g* for 15 min, and washed three times with PBS ($NaHPO_4$, $2H_2O$, 0.035%; $Na_2HPO_4$, $12H_2O$, 0.276%; NaCl, 0.9%; pH 7.4). The bacteria were resuspended in sterile water, circles of 0.5 cm diameter were placed on glass slides, and allowed to dry at room temperature. The bacteria were fixed with cold acetone for 30 min, and then the slides were wrapped in paper and kept at −20 °C until used. The patients' sera were diluted serially beginning with 1/10 down to 1/1280 in PBS pH 7.4. From the dilutions, 20 µl was added to the each circle and slides were incubated in a humid chamber for 30 min at 37 °C and then washed with PBS twice. Ten microliters of the 1/160 dilution of goat anti-human antibodies (Human IgG + IgM + IgA-heavy and light chain Antibody-FITC Conjugated, BETHYL Laboratories. INC) plus Evans Blue dye 1% was added to each circle followed by incubation and washing as before. The slides were allowed to dry, and then mounted with Glycerol 90% and examined under a fluorescent microscope at 1000X magnification. All the clinical samples were tested in duplicate, and negative and positive sera were included in all assays. The negative sera were from individuals residing in non-endemic areas for leptospirosis with no history of exposure to the disease. The positive sera were pooled samples from suspected individuals that were collected during transmission season in Gilan province and had anti-*Leptospira* antibodies titers ≥1/320 by IFA. The titers ≥1/80 in which at least ≈50% of spirochetes emitted fluorescence were considered positive.

### 2.4. Spiking experiments

A strain of *L. interrogans* was cultivated in liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) medium. After one week, the exponential phase bacteria were collected by centrifugation and washed by resuspension in PBS pH 7.4. Suspensions of live leptospires in PBS