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Diagnosis of Leptospirosis in Febrile Patients of Golestan Province, Iran: Serology versus PCR

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ABSTRACT

Introduction: Leptospirosis is a significant public health problem in the Caspian littoral of Iran comprising Gilan, Mazandaran, and Golestan provinces. In Golestan province, serology assays indicated anti-*Leptospira* antibodies in animals and humans; however, no reliable record of infections in patients with signs and symptoms of the disease is available. **Methods**: We employed the indirect immunofluorescent antibody assay (IFA), and two PCR assays, a real-time PCR (qPCR) and a nested-PCR targeting 16S rDNA (*rrs*) sequence for diagnosis of leptospirosis in febrile patients in Golestan province. **Results**: Out of 52 febrile patients, 25 (48.07%) had antibody titers ≥1/80 by IFA, and were defined as positive. In 7, 9, and 7 individuals, the antibody titers were 1/40, 1/20, and 1/10, respectively, and 4 had no antibodies. The qPCR and nested PCR detected leptospiral DNA in 55.75% and 67.3% of the patients' sera, respectively. The two PCR assays had a Kappa agreement of 0.53 ($P< 0.0001$), suggesting a moderate agreement, and showed a significant reverse association with the IFA titers ($P<0.05$). **Conclusion**: Our results suggest that higher antibody titers accompanied the spirochete's removal from blood by the patient's immune response. Hence, a reliable diagnosis of *Leptospira* infection necessitates deploying a DNA-based method alongside serology.

INTRODUCTION

Leptospirosis is the most widespread zoonotic disease in virtually all tropical and temperate areas worldwide. The pathogenic spirochetes in the genus *Leptospira* cause the disease, and humans acquire infection through direct contact with infected animals or contaminated waters with the urine of animal hosts [1-3]. The infections in humans present various clinical features ranging from a mild, flu-like illness to life-threatening manifestations, such as severe pulmonary hemorrhage syndrome and Weil's disease [4]. Annually, around 1.03 million humans acquire the infection worldwide, and some 58,900 dies [5]. The disease has a significant impact on people living in rural areas involved in farming, especially those working in paddy fields and urban settings with inadequate sanitary systems, e.g., slums [3, 5]. In Iran, the infection is endemic to the Caspian Sea littoral covering Golestan, Mazandaran, and Gilan provinces [6-9]. In this area, the disease appears as a seasonal infection, mostly affecting people in the countryside involved in agriculture and animal husbandry.

Previously, serology exhibited anti-*Leptospira* antibodies in horses [10], rodents [11], and humans [12] in Golestan province. However, no reliable record of symptomatic infections among individuals is available from this area. Here, we diagnosed the leptospirosis in suspected febrile patients in this province by the indirect immunofluorescent antibody assay (IFA) and two PCR assays, targeting the 16S rDNA (*rrs*) sequence.

MATERIAL AND METHODS

Study area. Golestan (before 1997, part of Mazandaran province) in the southeast of the Caspian Sea, is one of the Northern Provinces of Iran. It borders Mazandaran in the west, North Khorasan (Khorasan-e Shomali) in the east, and Semnan, in the south; it also shares a border with Turkmenistan in the north (Fig. 1). This province covers an area of 22,000 km², and, with 2,530,696 inhabitants, is one of the populous areas in the country [13]. According to the

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Köppen–Geiger classification, the Caspian Sea littoral has a temperate climate (C) with subdivisions hot-summer Mediterranean climate (Csa) and warm-summer Mediterranean climate (Csb) in Golestan province [14, 15]. With 60.000 hectares of rice paddies, this province, along with Gilan and Mazandaran, constitutes the country's leading rice cultivation center. In this province, the Caspian Sea coastal plains are mainly paddy fields, where different rodent species, mainly *Rattus norvegicus* and *rattus rattus*, the notorious reservoirs of leptospirosis abound. Hence, climate, environmental, and socioeconomic conditions favor the transmission of water-borne zoonotic diseases like leptospirosis in this area.

Clinical samples. Five ml of blood was obtained from 52 febrile patients residing in different counties of Golestan province (Fig. 1), from Jul. 2012 to Nov. 2013. The patients were referred to public health centers with fever and one other symptom, including headache and muscular pain. The samples were kept at 4ºC, and then transferred to the Department of Parasitology, Pasteur Institute of Iran, within 4–7 days under a cold condition. The sera were separated after incubation at 4 °C for 24 h and used for different assays. Informed consent was obtained from all the adult participants, and the parents, or the children's legal guardians. The Ethical Committee of Research, Pasteur Institute of Iran reviewed and approved this study (Project No. 626).

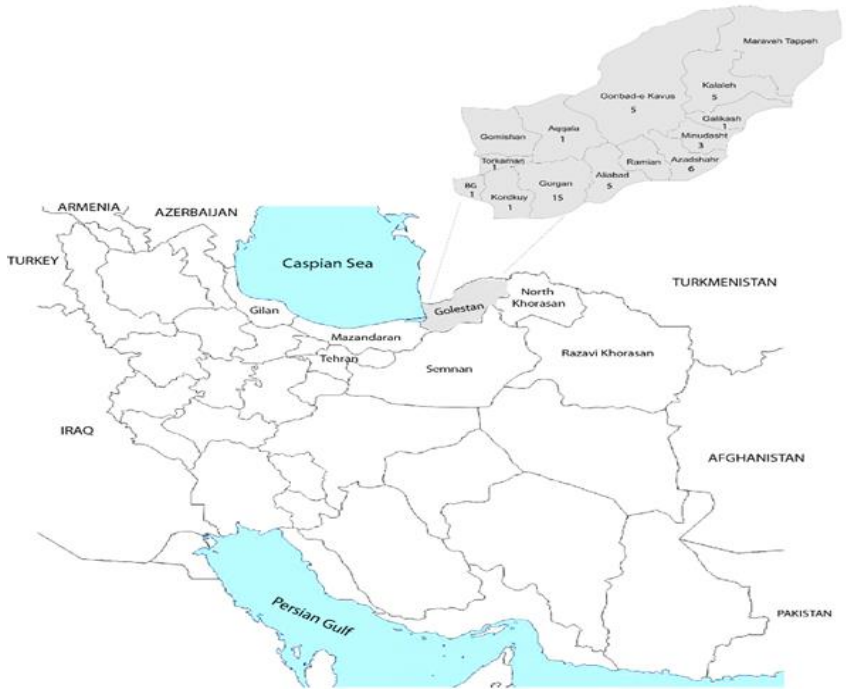


Fig 1. Map of Iran and the study area (Golestan province). The numbers indicate the patients in each county.

IFA. The serum samples were screened by a housemade IFA, as described previously [9, 16, 17]. The positive controls were the pooled sera from leptospirosis patients in the Caspian Sea littoral referred to health care centers during transmission seasons and had anti-*Leptospira* antibodies titers ≥1/160 by IFA. The negative control sera belonged to individuals residing in none leptospirosis endemic areas of Iran. The patients were defined as positive if their sera at titers ≥ 1/80 reacted with at least ≈50% of spirochetes in the microscopic fields resulting in fluorescence emittance. Regarding the occurrence of CCHF in the area [18], the sera were initially checked for anti-CCHF antibodies by the Department of Arboviruses and Viral Hemorrhagic Fevers Laboratory (National Reference Laboratory), Pasteur Institute of Iran as previously described [19].

DNA extraction. Amounts of 200 µl of sera were subjected to DNA extraction using the Miniprep DNA kit (QIAGEN, Hilden, Germany) with final elution using 100 µl of elution buffer. The extracted DNA quality was evaluated by the A260/A280 ratio using a small volume spectrophotometer (Picodrop Ltd, Cambridge, UK). The ratios with 1.7–1.9 range indicated pure and high-quality DNA samples.

Real-time PCR (qPCR). In qPCR assay, an 80-bp fragment was amplified by the primers, and the probe complementary to the *rrs* sequence (Table 1). The primers and the probe were designed and checked for specificity by the AlleleID software (http://premierbiosoft.com/bacterial-identification/index.html). The optimized primer/probe (metabion, Planegg, Germany) ratio and the annealing temperature were obtained by the Premix EXTaq™

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(TaKaRa Bio, Shiga, Japan). The final optimized 25 µl reaction tubes contained 1X Premix ExTaq™ (12.5 µl), 5 pmol (0.2 pmol/µl) of each primer, 2.5 pmol (0.1pmol/µl) probe, 5 µl of the template DNA, and Double distilled water (DDW) to the final volume. Amplification for all specimens was performed in duplicates in a Rotor-Gene 6000 instrument (Corbett Life Science, Sydney, Australia) programed for an initial denaturation at 95 °C for 1 min, followed by 40 cycles of denaturation at 95 °C for 10 sec, and annealing/elongation at 61 °C for 45 sec. DDW, as no-template controls (NTCs), were included in all assays to ensure DNA-free reagents. A ten-fold serial dilution of *Leptospira interrogans* containing approximately 7×10^5, 7×10^4, 7×10^3, 7×10^2, and 7×10^1, copy numbers of bacterial DNA per reaction, were used in the controls to measure the limit of detection. The PCR inhibitors and DNA efficiency in the extraction procedure were checked by amplifying a mammalian housekeeping β-actin gene in the clinical specimens by the primers previously used by others [20].

Nested PCR. A 291 bp sequence was amplified by a nested PCR assay using the primers complementary to the *rrs* sequence (Table 1), and the reagents and protocols successfully practiced by others [6, 16]. Previously, we could detect as low as ≥ 0.6 spirochete genome per reaction by this method [16].

Table 1. The primers and the probe used in qPCR and nested PCR assays.

Sequence	Primer	Amplicon size	Reference
rrs (qPCR)	5'-GTGA**H**CGGCCACA**W**TGGAA-3' 5'-TTC**S**CCCATTGAGCAAGATT-3' Probe: FAM 5'-ACACGGTCCATACTC CTACGGGAGGC-3' TAMRA	80 bp	**Present study**
rrs (Nested PCR)	5′- GGC GGC GCG TCT TAA ACA TG-3′ 5′-GTCCGCCTACGCACCCTTTACG-3 5'-CAAGTCAAGCGGAGTAGCAA-3' 5'-CTTAACCTGCTGCCTCCCGTA-3'	525 bp 291 bp	**[6]**

W is A, or T; **H** is A or C; T; **S** is G or C

Statistical analysis. Continuous data were described by mean ± SD, median and interquartile range (IQR). The agreement between the results of qPCR and nested PCR tests was estimated by the Weighted Kappa coefficient. Spearman's rho correlation coefficient was used to assess the correlation between qPCR threshold (C_t) values and IFA titers. Also, the median of C_t values was compared between different IFA titers using the Kruskal Wallis test. Statistical tests were considered significant at 0.05 levels. Analyses were performed in Stata software (version 11; Stata Corp, College Station, TX, USA).

Table 2. Diagnosis of leptospirosis by IFA and two PCR assays in febrile patients, Golestan Province, Iran

IFA Titer	No.	Threshold (C_t)			P_{ANOVA}	qPCR Positive samples	Nested PCR Positive samples
		Mean ± SD	Median (IQR)	Min, Max		No. (%)	No. (%)
1/320	6	35.9 ± 2.3	35.9 (34.3, 37.5)	34.3, 37.5	0.1912	2 (33.3)	3 (50.0)
1/160	6	31.9 ± 0.7	32.2 (31.1, 32.6)	31.1, 32. 6		3 (50.0)	3 (50.0)
1/80	12	36.0 ± 3.3	37.2 (33.9, 38.4)	30.4, 39.1		4(33.3)	3 (25.5)
1/40	8	34.5 ± 3.1	33.6 (33.0, 34.9)	31.4, 39.6		6 (75.0)	8 (100)
1/20	9	33.3 ± 2.6	33.5 (32.9, 35.1)	27.4, 35.8		8 (88.8)	9 (100)
1/10	7	34.6 ± 0.5	34.6 (34.2, 34.9)	34.2, 34.9		4 (57.1)	6 (85.7)
Negative	4	34.6 ± 2.7	32.7 (32.7, 36.5)	32.7, 36.5		2 (50.0)	3 (75.0)
Total	**52**					**29 (55.7%)**	**35 (67.3%)**
Spearman's rho: -0.0307; $P_{Spearman}$: 0.8817							

N: Number of tested sera. **SD:** Standard Deviation; **SEM**, standard error of the mean at the confidence level of 95%.

RESULTS

Patients. Febrile patients included 33 men and 19 women. For most of the patients, no data of the age and the occupation was available. Of the 44 patients that provided a resident address, 15 resided in Gorgan, 6 in Azadshahr, 5 in each county of Gonbad-e Kavus, Aliabad-e Katul, and Kalaleh, 3 in Minudash, and 1 in each county of Aqh-Qala, Kordkuy, Bandar Turkmen, Galikesh, and Bandar-e Gaz, (Fig. 1). Most people who lived in these counties had access to purified or clean water.

IFA. None of the patients was positive for CCHF virus antibodies. Among the 52 patients, 25 (48.07%) were positive for anti-*Leptospira* antibodies by the IFA with titers ranging from 1/80 to 1/320. In 7, 9, and 7 individuals, the antibody titers were 1/40, 1/20, and 1/10, respectively, and 4 had no antibodies (Table 2). All the positive control sera were positive with titers ≥1/160, and all negative control sera became negative.

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qPCR and nested PCR. The mean± C_t values for the spiked sera containing 7×10^5, 7×10^4, 7×10^3, 7×10^2, 7×101 copy numbers of bacterial DNA per reaction were 19.26±1.72, 24.82±2.20, 28.23±2.00, 33.22±2.87, and 36.92±2.6, respectively. Of the 52 clinical specimens, 29 (55.75%) became positive by the qPCR with the mean C_t value 34.11± 0.996. The C_t values for the sera with various IFA titers showed no significant difference (0.1912). In the nested PCR assay, 35 (67.3%) out of 52 clinical specimens, became positive (Table 2, Fig. 2), yielding the expected ≈290 bp amplicon.

Agreement between tests. Our results showed a weighted kappa coefficient of 0.53 between the results of qPCR and nested PCR assays, which shows a moderate agreement rate. The observed agreement rate was also significantly higher than the chance agreement (P< 0.0001, Table 3).

There was a significant discrepancy between IFA and PCR test results, in a way that 67.9% and 89.3% of IFA-negative cases were positive by qPCR and nested PCR tests, respectively. On the other hand, both the qPCR and nested PCR tests were negative in 62.5% of IFA-positive cases. The observed reverse association between IFA and molecular tests was statistically highly significant (Table 4). The percent of cases with positive qPCR and nested PCR results showed a gradual increase as the IFA titer increased up to 1/20 and 1/40, respectively. For both PCR tests, the percent of positive results were much lower in IFA titers ≥1/80 rather than titer ≤ 1/40 (Fig. 2).

There was also a week negative association between IFA titers and C_t values. This association, however, was not statistically significant (P_{Spearman} = 0.1912). The median C_t values were also not significantly different across different IFA titers (P_{ANOVA} = 0.1912, Table 2).

Table 3. Agreement between qPCR and nested PCR tests in diagnosing leptospirosis in febrile patients, Golestan, Iran.

	Nested PCR				
qPCR	No. of negative samples	No. of positive samples	Agreement (%)	Weighted Kappa	*P*-value
Negative	15	9	76.9	0.53	< 0.0001
Positive	3	25			

Kappa values ≤ 0 as indicating no agreement and 0.01–0.20 as none to slight, 0.21–0.40 as fair, 0.41– 0.60 as moderate, 0.61–0.80 as substantial, and 0.81–1.00 as almost perfect agreement

Table 4. Agreement between PCR and IFA tests in diagnosing leptospirosis in febrile patients, Golestan, Iran.

Molecular Test	**IFA§**		***P*-value**
	No. of negative samples (%)	**No. of positive samples (%)**	
qPCR			
Negative	9 (32.1)	15 (62.5)	0.0290*
Positive	19 (67.9)	9 (37.5)	
Nested PCR			
Negative	3 (10.7)	15 (62.5)	<0.0001**
Positive	25 (89.3)	9 (37.5)	
Total	28 (100)	24 (100)	

§ IFA titers ≤1/40 and ≥ 1/80 were considered as negative and positive, respectively. * *P*-value was generated by the Chi-Square test; ** *P*-value was generated by Fisher's exact test.

DISCUSSION

The Caspian Sea littoral, in the north of Iran, is an endemic area for leptospirosis. For a long time in this area, clinical features, especially manifestations, environmental conditions, and above all, seasonality, suggested an infectious disease that matched leptospirosis. However, due to the lack of access to reliable diagnostic tools, the disease remained neglected and underdiagnosed for many years. In the late 1990s, the disease received more attention in the country, and serological methods, mainly microscopic agglutination test (MAT), were employed to diagnose infection in both humans and animals. Early studies using MAT exhibited anti-*leptospira* antibodies in various animals, and later this method and ELISA were used to screen people for leptospirosis [20-21]. Most studies on human leptospirosis in the Caspian Sea littoral of Iran are from Gilan and Mazandaran, and the data on Golestan is very scarce. In the only survey on humans in 2012, out of 1028 randomly tested individuals, 10.4% showed anti-*Leptospira* IgG antibodies by ELISA in this province [12].

The MAT is still the gold standard for serodiagnosis of leptospirosis due to the high sensitivity and capability of detecting group-specific antibodies. However, it requires a continuous culture of various serovars to prepare live antigens, making this tool exclusive to well-equipped reference laboratories. The IFA exhibited a reliable specificity and sensitivity compared with the MAT for the initial diagnosis of leptospirosis [21-22]. Our housemade IFA was also shown to be a reliable tool for diagnosis and epidemiological surveys of leptospirosis [9, 16, 17].

In the present study, there was a significant discrepancy between IFA and PCR test results, in a way that 67.9% and 89.3% of IFA-negative cases became positive for qPCR and nested PCR assays, respectively. On the other hand, both types of PCR tests were negative in 62.5% of IFA-positive cases. The observed reverse association between serology

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and molecular tests was statistically highly significant (Table 4). The percent of cases with positive qPCR and nested PCR results showed a gradual increase as the IFA titer increased up to 1/20 and 1/40, respectively. The percent of positive results for both PCR tests were much lower in IFA titers ≥1/80 (Fig. 2).

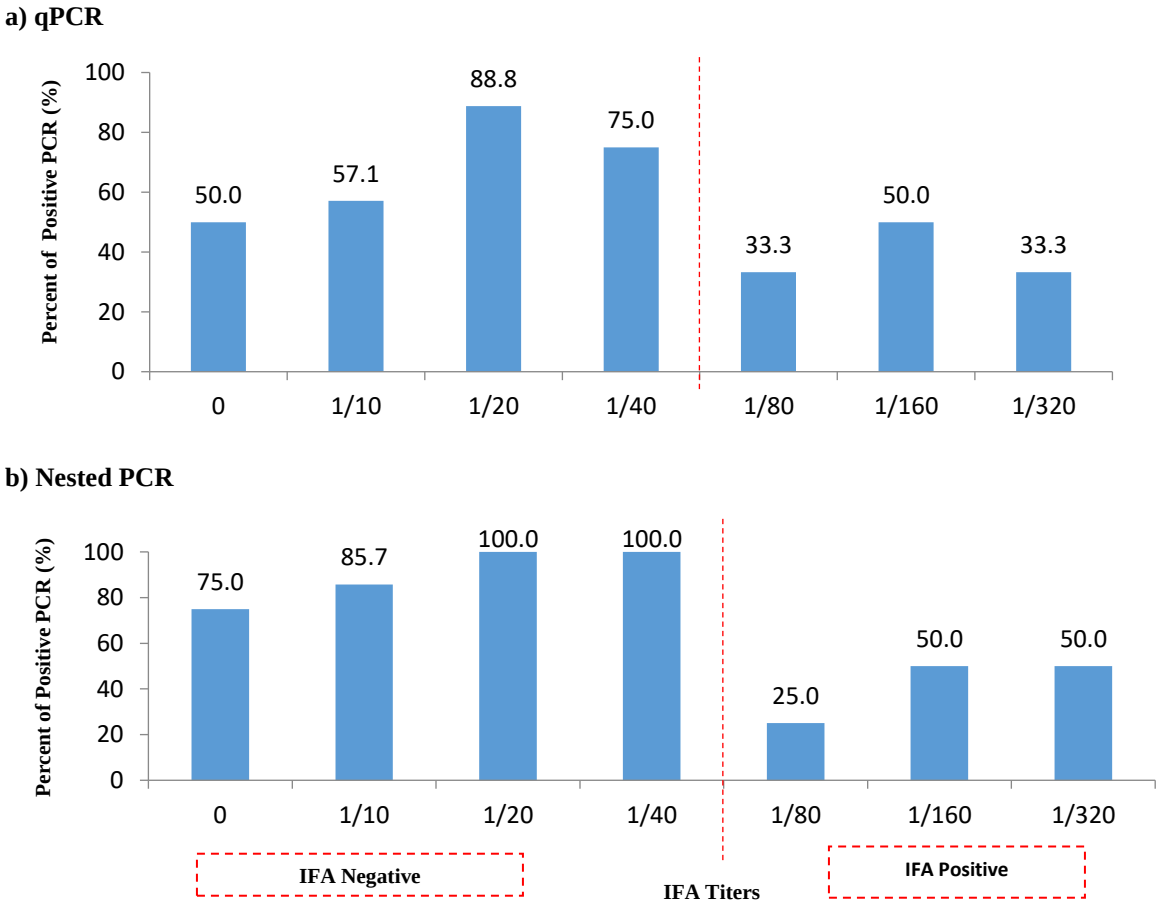


Fig 2. The percent of positive PCR test results by IFA titers. a) Percent of positive qPCR test results; b) Percent of positive nested PCR results.

In the current study, the IFA titers showed a slight inverse correlation with the qPCR C_t (Spearman's rho: -0.0307). Also, 67.9% and 89.3% of IFA-negative cases (titers ≤ 1/40) became positive for qPCR and nested PCR assays, respectively (Table 4). The observed association, however, was not statistically significant (*P*: 0.8817). Future studies with larger sample sizes may provide enough statistical power to detect real associations better. Our finding is in agreement with previous similar studies in the Caspian Sea littoral [9, 16], which showed that by the removal or reduction of *Leptospira* spirochete in blood by the patient's immune system, the antibodies titers increase. Previously, we showed that during the infection, the IFA becomes positive much later than PCR [16]. Hence, a reliable diagnosis of *Leptospira* infection necessitates deploying a DNA-based method alongside serology; the absence of either assay might result in underdiagnoses of infection.

We found both PCR assays reliable for early diagnosis of illness, but nested PCR was cheaper and appeared more appropriate for our laboratories in rural settings.

In the present study, most cases were from Gorgan, the Golestan province center, indicating more access to the health care centers by the inhabitants. Increased awareness of the infection in different parts of the province and strict surveillance, especially during transmission seasons, may result in the identification of more cases in remote areas of the province.

CONFLICT OF INTEREST

The authors declare there are no issues to be perceived as a conflict of interest with this article.

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