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Original Article

Seroepidemiology of leptospirosis in Guilan province, northern Iran: comparison between MAT and IgM-ELISA techniques

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Abstract

Introduction: Leptospirosis is a widespread zoonotic disease which is endemic in Guilan province, Iran. Besides economic losses in the dairy industry, leptospirosis is also considered an important public health problem. This study aimed to evaluate two serological techniques, MAT and IgM-ELISA for detection of leptospiral antibodies.

Methodology: A total of 185 samples were collected from individuals in Guilan province suspected of having leptospirosis from April 2016 to December 2016. Sera from participants were analyzed for *Leptospira* IgM antibodies using an available ELISA test and the MAT method. The specificity and sensitivity of the tests were calculated and compared.

Results: Of the 185 serum samples examined 114 (61.6%) and 94 (50.8%) samples were determined to be positive by MAT and IgM-ELISA, respectively. The results also showed that 17.5% of the sera that reacted positive in MAT were negative by IgM-ELISA, and 20.2% of IgM-ELISA positive sera were negative by MAT. We also showed that the MAT had specificity and sensitivity of 100%, when compared to leptospirosis-positive and negative serum samples. The specificity and sensitivity of IgM-ELISA was calculated as 78.8% and 82.4% respectively when compared with MAT. Bivariate analysis showed high correlation between the season, community of residence, possible reasons of pollution and leptospirosis ($P < 0.1$).

Conclusion: Rural areas of Guilan, especially rice farming areas, are endemic for leptospirosis. Rice farmers have a high risk of infection with leptospirosis; infection is associated with direct exposure to rodent urine, gender (male) and season (spring).

Key words: *Leptospira*; microscopic agglutination test; IgM-ELISA.

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Introduction

Leptospirosis as a serious zoonotic disease caused by pathogenic spirochetes belonging to the genus *Leptospira*. The most common etiological agent of this febrile illness is *Leptospira interrogans* (*L. interrogans*) and infection can lead to severe and potentially fatal illness [1]. It is estimated that over 500,000 new cases occur annually, with fatality range rising up to 70% in different cohorts [2]. Human hosts commonly acquire these pathogenic microorganisms through mucosal surfaces and skin abrasions following contact with soil or water contaminated with urine of infected rodents or other mammals [3]. It is believed that the initial presentation of leptospirosis in patients from an endemic area for is encephalitis [4].

Guilan province is located in the north of Iran, with an area of 14042 km^2 and a population of over 2.48 million. In regard to relative population density, Guilan is second after Tehran (capital) province in Iran. Human leptospirosis may be acquired through occupation or recreational exposures. Occupation risk factors can be divided into direct and indirect contacts. Direct contact with infected animals accounts for most infections in farmers, abattoir workers, veterinarians, rodent control workers, meat inspectors, and other occupations which contact directly with animals. Indirect contact is important for rice field workers, fish farmers and sewer workers. Farming and fishing are two main occupations of the people of the Guilan province. The seroepidemiology of leptospirosis is unknown in the Guilan province of Iran. Since rice farming in particular and agriculture in general are the two main jobs in the province contact with river and lake water is commonplace and transmission of *Leptospira* among the general population likely. There are not currently reliable statistics about the magnitude of *Leptospira* infection in the Guilan province. In local clinical laboratories there is limited availability of specific

diagnostic tests for leptospirosis, hence, treatment is often based on recognition of clinical manifestations to make a probable diagnosis [5]. Several methods have been proposed for laboratory detection of leptospirosis including detection of DNA by polymerase chain reaction (PCR), the microscopic agglutination test (MAT), detection of specific antibodies to the organism and culture methods [3]. Isolation of pathogenic *Leptospira* from the clinical specimens could be included along with the detection of DNA but is a technically demanding procedure.

The MAT is considered to be the reference immunological test for detecting both immunoglobulin M (IgM) and immunoglobulin G (IgG) class agglutinating antibodies. The test is performed in a few laboratories and requires a high level of technical expertise, and use of live pathogenic *Leptospira* standard cultures that increase the risk of laboratory acquired infection to the laboratory technicians [6]. The test has been established to be highly specific, although it has been demonstrated that there are some cross-reactions within serogroups and even between serogroups [7]. In spite of the limitations, the MAT is still considered the immunological "gold standard" or "reference standard" test for serological diagnosis of leptospirosis [8,3,9].

The ideal test for diagnosis of leptospirosis should have both high specificity and sensitivity during the acute phase of infection, be widely reliable and available, and give quick results [10]. IgM detectable enzyme-linked immune sorbent assay (IgM-ELISA) has these properties and is easier to carry out when compared with MAT. However, the diagnostic accuracy of IgM-ELISA has not been fully established [10]. Some studies have reported that IgM-ELISA has high specificity and sensitivity [11,12] and there remains much debate about the validity of using MAT as an immunological gold standard for evaluation of rapid diagnosis of leptospirosis [13]. In this study, we evaluated and compared two laboratory available tests, MAT and IgM-ELISA, for diagnosis of leptospirosis via detection of specific acute phase IgM antibodies. We also investigated the correlation between the behavioral, sociodemographic, and housing characteristics with leptospirosis - according to these two tests.

Methodology

Study population and serum preparation

In this study, a total of 185 patients with a presumptive diagnosis of leptospirosis were referred to the Reference Laboratory of the Guilan province. Patients were recruited from April 2016 to December 2016. Patients with suspected leptospirosis were diagnosed based on the World Health Organization-Leptospirosis Epidemiology Research Group (WHO-LERG) criteria [14], such as acute febrile illness with headache, jaundice, myalgia, arthralgia, oliguria, protreinuria, hematuria, anuria, meningeal irritation, conjuctival suffusion, cardiac arrhythmia, or a contact history of exposure to water or soil contaminated with urine of infected animals. Serum samples were taken during the symptomatic period of the disease and kept at -20 °C until performing of IgM-ELISA test. MAT was carried out immediately after recruitment.

For validation of the MAT test, eighty serum samples containing forty from negative controls and forty leptospirosis-positive serum samples (that kindly provided by Dr. Ali Afgar, Kerman University of Medical Sciences, Kerman, Iran) were used in this study.

Microscopic agglutination test

The *L. interrogans* serovars Canicola and Icterohaemorrhagiae (kindly provided from Department of Parasitology, Kerman University of Medical Sciences, Kerman, Iran) were used in this test. Each acute phase serum sample was initially diluted (1:25) with phosphate buffer saline (PBS, pH 7.2) in a 96-well round bottomed microtiter plate (Greiner-bio One, Frickenhausen, Germany) and 25 µL of PBS placed into each well of the plate and an equal volume of the diluted serum sample was placed in the first row of the plate. The diluted serum (1:50) was serially diluted (two-fold). Then, 25 µL of the live antigen (4-8 day-old cultures containing 10^8 CFUs/mL) was added to each well and incubated for 2 hrs at 37 °C. The test was examined by transferring a drop from each well onto a glass microscope slide. Agglutination was evaluated under a magnification of 200X using dark field microscopy. The reciprocal of the highest dilution agglutinating at least 50% or more of the antigen (live leptospires) was considered as the positive reporting titer. The end point titer was taken as the last well in which 50% or more agglutination was detected. The titer of ≥ 400 in acute samples were considered as MAT positive [3].

IgM-ELISA

The antigen used for ELISA was a genus-specific antigen (lipopolysaccaride) derived from boiling of pure culture of *L. interrogans*. This indirect ELISA technique was used based on protocols by Faezi *et al.* [15]. It was used with a working volume of 100 µL of

each reagent. The 96-well of microtitration plates (Immulon, Dynatech Laboratories, Alexandria, USA) were coated with 100 µL of 1:1600 dilution of LPS antigen in coating buffer (0.05 M carbonate/bicarbonate buffer, pH 9.6) for overnight at 4 °C. The plates were washed two times with washing buffer (PBS containing 0.05% (v/v) Tween 20) and then 100 µL of PBS-Tween 20 containing 2% bovine serum albumin (or BSA, Sigma, Steinheim, Germany) as blocking buffer was added to each wells to prevent non-specific binding and incubated for 2 hrs at 37 °C. After three times washing, 100 µL of known sera (1:200 diluted sera in PBS containing 0.03% Tween 20) as controls and the test sera were added to the wells in duplicate and incubated at 37 °C for 2 hrs. The plates were then washed four times and 100 µL of pre-diluted anti-human IgM alkaline phosphatase conjugated (Sigma, St. Louis, USA) in PBS-Tween 20 was added and incubated at 37 °C for 1 h. After four times washing, 100 µL of substrate p-nitrophenyl phosphate (Merck, Darmstadt, G ermany) was added to each well and incubated at 37 °C for 30 min. The optical density (OD) of each well was measured by microplate ELISA reader (LabSystems Multiskan, Houston, USA) at 450 nm wavelength. Sera were reported as positive if their OD readings were higher than the OD readings of the mean negative controls plus three standard deviations. Each test was performed with a positive control, negative control and cut-off calibrator (standards) in duplicate.

Interpretation of results was as follows: anti-Leptospiral IgM ≥ 20 IU/mL, positive result which is interpreted as a recent or current infection; 15–20 IU/mL, borderline result, suggesting that may be a recent infection and; < 15 IU/mL, negative result. suggesting no evidence of infection.

Specificity and sensitivity

The relative specificity and sensitivity (in percent) of the IgM-ELISA for the detection of leptospiral antibodies were calculated in comparison to the MAT as described below.

Sensitivity = a / (a + c) × 100, where "a" is the number of serum samples positive by the test and MAT, "c" the number of serum samples positive by MAT but negative by test.

Specificity = d / (b + d) × 100, where "d" is the number of serum samples negative by test and MAT, "b" the number of serum samples negative by MAT but positive by test [16].

Ethical Aspects

Ethics approval was obtained from the Ethics Committee of the Faculty of Medicine, Guilan University of Medical Sciences. The aims and procedures of the survey were explained to all participants and a written informed consent was taken from all of them. Informed written consent was obtained from parents or guardian on behalf of patients aged below 18 years.

Statistical analysis

We carried out the statistical analysis with the aid of the software SPSS version 18. The sample size was calculated with the following formula: a reference seroprevalence of 14.2% as the expected frequency for the factor under study, 2.48 million as the population size from which the sample was selected, 5% confidence limits, and a 95% confidence level. The result of the sample size calculation was 185 subjects. The results were analyzed by using the paired *t* test method. Housing, sociodemographic, and behavioral variables with a *P* value ≤ 0.1 obtained in the bivariate analysis were included in a multivariate analysis to verify their association with *Leptospira* seropositivity. We also determined the odds ratios (OR) and 95% confidence intervals (CI) by using logistic regression analysis with the backward stepwise method. The *P* value less than 0.05 was considered statistically significant [17].

Results

A total of 185 patients with acute fever and a diagnosis of suspected leptospirosis participated in this study. ELISA results were divided into three categories; positive, negative and borderline. Out of the 185 serum samples examined by IgM-ELISA, 73 (39.5%) of them had low (< 15 IU/mL), eighteen (9.7%) had borderline (15-20 IU/mL), whereas 94 (50.8%) had high (≥ 20 IU/mL) anti-*Leptospira* IgM antibody levels. Out of the 185 sera included in the final analysis, a total of 114 (61.6%) samples were determined to be positive for MAT as a reference standard which confirmed leptospirosis in the cohort, while 71 (38.4%) were negative. Among MAT positive serum samples, 24 (21%) were found to be infected by serovar Icterohaemorrhagiae, which was a predominant infecting serovar. Eleven (9.6%) were infected by serovar Canicola. When comparing the MAT and IgM-ELISA, 94 (50.8%) were positive to ELISA, while 114 (61.6%) were positive to MAT as an immuncapture test.

The MAT was compared to eighty serum samples, forty negative sera and forty leptospirosis-positive

serum samples. This comparison showed that the MAT has sensitivity and specificity of 100%. This value was considered the basis for comparison of sensitivity and specificity with IgM-ELISA test. Nineteen (20.2%) serum samples were positive by ELISA and negative by MAT. Twenty (17.5%) were positive to MAT and negative to ELISA. In the other hand, 20.2% and 17.5% of cases with ELISA were considered as false positive and false negative, respectively. Seventy-one (97.2%) were negative to ELISA and negative to MAT. Ninety-four (82.4%) which were positive tby MAT were positive by ELISA. According to the results of the paired *t*-test conducted at 95% confidence interval between MAT and IgM-ELISA, the *t* value was found to be 0.39, and correlation 0.499, therefore these two tests were statistically compatible. MAT had a sensitivity of 100%, while IgM-ELISA had a sensitivity of only 82.4%; there was a significant difference in sensitivity between the two methods ($P < 0.05$). The specificity of MAT (100%) was significantly ($P < 0.05$) higher than that of IgM-ELISA (78.8%). Taken all together, MAT retained good levels of specificity or sensitivity.

The behavioral characteristic, baseline demographic and housing conditions of participants and the correlation with *Leptospira* seropositivity are shown in Table 1 according to the results of IgM-ELISA and MAT as a standard test. The 97 subjects of enrolled participants had 45 or less than 45 years old; 72 subjects had 46 to 65 years old; and 16 subjects had > 65 years old. No significant difference was seen between age and leptospirosis by MAT ($P = 0.78$) and ELISA ($P = 0.74$) tests. With respect to the behavioral, sociodemographic and housing characteristics studied, a significant

Table 1. Baseline sociodemographic, housing, and behavioral variables and seroprevalence of *Leptospira* exposure by bivariate analysis. P values in red were considered.

Characteristics	Number of subjects tested	Positive MAT tested			Positive ELISA tested		
		Number	%	*P* value	Number	%	*P* value
Gender							
Male	114	69	60.5	0.69	57	50	0.78
Female	71	45	63.3		37	52.1	
Occupation							
Rice farmer	93	63	67.7		53	56.9	
House keeper	51	27	52.9		23	45	
Employee	14	8	57.1	0.45	6	42.8	0.5
Free worker	14	9	64.2		6	42.8	
Fish farmer	5	3	60		3	60	
Others *	8	4	50		4	50	
Season							
Spring	103	58	56.3		43	41.7	
Summer	74	51	68.9	0.023	47	63.5	0.017
Autumn	8	5	62.5		4	50	
Habitat							
Village	155	100	64.5	0.06	84	54.1	0.03
Town	30	14	46.6		10	33.3	
Drinking water							
Pipeline	133	87	65.4		71	53.3	
Well	45	23	51.1	0.22	21	46.6	0.35
Fountain	7	4	57.1		2	28.5	
Possible reason of pollution							
Work in farm	110	74	67.2		63	57.2	
Fish farming	1	0	0	0.04	0	0	0.22
Swimming	1	0	0		0	0	
Exposure to							
Dogs	1	0	0		0	0	
Animal urine	19	7	36.4		7	36.8	
Contaminated water	1	0	0	0.04	0	0	0.22
Cattles	1	1	100		1	100	
Stagnant water	8	3	37.5		2	25	
Others @	43	29	67.4		21	48.8	

* students, sellers, ranchers, coach, retired and drivers. @ work in farm as a second job.

difference ($P \leq 0.1$) was observed in the season and community of residence in the positive MAT and ELISA tests. Also, according to the positive MAT test, the results of possible reasons of exposure, such as work in the farm, contact with animal urine, stagnant water and individuals who work in the farm as a second job, was significant ($P \leq 0.1$) by bivariate analysis. Other sociodemographic, housing, and behavioral variables, including gender and drinking water sources such as pipeline, well and fountain had *P* values greater than 0.1 by this analysis. Multivariate analysis of sociodemographic, housing and behavioral characteristics with *P* values < 0.1 obtained by bivariate analysis showed that exposure to *Leptospira* was positively associated with community of residence (OR = 2.33; 95% CI: 1.41-3.13; $P = 0.066$), season (OR = 2.79; 95% CI: 1.67-5.92; $P = 0.017$) and the possible reasons of pollution (OR = 2.05; 95% CI: 1.91-4.48; $P = 0.042$).

Discussion

Leptospirosis can cause a significant economic impact, so serosurveillance and diagnosis are very important for any control program. Various serologic screening tests have been developed in the field for leptospirosis. The present study was designed to compare MAT and IgM-ELISA for better screening of human leptospirosis. The overall seroprevalence rate of leptospirosis in the Guilan province was 61.6% and 50.8% according to MAT and IgM-ELISA tests, respectively. These results indicate that leptospiral infection is a widespread infection that should be considered. This finding, along with other reports of leptospirosis suggests that leptospirosis may be a more frequent infection in the rural areas of Guilan than previously described [18,19]. IgM-ELISA showed 82.4% sensitivity compared to MAT. Nineteen samples positive to IgM-ELISA were negative to MAT (false positive). On the other hand, twenty samples positive to MAT were negative to IgM-ELISA (false negative).

Rural inhabitants and rice farmers were dominant in the study; therefore, all of them could be affected by direct contact with animal urine. We also demonstrated that the highest prevalence of leptospirosis was in the first six months of the year when the weather is warm and farmers work barefoot during a day. Leptospirosis is also called Paddy fever in north of Iran, where rice is the most frequent crop of the farmers (more than 80% of the cases). The results of this study suggested that leptospirosis is endemic in the rural areas of Guilan in people engaged in rice production, as well as the north of Iran. Previous studies from other rice-producing countries such as Sri Lanka, Thailand, Brazil and India have documented leptospirosis as an occupational infection of rice farmers [20,21,3,22]. The current study also revealed that living in villages and rural areas is associated with a high risk of contact with rodents and rats, exposure to the river and stream water with high probability of contamination with urine of rodents/rat or other animals suspected to leptospirosis, which is in agreement with other studies [23-25]. The infection rate in men was higher than women, but the effect was not statistically significant. Individuals less than 45 years were the most affected among the study population. The results are in agreement with the results of some reports [26,19], but are different from some other reports [20,24].

Conclusion

In conclusion, the findings of this survey indicate that rural areas of Guilan province especially in rice farming areas are endemic for leptospirosis. We demonstrated serological evidence of *Leptospira* exposure in the rural population in the northern Iran. The contributing factors associated with Leptospirosis found in this study may be useful for optimal planning of control programs against *Leptospira* infection. Further research on the epidemiology of *Leptospira* infection in the northern Iran is needed.

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Authors' contributions

AA; execution of this study and manuscript preparation, AE; statistical analysis of data, MF; sampling and statistical analysis of data, SH; scientific advisor, SF; corresponding author and planning of study.

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