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Simultaneous Detection of Pathogenic and Saprophyte Leptospira in Human Plasma by Multiplex Taqman Real Time PCR

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Abstract

Background: Leptospirosis is an infectious disease caused by pathogenic and saprophytic Leptospira species. The clinical and laboratory diagnosis of this infection is complicated. However, timely diagnosis of leptospirosis is essential for treatment of this disease. Conventional laboratory methods are incapable in the early diagnosis of it. Molecular tests such as real time PCR are very efficient when diagnosing it.
Objectives: In this study, we designed and developed a multiplex Taqman real time PCR to simultaneously detect saprophyte and pathologic Leptospira in clinical samples.
Methods: 250 human plasma samples were obtained from suspected patients. Two pair specific primers and the corresponding probe for detecting pathogenic and saprophytic Leptospira were designed and established in a single tube. The developed tests were run on all DNA extracted from the samples.
Results: Of the 250 samples, 93 (37.2%) were positive for pathogenic and 15 (6%) for non-pathogenic cases. In two samples, pathogenic and non-pathogenic DNA strains were simultaneously positive.
Conclusions: Based on our finds, the real time PCR is a suitable test for the diagnosis of leptospirosis and differentiation between pathogen and saprophyte Leptospira simultaneously.

Keywords: Leptospira, Real Time PCR, Saprophyte, Leptospirosis

1. Background

Leptospirosis is a zoonotic disease scattered throughout the world (1, 2). For the first time, Adolf Weil explained an acute and severe form of the disease with jaundice, which was later known as the Weil's disease (3). The agent of Leptospirosis is a spirochete, which belongs to genus Leptospira. In fact, Leptospirosis is an occupational disease and is generally common between farmers, herders, veterinarians, fishers and slaughterhouse workers (4, 5). In addition to ones occupation, water games, swimming in contaminated waters and migration to endemic countries are also considered risk factors (6). Bacteria is transferred to humans through direct contact with urine of infected animal or indirect contact with water and soil contaminated with the urine of these animals. The bacteria enters the body through broken skin or hair follicles as well as the blood stream and then spread to organs such as liver, brain and other tissues. The bacteria then stays there and eventually causes an infection to the organs (1).

The differential diagnosis of leptospirosis with other febrile infectious diseases is difficult for the physician and laboratory diagnosis of this disease, which has changed it to a neglected disease (7). However to date, using molecular techniques and DNA analysis of identified Leptospires, 20 pathogenic, 6 saprophytic species and 1 intermediate species have been identified in humans, animals, soil and surface water (3). Saprophyte species are commonly found in soil, surface and environment water (8). Main pathogenic Leptospira belong to *Leptospira interrogans* and main saprophytic Leptospira belong to *Leptospira biflexa* that each have many strains (9).

Recently, some reports have been published that some saprophytic strains have isolated from patients (3, 10). Saprophytic and pathogenic Leptospira have similar appearances and morphology, however only laboratory tests can distinguish them from each other. Fast and accurate differential diagnosis of pathogenic and saprophytic strains from each other is essential for treatment management, epidemiologic planning and controlling infection (11).

Up to now, microscopic agglutination test (MAT) is known as the gold standard serological test for the diagnosis of Leptospirosis. This test is incapable in the early diagnosis of the disease and can only detect the disease at least one week after the establishment of Leptospira and antibodies' production. Other serological methods, similar to MAT, also have inappropriate diagnostic sensitivity and specificity (12). For a more accurate and earlier diagnosis of leptospirosis, more sensitive and faster easily accessible methods that can be simply performed are required to manage the treatment strategy of patients better as well as to control the epidemiologic of the disease more successfully (11).

In recent years molecular biological methods such as PCR and real time PCR, looking for DNA and 16s rRNA of microorganisms, have been used as a useful tool to detect microorganisms in a variety of biological samples. According to the available information to date, Real Time PCR technique have been used for a number of tests to identify the pathogenic and saprophytic strain of Leptospira, which have been used for the diagnosis of Leptospirosis (12). Up to now, only a few tests are available for the differential diagnosis of pathogenic and saprophytic strains of Leptospira at the same time (13, 14). The importance and necessity of early detection and diagnosis of pathogenic and saprophytic strains in human Leptospirosis is very obvious for treatment and epidemiologic studies. Thus, in this study a Taqman multiplex real time PCR was designed to simultaneously identify pathogenic and saprophytic strains in one reaction tube. For this purpose, LipL32 and 16s rRNA genes were aimed as pathogenic and saprophytic strains targets. LipL32 genes encode a lipoprotein that can be used as an adhesion molecule to laminin-collagens and fibronectin bands (15-17). LipL32 lipoprotein is a major outer membrane protein of Leptospira, which can only be found in pathogenic strains and absent in saprophytic bacteria (3, 18, 19).

In the present study, Taqman primers and probes that are specifically for LipL32 and 16s rRNA genes were designed so that it becomes possible to separately identify pathogenic and saprophytic Leptospira targets. Appropriate multiple reactions for the primer and probe were optimized and then performed on human samples suspected to Leptospirosis.

2. Methods

2.1. Leptospira Strain

A panel of reference strains of pathogen Leptospira intrroganse included in Pomona, Grippotyphosa, Icterohaemorrhagiae, Hardjo, Ballum, Canicola and *Leptospira biflexa* (saprophytic) were obtain from the Leptospira research laboratory of veterinary faculty of Tehran University, collaborating center of WHO, Tehran, Iran. All Leptospira strains were subcultured in Ellinghausen Mccullough Johnson Harris broth media (EMJH) (Difco; USA) and enriched with 10% rabbit serum at 30°C for one week.

2.2. Clinical Samples

Two hundred fifty samples were calculated based on the sample size statistical formula (Equation 1). These ETDA plasma samples were collected from patients with Leptospirosis clinical signs that had been referred to the approval laboratory of Guilan University of Medical Sciences for Leptospirosis. Previous studies were reported that EDTA plasma samples gave good results for DNA amplification tests in leptospirosis (17). About one milliliter of remained plasma specimen from each patient was centrifuged at high speed for 30 minutes and then the entire sediment of each sample was processed for DNA extraction. The supernatant were used for serological testing such as microscopic agglutination test (MAT).

$$n = \frac{\left[Z_{1-\frac{\alpha}{2}}(c+1) + Z_{1-\beta}\sqrt{(c+1)^2 - (c-1)^2 \times P}\right]^2}{(c-1)^2 \times P} = \frac{\left[1.96(3+1) + 0.84\sqrt{(3+1)^2 - (3-1)^2 \times 0.22}\right]^2}{(3-1)^2 \times 0.22} = 154 \quad (1)$$

(Note: P = 0.22, C = 3, 1-α = 0.8 and 1-β = 0.95).

2.3. Microscopic Agglutination Testing (MAT)

Based on the standard method, microscopic agglutination testing was performed at the laboratory of veterinary medicine faculty of Tehran University, using live cultured of *Leptospira interrogans* serovars panel consist of six antigens (mentioned above). Members of this panel were predominantly isolated from the geographical area where our samples had been collected in the previous study (20).

2.4. DNA Extraction

The genomic DNA from standard *Leptospira interrogans* serovars (as pathogenic strain) and *Leptospira biflexa* (as saprophytic strain) were extracted from the pellet of one ml broth cultured medium using the QIAamp DNA mini kit according to its manual (Qiagen; Germany). The DNA of clinical plasma samples were extracted from their prepared sediments. The concentrations of extracted DNA from all samples were measured and equivalent (identical

value) by the Spectrophotometer ND-1000 Nanodrop (3411 Silverside Rd, Bancroft Building, Wilmington, DE 19810, USA) and then applied in Taqman real time PCR reactions.

2.5. Taqman Real Time PCRs Set Up

The LipL32 genes of pathogenic Leptospira and the 16s rRNA gene of saprophytic strain sequences were aligned for findings of the conserved sequences by the Allele ID 7.6 software. The specific primer and Taqman probes for this region were designed by the software and checked by blastn on the NCBI database (Table 1). The chemical and physical conditions of Taqman Real time PCRs were optimized by ABI one step real time PCR equipment using extracted DNA from standard strains mentioned above as well as a Takara master mix (Takara, Japan).

Table 1. Primers and Probe Sequence for Detection of Pathogen and Saprophyte *Leptospira* spp.

Name	Primer or Probe Sequence	Target Gene
Patho_F	5'-GCGATTCAGTTTAATCCT-3'	LipL32-Pathogen
Patho_R	5'-AATGTATTCTTTTGTGTGAG-3'	LipL32-Pathogen
Patho_Probe	5'-Fam-AGAATTGGCTGAGAATTTGAAA-Tamra-3'	LipL32-Pathogen
Sapro_F	5'-GGATAACCTACCTAGAAGTT-3'	16s rRNA-Saprophyte
Sapro_R	5'-CATTGCTGCTTTAACCAA-3'	16s rRNA-Saprophyte
Sapro_Probe	5'-Hex-CGAATGTGACGGTTCCTGGTAG-Tamra-3'	16s rRNA-Saprophyte

In preparation, one microliter of genomic DNA (about 100 ng) was amplified in 20 μL of a reaction mixture containing 10 μL master mix, 0.5 μL of 10 pmol of each primers (Pathogen and saprophyte) and 0.3 μL of 10 pmol of each Taqman probs (Pathogen and saprophyte). The optimized Taqman real time PCR conditions began with 10 minutes of the initial denaturation step at 95°C, 40 cycles of comprised denaturation at 94°C for 30 seconds and then annealing and extension at 60°C for 45 seconds. Then the Optimized multiplex Taqman real time PCRS were run for all clinical DNA samples. All reactions with a CT over 35 were considered as negative (Figure 1).

In addition, the detection limit of our Real Time PCR assays were also found using the serial 10-fold diluted of the genomic DNA from leptospires in either Ellinghausen-McCullough-Johnson- Harris medium and spiked plasma specimens as a minimal number of bacteria that could be visually detected. Moreover, some another bacteria that cause disease similar to Leptospira infections also were tested.

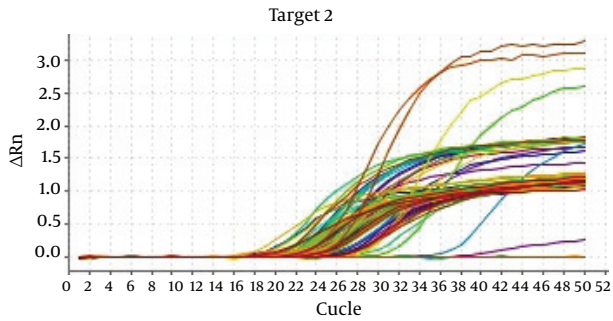

Figure 1. Ampliphication Plot of Multiplex taqman Real Time PCR

2.6. Assessing the specificity of PCR reaction

In order to assess the specificity of the used primers, DNA extracted from 6 reference pathogenic strains (said above) and DNA of reference saprophytic strains were used for the PCR test. All mentioned DNAs entered the multiplex Taqman real time PCR reaction using specific primers and probs of pathogenic and saprophytic strains. In addition, DNA of some febrile-causing non-Leptospira bacteria such as *Salmonella typhimurium*, *Shigella dysentery*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Legionella pnemeuphila* and *Streptococcus pyogenes* were also used in the above mentioned reactions (2, 12, 21).

2.7. Assessing the Sensitivity of PCR Reactions

To determine the sensitivity of the primers, pure cultures of pathogenic and non-pathogenic strains were used. The Leptospira strains were grown in special culture mediums and were diluted by negative plasma by serial dilution. Using the 0.5 McFarland turbidity, the standard concentrations were prepared and diluted serially for up to 10 bacteria per ml plasma. The pellet obtained through one mL of each prepared dilution was entered in the DNA extraction reaction. Then, optimized reactions of multiplex taqman real time PCR were done on the extracted DNA (2, 12, 21).

2.8. Statistical Analysis

All results were analyzed by the SPSS16.0 software.

3. Results

3.1. Clinical Samples

Of the 250 plasma samples that were collected from patients suspected with leptospirosis who referred to the central laboratory of Leptospirosis diagnosis in Guilan

province, 142 were male and 108 were female with a mean age of 46 years. All patients worked as farmers on fields. All samples were obtained from patients in the first week of the disease.

3.1.1. Assessing the Specificity of PCR Reaction

DNA obtained from references trains were only multiplied by their specific primers while non-specific DNAs (obtained from non-Leptospira) had no proliferative reaction. In addition, the DNA of some febrile-causing non-Leptospira bacteria (stated before) were also used in the above mentioned reactions, in which no proliferative reaction was observed. In other words, specific primer and probs for saprophytic and pathogenic Leptospira had no reaction on bacterial genome other than Leptospira.

3.1.2. Assessing the Sensitivity of PCR Reactions

Both primer and probe series, specific of pathogenic and saprophytic strains proliferated up to 50 bacteria per ml in the multiple response and the proliferative responses were clearly positive.

3.2. Recognition of DNA of Pathogenic and Non-Pathogenic Leptospira Bacteria in Patients' Samples

There were a total of 250 samples from suspected patients of Leptospirosis from different genders and ages. Multiplex Taqman real time PCR reactions, which were previously optimized by DNA of pathogen and saprophyte Leptospira, were run on DNA samples from all patient's plasma specimens. Of the 250 samples, 93 (37.2%) were positive for pathogenic and 15 (6%) for non-pathogenic cases. In two samples, pathogenic and non-pathogenic DNA strains were simultaneously positive.

4. Discussion

Routine diagnosis of Leptospirosis is usually made by serological based methods (5). The most common tests for the diagnosis of this disease include MAT and ELISA, which are widely used (3, 15). Medical diagnosis laboratories mostly use ELISA tests because they are easily available. On the contrary, the MAT test has performance limitations and is not easily applicable in clinical laboratories, however it is still considered and used as the gold standard test for Leptospirosis diagnosis. Since all serological based tests depend on the immune system's response, they become applicable after a while and the duration of time depends on the system reaction and antibodies' production (3, 22, 23). Another problem of serological tests is that they do not represent the real infection, due to the long half-life of antibodies and the fact that they may have remained from the previous infections. Therefore, their presence cannot indicate the current infection and the increase in antibody concentration must be checked, which require two samples with an interval that is also time-consuming (24). However, due to all the problems that late diagnosis of Leptospirosis creates for patients, including the relatively high mortality rate of the disease, lack of techniques that can detect the infectious disease fast and accurately have kept the field of study and research open in this regard. Researchers have conducted numerous studies on fast and on-time diagnostic test for this disease but in fact, an appropriate test confirmed to supply physicians' needs has not been provided yet. Some part of this issue relies on the nature of the disease that is not easily identifiable by routine standard bacteriologic methods; for example, Leptospira is not easily cultivated in the laboratory and requires a long time. Therefore, when bacteria cannot easily be cultivated with microbiological techniques and direct method, it should be done with other appropriate techniques. In the early days of entry of Leptospira into the body, the number of bacteria is low (3) in the blood and detection of this amount of bacteria is also one of the problems for the diagnosis of Leptospirosis. Molecular-based techniques such as PCR are able to detect and identify infectious agents through tracking and multiplying DNA of microorganisms. These techniques never depend on the growth of microorganisms and work by identifying genomes of infectious agents and can identify them in the early days of microbes entering the body with high efficiency. On this basis, different molecular techniques have been developed and used in the diagnosis of multiple infectious agents such as Leptospira. Molecular tests such as Taqman Real Time PCR, have high sensitivity and specifity and can detect the infectious agents in the early days of entry to the body and can also track small amounts of these factors (25, 26). The present study used the Taqman Real Time PCR technique to evaluate its diagnostic power for Leptospirosis. A number of patients who were referred from different cities for confirmation of the diagnosis of Leptospirosis to reference laboratories of Guilan province were selected and blood samples were taken from them. The samples were tested by commonly available serological tests, such as ELISA and MAT and the tested by the optimized Taqman Real Time PCR reaction to track the Leptospira bacteria. The results were remarkable. Some of the samples with positive serological test results had no proliferative reaction in the molecular test and conversely a number of samples that had Leptospira bacteria genome (Pathogen Real Time PCR positive) had a negative serological test result. The time of taking samples with positive Leptospirosis DNA in plasma were closer to the beginning point of exposure of infectious agent

and vice versa, samples with positive serological test result had a greater distance from the beginning of the infection. A noticeable fact regarding Leptospirosis is that recently some researchers have reported the presence of some saprophytic Leptospira strains in some patients with Leptospirosis (11, 13, 27). This may be the reason for the non-response of the Leptospirosis diagnostic tests that are designed only for pathogenic strains. Therefore, in this study, the simultaneous presence of both pathogenic and saprophytic strains in patients with suspected Leptospirosis was assessed using Multiplex Taqman real time PCR techniques to elucidate this ambiguity. Thus, in the designed multiple reactions, DNA extracted from plasma samples obtained from patients with suspected Leptospirosis entered one test tube at the same time using primers and probes for pathogenic and saprophytic strains for DNA amplification. One of the interesting results was that a number of selected patients with clinical signs of Leptospirosis were positive for saprophytic Leptospira in the real time PCR test while pathogenic Leptospira and other non-Leptospira agents were also positive. Perhaps these results explain Leptospirosis in them. However, it is required that this issue is also evaluated in other geographical areas, both within the country and in other countries, by similar studies and also have their results be evaluated.

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Footnote

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