

Simultaneous Detection and Differentiation of Pathogenic and Nonpathogenic *Leptospira* spp. by Multiplex Real-Time PCR (TaqMan) assay

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Abstract

Leptospirosis, caused by pathogenic *Leptospira*, is one of the most important zoonoses in the world. Several molecular techniques have been developed for detection and differentiation between pathogenic and saprophytic *Leptospira* spp. The aim of this study was to develop a rapid and simple assay for specific detection and differentiation of pathogenic *Leptospira* spp. by multiplex real-time PCR (TaqMan) assay using primers and probes targeting *Leptospira* genus specific 16S ribosomal RNA gene, the pathogen specific *lig A/B* genes and non-pathogenic *Leptospira biflexa* specific 23S ribosomal RNA gene. Sixteen reference strains of *Leptospira* spp. including pathogenic and nonpathogenic and ten other negative control bacterial strains were used in the study. While the 16S primers amplified target from both pathogenic and non-pathogenic leptospires, the *ligA/B* and the 23S primers amplified target DNA from pathogenic and non-pathogenic leptospires, respectively. The multiplex real-time PCR (TaqMan) assay detection limit, that is, the sensitivity was found approximately 1×10^2 cells/ml for *ligA/B* gene and 23S ribosomal RNA gene, and 10 cells/ml 16S ribosomal RNA. The reaction efficiencies were 83–105% with decision coefficients of more than 0.99 in all multiplex assays. The multiplex real-time PCR (TaqMan) assay yielded negative results with the ten other control bacteria. In conclusion, the developed multiplex real-time PCR (TaqMan) assay is highly useful for early diagnosis and differentiation between pathogenic and non-pathogenic leptospires in a reaction tube as having high sensitivity and specificity.

Key words: *Leptospira* genus, leptospirosis, multiplex real-time PCR (TaqMan) assay, pathogenic *Leptospira*, saprophytic *Leptospira*

Introduction

Leptospirosis is caused by spirochetes of the genus *Leptospira* (Guerra, 2009). The *Leptospira* genus has been classified into 17 species by DNA-DNA hybridization. This genus is further divided into three groups as pathogenic, nonpathogenic, and opportunistic/possibly pathogenic. The pathogenic leptospires include eight species: *Leptospira interrogans*, *Leptospira kirschneri*, *Leptospira borgpetersenii*, *Leptospira santarosai*, *Leptospira weilii*, *Leptospira alexanderi*, *Leptospira genomospecies 1* and *Leptospira noguchii* (Brenner *et al.*, 1999). The *Leptospira* genus has also been classified on the basis of surface antigen patterns. They are divided into at least 250 serotypes that have major antigens in common and are combined into 24 serogroups (Dutta and Christopher, 2005).

Leptospirosis is identified as one of the emerging infectious diseases and a major public health concern worldwide. The organism affects virtually any mammal, including humans. Humans acquire the organisms through contact with contaminated soil, water, vegetation or with the body fluids of animals harboring leptospires (Palaniappan *et al.*, 2005). More than 500 000 severe leptospirosis cases have been reported each year around the world (WHO, 1999). The rate of leptospirosis cases varies depending on the climate and animal reservoirs. The incidence of leptospirosis is especially highest during the summer season with heavy rains and floods (Xue *et al.*, 2008).

Since the currently available microscopic agglutination test (MAT) that is a known gold standard does not permit early diagnosis and other serologic methods have low sensitivity, more rapid and sensitive methods

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are needed for detection of *Leptospira* as well as the ability to distinguish pathogenic and nonpathogenic *Leptospira*. Therefore, a number of molecular methods such as conventional multiplex PCR (Kositanont *et al.*, 2007), real-time PCR (Slack *et al.*, 2007; Levett *et al.*, 2005), nested-PCR (Bomfim *et al.*, 2008), loop mediated isothermal amplification (LAMP) (Lin *et al.*, 2009), nested PCR-restriction fragment length polymorphism (RFLP) (Djadid *et al.*, 2009) have been developed for specific detection of pathogenic *Leptospira* spp. in diagnostic laboratories. The TaqMan real-time PCR method has also been used for detection of pathogenic *Leptospira* spp. based on specific target sequences including the ribosomal 16S ribosomal RNA gene, and the *Leptospira* immunoglobulin-like protein A and B gene (*lig A* and *lig B*) (Palaniappan 2005). The *lig A* and *lig B* genes encode amino-terminal lipoprotein signal peptides followed by 10 or 11 big domain repeats. The *lig* genes are only detected in pathogenic *Leptospira* spp. as a unique virulence factor. Conversely, these genes are not found in nonpathogenic *Leptospira* spp. (Matsunaga *et al.*, 2003).

The aim of this study was to develop a rapid and simple assay for the specific detection and differentiation of pathogenic *Leptospira* spp. by multiplex real-time PCR TaqMan method using primers and probes specific for *Leptospira* genus 16S ribosomal RNA gene, pathogen specific *lig A/B* genes and non-pathogen *Leptospira biflexa* specific 23S ribosomal RNA gene.

Experimental

Materials and Methods

***Leptospira* reference strains and control bacterial strains.** Sixteen reference strains of *Leptospira* spp. including pathogenic and nonpathogenic were obtained from the Etlik Central Veterinary Control and Research Institute (ECVCRI), WHO Collaborating Center, Ankara, Turkey (Table I). All strains were stored in Fletcher media (Difco, Detroit, MI, USA) and then cultured in liquid Ellinghausen McCullough Johnson Harris (EMJH) (Difco) media supplemented with 10% serum of hemolysed rabbit blood at 30°C for 7 days. Other control bacterial strains including *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 13883, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 27270, *Salmonella typhimurium* NCTC 12023, *Legionella pneumophila*, *Neisseria gonorrhoeae* NCTC 8375, *Borrelia burgdorferi* strain B31, and *Streptococcus pyogenes* NCTC 12696 were selected from stock reference culture collection in our laboratory. The isolates were stored at -70°C

in trypticase soy broth (Merck, Darmstadt, Germany) supplemented with 15% glycerol before being tested.

Design of primers and probes. Oligonucleotide primers and probes for multiplex real-time PCR (TaqMan) assay were designed based on a particular region of the *ligA/B* gene sequence (*ligA/B*-P1-5'-cgggtc tcactctattcaaa-3', *ligA/B*-P2-5'-attgaagaatcgat gagaa-3', and *ligA/B*-Probe-Texas red-5'-atcctgtaaa tcctt ctcttgcaaa-3'-Bhq-2) for pathogenic *Leptospira* spp. (Genbank accession nos FJ030916, EF517920, AF534640, AF368236, and AY221109), the 16S ribosomal RNA gene sequence (16S-P1-5'-tagtgaacgg gatagatac-3', 16S-P2-5'-ggtctacttaacccgttagg-3', and 16S-Prob-Fam-5'-aatccacgcctaacacgttgctac-3'-Bhq-1) for *Leptospira* spp. genus (Genbank accession nos FJ154560, FJ154600, FJ154577, FJ154571, FJ154569, FJ154568, FJ154564, FJ154563, FJ154556, FJ154555, and FJ154553), and the 23S ribosomal RNA gene sequence (23S-P1-5'-acaattctacaaaccctatc-3', 23S-P2-5'-ttaccactagcgtagatt-3', and 23S-Prob-Joe-5'-tcgaactgtgaactgaagtactgca-3'-Bhq-1) for non-pathogenic *L. biflexa* (Genbank accession no CP000786) by using oligo analysis and design program Oligaware 3.0 developed in our institution (Table II). BLAST program was used to initially assess the ability of the primers and probes to identify target sequences (Smythe *et al.*, 2002; Altschul *et al.*, 1997). The primers and probes were synthesized by Metabion International, Germany.

DNA extraction. DNA was extracted from the samples by treatment with 1% SDS and 100 mg proteinase K (Sigma Chemicals, St. Louis, Missouri, USA) in a buffer containing 50 mM Tris (pH 8.0), 50 mM EDTA, 100 µM NaCl. After 2 hours of incubation at 55°C in a waterbath, the DNA was purified by repeated extraction with phenol/chloroform/isoamyl alcohol (25:24:1). The DNA was concentrated by precipitation with 99% ethanol. The precipitate was collected by centrifugation, then dried and resuspended in deionized sterile water (Veloso *et al.*, 2000).

Multiplex real-time PCR (TaqMan). The multiplex real-time PCR (TaqMan) method was performed by using a 7500 ABI Prism Sequence Detector (Applied Biosystems, Foster City, Calif., USA). In brief, 2 ml of the extracted nucleic acid solution was added to 23 µl of reaction mixture containing 0.8 µM of each primer and 0.4 µM each fluorophore probe (final concentration), and mixed with 12,5 µl of TaqMan Universal PCR Master Mix (Applied Biosystems). The TaqMan cycling conditions included a 10 min degradation of the preamplified templates at 95°C and then 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 60 s. All experiments were repeated at least twice for testing the reproducibility of the assay.

Detection limits determination and assay validation. Pathogenic *L. interrogans* strain Wijnberg and

Table I
Leptospira strains used in this study

Genomospecies	Serovar	Strain
<i>Leptospira interrogans</i>	australis	Ballico
<i>Leptospira interrogans</i>	autumnalis	Akiyami A
<i>Leptospira interrogans</i>	bataviae	Swart
<i>Leptospira interrogans</i>	bratislava	Jes Bratislava
<i>Leptospira interrogans</i>	hebdomadis	Hebdomadis
<i>Leptospira interrogans</i>	icterohaemorrhagiae	Wijnberg
<i>Leptospira interrogans</i>	hardjo	Hardjoprajitno
<i>Leptospira. interrogans</i>	canicola	Hond Utrecht IV
<i>Leptospira interrogans</i>	icterohaemorrhagiae	RGA
<i>Leptospira interrogans</i>	pomona	Pomona
<i>Leptospira interrogans</i>	pyrogenes	Salinem
<i>Leptospira borgpetersenii</i>	ballum	Mus 127
<i>Leptospira borgpetersenii</i>	javanica	Veldrat Batavia 46
<i>Leptospira borgpetersenii</i>	tarassovi	Perepelitsin
<i>Leptospira kirschneri</i>	grippotyphosa	Moskova V
<i>Leptospira biflexa</i>	patoc	Patoc I

Table II
Oligonucleotide sequence of the primers and probes used in this study

Target region	Oligo Name	Sequence (5'-3')	Genbank accession no
<i>ligA/B</i> gene for pathogenic <i>Leptospira</i> spp.	<i>ligA/B</i> -P1 (Forward)	5'-cggttctcacttcttctcaa-3'	FJ030916, EF517920, AF534640, AF368236, and AY221109
	<i>ligA/B</i> -P2 (Reverse)	5'-attgaagaatcgatgagaa-3'	
	<i>ligA/B</i> -Prob	Texas Red-5' -atcctgtaaatccttctcttgcaaa-3'-Bhq-2	
23S rRNA gene for non-pathogenic <i>Leptospira</i> spp.	23S-P1 (Forward)	5'-acaatcttaccacacctatc-3'	CP000786
	23S-P2(Reverse)	5'-ttaccacttagcgtatgatt-3'	
	23S-Prob	Joe-5'-tccgaactgtactgaactgaagtactgca-3-Bhq-1	
16S rRNA gene for <i>Leptospira</i> genus	16S-P1(Forward)	5'-tagtgaacgggattagatagac-3',	FJ154560, FJ154600, FJ154577, FJ154571, FJ154569, FJ154568, FJ154564, FJ154563, FJ154556, FJ154555, and FJ154553
	16S-P2 (Reverse)	5'-ggtctacttaatccgtagg-3',	
	16S-Prob	Fam-5'-aatccacgccctaactgtgtctac-3'-Bhq-1	

L. biflexa strain Patoc I were cultured in EMJH (Difco) media to obtain a cell density of 1×10^8 cells/ml for each of the target species, and then total DNA was extracted. Serial 10-fold dilutions of extracted DNA were prepared ranging from 1×10^6 cells/ml to 1×10^0 cells/ml by using sterile ddH₂O. The threshold cycle (Ct) value of each dilution was recorded. The lowest number of leptospires detected visually was defined as the detection limit of the assay.

Results

Multiplex real-time (TaqMan) PCR assay using LigA/B, 16S, and 23S primers and probes. The sixteen standard strains of *Leptospira* spp. were tested for all genes. The multiplex real-time PCR (TaqMan) assay was used to amplify specific leptosiral sequences including *ligA/B* genes, 16S ribosomal RNA gene and

23S ribosomal RNA gene simultaneously in a single tube. The *ligA/B* primers and probe designed based on particular region of *ligA/B* of pathogenic leptospires gave a detectable product, Ct values ranged from 17 to 39 and averaged 24 for only all pathogenic leptospires, whereas the 23S primers and probe designed based on the particular region of 23S ribosomal RNA non-pathogenic leptospires gave a detectable product Ct values ranged from 18 to 36 and averaged 23 for only non-pathogenic leptospires. The 16S primers and probe designed from the conserved region of 16S ribosomal RNA of *Leptospira* genus amplified genomic DNA from both pathogenic and non-pathogenic leptospires.

Sensitivity and specificity of multiplex real-time PCR (TaqMan) method. The reaction efficiency of each assay was counted from the slopes of standard curves. The reaction efficiencies were found to be 83–105% with decision coefficients of more than 0.99 in all multiplex assays. To determine assay sensitivity,

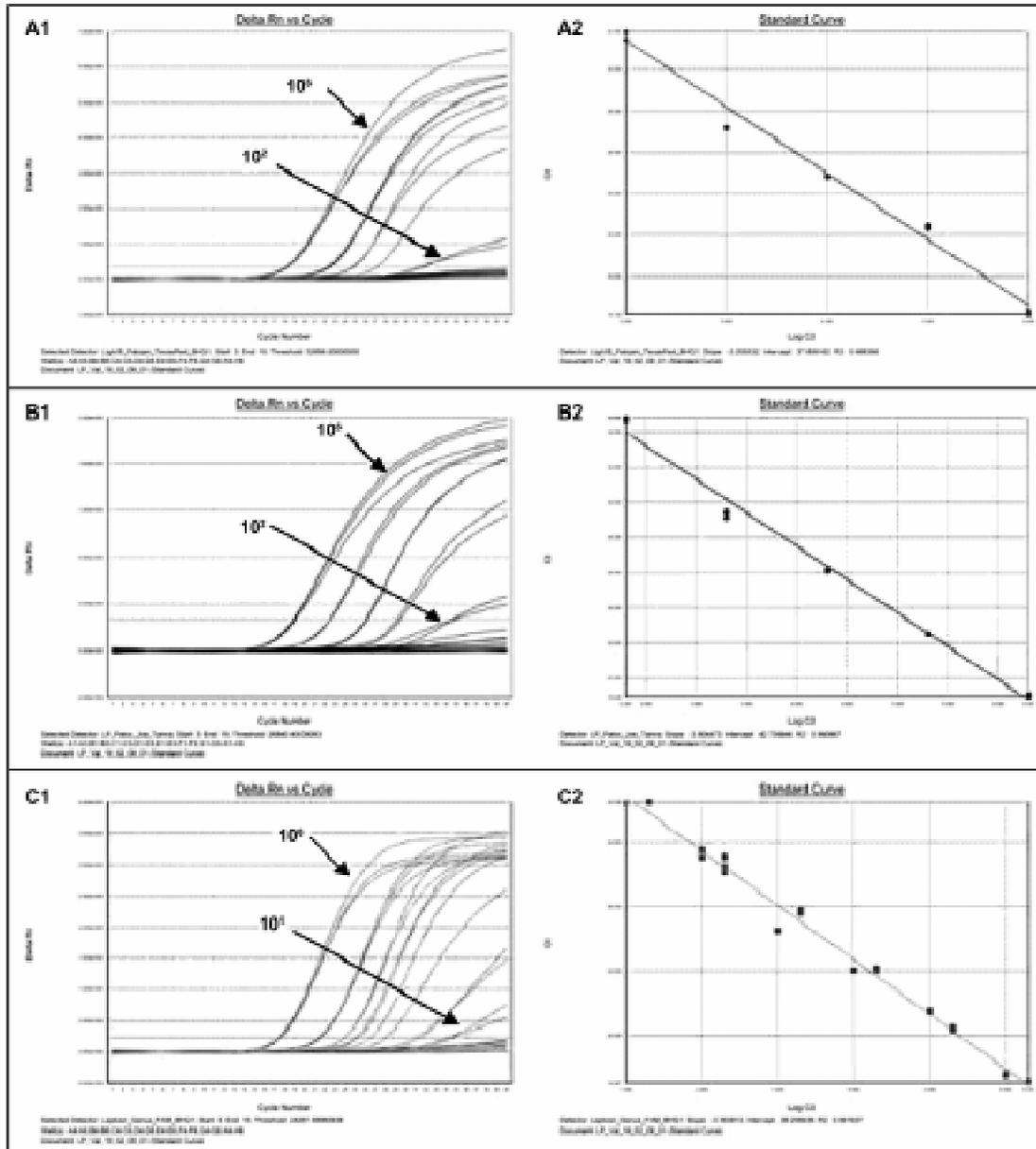


Fig. 1. Amplification curves and dilution end point standard curves of log₁₀ genome equivalents versus CT cycle number. The analytical sensitivity of this assay for *ligA/B* (A1-A2) and 23S ribosomal RNA (B1-B2) assays were approximately 100 genome per ml and 16S rRNA (C1-C2) assay was approximately 10 genome equivalents per ml.

Leptospiral DNA was adjusted by 10-fold serial dilution ranging from 1×10^6 cell/ml to 1×10^0 cells/ml from both pathogenic *Leptospira interrogans* strain Wijnberg and *Leptospira biflexa* strain Patoc I. Linear dynamic range of multiplex real-time PCR (TaqMan) assay was performed with triplicates of 10-fold dilutions of the purified genomic DNA extracted from pathogenic and nonpathogenic leptospires, and was observed constantly (Fig. 1). The standard curves obtained with 10-fold serially diluted genomic DNA preparations were linear over seven orders of magnitude for targeting sequence. The assay detection limit, that is the sensitivity, was found to be approximately 1×10^2 cells/ml for *ligA/B* gene and 23S ribosomal RNA gene, and 10 cells/ml 16S ribosomal RNA. To

determine the assay specificity of multiplex real-time PCR (TaqMan) assay, other control bacteria were tested with *ligA/B*, 23S and 16S primer and probes. The multiplex real-time PCR (TaqMan) assay yielded negative results with the ten other control bacteria. The *ligA/B*, the 16S, and 23S primers and probes were specific for pathogenic leptospires, *Leptospira* genus, and nonpathogenic leptospira, respectively (Fig. 2).

Discussion

Leptospirosis is known to be an emerging zoonotic disease caused by the genus *Leptospira* worldwide (Guerra, 2009). Since there is no national *Leptospira*

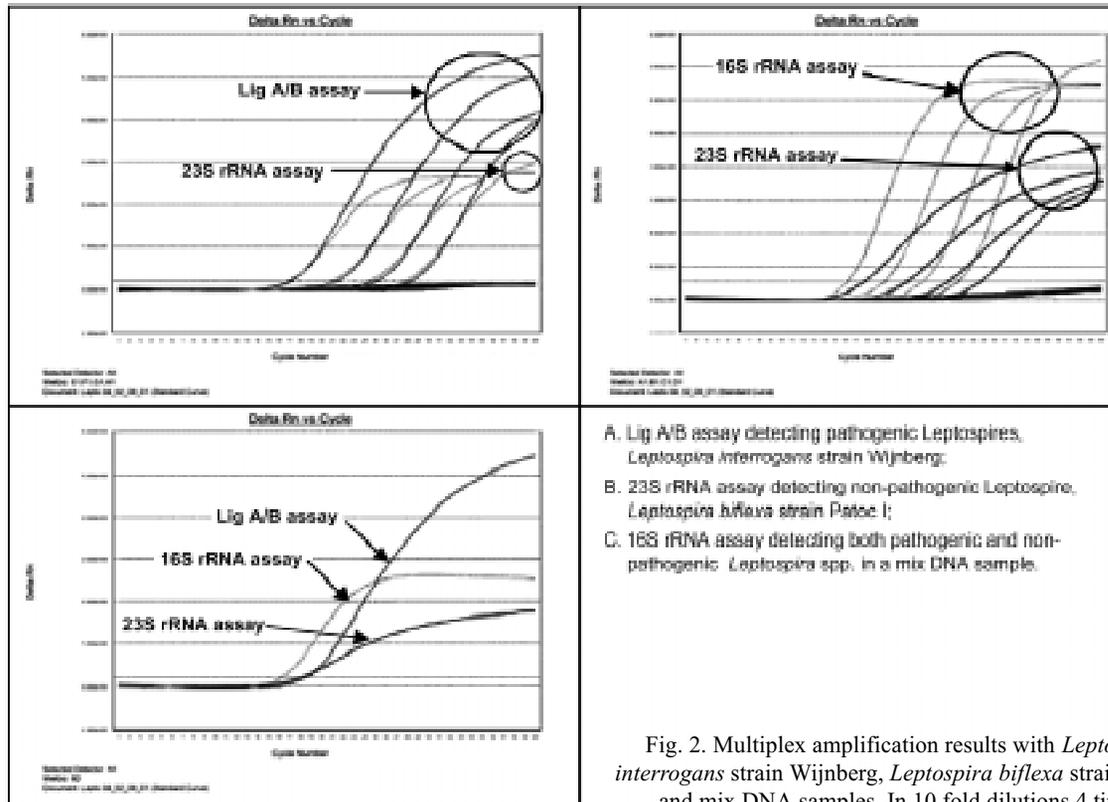


Fig. 2. Multiplex amplification results with *Leptospira interrogans* strain Wijnberg, *Leptospira biflexa* strain Patoc I and mix DNA samples. In 10 fold dilutions 4 times.

reference center in Turkey, reliable data about the incidence of leptospirosis are not available. Twelve Turkish cases were reported from south region of Turkey in 1997 (Saltoglu *et al.*, 1997). In 2005, three cases of anicteric leptospirosis with mild and severe complications, a case of icteric leptospirosis with resistant immune hemolytic anemia, and four cases of anicteric leptospirosis with mania and psychosis were reported from the central Anatolia and northwest region of Turkey (Erdinc *et al.*, 2006; Solmazgul *et al.*, 2005; Semiz *et al.*, 2005). Recently a Turkish leptospirosis case complicated with cerebral venous thrombosis was reported from the northwest region of Turkey (Turhan *et al.*, 2006).

A spectrum of human leptospirosis is recognized ranging from subclinical infection to a severe syndrome of multiorgan infection such as severe vasculitis and intravascular coagulation (Guerra, 2009). However, the symptoms of leptospirosis at the prodromal stage are almost indistinguishable from various other bacterial and viral febrile infections such as salmonellosis and dengue virus infection (Srimanote *et al.*, 2008). In addition, the lack of reliable techniques for rapid diagnosis of leptospirosis may cause delay in treatment of patients and lethal sequela (Djadjid *et al.*, 2009). In diagnosis of leptospirosis, serological methods such as MAT and enzyme-linked immunosorbent assay (ELISA) based on the immunogenic response of the host and culture method to observe the organisms from clinical samples are usually per-

formed in many laboratories. MAT is used to detect leptospiral antibody from the patient sera using live organisms as sources of antigens. Since antileptospiral antibody becomes detectable only after 8–10 days from the onset of illness, MAT cannot provide early diagnosis of leptospirosis (Smythe *et al.*, 2002). In addition to MAT, other serologic tests detecting leptospires from clinical samples are not appreciable, especially during the early phase of the infection (Palaniappan *et al.*, 2005; Srimanote *et al.*, 2008). *Leptospira* is a fastidious microorganism and difficult to grow in culture medium. Moreover, culture which requires a special medium and at least two weeks to yield the organisms, cannot provide an early diagnosis. Thus, these techniques are used mainly for epidemiological purposes (Palaniappan *et al.*, 2005; Srimanote *et al.*, 2008). Therefore, there is a need for a reliable and accurate method for detection and differentiation of leptospires from clinical samples. The multiplex real-time PCR (TaqMan) assay is a rapid, sensitive and specific assay for detection of leptospires during early stage of infection (Palaniappan *et al.*, 2005; Smythe *et al.*, 2002; Slack *et al.*, 2006). This assay has also been used as a diagnostic tool for the discrimination of pathogenic and non-pathogenic leptospires using particular gene sequence (Smythe *et al.*, 2002).

In this study, we developed a multiplex real-time PCR (TaqMan) assay to detect infection and the same time distinguish pathogenic leptospires from non-pathogenic leptospires in same reaction tube. The

ligA/B, 23S, 16S primers and probes were used for specific detection of pathogenic, non-pathogenic leptospires and leptospira genus. This assay was able to detect rapidly leptospira genus and distinguish pathogenic leptospires from non-pathogenic ones. The detection limit of the assay was 100 leptospira cells/ml for pathogenic, non-pathogenic leptospires, and 10 cells/ml for leptospira genus. While the 16S primers amplified the target from both pathogenic and non-pathogenic leptospires, *ligA/B* amplified target DNA only from pathogenic leptospires and the 23S primers were specific for non-pathogenic ones. Thus, this assay may enable the recognition and discrimination between pathogenic and non-pathogenic, environmental contaminant leptospires in the same reaction tube.

A number of gene sequences have been used to detect and distinguish pathogenic leptospires from non-pathogenic ones. Woo *et al.* developed a real-time PCR (TaqMan) method using 23S rRNA gene sequence for identification of pathogenic *Leptospira* (Woo *et al.*, 1996). Levett *et al.* evaluated a highly sensitive and specific real-time PCR (TaqMan) method for *lipL32* gene, which is a probable virulence gene, for detection of pathogenic leptospires (Levett *et al.*, 2005). Slack *et al.* used DNA gyrase subunit B gene for identification of pathogenic *Leptospira* spp. When compared to the 16S rRNA gene, the *gyrB* gene showed greater nucleotide/evolutionary divergence allowing superior identification (Slack *et al.*, 2006). Kawabata *et al.* used *flab* gene and suggested that PCR-RFLP was an efficient tool for rapid detection and identification of species of *Leptospira* from clinical specimens (Kawabata *et al.*, 2001). Djadid *et al.* developed a nested PCR-RFLP assay using 16S rRNA as a rapid and specific available technique for differentiate pathogenic and non-pathogenic *Leptospira* spp. in the early stage of infection (Djadid *et al.*, 2009). In this study, the *ligA/B* primers were used in a multiplex real-time (TaqMan) PCR assay to detect and distinguish pathogenic *Leptospira* serovar. The *lig* genes encode surface proteins containing immunoglobulin-like repeat predicted to play a role in adhesion to host tissues. Previous studies have demonstrated that pathogenic leptospires contain these genes, while they are absent from the non-pathogenic saprophyte (Xue *et al.*, 2008). Diagnostic methods such as PCR and ELISA targeting *lig* genes have been recently developed to discrimination pathogenic and non-pathogenic leptospires (Palaniappan *et al.*, 2005; Srimanote *et al.*, 2008). Palaniappan *et al.* developed a real-time PCR (TaqMan) assay using *lig1/lig2* primers targeting the conserved region of *ligA* and *B* as a sensitive and rapid tool for early diagnosis of leptospirosis (Palaniappan *et al.*, 2005). One of the most significant advantages of our multiplex real-time PCR (TaqMan) assay is that both pathogenic and non-patho-

genic leptospires were detected at the same cycling conditions allowing all three reactions to be performed in a single PCR tube simultaneously. We also analyzed leptospires based on the genus in the same reaction tube as an internal control. To our knowledge, this is the first study detecting three leptospire gene sequences in the same reaction tube. The efficiency of each assay ranged between 83 and 105%; the accepted ranges for PCR efficiency are generally between 80.0 and 110.0%.

In conclusion, the developed multiplex real-time PCR (TaqMan) assay targeting *ligA/B* gene, 16S ribosomal RNA and 23S ribosomal RNA sequences is highly useful for early diagnosis of leptospirosis and differentiation between pathogenic and non-pathogenic leptospires in the same reaction tube with high sensitivity and specificity.

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