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Original Article

Application of a Multiplex PCR Assay for Molecular Identification of Pathogenic and Non-Pathogenic Leptospires based on lipL32 and *16S rRNA* Genes

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Abstract

Leptospirosis is a serious zoonotic infection and the most prevalence disease is in the tropical and subtropical region. The definitive diagnosis of Leptospirosis, caused by spirochetes of the genus *Leptospira* infection is already using culture methods, serological tests such as the microscopic agglutination test (MAT) and molecular detection methods (PCR) are possible. In this study, we used multiplex PCR method for detection of pathogenic and non - pathogenic *Leptospira* based on *lipL32* and *16S rRNA* genes. All serovars were obtained from the *Leptospira* Reference Laboratory of Microbiology Department, Razi Vaccine and Serum Research Institute, Karaj, Iran. The PCR product for the *lipL32* and *16S rRNA* genes was 272 bp and 240 bp respectively. The sensitivity amplification for the multiplex assay was 10^{-6} pg / µl for *16S rRNA* gene and 10^{-4} pg / µl for *lipL32* gene. The sensitivity for multiplex PCR was 10^{-3} pg / µl. The results supported the idea that multiplex PCR can be used to detect *Leptospira* samples. This method was also able to differentiate between saprophytic and pathogenic leptospires and was able to do so much easily than conventional methodologies. Due to the slow growth of *Leptospira* and the importance of time in diagnosis, molecular methods such as PCR are suggested.

Keywords: *Leptospira*, 16S rRNA gene, lipL32 gene, Multiplex PCR, Molecular Identification

1. Introduction

Leptospirosis is an acute febrile illness caused by pathogenic Leptospires. This zoonotic disease has a worldwide distribution but is most common in tropical and subtropical regions and has the greatest impact on public health in developing countries (1, 2). The infection of humans occurs by direct or indirect contact with the urine of infected animal (3, 4).

Laboratory diagnosis of leptospirosis is based on several methods such as culture, serology methods like MAT and ELISA, molecular detection (PCR) (5-7). Culture has low sensitivity and requires specialized expertise and is time consuming (6, 8). Although MAT is used as a reference method for diagnosis in various studies, it is not a suitable method because it is based on the use of a live bacteria, with false negative results (low sensitivity) in the early course of infection and is time consuming (9, 10).

PCR has been used to detect leptospiral DNA in samples obtained from animals (11, 12) and human (13, 14), with quick results and high sensitivity. This method is superior to the methods of culture and MAT (13, 14). The multiplex PCR method is used for rapid and simultaneous detection. This method allowed detection of several PCR products (3).

The *lipL32* gene is a major outer-membrane lipoprotein from the genus *Leptospira*. (15) LipL32 is highly conserved among pathogenic *Leptospira* species (15, 16) but has no present in the saprophyte *Leptospira* biflexa (17). The *16S rRNA* gene is used for phylogenetic studies (18) because it is highly conserved between different species of bacteria (19). However, *16S rRNA* gene sequence has hypervariable regions, which can be used to identify bacteria. Identification of pathogenic and non-pathogenic *leptospirea* has been used by *16S rRNA* gene (20, 21).

Therefore, the purpose of this study was molecular detection of pathogenic and non-pathogenic *Leptospira* by Multiplex PCR assay based on *lipL32* and *16Sr RNA* genes.

2. Materials and Methods

2. 1. Bacterial Serovars

The bacterial serovars used in the study are presented in table 1. All serovars obtained from the *Leptospira* Reference Laboratory, Department of Microbiology, Razi Vaccine and Serum Research Institute, Karaj, Iran.

Table 1. Bacterial serovars used in the study

Serogroup	Serovar	Code NO.(RTCC)
Pathogenic *L.interrogans*	Autumnalis	2802
L. interrogans	Canicola	2805
L. interrogans	Grippotyphosa	2808
L. interrogans	Serjo	2821
L. interrogans	Pomona	2822
L. interrogans	Grippotyphosa	2825
L. interrogans	Celledoni	2832
L. interrogans	Canicola	2836
L. interrogans	Icterohaemorragiae	2837
L. interrogans	Ballum	2838
L. interrogans	Australis	2840
L. interrogans	Serjo	2843
Nonpathogenic *L. biflexa*	Semaranga	2819
L. biflexa	Semaranga	2828
Negative control *S. enterica*	Enteritidis	1621

2. 2. Culture

The bacteria were subcultured in a selective culture medium of EMJH with rabbit serum and enrichment supplements under aerobic conditions at 28 ° C for 7 - 10 days. Their growth was investigated by a dark field microscope.

2. 3. DNA Extraction

Culture isolate were centrifuged at 17000 X g for 20 min at 4 ° C. The genomic DNA was extracted by the standard phenol-chloroform method. The quality and quantity of the extracted DNAs were evaluated by electrophoresis and by UV spectrophotometry and analyzed by 1% agarose gel electrophoresis. DNA was stored at -20°C until used for PCR.

2. 4. Multiplex PCR

In this study, we have used the Multiplex PCR method with 2 sets of primers, which will aid in the specific and sensitive detection of *Leptospira* species.

The characteristics of primers used in this study are presented in table 2.

The LipL32 PCR assay was done to amplify a 272bp fragment from pathogenic *Leptospira*. The *Lipl32* gene is specifically pathogenic leptospiral and is present only in pathogenic serovars.

The 16S rRNA PCR assay was done to amplify a 240bp fragment from saprophytic *Leptospira*, using previously described specific primers (20). These primers were expected to amplify saprophytic leptospiral DNA.

These two sets of primers allowed us to distinguish between pathogenic and saprophytic *Leptospira* species. *Lipl32* gene is amplified in pathogenic species and *16S rRNA* is amplified in the saprophytic species.

To determine the proper concentration of each primer, the values of 5, 10, 15 and 20 pmol of primers were evaluated in the PCR test. Sensitivity testing was performed for both pairs of primers. The sensitivity of the PCR was determined using a serial dilution of genomic DNA leptospiral.

Multiplex PCR amplification was performed in a final reaction volume of 16 µL. It consisted of 8 µl 2X Master Mix, 1 µl (10 pM) from each of the forward and reverse primer and 10 ng of template DNA. The PCR was performed in thermocycler (Eppendorf, Germany). The reaction mixture was initiated by incubation at 95 ºC for 5 minutes, followed by 35 cycles of denaturation

at 95 ºC for 30 seconds, annealing at 61 ºC for 30 seconds, extension at 72 ºC for 1 minute, and a final extension at 72 ºC for 10 minutes.

The PCR products were electrophoresed on 1% agarose gels and stained with safe staining (Sinagen). Gels were photographed using a gel documentation system (Bio-Rad Chemi XRS Gel Documentation system, UAS).

Table 2. Primers for the multiplex PCR amplification of *Leptospira* species

Oligonucleotide sequence 5'-3'	size (bp)	annealing temperature	Ref.
Lipl32 F: GAATCAAGATCCCAATCCTC Lipl32 R: TTACTTAGTCGCGTCAGAAGC	272 *bp*	50-62 ℃	This study
16Sr RNA F: AGAAATTTGTGCTAATACCGAATGT 16Sr RNA R: GGCGTCGCTGCTTCAGGCTTTCG	240 bp	50-62 ℃	20

3. Results

Initially, the PCR was performed separately with each primer. The PCR products of pathogenic *Leptospira* amplified by lipl32 primer visible bands with molecular size of 272bp. The bond was not observation in non-pathogenic *Leptospira* and *Salmonella enteritidis* (as negative control). For detection of saprophytic *Leptospira*, *16S rRNA* gene were used with molecular size 240bp. This fragment was not detected in pathogenic *Leptospira* and *Salmonella enteritidis*. The sensitivity for *lipl32* and *16S rRNA* primers were 10^{-4} pg / µl and 10^{-6} pg / µl respectively. The sensitivity for multiplex PCR was 10^{-3}pg / µl.

3. 1. Multiplex PCR

In multiplex PCR, were used two pairs of primers simultaneously. The PCR products of pathogenic *Leptospira* amplified by lipl32 primer (272bp) whereas non-pathogenic *Leptospira* showed the 240 bp of 16S rRNA only (Figure 1).

In this Multiplex PCR reaction, pathogenic and non-pathogenic serovars were separated from each other.

According to the figure 1, The PCR products of pathogenic and non-pathogenic *Leptospira* by lipl32 and 16S rRNA primers, were respectively produced visible bands with molecular size of 272 bp and 240 bp. In this way, pathogenic and non-pathogenic serovars were separated. The Figure lanes 2, 3 shows the positive amplification of only the *16S rRNA* gene for non-pathogenic *Leptospira* (240bp) and lanes 4, 5 shows the positive amplification of only the *lipL32* gene for pathogenic *Leptospira* (272bp).

These two sets of primers allowed us to distinguish between pathogenic and saprophytic *Leptospira* species. Both genes are amplified in *Leptospira* genus (lipL32 in pathogenic *Leptospira* and 16S rRNA in non-pathogenic *Leptospira*).

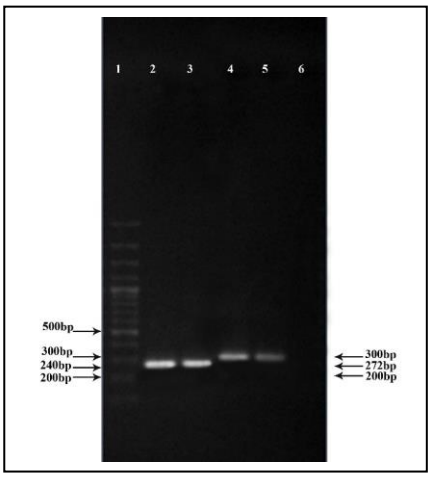

Figure 1. Agarose gel electrophoresis of multiplex-PCR products showing the detection of pathogenic and non-pathogenic leptospires. Lane 1:100 bp DNA ladder; Lan 2: non-pathogenic *Leptospira* (*L.biflexa* Serovar *Semaranga* RTCC 2819); Lan 3: non-pathogenic *Leptospira* (*L.biflexa* Serovar *Semaranga* RTCC 2828); Lane 4: pathogenic *Leptospira* (*L.interrogans* Serovar Canicola RTCC 2805); Lan 5: pathogenic *Leptospira* (*L.interrogans* Serovar *Grippotyphosa* RTCC 2808); Lan 6: *Salmonella enteritidis (RTCC 1621)*

4. Discussion

Leptospirosis is one of the most common zoonoses in the world. Non-pathogenic and pathogenic leptospires are morphologically similar to each other and culture is very slow and time consuming method. PCR is used to identify a large number of microorganisms, including important clinical microorganisms. The PCR reaction has a high sensitivity and no need culture microorganisms. As a result, PCR is a good way to identify the organisms involved in acute infections (2).

The advantage of PCR for detecting leptospirosis is due to its ability to detect *Leptospira* in the early stages of the disease. Leptospirosis is an acute illness and spreads rapidly. Therefore, an appropriate laboratory test such as PCR with high sensitivity and specificity is required for diagnosis (22).

The purpose of this study was to evaluate Multiplex PCR method for identifying pathogenic and non-pathogenic *Leptospira* simultaneously using two pairs of primers. The Multiplex PCR used in this study offers further advantage over that of conventional PCR because it uses 2 sets of primers that increase the specific amplification (272 bp and/or 240 bp).

The targeting of the two genes in the Multiplex PCR allows us to more precisely reach the final diagnosis. In this study, using multiplex PCR method and using two pairs of specially designed Lipl32 primers of pathogenic *Leptospira* and specific 16S rRNA of non-pathogenic *Leptospira*, these two species of *Leptospira* were found to be much easier than conventional methods.

Tansuphasiri, Thipsuk (3) in 2006 for detected pathogenic serovars and genus *Leptospira* used LipL32 primers with lengths of 279 bp and 16S rRNA with 430 bp length. The sensitivity of primers in this experiment was 1pg. In this study, two pairs primers (LipL32 and 16S rRNA) were identified as pathogenic and non-pathogenic serovars. The sensitivity of the primers LipL32 and 16S rRNA was 10^{-4} pg and 10^{-6} pg respectively. The sensitivity of our research was more than research Tansuphasiri et al. In addition, the 16S rRNA primer in the Tansuphasiri study could not detect non-pathogenic serovars (3).

In 2006, Leon, Pronost (23) performed a multiplex PCR test using three primer pairs of hap1 (262bp), 23S rRNA (248bp) and 16S rRNA (240bp). The hap1 primer was able to detect pathogenic serovars. 16S rRNA Primer detected non-pathogenic serovars and the 23S rRNA primer was effective in identifying the genus *Leptospira* (23).

The distinction between the findings of this study and Leon's studies was that we used LipL32 to detect pathogenic serovars, but Leon used hap1. Subscription these two studies were based on the use of the 16S rRNA primer that was able to detect non-pathogenic serovars. The results of the 16S rRNA primers are common in both studies.

Moreno and Agudelo-Florez (24) in 2010 to implement and validate conventional and multiplex PCR methods using *lipL32* and *secY/flaB* genes to assess the capacity of PCR methods to identify pathogenic and saprophytic species of *Leptospira ssp*. In this study 22 international reference strains and 12 colombian isolates were used. Best results were obtained with the lipL32 PCR, which displayed a better sensitivity and a better capacity to detect different strains than the multiplex PCR. The secY primers showed a poor specificity to pathogenic species and a poor sensitivity. Thus, lipL32 primers show high potential for molecular diagnosis of *Leptospira spp* in clinical and environmental samples (24).

Ahmed, Sandai (15) in 2012 developed a multiplex PCR assay for detecting Leptospira's DNA. The multiplex PCR assay detected both the LipL32 primers with lengths of 330 bp and 16S rRNA with 660 bp length. In
this method from 10 species of *Leptospira* and 23 other species of bacteria were used. A positive result was obtained from all leptospiral serovars. In this study, pathogenic and non-pathogenic serovars were not distinguished from each other, but in the present study pathogenic and non-pathogenic serovars were identified from each other (15).

Our results suggested that that multiplex PCR can be used to detect *Leptospira* samples. This method was also able to differentiate between saprophytic and pathogenic leptospires and was able to do so much easily than conventional methods such as culture and MAT.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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