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Original Article

Molecular detection of pathogenic leptospiral serovars by PCR, based on *lipL21* gene

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ABSTRACT

Leptospirosis is a zoonotic disease with global distribution that caused by pathogenic spirochetes of the genus *Leptospira*. Accurate diagnosis for differentiation of leptospirosis from other pyrogenic infections prevailing in the same locality and is imperative for proper treatment. Therefore a molecular diagnostic test with high specificity and sensitivity such as PCR is essential. Gene encoding of outer membrane proteins of *Leptospira* are potential candidates that may be useful as diagnostic and analysis of the disease. In this study, *lipL21* gene was used for detection and differentiation of pathogenic from saprophytic leptospiral serovars in PCR assays. The leptospiral *lipL21* gene expressed only in pathogenic *Leptospira* spp. The bacteria were inoculated into the EMJH (with 5% rabbit serum) and extraction of the genomic DNA was done by standard Phenol-Chlorophorm method. The specific primers for proliferation of *lipL21* gene were designed. The *lipl21* gene was observed in pathogenic *leptospira* and was not in saprophytic leptospires. The specificity and sensitivity of PCR was evaluated. PCR assay with high specificity and sensitivity may prove to be a rapid method for diagnosing acute leptospirosis and designed a positive control to optimize this diagnostic test. The results showed that molecular detection of pathogenic leptospiras based on *lipL21* gene can be used for laboratory diagnosis of leptospirosis.

Keywords: leptospirosis, *lipL21* gene, PCR, pathogenic leptospires

INTRODUCTION

Leptospirosis, a zoonotic disease in humans and animals is caused by pathogenic *Leptospira interrogans* serovars (Bharti *et al* 2003, Koizumi & Watanabe 2005, Vedhagiri *et al* 2009, Vijayachari *et al* 2008). The disease has a worldwide distribution but is most common in temperate regions with high rainfall (Branger *et al* 2005, Cheema *et al* 2007). Studies suggest an increase in the disease in Iran the disease has been reported in different parts of the country (Rafiei *et al* 2014). Early detection of leptospiral infection in humans because of its symptoms similarities to other febrile diseases such as influenza, dengue fever, meningitis and hepatitis is very important (Faine 1994). Due to the variable clinical symptoms, the diagnosis of leptospirosis is difficult and using different laboratory tests is necessary (Bharti *et al* 2003, Faine 1994, Hookey 1991). Structural and functional proteins are part of the outer membrane of

leptospiral bacteria, where lipoproteins are comprised a large part of them and their abundance on the cell surface is as follows: LipL32> LipL21> LipL41 (Cullen *et al* 2005). Microscopic agglutination test (MAT) is the most common diagnostic test which has the advantage of making it possible to determine the serovar or serogroup of bacteria. But due to the need to culture the bacteria, the problems encountered (Fraune *et al* 2013, Mulla *et al* 2006, Murray *et al* 2011). Recently molecular method such as Polymerase chain reaction (PCR) is used for detection of *leptospira* DNA and will be positive in blood and spinal fluid within the first 7 to 12 days of the disease and after 2 weeks that followed in the urine. This method is rapid and its results are reliable (Brown *et al* 1995, Kee *et al* 1994, Merien *et al* 1992, Smythe *et al* 2002, Yasouri *et al* 2013). One of the genes identified in *leptospira*, *lipL21*, is only found in pathogenic serovars of *leptospira* (Cullen *et al* 2003). In this study, *lipL21* gene was used for detection and differentiation of pathogenic from saprophytic leptospiral serovars by PCR assays for first time in Iran. Moreover, due to the difficulties encountered in cultivation and maintenance of *leptospira*, and is performed in a few laboratories, design of a positive control to optimize this diagnostic test is necessary.

MATERIALS AND METHODS

Bacterial strains. In this study, the five pathogenic Leptospiral serovars including: *L. interrogans* Sejroehardjo (RTCC2810), *L. interrogans* Canicola (RTCC2806), *L. interrogans* Icterohaemorrhagiae (RTCC2823), *L. interrogans* Pomona (RTCC2822), *L. Interrogans* Grippotyphosa (RTCC2808), and a saprophytic serovar *L. biflexa* (RTCC2819) maintained in the *Leptospira* Reference Laboratory, Razi Vaccine and Serum Research Institute, Karaj, Iran were used.

Culture and DNA extraction. The bacteria were inoculated into the selective culture (EMJH) medium (Difco, Sparks, USA) containing 10% rabbit serum and enrichment supplements in aerobic conditions at 28 °C and the growth evaluatedafter 7-10 days with dark field microscope. The samples were then precipitated at 15000×g for 20 min and centrifuged at 4 °C. The leptospiral genomic DNA was extracted by proteinase K treatment and Phenol-Chloroform extraction method (Sambrook & DW 2002). The extracted DNA was resuspended in 20µl of TE buffer (pH 8.0) and stored at -20 °C. The quality and quantity of extracted DNA was then evaluated by agarose gel electrophoresis and spectrophotometry, respectively.

PCR. The specific *lipL21* gene primers for amplification were designed using Vector NTI software. The forward and reverse primer had sequences of 5' CGACATGATCAATAGACTTATAG CTC 3' and 5' CAGTTATTGTTTGGAAACCTCTT GAG 3' respectively. PCR reaction was performed in volume of 20 µl [10µl Mastermix (Ampliqon), 1 µl (100 ng) DNA template, 1 µl primer forward (10 pmol), 1 µl primer reverse (10 pmol), and 7 µl nuclease free water] with the following program. For initial denaturation, DNA was placed for 5 min at 94 ˚C and then denaturing at 94 °C for 1 min, annealing of the primers to the DNA target at 55 °C for 1 min, and amplifying DNA at 72 °C for 1 min, which were repeated in 35 cycles and finally 10 min final extension at 72 °C was used. PCR product was evaluated in all samples and confirmed by 1.5% agarose gel electrophoresis.

Specificity and sensitivity analyses. To determine the *lipL21* primers specificity for pathogenic leptospiral strains, PCR assay with specific primers *lipL21* was performed on the DNA extracted from five pathogenic serovars of *leptospira* including: *L.Interrogans* Sejroehardjo, *L. Interrogans* Canicola, *L. Interrogans* Icterohaemorrhagiae, *L. Interrogans* Pomona and *L. Interrogans* Grippotyphosa and a saprophytic serovar *L. biflexa*. DNA extracted from *Salmonella enteritdis* (RTCC1621) was used for further confirmation. In order to obtain the lowest amount of DNA for amplifying and detecting with the PCR primers, a sample of extracted DNA concentration was determined by Spectrophotometer device and diluted

up to 0.01pg/µl. PCR assay was eventually performed on each sample diluted.

Design of a positive control to optimize the test. PCR product of *lipL21* gene leptospiral serovar *L.interrogans* Pomona was purified using kit (Fermentas, Germany) and then incorporated into the vector pTZ57R/T, then transformed into *E. coli* (DH5α). The cells were placed on ice for 1 hr, and then heat shocked in 42 °C water bath for 90 s and after that immediately placed on ice again for 5 min. The cells were ultimately grown on LB agar plates containing ampicillin at 37 °C for an overnight. Then, *lipL21* gene in recombinant colonies was confirmed by PCR. Recombinant colonies were grown on ampicillin-containing LB Broth and plasmid purified using kit (Roche, Germany).

Sequencing. The extracted recombinant plasmid containing the desired, *lipL21* gene, was sequenced by Macrogen Company (South Korea) to confirm the correct gene sequence. The sequence was deposited in the Genbank database of NCBI with the accession number KM817034. The homology of sequence of our serovar was evaluated with the BLAST program of NCBI.

RESULTS

The results of PCR products revealed a 561 bp fragment that represented the gene amplification *lipL21* which followed and approved by 1% agarose gel electrophoresis. Results of the primer sensitivity analyses were also showed that the gene-specific primers *lipL21*, could be amplified in DNA concentrations up to 1pg/µl and used in the molecular detection of pathogenic leptospiral with high sensitivity rate (Figure 1). The amplified gene was successfully cloned in pTZ57R/T vector and transformed into *E. coli* DH5α cells. Analysis of the sequence by using the NCBI database and BLAST revealed high homology (>96%) among our leptospiral serovar and reference sequences submitted in Genbank.

DISCUSSION

Leptospirosis is a zoonosis with a worldwide distribution (Pappas *et al* 2008, Vijayachari *et al* 2008). According to the reports obtained from various parts of Iran, the increasing incidence of the disease and the importance of health and economic aspects of leptospirosis, study for a rapid diagnosis of this disease for treatment, control and prevention is important. Culture method for the detection of leptospirosis is expensive, time-consuming and difficult for the reason that the cultivation requires the use of specific techniques which are not possible in all laboratories.

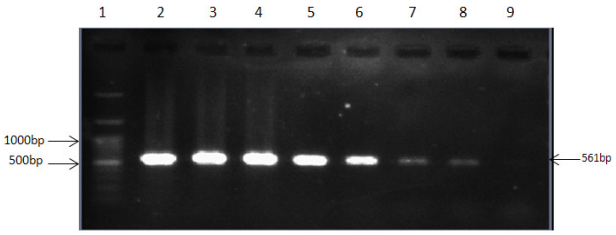

Figure1. Results of the primer sensitivity determination; lane1, DNA ladder 100bp; lane2, positive control; lane3, 100 ng/µl; lane4, 10 ng/µl; lane5 ,1 ng/µl; lane6, 100 pg/µl; lane7, 10 pg/µl; lane8, 1 pg/µl

MAT standard serological testing is also dangerous, since the culture of live Leptospires as antigen used and convalescent serum of the patients needed, so the use of this test is limited to specific laboratories (Levett *et al* 2005). Recently, several PCR protocols for the detection of *Leptospira* DNA in clinical samples have been used which most of them showed high sensitivity (Bal *et al* 1994, Brown *et al* 1995, Chan *et al* 2014, Cheema *et al* 2007, Perez & Goarant 2010, Smythe *et al* 2002, Villumsen *et al* 2012). Therefore, PCR method can be used for rapid and accurate detection of this bacterium. Zhang, et al. in (2005) analysed the gene encoding the outer membrane proteins of *leptospira* endemic in China. The genes encoding LipL21, LipL32 and OmpL1 from the complete genome sequence of the *Leptospira intterogans* serovar Lai, strain Lai cloned and expressed *in vitro*. Comparative sequence analysis showed that these three genes are highly consistent among different epidemic leptospires (Zhang *et al* 2005). In another study conducted by Cullen in 2003,

alignments on sequence of *lipL21* in six pathogenic strains showed 96 to 100% similarity among these strains (Cullen *et al* 2003). Given these facts, *lipL21* gene as a gene conserved in pathogenic strains can be used for PCR, and our study was conducted for the first time on this gene in Iran. In the present study, it was found that the *lipL21* gene was present in pathogenic leptospiral serovars whereas absent in saprophytic *Leptospira biflexa*. In another study conducted by Cullen and colleagues showed that PL21 peptide sequences derived from the gene of *lipL21* was the second frequency in outer membrane proteins of *Leptospira intterogans* serovar Lai (Cullen *et al* 2002). Similar to results that reported by others (Dezhbord 2012, Fotohi *et al* 2012), PCR based on the *lipL21* gene in identification of pathogenic *Leptospires* was in high sensitivity and specificity. The entire *lipL21* gene was considered for the primers designed in this study. Accordingly, the resulting amplicon and cloned can be used as a positive control for all primers using whole-genome or partial primers designed for use in PCR tests in all laboratories.

According to the studies conducted around the world on this gene, and the similar results to the current research, regarding the presence of *lipL21* gene in pathogenic leptospiral serovars, this gene can be used to differentiate pathogenic from saprophytic leptospires.

Ethics

I 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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