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Molecular detection of pathogenic *Leptospira* in Iran

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ABSTRACT

Leptospirosis is an acute infectious, systemic and septisemic disease which had recent outbreaks in some parts of Iran especially in north provinces. Rapid detection is a critical step for treatment and control of this disease. In this research a PCR based method was evaluated for detection of Iranian local endemic serovars. All reported endemic serovars of *Leptospira* including *Leptospira grippotyphosa, Leptospira canicola, Leptospira sejreo hardjo, Leptospira pomona* and *Leptospira icterohaemorrhagiae* which at present are used for Leptospira micro agglutination test (LMAT) in Iran, were subjected to this PCR. Standard representative serovars from ATCC studied in parallel to local serovars. DNA extracted by phenol-chloroform-isoamyl alcohol precipitation. After optimization, the sensitivity of PCR was about 1 fg equal to DNA of 1 *Leptospira*. All studied serovars including non pathogenic *L. biflexa* produced the reported 285 bp fragment. Restriction analysis with *Mbo*I confirmed the PCR product accuracy. In case of *L. biflexa* the pattern was completely different from the pathogenic ones. None of near matching bacteria had product in this method. This system was able to detect the existence of *Leptospira* DNA in all of studied LMAT positive serums.

Keywords: *Leptospira*, PCR, Molecular diagnosis, MAT and Leptospirosis

INTRODUCTION

Leptospirosis is a zoonotic infection caused by spirochetes of *Leptospira* genus. Infection usually is through direct contact via injured skin or mucosal membrane (Levett 2001). *Leptospira* genus comprises both pathogenic and non-pathogenic species. Differential diagnosis of leptospirosis is difficult due to the wide spread and often "flu like" symptoms which may result in a missed or delayed diagnosis (Plank & Dean 2000). Rapid diagnosis of leptospirosis is important for control and treatment. Today's, culture and isolation of bacteria, serologic methods like microscopic agglutination test (MAT) immuno fluorescence assay (IFA), slide agglutination test (SAT) and enzyme-linked immuno sorbent assay (ELISA) in addition to molecular methods like PCR used for detection of *Leptospira*. Confirmatory serological diagnosis of leptospirosis is usually made by MAT. This method is able to detect specific antibody produced against the infecting leptospiral organism (Ahmad *et al* 2005). MAT is based on the use of live *Leptospira* cultures as the source of antigen with a panel of representative local serovars. A specific antibody response detectable by MAT, generally occurs

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around 8-10 days after onset of disease(Plank & Dean 2000, Ahmad *et al* 2005). MAT is generally performed by reference laboratories due to the safety risks of leptospiral live culture handling, the high cost of commercial media, and the need for ongoing maintenance of representative serovars or serogroups. Culture may take up to eight weeks with weekly inspection and examination. In the acute phase, which lasts up to about 10 days, leptospires may be cultured from blood or cerebrospinal fluid (CSF). The failure of serologic methods in rapid detection of these bacteria (These methods need to onset of disease and development of antibodies in the patient blood) have caused PCR to be potentially as a very sensitive and precise rapid candidate for this purpose. Clearly, current diagnosis methods for leptospirosis lack sensitivity, specificity and are time consuming. Polymerase chain reaction (PCR) has been used to detect a large number of microorganisms, including those of clinical significance. Sensitivity of PCR often precludes the need for isolation and culture, thus making it ideal for rapid detection of organisms involved in acute infections (Gravekamp *et al* 1993, Brown *et al* 1995, Veloso *et al* 2000, Lucchesi *et a*l 2004, Fonseca *et al* 2006, Safavieh & Aghaiypour 2007).

Considering the current situation of acute and chronic leptospirosis in Iran, it is necessary to study and invest on its control and treatment (Hoshmand Rad & Maghami, 1976, Maghami & Hoshmand Rad, 1977, Vand Yousefi *et al* 1995, Safavieh & Aghaiypour, 2007). The first step could be introducing accurate and rapid detection methods for these bacteria. In this research it was tried to study and set up a PCR method for detection of iranian local pathogenic leptospira in order to have a rapid and sensitive method for diagnosis of these bacteria.

MATERIALS AND METHODS

***Leptospira* and other bacterial strains.** Local serovars of *Leptospira* species used in this work are listed in the table. These strains in addition to other microorganisms including *S. aureus, B.cereus, L. monocytogenes* and *E.coli* provided from Razi Type Culture Collection (RTCC), Razi Vaccine and Serum Research Institute, Karaj, Iran. Five ATCC representative references *Leptospira* strains including a non pathogenic *Leptospira biflexa* obtained from American Type Culture collection (Table).

Table. Leptospira serovars used in this study

GENOMIC PECIES	Serovar	STRAIN	REFERENCE
L.interogans	canicola	Hondutrecht IV	RTCC[1]
L.interogans	canicola	Hondutrecht IV	ATCC[2]
L.borgpeterseni	hardjoe	Hardjoprajitno	RTCC
L. kirschneri	grippotyphosa*	Moskva	RTCC
L. kirschneri	grippotyphosa*	Moskva	ATCC
L. interogans	pomona	Pomona	RTCC
L. interogans	pomona	Pomona	ATCC
L. interogans	icterohaemorrhagiae	RGA	RTCC
L. interogans	icterohaemorrhagiae	RGA	ATCC
L. biflexa	patoc	Patoc1	ATCC

*: Grippotyphosa is classified as L. interogans by Yasuda *et al.*
1. Razi Type Culture Collection (RTCC).
2. American Type Culture Collection (ATCC).

Leptospires were grown in liquid medium of Ellinghausen and McCullough, as modified by Johnson and Harris (EMJH medium) for five to ten days under aerobic conditions at 27 °C and periodically subcultured in fresh medium. Growth of bacteria was followed by direct examination under dark field microscope.

DNA extraction. Exponentially growing organisms were centrifuged at 13,000 g for 30 min at 4 °C. The pellet was washed twice in phosphate-buffered saline (PBS) and DNA released from leptospires with incubation of the bacteria in 200 µl of buffer as described by Veloso *et al* (2000). The released DNA was extracted with equal volume of phenol/choloroform/isoamyl alcohol (25:24:1) and

precipitated with ethanol 70% v/v. After complete removal of ethanol, the percipitated DNA was resuspended in 100 µl TE buffer (10 mM Tris-HCl and 1 mM EDTA) pH 8.0.

Polymerase chain reaction. The primers used in this study were G1 (upstream) and G2 (downstream) which previously described by Gravekamp *et al* (1993). The reaction mixture (25 µl) contained 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3, 400 µM dNTP, 0.6 µM from each of the above primers and 1 U of Taq DNA polymerase (Roche Applied Science, D-68298 Mannheim, Germany). PCR was performed in Eppendorf mastercycler gradient (Eppendorf-Netheler-Hinz GmbH, 22331 Hamburg, Germany) with initial denaturation at 95 °C for 5 min and 30 cycles of the following steps: denaturation at 95 °C for 60S, annealing at 51.5 °C for 60 S and elongation at 72 °C for 90 S. The last elongation step was for 10 min at 72 °C.

PCR optimization. Optimization of PCR affecting parameters was performed on genomic DNA from *L. canicola Hond Utrecht IV*.

Electrophoresis and visualization. PCR products were run on 1% agarose with TBE buffer, stained in 1 µg/ml ethidium bromid solution for 15 minutes(Romero *et al* 1998) and visualized on Bio Rad Chemi Doc (Bio-Rad Laboratories, Inc., Hercules, CA 94547 USA).

Sensitivity determination. The sensitivity of method was determined on extracted genomic DNA from *canicola Hond Utrecht IV* by 10 fold serial dilution and checked with other serovars. Restriction enzyme analysis of PCR product was investigated with *Mbo*I (Roche Applied Science, D-68298 Mannheim, Germany)(Brown and Levett, 1997).

Clinical samples. Eighteen human and cattle's *Leptospira* MAT positive sera from Gilan, a province in north of Iran, were used as clinical samples. These samples had been tested routinely by Leptospira microscopic agglutination test (LMAT) with a panel of five local *Leptospira* serovars (Table) including: *L. canicola, L. grippotyphosa, L. pomona, L. icterohaemorrhagiae and L. sejreohardjo (Hardjoprajitno)* in the form of live antigens of a standard density (Vand Yousefi *et al* 1995) Normal sera with negative MAT test were used as negative controls.

RESULTS

All studied strains, local *Leptospira* and ATCC reference serovars, were cultured and used for DNA extraction. The quality and quantity of extracted DNA were evaluated on agarose gel electrophoresis and with spectrophotometer. DNA was extracted by phenol-chloroform-isoamyl alcohol and purified two times with ethanol 70%. The results with application of an internal control, showed that two times washing of isolated DNA with ethanol is necessary to ensure successful PCR in all kind of samples regardless of *Leptospira* serovar or sample origin. In order to optimize PCR, various factors such as different sources and concentration of DNA template, concentration of $MgCl_2$, dNTPs, primer, DNA polymerase, temperature and duration of PCR steps were optimized. Final optimized concentrations for detection of *Leptospira* in this system were above mentioned.

To assess the specificity of these primers and PCR system in detection of Iranian local endemic serovars, five ATCC reference strains including the non pathogenic Leptospira biflexa and five local Leptspira strains (used for production of vaccine) (Table) and four other bacteria (*S. aureus, B. cereus, L. monocytogenes* and *E. coli*) were subjected to this PCR. A detectable PCR product was observed for all of ten pathogenic *Leptospira* strains and the non-pathogenic ones (Figure1). Other bacterial strains had no products.

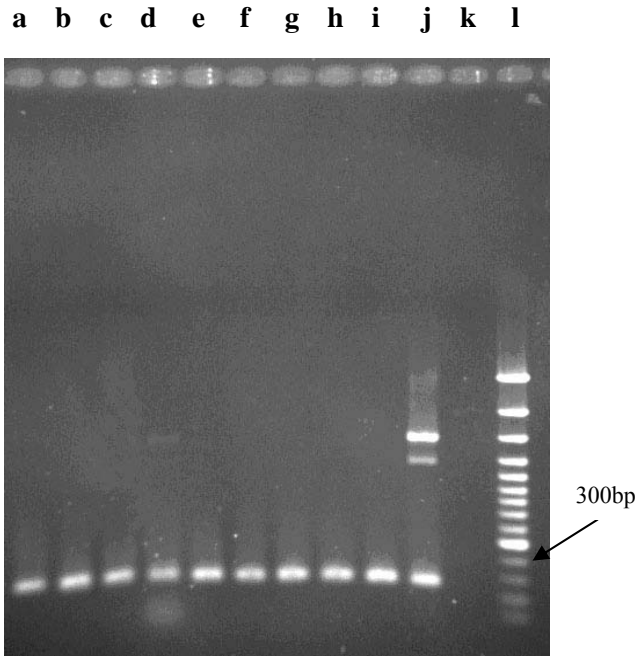

Figure 1. Gel electrophoresis of PCR products by G1/G2 primers on 1% agarose. L. serovars are *canicola* ATCC (lane **a**), *canicola* RTCC (lane **b**), *hardjo* RTCC (lane **c**), *grippotyphosa* ATCC (lane **d**), *grippotyphosa* RTCC (lane **e**), *pomona* ATCC (lane **f**), *pomona* RTCC (lane **g**), *icterohaemorrhagiae* ATCC (lane **h**), *icterohaemorrhagiae* RTCC (lane **i**), *patoc* ATCC (lane **j**). Lane **k**, control negative and lane **l**, 100 bp ladder.

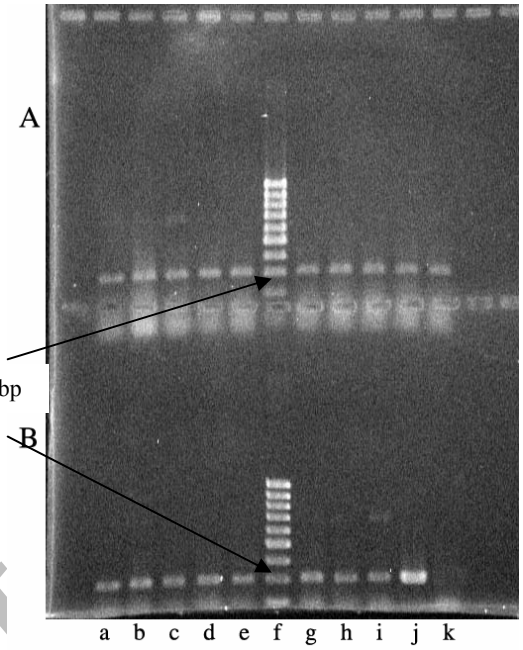

Figure 2. Leptospira detection in MAT positive serum samples by G1/G2 pair of primers. Eighteen MAT positive serum samples in addition to positive (Row **A**, lane **j)** and negative (Row **B**, lane **k**) controls, **f**: 100bp DNA ladder. A PCR product about 300bp on 1% agarose obtained for all of positive samples.

The sensitivity of assay was evaluated with extracted DNA from 1 ml reference culture of exponentially growing organisms containing 10^6 - 10^8 cells from 7-10 day growth of *L. canicola*. Different concentrations of DNA were prepared with serial ten fold dilution. Various concentrations of the purified DNA were studied in order to determine the minimal detection limit of the Leptospiral DNA in this PCR system. A minimum detection limit up to one cell in the PCR reaction mixture was achieved. This sensitivity was repeated more or less in all of other studied serovars.

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as expected. The reaction performed on purified and unpurified PCR products. Based on agarose gel visualization, for both cases no difference in the efficacy of reaction or quality of results was observed. Eighteen serum samples which were positive in Leptospira microscopic agglutination test (LMAT) subjected to this PCR system in addition to positive and negative controls. These samples were from clinically suspected human or animals which their infection had been confirmed by LMAT. The existence of Leptospiral DNA was detected in all of them (Figure 2) while no product observed for negative controls. These results suggest that this method could be a very sensitive and rapid diagnosis system for detection of the endemic local serovars of these bacteria in Iran.

DISCUSSION

During this study, it was tried to study on a rapid detection method for pathogenic *Leptospira* for Iranian endemic strains. The first step was to achieve a standard method for isolation and purification of DNA in order to detect pathogenic *Leptospira* by PCR. *Leptospira* has been diagnosed in Iran for many years by MAT and culture methods (Maghami & Hoshmand Rad 1977, Abdollahpour 1990, Vand Yousefi *et al* 1995). Recently, A preliminary study was performed on detection of *Leptospira* genous by PCR (Safavieh & Aghaiypour 2007). In this paper we continued the detection of pathogenic ones. Nowadays, PCR is applied for detection of various microorganisms, including clinical bacteria and viruses. Sensitivity of PCR is so high, that other methods such as isolation and culture of organisms could not compete with this method anymore. Therefore, this method is a suitable approach to identify microorganisms in acute infections(Levett 2001). Practical value of PCR in diagnosis of leptospirosis is due to its ability in detection of the bacteria in early phase of disease. Rapid diagnosis of this disease has very important role in its treatment and control. Thus, only rapidity and sensivity of PCR test is able to solve the problem(Levett *et al* 2005). Leptospirosis is known as an acute and very contagious disease. Previously the diagnosis of leptospirosis had relied mainly on the detection of antibodies with either ELISA or MAT. In many cases, particularly in the early phase of infection, the lack of antibodies in patient sera results in diagnosis failure. In PCR there is an opportunity for early detection on a single specimen. Thus, PCR has the potential to enhance greatly patient management through timely commencement of medical treatment. Therefore, several studies were performed to identify *Leptospira* bacteria by PCR. The ability of PCR to detect a wide range of pathogenic serovars and also distinguish between pathogenic and non-pathogenic *Leptospira* species have been discussed and studied in many papers. The non-pathogenic representative of *L. biflexa* which was used in this study is the most commonly described environmental contaminant and frequently confuses identification of pathogenic strains from environmental samples. In this study the pathogenic ones were Iranian abundant endemic serovars that used for MAT. Culture under special condition facilitates discrimination between *L. biflexa* and other pathogenic species, but PCR could provide an alternate and rapid method for identification.

As DNA extraction is a critical step in molecular diagnosis method and could cause in false negative results easily(Veloso *et al* 2000), we studied some versions and modifications of phenol chloroform isoamyl alcohol extraction. It was found that this could be a good method if following by two times ethanol 70% washing. In a previous report in spite of very high sensitivity of PCR, it failed to amplify *Leptospira* DNA in some sera of proven patients(Gravekamp *et al* 1993). There are many references about inhibition of Taq DNA polymerase by several factors such as chelation of free magnesium ions, hemoglobin, bile salts, acidic

polysaccharides from glycoproteins and extreme pH variations. Phenol and chloroform, which are used for DNA extraction and purification, are considered to be inhibitors (Brown *et al* 1995). As a consequence of the presence of inhibitors, some extra DNA purification steps are necessary before performing PCR amplification for clinical purposes. In this PCR system for detection of *Leptospira*, two times washing of isolated DNA with ethanol could remove all of these inhibitors from most kind of samples like cultured medium, serum and whole blood. In previous reports the detection limit of PCR was estimated to be up to 1-10 leptospires in 1 ml spiked serum (Gravekamp *et al* 1993). In a real-time PCR the limit of detection of leptospires extracted from urine reported approximately equal to 10 cells and for serum approximately 3 cells(Levett *et al* 2005). The final optimized PCR in this study was able to replicate up to a single DNA molecule, in femtogram quantities of template DNA. Thus, sensitivity of this optimized PCR was comparable with other reports. In a previous study, reference strains belonging to pathogenic *Leptospira* species such as *L. interrogans, L. borgpetersenii, L. weili, L. noguchi, L. santarosai* and *L. meyeri* except *L. kirschneri* were amplified by primers G1 and G2 (Gravekamp *et al* 1993). In this study, we were able to amplify all of studied pathogenic species even *L. krischneri* (*L. grippotyphosa* ATCC 23469 & RTCC strain) and non-pathogenic *Leptospira* (*L. biflexa patoc* ATCC 23582). These results were similar to a recent report (Oliveira *et al* 2003). In case of *Leptospira biflexa*, in addition to 285bp band, two 1200 and 1500 bp extra bands were appeared. Observation of these two bands, could distinguish between non-pathogenic and pathogenic species. There are some discrepancies between researchers. Some papers reported detection of pathogenic and non pathogenic *Leptospira* serovars like *grippotyphosa* and *biflexa* (Oliveira *et al* 2003) while others reported only detection of some pathogenic *Leptospira* serovars (Gravekamp *et al* 1993). We found the 285 bp band for all of studied serovars but in case of non pathogenic *L. biflexa* the pathern was completely different from pathogenic ones. None of other studied bacteria including *B. subtilis, S. aureus, E.coli* and *L. monocytogenes* can be detectable in this system. Restriction analysis is a routine method for detection and confirmation of PCR product. *Mbo*I cut the product in position 198 into two fragments. In addition to product confirmation, no polymorphism for this site observed. The buffer solution of *Mbo*I was similar to Taq DNA polymerase buffer. The reaction performed on purified and unpurified PCR product. Based on agarose gel visualization, no difference in the efficacy of reaction or quality of results was observed. Thus for rapidity in continuation of our studies *Mbo*I directly added to PCR tube without need to further purification. For all of MAT positive serum samples we had the PCR product and the expected band, while for none of the negative samples and controls, the band observed. This study suggests that G1/G2 PCR system could be a rapid and sensitive method for detection of main pathogenic *Leptospira* serovars in Iran by considering some recommendations and is able to promote environmental and public health. In continuation of our studies, we are focused on differentiation and identification of local pathogenic serovars to characterize and distinguish them from each other by molecular method.

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