Full Length Research Paper

Detection of *Leptospira interrogans* in plasma and urine samples by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

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Acute phase of leptospirosis has high mortality. A diagnosis can be designed with high sensitivity by testing blood and urine samples by polymerase chain reaction (PCR) for early diagnosis of this phase of disease. The aim of this research was to detect *Leptospira interrogans* by PCR technique in plasma and urine samples which were taken from symptomatic patients. Clinical isolates were obtained in hospitals of north Iran, and analyzed by PCR method and confirmed with restriction fragment length polymorphism (RFLP). DNA was extracted from plasma and urine samples and the quantity of *L. interrogans* DNA was determined by using PCR technique with LP1 and Lp2 primers. RFLP technique was used by Alu I restriction enzyme for confirming of detection of one of the prevalent serogroup of leptospira in Mazandaran Province. In this study, more than 90 blood and urine samples were examined, 10 (9%) blood and 3 (3%) urine samples became positive by molecular method. The high specificity and sensitivity of molecular assay provide valuable tools for the early diagnosis of acute leptospirosis.

Key words: Restriction fragment length polymorphism (RFLP), *Leptospira interrogans*, polymerase chain reaction.

INTRODUCTION

Leptospira is a genus of spirochete which causes disease. Leptospirosis is one of the most common zoonotic diseases which are common between humans and animals; this disease is common in tropical and subtropical humid areas of the world (Levett et al., 2005; Sharma and Kalawat, 2008; Bharti et al., 2003; Ricaldi and Vinetz, 2006; Plank and Dean, 2000). All Leptospira serovars may cause Leptospirosis in humans. Some cases are without jaundice

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(illness without jaundice) and some cases are with jaundice (Weil's disease, the jaundice form of the disease). Basically, animals get infected with Leptospirosis (Levett et al., 2005; Sharma and Kalawat, 2008).

Human infections are caused incidentally and subsequent to a contact with water or other materials which are infected with contaminated secretions of host animals (Levett et al., 2005; Sharma and Kalawat, 2008; Bharti et al., 2003; Ricaldi and Vinetz, 2006; Plank and Dean, 2000; Park et al., 2006).

Those people who are exposed to contaminated water by voles (such as mine workers, farmers and fishermen) have the highest risk of infection. Human infection is mostly caused by drinking infected water or eating infected food. In rare cases, the organism may enter the body through mucous membranes or skin.

Leptospirosis is diagnosed based on identifying the patient's clinical picture and showing the antibodies against Leptospira. Isolating the bacteria from clinical samples through sample culture methods is very difficult, time consuming, lengthy and, most of the time, unsuccessful (Ricaldi and Vinetz, 2006; Park et al., 2006)

Human acute leptospirosis mostly is caused by infection with strains of the icterohaemorrhagiae serogroup of *L. interrogans* (Kee et al., 1994).

Pathogenic Leptospira are very fastidious and slow-growing and their isolation from clinical samples is very difficult and time consuming (Fonseca et al., 2006). In the first week of the disease, in which specific antibodies do not exist yet, serology is not useful; in this week, sample culture-serology methods and direct observation are not useful in early and rapid diagnosis of Leptospirosis.

Early diagnosis of leptospirosis is critical because of the risk of severe complications, such as pancreatic islet and vasculitis, as well as lung and intracranial haemorrhages, which require intensive care therapy.

In addition, leptospirosis is usually misdiagnosed because of its variable and non-specific clinical symptoms that has over-laps with other febrile diseases, such as dengue fever or other haemorrhagic fevers (Fonseca et al., 2006). Detection of small numbers of leptospires in clinical samples has become practical due to specific polymerase chain reaction (PCR). This is important as leptospirosis can run a fulminant course and patients may die before the development of the characteristic clinical protests of leptospirosis or the appearance of leptospirosis antibodies or both, and, therefore, the disease may go unrecognised. Post-mortem diagnosis may fail because leptospires may die before inoculation of culture medium and specific antibodies may not yet be demonstrable in serum samples.

Therefore early diagnosis of leptospirosis is important because severe leptospirosis infection can run a fulminant course. The polymerase chain reaction (PCR) was evaluated for the detection of leptospires in clinical samples from patients with acute leptospiral infection ("Kee et al., 1994).

**MATERIALS AND METHODS**

**Subjects and Methods**

In this study, blood and urine samples of 90 suspected patients, who were hospitalized based on clinical symptoms, were collected from May to late September in 2012. The blood and urine samples of the first and the second weeks of the disease were used for detection. All the patients were 60 men and 30 women. 4 ml of blood and 10 ml of urine samples were obtained from all suspected patients, who were admitted in the infectious diseases wards of Ayatollah Rohani Hospital in Babol and Razi Hospital in Gha'emshahr.

**Plasma separation**

The patients' bloods were poured into twisty glass flotation tubes (Isolab, Germany) containing EDTA. Then, they were centrifuged at 2556 Rcf (g) for 30 min after that, the separated plasmas were put into micro-tubes and placed in a -20°C freezer (Honarmand et al., 2005).

**Urine separation**

10 ml of patient's urine was poured into a sampling tube. 10 μl of formalin was added to make the bacteria fixed and motionless. Then it was centrifuged at 2556 Rcf (g) for 30 min. After that, the supernatant liquid was thrown away and 500 μl PBS was added to the remaining sediment; then, it was well mixed with vertex and was placed in a -20°C freezer (Honarmand et al., 2005).

**PCR analysis**

High Pure PCR Template Preparation Kit (Roche from German company) was used to extract the bacterial DNA. 200 μl of each sample was used for this extraction.

Since the amount of the separated DNA was very little and it was not enough for PCR, the extraction method was carried out in this way: 600 μl of each sample was used in separated micro-tubes; we had three 200 μl micro-tubes and each of them was filled with separated materials; when it was time for the stage of passing through a filter, these three micro-tubes were passed through the same filter; the rest of the operation was carried out according to the kit instructions. LP1 and LP2 primer pairs are exclusively designed for such *L. interrogans*. The LP1 and LP2 primer pairs generate 274 base-pair sequences (Kee et al., 1994).

LP1 :5'- ATA CAA CTT AGG AAG AGC AT-3'
LP2 :5'- GCT TCT TTG ATA TAG ATC AA-3

To optimize the amplification conditions, the parameters that affect the PCR including buffer, primer concentrations, MgCl2 concentration, and annealing temperature were checked. The final optimized PCR Reaction consists of 0.4 μM of each prime, 3 μL dNTP (10 mM), 2 μL MgCl2, 0.3 unit Taq polymerase (Metabion, Martinsried, Germany), 5 μl PCR buffer, and 5 μL of DNA template in total volume of 50μl with double distilled water. The cycling program was adjusted as follows: initial denaturation at 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 48°C for 45 s and 72°C for 30 s, with final extension 72°C for 7 min in 1 cycle.

The PCR product was electrophoresed on 1/5% agarose gel containing ethidium bromide staining for 1 hour and then it was photographed (Figure 1). Extracted DNA of *Leptospira interrogans*, used as the positive control, and water used as negative control. We used 100 bp molecular markers size (made by Fermentas Company) and...
 thermo-cycler were amplification, respectively. To check the specificity of our primers used, several Gram positive and negative bacteria, such as DNA from E. coli (NTCC21157), S. aureus (ATCC29213), E. faecalis (ATCC292) P. aeruginosa (ATCC49189), A. baumannii (NTCC12156) and K. pneumoniae (NTCC5056), were used for PCR.

RFLP method was used to confirm the PCR production and to digest 274 bp sequences enzymatically. The RFLP method was performed according to the following protocol: 7 μl of deionized water, 2 μl of buffer, 10 μl of the PCR production, and 1 μl of Alu enzyme were mixed together. Then, it was placed in incubator (37°C) for 30 min to be incubated and the enzyme to be activated (Kee et al., 1994). After that, it was incubated at 65°C so that the enzyme gets disabled. Then, it was loaded on 2% agarose gel.

**RESULTS**

From the total of clinical specimens using primers LP1 and LP2, we had 13 (14.4%) positive case in PCR. For the results of 90 plasma samples, we had 10 (11.1%) and in 90 urine samples, we had 3 (3.3%) positive results in PCR detection (Table 1). On the other hand, one of the samples had plasma and urine positive PCR result. The above sample belongs to a patient with clinical and epidemiological history (Fever (39°C), respiratory insufficiency, blood icterus, thrombopenia, renal insufficiency, bathing in river). The remarkable thing in our result is that we have 11 positive cases in men and 2 positive samples in woman.

In our study we used samples from 60 male and 30 female, therefore, our molecular analysis determined 18.3% leptospirosis in men group and 6.6% leptospirosis in women group. As a result of RFLP, Alu lenzyme cleaved the sequence at two points, 203 and 71 bp and therefore confirms our PCR analysis (Figure 2).

**DISCUSSION**

Leptospirosis is a zoonotic disease with a worldwide distribution. Based on the receiving reports from different parts of our country regarding the increasing incidence of the disease and according to the importance of the health aspects of Leptospirosis (Bharti et al., 2003; Dezfully and Mehrbayan, 2012), the study of rapid diagnosing methods of this disease is important.

Nowadays, culture and serological methods are used for diagnosing Leptospira; serological methods need at least one or two weeks and they cause humoral response in patients, and in culturing the mentioned period expands to several weeks (Organization, 2000; Guidugli et al., 2000; Vinetz, 2004; Merien et al., 1995; Turhan and Sezer, 2012; Brown and Levett, 1997).

PCR is being used for detection of a large number of microorganisms, including clinically important ones. The sensitivity of PCR is such that there will be no need for organism culture and separation. As a result, this method is the ideal method for rapid detection of the organisms involved in acute infections (Turhan and Sezer, 2012).

The practical value of PCR in diagnosing Leptospirosis is due to its ability in the detection of Leptospirosis patients at the early stages. Leptospirosis is an acute disease and spreads quickly. Thus, its early and rapid diagnosis is very important in treatment.

It should be noted that applying sero-logical methods requires proper immunologic response and the passage of the course of the disease. On the other hand, the available serologic methods show the antibodies against Leptospira, but they cannot show Leptospira, while PCR
can show Leptospira even within the first days of infection.

In a study, Brown (1995) concluded that PCR can show Leptospira in acute stages of Leptospiral infection and it's a valuable method in diagnosing leptospirosis at the early stages (Organization, 2000).

The inability of serological methods in rapid and accurate diagnosing of this bacterium has led to the consideration of PCR as an appropriate method due to its high sensitivity and accuracy. Since there is no sensitive diagnosing method for this bacterium and for determining its genotype in Mazandaran, this study was used as a rapid and accurate way of diagnosing and starting of a sensitive PCR technique. In this study, the isolated Leptospires were detected by PCR-RFLP as well.

In a study by Sun Huki in 1993 in Korea, the leptospira interrogans antibodies were detected by microscopic agglutination seven days after the infection. Then, the L. interrogans was detected by the use of LP2 and LP1 primers with 274 bp bonds (Kee et al., 1994).

In our study, it was detected by using LP2 and LP1 primers with 274 bp bonds, which were relevant to L. interrogans, as well. An important advantage of PCR-RFLP is that it can be applied directly to clinical samples, and immediately after the PCR of the clinical sample and the observation of the specified bond, RFLP can be implemented. Thus, the diagnosis will be approved as well (Savio et al., 1994; O'Keefe, 2002; Heinemann et al., 2000).

In comparison with Protat's and Elis's studies, in our study, PCR was used for clinical samples and RFLP was implemented for approval. It seems that the negative cases of PCR belonged to those patients who were treated arbitrarily or tentatively. The response of Leptospira to antibiotic is as follows: leptospira escapes rapidly from the blood and migrates to Parenchymal tissue and high water content tissues, especially kidneys, lungs, liver and brain, and resides in there; thus, leptospirosis subsides to antibiotic is as follows: leptospira escapes rapidly from the blood, and migrates to Parenchymal tissue and high water content tissues, especially kidneys, lungs, liver and brain, and resides in there; thus, leptospirosis subsides and the number of bacteria of the blood decreases greatly (Brown et al., 1995; Perolat et al., 1990), since the Leptospira resulted bacteremia does not last more than 7-10 days in human and with the advent of antibody and treatment (antibiotics); the bacteria escape from the blood, thus there will be no bacteria and the problem of false-positivity of PCR is associated with other issues and mainly with saprophytic leptospira infection which are abundant in the environment (Turhan and Sezer, 2012; Organization, 2003).

This study shows that PCR can be effective in rapid diagnosis of the disease, but it is also likely to show false-negativity. In 90 patients suspected to Leptospirosis, only 13 of them had positive PCR; this may be due to the following reasons:

1) Leptospiemia subsided due to taking antibiotics. Some individuals of our sample had taken antibiotics.

2) Its low accuracy may be attributed to technical errors, inefficiency of the used chemicals, low amounts of DNA (or low amounts of bacteria) of the sample. In all these cases, PCR may generate false-negative responses.

3) The DNA extraction method and the kits which were used for this purpose may also affect the accuracy of PCR (Perolat et al., 1990).

Tony et al. (1997) and Savio et al. (1994) applied PCR-RFLP method to separate a large number of standard and isolated strains; they found it to be useful for rapid detection of subspecies of L. interrogans (Savio et al., 1994).

This study shows that men more than women are exposed to leptospirosis infection, perhaps it is for this reason that compared with women, most men work in agriculture and animal husbandry and generally can be stated that leptospirosis is a job-related illness.

In this study, PCR-RFLP was detected by LP2 and LP1 primers which were related to Leptospira interrogans. In our study, the evaluation criteria were very similar to the mentioned studies.

Conclusion

Of the 90 samples with primer LP1, LP2, 13 samples (4/14 of total) have been the piece. Thus, according to the high efficacy of PCR-RFLP method, it seems that it can be used directly for clinical samples and carry out the rapid diagnosis and identification simultaneously.

Conflict of interests

The authors have not declared any conflict of interest.

REFERENCES


