MULTIPLEX PCR ON LEPTOSPIRAL ISOLATES FROM KOLENCHERY, KERALA, INDIA

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Abstract

Human leptospirosis causes severe multiorgan dysfunctions that may end in multiorgan failure and death. The methods in hand for diagnosis of leptospirosis like culture, ELISA and MAT only help to confirm the disease, and are of little value in early detection. The aim of this study was to find out if the two sets of primers described earlier could detect all the isolates from the area, for the purpose of using the resultant database for early detection of leptospires in future from clinical specimens. The study was done on culture isolates from Jan 2000 to June 2002 attending the department of medicine, MOSC medical college hospital, Kolenchery, Kerala, India. DNA of 45 culture isolates were amplified by multiplex PCR using two sets of previously described primers, G1, G2 and B 64-I, B 64-II. Specific amplifications of either 285 or 563 bp size were obtained from all isolates included in the study indicating the utility of the multiplex PCR in the rapid detection of leptospires in clinical samples.

Key words: Leptospira, multiplex PCR

Leptospirosis is a widespread zooanthroponosis caused by *Leptospira interrogans*. The clinical spectrum of infection ranges from subclinical to severe illness with high mortality rate. Human leptospirosis causes severe multiorgan dysfunctions that may end in multiorgan failure and death. Refractory hypotension, acute renal failure (ARF), adult respiratory distress syndrome (ARDS), myocarditis, severe thrombocytopenia leading to haemorrhage in vital organs are some of the main causes of death in the hospital where the study was carried out. Early diagnosis is important for the management of patients since leptospirosis is treatable.

In this study we wished to standardise multiplex PCR as a tool for diagnosis for identification of the strains belonging to the *L. kirschneri* genomospecies and others.

G1 and G2 amplifies DNA from strains of *L. interrogans*, *L. borgpetersenii*, *L. weilii*, *L. noguchii*, *L. santarosai* and *L. meyeri* (strain ICF)1 whereas B64-I and B64-II amplifies DNA from strains of *L. kirschneri*.2 We combined these two sets of primers and tested them on several serotypes of *Leptospira*.

Materials and Methods

Forty five leptospiral isolates from human patients with suspicion of leptospirosis attending the department of medicine, MOSC medical college hospital, Kolenchery, Kerala, India, during the period January 2000 to June 2002, were included in the study. Cultures were maintained in semisolid Ellinghausen Brief Communication - McCullough - Johnson - Harris (EMJH) medium, till the time of carrying out the DNA typing.

Microscopic agglutination test

Primary serogrouping of all the isolates was done using Microscopic agglutination test (MAT)3 with monoclonal antibodies/antiserum obtained from WHO collaborating centre for reference and research on leptospirosis, Amsterdam. Beginning at 1:50 dilution, equal amounts of series of antiserum/monoclonal antibody and 10⁸ organisms/mL of live, 4 to 14-day-old leptospiral cultures in liquid EMJH medium, were mixed, incubated for 2-4 hrs at 28-30°C and agglutination noted microscopically using 20X dry, dark-field objective. The reacting serogroup was noted for each.

Reference strains

The nine reference strains used in the study were obtained from WHO Collaborating Centre Brisbane, Queensland, Australia, and consisted of *andamana* (strain CH 11), *ballum* (strain Mus 127), *canicola* (strain Hond Utrecht IV), *grippotyphosa* (strain Moskva V), *hardjo* (strain Hardjoprajitno), *icterohaemorrhagiae* (strain RGA), *mini* (strain Sari), *pomona* (strain Pomona) and *panama* (strain CZ214K).

DNA extraction

Culture isolate (3 mL) was centrifuged at 10,000 rpm for 25 minutes at 4°C. Pellets were washed with 1 mL of lysis buffer (10mM Tris HCl, 10mM KCl, 10mM MgCl₂, 2mM EDTA, pH 7.6) and 0.5 mL of a second lysis buffer (10mM Tris HCl, 10mM KCl, 10mM MgCl₂, 2mM EDTA, 0.4mM NaCl, pH 7.6) was added. 50 µL of lysozyme (5 mg/mL) was mixed and kept at 37°C water bath followed by addition of

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Received: 30-03-2004
Accepted: 31-07-2004
50µL of 10% Sodium dodecyl sulphate and mixed. To this 250µL of 6 M NaCl was added and mixed slowly and centrifuged at 10,000 rpm for 15 minutes at 4°C. Two volumes of ethanol was added at room temperature, mixed and centrifuged at 10,000 rpm for 20 minutes at 4°C. The pellet was washed with 0.5 mL 75% alcohol by centrifuging for 20 minutes at 10,000 rpm at 4°C, dried and resuspended in 20 µL Tris EDTA buffer (pH 8.5), and kept in 57°C water bath for 10 minutes. This DNA extract was electrophoresed using 0.8% agrose gel containing ethidium bromide (0.5 µg/mL). The extracts were kept at -20°C until used.

**Multiplex PCR**

The combined sets of primers G1, G2 and B64-I, B64-II as previously described by Gravekamp et al in the same mixture, were used in the study. The primers G1 and G2, derived from the 5' end (nucleotides 1-20) and the 3' end (nucleotides 264-285) respectively, of the nucleotide sequence of the recombinant plasmid pLIPs60 selected from the genomic DNA library of *L. interrogans* strain RGA were used. This clone reacted with representatives of all pathogenic *Leptospira* species with the exception of *L. kirschneri*. The primers B 64-I and B 64-II were derived from 5' end (nucleotides 1-20) and 3' end (nucleotides 542-563) respectively, of the nucleotide sequence of the recombinant plasmid pBIM64 selected from genomic DNA library of *L. kirschneri* thus helping in the detection of all pathogenic strains of *Leptospira* so far isolated from clinical samples.

Following are the primers used in the study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>G1</td>
<td>(5' CTG AA T CGC TGT A TA AAA GT)</td>
</tr>
<tr>
<td>G2</td>
<td>(5' GGA AAA CAA A TG GTC GGA AG)</td>
</tr>
<tr>
<td>B 64-I</td>
<td>(5' CTG AA T TCT CA T CTC AAC TC)</td>
</tr>
<tr>
<td>B 64-II</td>
<td>(5' GCA GAA ATC AGA TGG ACG AT)</td>
</tr>
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50µL of PCR mixture contained 2µL of purified DNA extract, of 100 pmol solution of each primer, 250mM each of dNTP (dATP, dCTP, dGTP and dTTP), Taq DNA polymerase (0.5 U) (Finnzymes), in 100mM Tris HCl (pH 9.0), 15 mM MgCl₂ and 500mM KCl.

The DNA sample was first subjected to 5 minute denaturation at 94°C, followed by 34 cycles of denaturation at 94°C for 1.5 minutes, annealing at 55°C for 1 minute and extension at 72°C for 2 minutes. This was followed by a final elongation step at 72°C for 7 minutes. The PCR amplification was carried out in a thermal cycler (DNA Engine, MJ Research, USA) using 0.5mL microfuge tubes. Electrophoresis of the PCR product was carried out using 1.5% agarose gel in Tris borate buffer (pH 8.2). The gel was visualised under UV transilluminator (Fotodyne) and photographs were obtained using polaroid film.

**Results**

All PCR products of isolates using the two sets of primers invariably yielded single amplification either of 285 bp or 563 bp (Fig.1 and 2), indicating that they were all pathogenic. The saprophytic leptospiral strain CH 11 used as control, did not give any amplification.

MAT was carried out on all the 45 isolates. They were classified as belonging to serogroups *Autumnalis* (17), *Bataviae* (5), *Sejroe* (1), *Grippotyphosa* (6), *Canicola* (2), *Pyrogenes* (7), *Australis* (3), *Hebdomadis* (1), *Icterohaemorrhagiae* (2), and *Louisiana* (1). Of the 45 isolates, 41 (91.1%) gave amplification of 285bp. Only 4 isolates (8.89%), all belonging to serogroup Grippotyphosa, gave amplicons of 563 bp assigning them to *L. kirschneri* genospecies. Two isolates, out of the total six belonging to
serogroup Grippotyphosa, gave amplification of 285 bp, indicating that they belonged to genomospecies other than *L. kirschneri*. No mutual interference bands were observed with the mixture of two sets of primers used in the study.

**Discussion**

As multiplex PCR using two sets of primers gives specific amplification either in the 285 or 563 bp region, pathogenic strains can be detected rapidly for diagnostic purpose. Basic classification of designating the leptosporial isolates to the pathogenic or saprophytic group, is taken care of with multiplex PCR.

Isolates belonging to the same serogroup may belong to different genospecies as seen in this study with the isolates from *Grippotyphosa* serogroup. Differentiation into *L. kirschneri* and non-*L. kirschneri* group, narrows the genomic classification further.

In a study using the same set of primers, Bal et al. explored the use of PCR analysis and were able to detect leptosporial evidence in twice as many urine samples as by culture. All of their samples were amplified by G1-G2 only, indicating none of them belonged to *L. kirschneri*.

Noubade et al conducted PCR studies at IVRI, Izatnagar, Bareilly, India, on pathogenic and non-pathogenic leptospires from various sources like blood, urine, kidney, milk of infected animals and environmental specimens like soil and water. They used only one set of oligonucleotide primers G1 and G2, which amplified the DNA, producing an amplicon size of 285 bp to detect the pathogenic leptospires.

Use of only the primers G1 and G2, may miss out the *L. kirschneri* group of pathogenic leptospires, which is very much present in this specific geographical area, as evidenced from our studies and may be present in other parts of Kerala and India as well. Hence inclusion of B64I and B64II will help in identifying the *L. kirschneri* group of pathogenic leptospires.

As this methodology is not technically demanding and uses only commercially available reagents, it can be applied to diagnostic laboratories. Only single bands are formed and the results are easy to interpret. Use of two sets of primers in the same reaction mixture saves time and expense.

Though this test began for species detection as a prime step towards characterization, the results were very encouraging. We believe this can be used as a diagnostic test for leptospirosis. Since MOSC medical college Hospital, Kolenchery, is the only hospital in Kerala culturing leptospires from humans, we believe that the strains used in this study represent strains from all over Kerala. Only *Pomona* and *Javanica* were not included in this study, however, they have been isolated earlier from this area. These two primer pairs may be employed till a strain not detected by them is isolated. To conclude, multiplex PCR using both the primer pairs is a useful tool in diagnosing leptospirosis and additionally in delegating them to the *L. kirschneri* or non-*L. kirschneri* groups.

**Acknowledgement**

We acknowledge the management, Dr. CK Eapen, Dr. C Radhakrishnan, Dr. G Radhakutty amma, MOSC medical college, Kolenchery, for supporting the project financially and Dr. Mariamma Kuriakose and Dr. CK Eapen for referring the cases for culture. We also thank the Dean, University of Animal Sciences, and HOD Dept. of Animal Biotechnology for permitting us to carry out PCR studies at the Dept. of Animal Biotechnology, Madras Veterinary College (MVC), Chennai. We thank Professor P Ramadass, Dr. Senthil Kumar and Mr. Krishnakumar of Dept. of Animal Biotechnology, for help with the PCR work.

**References**


