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PCR-SSCP: A TOOL FOR MOLECULAR DIAGNOSIS OF LEPTOSPIROSIS

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ABSTRACT

Leptospirosis, a disease with protean manifestations, is caused by *Leptospira* species. Early and reliable identification of *Leptospira* serovars remains to be a challenge due to diversity at serovar level. The potential of polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP) was assessed for detection of sequence variation in pathogenic specific 631 bp fragment of 16S rDNA of *Leptospira* for serovar typing. Standardization of this technique on reference serovars of *L. interrogans* (Canicola, Pomona and Icterohaemorrhagiae) and of *L. borgpetersenii* (Ballum, Tarassovi and Javanica) yielded distinct SSCP profiles. Employing this technique 15 isolates could be characterized to serovars Canicola, Icterohaemorrhagiae, Pomona and Javanica. Similar results were obtained when a panel of monoclonal antibodies in ELISA typed these isolates. In addition, this technique was applied on blood samples of human leptospirosis for direct identification and characterization of *Leptospira* serovars. Five of 50 samples were characterized as serovar Tarassovi. DNA sequencing of 631bp amplicons of these samples validated the results obtained with PCR-SSCP. This PCR-SSCP was therefore, able to identify and characterize the *Leptospira* to serovar level obviating the need for isolation of leptospires before testing.

KEYWORDS : ***Leptospira*** **spp., PCR, SSCP**

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INTRODUCTION

Leptospirosis is one of the most widely distributed bacterial zoonosis. This eminently treatable infection of ubiquitous distribution is caused by pathogenic *Leptospira* species; a spirochete that has been classified into many serovars based on their antigenic characteristics and recently adapted genomic studies [1]. The protean manifestations of this disease ranging from undifferentiated fever to fulminant disease may present a confusing picture to the clinicians. The diagnosis of leptospirosis is made even more difficult by the lack of sensitive and readily accessible diagnostics. Definite diagnosis of leptospirosis during the first week of the illness is a major challenge to investigating laboratories owing to the difficulty in detecting the organism from clinical specimens by microscopy and/or culture [2]. Conventionally, culture positivity, recent sero-conversion, or a four-fold rise in the microagglutinin titres (MAT) is considered diagnostic of the disease [3, 4, 5]. PCR is a rapid, sensitive and specific means of diagnosing *Leptospira* infections especially during the first few days of the disease [6, 7, 8]. Apart from the early diagnosis of the disease, the identification of the causative serovar is equally important for proper management of the disease. Traditionally MAT and cross agglutination absorption test are employed for serovar identification of *Leptospira* isolates. However, these standard classical methods are troublesome and highly complicated besides being time consuming. There has been a great interest in molecular methods for identification and sub-typing. Methods employed have included restriction endonucleases (REA), restriction fragment length polymorphism (RFLP), ribotyping, pulsed field gel electrophoresis (PFGE) and random amplified polymorphic DNA (RAPD) [9, 10, 11, 12, 13]. A limiting factor in all methods is the requirement for pure culture and also at times reproducibility is difficult to achieve [14, 15, 16]. The SSCP method offers a sensitive, inexpensive and rapid alternative determining differences in DNA sequences. A major advantage of using this methodology is that polymorphism at genetic level can be appreciated even at a single nucleotide change [17].

In the present study, a 631bp specific 16S rDNA fragment of pathogenic *Leptospira* species was employed in PCR-SSCP for serovar identification of leptospires from culture and clinical samples.

MATERIALS AND METHODS

Leptospira serovars and isolates:

Pathogenic serovars of *Leptospira* species, viz, *L. interrogans* serovars Canicola (strain Hond Utrecht IV) and Icterohaemorrhagiae haemorrhagiae (strain RGA) and *L. borgpetersenii* serovars Ballum (strain Ballum), Javanica (strain Poi) and Tarassovi (strain Tarassovi) were obtained from the WHO Collaborating Center, Netherlands. Pathogenic serovar of *Leptospira* species *L. interrogans* serovar Pomona (strain Pomona) was obtained from the National Leptospirosis Reference Center, Regional Medical Research Center (ICMR), Port Blair India. These serovars were maintained in Ellinghausen McCollough, Johnson and Harris (1967) (EMJH, Difco, USA) media for use in the present work.

Nine isolates of *Leptospira* recovered from hospitalized patients with pyrexia of unknown origin (PUO) and/or febrile jaundice and six isolates recovered from trapped wild rodents from Gwalior City were utilized in this study.

Clinical Samples:

Fifty hospitalized patients admitted in Birla Medical Research Institute, Gwalior, having fever (pyrexia) for more than 3 days of unknown origin (PUO) with or without jaundice were included in this study. Two milliliters of blood was collected from these patients in heparinized vials.

PCR-SSCP:

For extraction of *Leptospira* genomic DNA, exponentially growing cultures were pelleted at 12000g for 30 min at 4°C, then the cells were

washed with sterile PBS and DNA was then extracted with Wizard genomic DNA purification kit (Promega USA) according to the manufacturer's instruction.

PCR was carried out to amplify 631bp pathogenic specific 16S rDNA gene segment from all the reference strains, isolates and blood samples of suspected cases of human leptospirosis using following primers [6] and the method described by Nizamuddin et al [18].

p16SF 5'- CGCTGGCGGCGCGTCTTAAA-3'

p16SR 3'- AAGGTCCACATCGCCACTT-5'

Briefly the PCR reaction was carried out in a total volume of 25 µl including 1.5mM $MgCl_2$, 200µM of each dNTP, 0.1µM of each primer, 100ng of template DNA and 0.5U of DNA Taq polymerase (Fermentas). The thermocycler conditions for the amplification were as follows: an initial denaturation step at 95°C for 4 min, then 30 cycles of amplification with denaturation at 95°C for 50 sec, annealing at 68°C for 1 min and extension at 72°C for 2 min and final extension of the incompletely synthesized DNA at 72°C for 7 min. The amplified products were detected in 0.8% agarose gel electrophoresis following staining with ethidium bromide. The products were purified using the commercially available Gel Extraction kit (Quiagen).

For PCR-SSCP, each 5 µl of the purified PCR product (10ng/µl) was denatured at 95°C for 5 min with same volume of 2X loading buffer (containing 95% formamide, 25mM EDTA and 0.05% bromophenol blue). Then the samples were subjected to 10% acrylamide gel (acrylamide / bisacrylamide 37.5:1) electrophoresis with 10% glycerol. The electrophoresis was carried out using 1X Tris Borate buffer (TBE), pH-8.0 for 6 hrs at 100V at room temperature. The gel was then subjected to silver staining using commercial available Proteo Silver plus Silver Stain kit (Sigma) according to the manufacturer's instruction to visualize the separated single strands.

Serovar identification of *Leptospira* isolates by ELISA

Of the 15 leptospiral isolates, 9 recovered from hospitalized patients with pyrexia of unknown origin (PUO) and/or febrile jaundice and 6 recovered from trapped wild rodents were subjected to serovar typing by ELISA. For this all the isolates were killed by keeping in a boiling water bath for three minutes and tested with a panel of serovar- specific monoclonal antibodies, using protocol described by Nizamuddin et al [18]. The panel consisted of monoclonal antibodies against the following serovars- Australis (Aus), Autumnalis (Aut), Bataviae (Bat), Canicola (Can), Grippotyphosa (Grip), Hebdomadis (Heb), Icteroicterohaemorrhagiae (Ict), Javanica (Jav), Pomona (Pom), Tarassovi (Tar) and Hardjo (Hard). Detection was done by rabbit anti-mouse peroxidase conjugate, using ortho-phenylenediamine hydrochloride and hydrogen peroxide as substrate.

DNA Sequencing

Double stranded sequencing of the amplified products of 631bp from isolates and positive blood samples was performed on an ABI 10 sequencer (Applied Biosystems, USA) employing Big dye terminator cycle sequencing ready reaction kit. Briefly, 2 µl (25 ng) of the purified PCR products was mixed with 3.2 pmol of respective primer and a reaction mixture containing the four dye-labeled dideoxynucleotide terminators. Cycle sequencing was then performed as follows: 25 cycles at 96°C for 30 secs, 50°C for 1 min and 60ºC for 4 min. The reaction mixture was purified by ethanol precipitation and the DNA was vacuum dried. The DNA pellet was resuspended in 10 µl of template suppression reagent (TSR) and preheated before loading onto the DNA sequencer.

RESULTS

The 631bp pathogenic specific 16S rDNA gene fragment of *Leptospira* species produced an appreciable resolution when subjected to 10% acrylamide gel electrophoresis with 10% glycerol for 6 hrs at 100V at room temperature.

PCR-SSCP could yield distinct profiles among the serovars of *L. interrogans* (Canicola, Pomona and Icterohaemorrhagiae) and of *L. borgpetersenii* (Ballum, Tarassovi and Javanica) undertaken for this study (Fig.1). When this PCR-SSCP was tested onto the fifteen isolates of *Leptospira* species, four different serovars were characterized namely serovar Canicola, Icterohaemorrhagiae, Pomona and Javanica based on similar SSCP profiles to their respective reference serovars. Seven isolates (six from patients and one from rodent) were characterized as serovar Canicola, five isolates (three recovered from patients and two from rodents) were characterized as serovar Icterohaemorrhagiae, two of the rodent isolates were characterized as serovar Pomona and one of rodent isolate was characterized as serovar Javanica. The serovar typing of these isolates by a panel of monoclonal antibodies revealed the similar results as obtained with PCR-SSCP. Table1 depicts the serovar identification of *Leptospira* isolates by PCR-SSCP and ELISA.

Table 1

Identification and characterization of Leptospira isolates by PCR-SSCP and ELISA

Isolate	PCR-SSCP	ELISA										
		Aus	Aut	Bat	Can	Grip	Heb	Ict	Jav	Pom	Tar	Hard
H2*	Icterohaemorrhagiae							+				
H7*	Icterohaemorrhagiae							+				
H8*	Icterohaemorrhagiae							+				
H13*	Canicola				+							
H29*	Canicola				+							
H31*	Canicola				+							
H1029*	Canicola				+							
H1167*	Canicola				+							
H1172*	Canicola				+							
R2#	Pomona									+		
R3#	Canicola				+							
R4#	Pomona									+		
R11#	Icterohaemorrhagiae							+				
R15#	Icterohaemorrhagiae							+				
R19#	Javanica								+			

*** Isolates recovered from patients**

 # Isolates recovered from environmental rodents

Figure 1
PCR-SSCP Profiles Of Reference Serovars of Leptospira

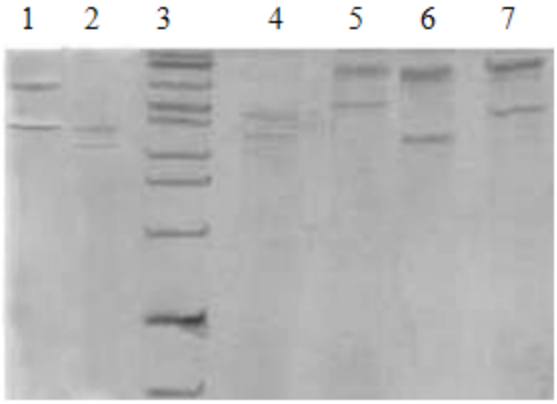

Figure 1: PCR-SSCP Profiles Of Leptospira species.
Lane 1: standard serovar Canicola; Lane 2: standard serovar Javanica; Lane 3**:** 1Kb DNA marker standard serovar; Lane 4: standard serovar Pomona; Lane 5: standard serovar Ballum; Lane 6: standard serovar Tarassovi; Lane 7: standard serovar Icterohaemorrhagiae haemorrhagiae

Further, evaluation of this technique was performed directly on to the blood samples of 50 suspected cases of human leptospirosis. Five of them were found positive for *Leptospira* by culture and 631bp 16S rDNA PCR. The 631bp amplicons of these samples when subjected to SSCP yielded the profile similar to the reference serovar Tarassovi (Fig. 2). DNA sequencing of the PCR amplicons of these blood samples validated the results of PCR-SSCP (Data not shown).

Figure 2
Application of PCR-SSCP on blood samples of suspected cases of human patients with leptospirosis

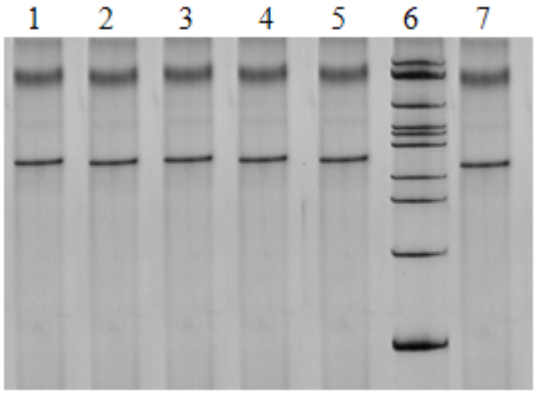

Figure 2: PCR-SSCP profiles of blood samples.
Lane 1: reference serovar Tarassovi; Lanes 2,3,4,5,7: PCR positive clinical samples; Lane 6: 1Kb DNA marker

DISCUSSION

Leptospirosis, caused by the pathogenic *Leptospira* species, is an important re-emerging zoonotic disease worldwide. The existence of a large number of serovars with extensive genomic homology shared among pathogenic and saprophytic serovars complicates the precise identification of this organism. Several primer pairs for PCR detection of leptospires have been described, some based on specific gene targets [19], most frequently 16S or 23S rRNA genes [6, 20, 21, 22, 23, 24, 25, 28]. A limitation of PCR-based diagnosis of leptospirosis is the inability of most PCR assays to identify the infecting serovar. Furthermore, with the lack of association of serovars with clinical syndrome manifestations, the reliable identification of the organism during disease condition, particularly during an outbreak is warranted for epidemiological assessment, effective management and response to treatment. Existing methods of identification and also of typing, including serological typing and molecular typing, normally are time consuming and tedious procedures that are possible once the culture is obtained in a pure form that might take 6-8 weeks and therefore, not of much help to clinicians.

SSCP takes advantage of the fact that the mobility of single stranded nucleic acids, when electrophoresed under non-denaturing conditions, is determined both by their fragment length and sequence-dependent secondary structures. PCR-SSCP provides powerful tools to study sequence variation and allows variants to be “typed” from culture or direct sample without the need for restriction digestion, southern blot or sequencing methods [17].

In the present study, we have assessed the potential of PCR-SSCP for detection of sequence variation in pathogenic specific 631bp fragment of 16S rDNA for serovar characterization of *Leptospira* spp. 16S rRNA was selected in this study because it is found in all organisms from bacteria to higher organisms and the 16S rDNA genes include, slowly evolving or conserved regions as well as rapidly evolving or variable regions. Thus this gene sequence differs between species. In our study, the 16S rDNA PCR-SSCP could yield distinct profiles among the tested reference strains of pathogenic *Leptospira* species *L. interogans* and *L. borgpetersenii*. In an earlier study, SSCP analysis of the G1-G2 amplicon allowed serovar identification within each genomospecies except the serovars of *L. kirschneri*. Moreover, the G1 and G2 primers amplified all species, including *L. biflexa* [2]. A similar study was carried out in China where the DNAs of reference strains from 15 serovars (14 serogroups) of pathogenic leptospires were characterized [22]. In our study we could find the utility of the PCR-SSCP method beyond the reference strains wherein 15 isolates recovered from human patients and rodents could easily be characterized to serovar level. Four different serovars were characterized namely serovar Canicola, Icterohaemorrhagiae, Pomona and Javanica based on similar SSCP profiles to their respective reference serovars. Similar results were obtained when these isolates were typed by a panel of 10 monoclonal antibodies in ELISA. In addition, it was possible to detect and characterize the infecting serovar in cases of human leptospirosis directly from blood samples. DNA sequencing of PCR amplicons of 631bp from blood samples validated our results.

Typing of *Leptospira* serovars based on MAT, CAAT phenotyping and ELISA requires the maintenance of a comprehensive collection of reference strains, their corresponding rabbit immune sera or monoclonal antibodies and extensive testing procedures of cross adsorption [26]. To overcome this problem, a number of genetic tests have been developed, including RAPD, restriction endonuclease analysis (REA) and

PFGE [9, 10, 27]. These molecular techniques are very sensitive to changes in reaction conditions and the presence of contaminants hence reproducibility is difficult to achieve without absolute standardization of experimental procedure. Profiles are affected markedly by the primer used, the quantity and quality of the DNA template and the electrophoresis conditions [14, 16]. Time taken to obtain culture in pure form is of no practical implication to the clinicians. Our results suggest that 16S rDNA gene SSCP is a simple, easy and rapid method of serovar characterization. The method can identify the *Leptospira* serovars directly from patients sample without culturing them .

CONCLUSION

Since serovar identification is important especially for epidemiological investigations to trace the source and spread of infection and may be for the prognosis of disease, precise identification of the infecting serovar can surely be of value for an effective treatment and control strategy. Epidemiological data on the type, the seasonal prevalence of the serovars and the reservoirs involved would definitely help in selecting the right kind of strains for vaccine preparations. The PCR-SSCP based characterization as described in the present study can be a useful tool for this purpose. Therefore, this technique is required to be tested and analyzed on a larger proportion .

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