

# Characterization of Leptospiral isolates by using PCR-Restriction Fragment Length Polymorphism analysis

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## Abstract

Leptospirosis is a disease of cosmopolitan distribution caused by bacteria under the genus *Leptospira*. Currently DNA based techniques used for leptospiral identification are Restriction fragment length polymorphism analysis, DNA-DNA hybridization, pulsed field gel electrophoresis, PCR followed by RFLP analysis and Random amplified polymorphic DNA fingerprinting. In the present study eight reference strains were used along with two human and two rat isolates. The strains and isolates were subjected to *flaB* PCR and it was found that the isolates and strains amplified a 793 bp fragment expect for the non-pathogenic strain Semaranga patoc. The amplified products were subjected to digestion with *HindIII* and *HaeIII* enzymes. Differences between various reference strains were more with *HaeIII* than with *HindIII*. The isolates regardless of the sources they were isolated from showed identical RFLP patterns. This can be an indication that the four isolates belong to same genomic species. This study shows that *flaB* RFLP can be used as a tool for classification of leptospires from pathogenic and non-pathogenic. The molecular characterization of human isolates showed close similarities with isolates from rodents indicating that rodents could be responsible for the transmission of infection in the islands.

**Key words:** PCR, Restriction fragment length polymorphism analysis, restriction enzymes, zoonosis.

## Introduction

Leptospirosis is considered the most common zoonosis worldwide and is endemic in tropical environments (Katz *et al.*, 2002). It has recently been classified as a re-emerging disease, largely because of increased recognition and recent rediscovery that it can present as a severe hemorrhagic illness, easily confused with some viral hemorrhagic fever (Monsuez *et al.*, 1997). Leptospirosis is emerging as an important public health problem world over and in India particularly outbreaks are frequently being reported from different regions of the country during last few years (John, 1996).

Leptospirosis gain extreme public health importance in countries like India, due to

close association of man with animals. In humans, leptospirosis occurs sporadically in temperate climates but is a common zoonosis in tropical regions. Leptospirosis has been recognized as an important occupational hazard of agriculture manual laborers, sewage workers, animal handlers, forestry workers and other outdoor workers, who work in wet conditions and butchers. Natural disasters such as floods and hurricanes increase the risk for human exposure to leptospires through contact with contaminated water or mud. In India the cases of leptospirosis are increasing as it is observed in recent outbreak in 1999 in Orissa, 2000 in Mumbai, 2002 in Kerala, 2005 again in Mumbai and 2006 in Gujarat. Although many infected persons recover spontaneously, 5% to 10% of cases can progress to a more serious and potentially

fatal second stage of illness that affects organ systems. Hence, quick and precise diagnosis of leptospirosis is important to provide early and specific treatment and control of infection. Conventional methods to detect leptospires in blood using dark field microscopy or sub culturing are unreliable, less sensitive and lengthy. Common circulating serovar of leptospires in India differs from region to region. Study among different population has shown that serovar Grippityphosa is common among north Andaman and Icterohaemorrhagiae is common among south Andaman. Similarly Autumnalis is common circulating serovar in southern part of India and Pyrogene is common in Maharashtra and Gujarat. Clinical manifestation in leptospirosis depends on the infecting serovar.

Detection of leptospires in the clinical samples using PCR is one of the most valuable additions in the diagnosis of leptospirosis. This assay is able to detect small number of leptospires in clinical sample (Terpstra, 2003 and Brown *et al.*, 1995). The PCR assay can be applied to selectively amplify specific DNA sequences by more than  $10^6$  fold (Saiki *et al.*, 1988). PCR has the advantage that it does not require the isolation of the organism and detects DNA from both viable and non-viable organism. PCR was found to be a simple and rapid method for differentiation of pathogenic and saprophytic leptospires (Noubade *et al.*, 2002). However, the determination of serovar in isolated strains burdens examiners with its intricacy and maintenance of a comprehensive collection of strains and more over takes several months to complete. Hence, the search for alternative

method of identification and characterization of isolates have been focused on DNA based techniques like Restriction Length Fragment Polymorphism, Random amplified polymorphic DNA, Fluorescent amplified fragment length polymorphism, DNA hybridization etc. Keeping this in view, the present study has been proposed to carry out the standardization of RFLP and characterize leptospiral isolates from animals and humans by using PCR-RFLP.

## Materials and Methods

The present study has been undertaken for characterization of leptospire by *flaB* - PCR RFLP.

### Bacterial strain and leptospiral isolates

All the strains and isolates of *Leptospira* used in the study were provided by the leptospira repository of RMRC (ICMR), Port Blair.

### Medium

EMJH (Ellinghausen McCullough Johnson Harris) (DIFCO, USA), media were used for the cultivation of *Leptospira* for the study.

### Reagents and Media

Selective EMJH medium, agarose (sigma), dNTPs Mix (mix of dATP, dGTP, dCTP, dTTP) (Bangalore Genei), Taq DNA polymerase (Bangalore Genei) and 10X Buffer (Bangalore Genei) were used. Various chemicals used in the study were of analytical reagents and guaranteed reagents grade. The glassware's and plastic were used from Schott Duran, Borosil, Corning and Tarson.

### Standard markers

DNA molecular weight marker used was 500bp DNA ladder (Bangalore Genei).

### Primers: (Sigma)

*flaB* primers:

Forward: 5'  
TCTCACCGTTCTCTAAAGTTCAAC 3'

Reverse: 5'  
CTGAATTCGGTTTCATATTTGCC 3'

### Restriction Enzymes

*Hae*III (New England Biolabs) and *Hind*III (MBI Fermentas).

### Isolation of *Leptospira* from human patients and animals Selection criteria

All the leptospiral isolates and reference strains were provided by the RMRC, ICMR leptospiral repository. Briefly, Patients with suspected leptospirosis attending a rural primary health centre (PHC) or admitted to a referral hospital were included for the isolation of *Leptospira*. Acute onset of fever, headache and body aches associated with jaundice, reduced urine output, cough, haemoptysis and breathlessness, any hemorrhagic tendencies including sub-conjunctival hemorrhage or severe calf muscle tenderness was the criterion for clinical suspicion of leptospirosis. Blood culture was attempted on all patients and isolates were identified using conventional and molecular method.

### Isolation of leptospire from the samples

Leptospire were isolated from blood. Few drops of blood specimen from patients or a small pin head size kidney tissue specimen from rat were inoculated in 5ml of EMJH semi solid selective medium containing 200 mg/ml of 5 fluorouracil. Incubation was carried out at 28° C and tubes were screened for leptospiral growth under dark field microscope at every 10 days interval for the period of 3-6 months.

### Maintenance of leptospiral isolates and reference strain for characterization

Strains and isolates from humans and animals provided by leptospiral repository of RMRC (ICMR), Port Blair, for characterization by *flaB* RFLP were maintained in liquid EMJH

List of isolates and strains used in PCR RFLP for characterization:		
SEROGROUP	SEROVAR	SOURCE
Isolate 1	-	Human
Isolate 2	-	Human
Isolate 3	-	Rat
Isolate 4	-	Rat
Shermani	Shermani	Reference strain
Grippotyphosa	Grippotyphosa	Reference strain
Pomana	Pomana	Reference strain
Ranarum	Ranarum	Reference strain
Autumnalis	Rachmati	Reference strain
Pyrogens	Salinem	Reference strain
Tarassovi	Tarassovi	Reference strain
Semarang	Patoc	Reference strain

medium dispensed in 5ml amount in screw capped test tube. Duplicate tube was inoculated. The cultures were frequently sub cultured to get an optimum growth of 1-2 x 10<sup>8</sup> cells/ml. The above said growth was obtained by three sub culture in 20 -21 days.

### Isolation of Genomic DNA (Boom *et al.*, 1990)

DNA was isolated following standard procedure by Boom R *et al.*, 1990. Exponentially growing 4 ml leptospiral culture was centrifuged at 13000 rpm for 20 minutes at 4° C. The pellet was suspended in 50 µl of solution I (see appendix) and 50 µl lysosyme (Sigma) and incubated at 37° C for 15 minutes. Then 50µl of 10% SDS (Appendix) and 5 µl of proteinase K (Sigma) was added and incubated at 65° C for 30 minutes. 40 µl of 5 M NaCl and 32 µl of CTAB NaCl were added (see appendix) and incubated at 65° C for 30 minutes. Equal volume of chloroform: isoamyl alcohol (24:1) was added and was vortexed well and centrifuged at 10,000 rpm for 15 minutes. The supernatant was taken out in fresh tube and 180 µl of chilled ethanol was added and shaken well. The tubes were incubated at 70° C for one hour and then centrifuged at 10,000 rpm for 30 minutes. The pellet was allowed to dry and then 50 µl of TE Buffer.

### *flaB*-PCR-RFLP for characterization of leptospiral isolates:

The genomic DNA was isolated from human and animal isolates and reference strains to be used in the study following standard protocol as described earlier.

### PCR:

Amplification of isolated DNA was carried out in 50 µl volume with about 50 ng of chromosomal DNA, 10 mM tris HCl (pH 9.0), 50mM KCl, 4mM MgCl<sub>2</sub>, 0.1mM each of the four deoxynucleotide triphosphate (dNTPs), 300 pm of each primer and 0.5 unit of Taq DNA polymerase L *flaB* F11 µl, L-*flaB* R1 1µl, dNTPs1µl, Taq DNA polymerase 0.5µl, Taq buffer (10X) 5 µl Milli Q water 39.5 µl, arget DNA2 µl and PCR reaction was carried out in a DNA engine (MJ Research PTC 200, USA). PCR amplification was performed initial denaturation 95° C for 5 min, denaturation. 95° C for 30 sec, annealing 50° C for 30 sec,

primer extension 72° C for 60 sec, repeat steps 2-4; 41 times and final extension 72° C for 10 min. After amplification 10 µl of PCR product was run on 1 % agarose gel for analysis of PCR product.

### Analysis of Amplified DNA

The amplified DNA (10 µl) was mixed with 5X loading buffer (2 µl) and run on 1% agarose gel using 1X tris acetate EDTA (TAE) running buffer system. The ethidium bromide (10 mg/ml) stained gel was visualized under UV transilluminator connected to gel documentation system (BIO-RAD) and results were documented.

### Purification of Amplified DNA

The amplified DNA was purified using PCR product purification Kit (Millipore).

### Restriction Digestion

The purified DNA was incubated overnight with the restriction enzymes *Hae*III and *Hind*III in water bath at optimum temperature of 37° C for 16 hours. After incubation, the reaction was stopped by heat inactivation. The reaction containing *Hae*III was inactivated by keeping in water bath at temperature of 80° C for 20 minutes and *Hind*III reaction was kept at a temperature of 65° C for 20 minutes.

### Analysis of Digested DNA

The digested DNA (10 µl) was mixed with 5 X loading dye and run on 1% agarose gel using tris acetate EDTA (TAE) running buffer system. The ethidium bromide (10 mg/ml) stained gel was visualized under UV transilluminator connected to gel documentation system and results were documented.

## Result

This outcome of present study, which deals with the characterization of leptospiral, isolates by *flaB* RFLP. Two leptospire were isolated each from seventy patient blood samples and from forty rat kidney samples respectively. These isolates were maintained at the centres leptospiral repository. After repeated sub culture, once the isolates attained a concentration, which can be utilized for genomic DNA isolation and later for characterization.

Genomic DNA was successfully isolated from four leptospiral isolates and eight reference stains used in the study. The isolated genomic DNA was amplified using the leptospira specific primers (*L-flaB*-F1 and *L-flaB*-R1). All four isolates and reference strain generated a fragment of 793 base pair amplified product observed in 1% agarose gel (Fig. 1). The serogroup Semarang a non-pathogenic *Leptospira* did not show any amplification.

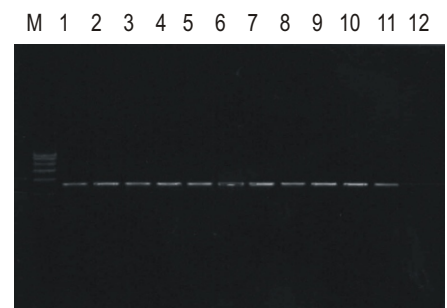


Fig 1: Amplification of Leptospiral DNA using *fla B* primer  
 Lane M: Marker 500 bp DNA ladder;  
 Lane 1 & 2: Human Isolate; Lane 3 & 4: Rat Isolate; Lane 5: Shermani; Lane 6: Grippotyposa;  
 Lane 7: Pomana; Lane 8: Ranaran;  
 Lane 9: Autumnalis; Lane 10: Pyrogenes;  
 Lane 11: Tarassovi; Lane 12: Semarang

The amplified products were purified using commercially available kit (Millipore) following manufacturers instruction. The purified product of all four isolates and eight reference strains were later subjected to restriction digestion with restriction enzymes *Hae*III and *Hind*III. The entire PCR product

was initially digested with restriction enzyme *Hind*III. This yielded two fragments with one band slightly above 500 base pair and the other two was below 500 bp (Fig. 2). The two human and two animals isolates showed a similar banding pattern. The banding pattern of four isolates was also similar to serogroups Shermani, Ranarum, Autumnalis and Pyrogenes (Fig. 3). Three serogroup Tarassovi, Panama and Grippotyphosa showed a different banding pattern when compared to the isolates.

When the PCR product was digested with restriction enzyme *Hae*III, this yielded a maximum of three bands. All the four isolates yielded three bands, which were below 500bp and showed a similar pattern. The banding pattern of one of the reference serogroup Autumnalis was similar to that of the isolates from humans and animal. Serogroup Panama showed single band of size 793 bp as *Hae*III did not digest the PCR product. Serogroup Salinem, Grippotyphosa, Tarrasovi, Ranarum and Shemani showed different banding pattern among themselves and also with the isolates. Autumnalis generated three bands when digested with *Hae*III restriction enzyme and the bands were below 500 bp.

Banding pattern yielded by both the restriction enzymes *Hae*III and *Hind*III had showed that all four isolates belong to same serogroup. The banding pattern of isolates was similar to that serogroup Autumnalis

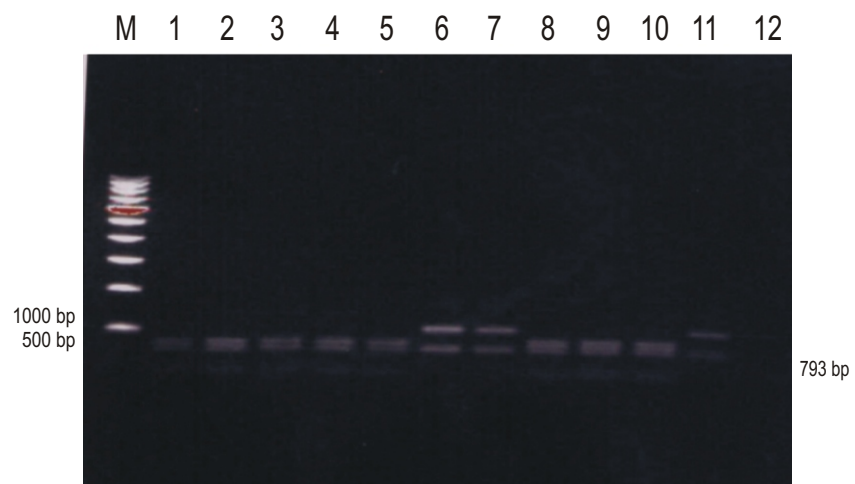


Fig 2: Restriction digestion with *Hind III*

Lane M: Marker 500 bp DNA ladder; Lane 1 & 2: Human Isolate; Lane 3 & 4: Rat Isolate; Lane 5: Shermani; Lane 6: Grippotyposa; Lane 7: Pomana; Lane 8: Ranaran; Lane 9: Autumnalis; Lane 10: Pyrogenes; Lane 11: Tarassovi; Lane 12: Semarang

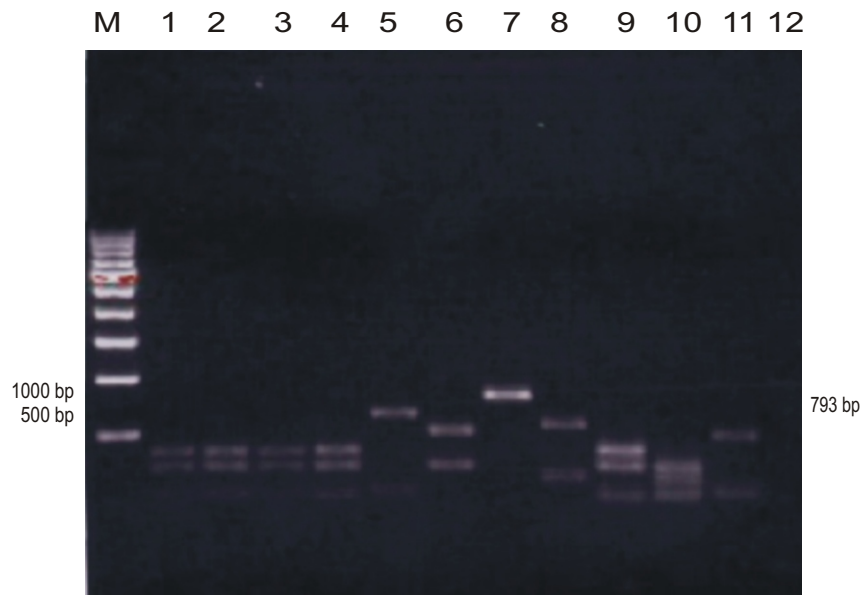


Fig 3: Restriction digestion with *Hae III*

Lane M: Marker 500 bp DNA ladder; Lane 1 & 2: Human Isolate; Lane 3 & 4: Rat Isolate; Lane 5: Shermani; Lane 6: Grippotyposa; Lane 7: Pomana; Lane 8: Ranaran; Lane 9: Autumnalis; Lane 10: Pyrogenes; Lane 11: Tarassovi; Lane 12: Semarang

under genome - species *L. interrogans*. Further characterization tests are required to come to a definite conclusion.

## Discussion

Leptospirosis is considered the most common zoonosis worldwide and is endemic in tropical environments (Katz *et al.*, 2002). It has recently been classified as a re-emerging disease, largely because of increased recognition and recent rediscovery that it can present as a severe hemorrhagic illness, easily confused with some viral hemorrhagic fever (Monsuez *et al.*, 1997). Leptospirosis is emerging as an important public health problem world over and in India particularly outbreaks are frequently being reported from different regions of the country during last few years (John, 1996). Due to their different clinical manifestation from mild to a severe one and due to their different clinical presentation from region to region it has become very important to characterize the isolates to understand the disease deeply.

The result of this study showed that *flaB* PCR can be used for characterization of leptospiral isolates and is capable of detecting pathogenic leptospire as shown in the study. On the other hand, reference strain Patoc 1 (Serovar Patoc) used in the study did not produce any *flaB* -

PCR product because it belongs to genome-species *L. biflexa*, which is a non-pathogenic leptospira.

All the isolates along with reference strains produced 793 bp amplified products. *flaB* RFLP can be used as a tool for classification of leptospire from pathogenic and non-pathogenic. The leptospiral isolates both from animal and human showed similar banding patterns with both the enzymes *HindIII* and *HaeIII* that showed two restriction sites at amplified product of *flaB* gene. The *HindIII* RFLP patterns of the seven reference strains showed minor differences as compared to *HaeIII* - RFLP patterns. This showed differences in restriction pattern in all reference strains. *HaeIII* when used in serogroup Panama yielded single band, which is an indication of no restriction site of *HaeIII* in *flaB* gene. Differences between the various reference strains were more apparent with *HaeIII* than with *HindIII*.

All the isolates, regardless of the sources they were isolated from, showed identical RFLP patterns. Similar type of results reported by Savio *et al.*, (1994) amplified a specific DNA fragment from genomic DNA of serovar hardjo type hardjoprajitno and nine serovars belonging to *L. interrogans*. The amplified DNA was digested with enzyme *HinfI* and

*DdeI* which yielded specific polymorphic patterns, allowing discrimination among majority of serovars. They concluded that the method was suitable for simple and rapid detection of *L. interrogans* and for serovar identification.

On the other hand several authors (Ellis *et al.*, 1991; Van Eys *et al.*, 1991; Zuerner *et al.*, 1990) approached (which also includes a number of techniques such as restriction enzyme analysis (REA) and pulsed field gel electrophoresis), restriction enzymes are used to cut leptospiral DNA into fragments (called RFLPs), which may be characteristic at strain level.

These fragments are then separated by agarose gel electrophoresis, forming characteristic banding patterns (Ellis *et al.*, 1991). Relationships between bacterial strains can then be established by comparing the patterns of unknown strains with those of known strains. Hybridization of labeled DNA probes to RFLP blots greatly simplifies the visualized banding patterns facilitating interpretation (Van Eys *et al.*, 1991) and allows serovars with similar RFLP banding patterns to be differentiated (Zuerner *et al.*, 1990).

Thiermann *et al.*, (1985) developed a new classification method in which genomes of leptospiral field isolates belonging to serogroup pomona were analyzed and compared with those of type strains by cleavage with restriction endonuclease. This method shows differences among these organisms not indicated by conventional serological typing method. The results of the study showed that restriction endonuclease analysis could be used as a reliable tool to classify leptospire for serogroup levels.

Bolin and Zuerner (1996) analyzed isolates of *L. interrogans* serovar pomona type kennewicki from cattle, swine, horses and wildlife by DNA restriction endonuclease analysis. Restriction fragment length polymorphisms in DNA digested with *HpaII* were correlated with host animal source of the isolates.

They reported that several type kennewicki variants, with different but stable REA profiles, exist and circulate primarily in swine or cattle populations and the restriction

fragment length polymorphisms identified in the present study are result of host adaptation of serovar pomona type kennewicki. They concluded that there may be a direct link between the profiles and genetic differences that make the variants more able to survive and grow in different hosts.

This could be an indication that all four isolates belongs to same genomo-species. It is further necessary to characterize them by different methods, both serological characterization method and other molecular methods like RAPD etc.

The present technique used in the study initially gives an idea about their genomo-species status and can be further strengthened by other techniques. In the present study two isolates both from humans and animals (Rat) are used and the technique used also gives you some clue regarding the transmission dynamics of *Leptospira* in these Islands. Rat being the reservoir host, sheds *Leptospira* in their urine through out their life without being infected.

The molecular characterization of the isolates recovered from the human showing close similarities with the isolates recovered from the rodents indicating that rodents could be one of the animals responsible for transmitting infection in these Islands among the human population. RFLP analysis is generally more sensitive and discriminatory than serotyping and high degrees of resolution are possible with RFLP, often surpassing those of serological methods and may reveal small differences between leptospiral strains. Further study is required to completely understand the transmission dynamics of leptospirosis in Andaman Islands.

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