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Use of *rpoB* gene analysis for detection and identification of *Leptospira* species by direct sequencing

[1] Chandan Shivamallu, [2] Sharanaiah Umesha, [3] Govindaraju Shruthi, [4] Vinayaka Balamurugan

[1] Faculty of Life Sciences, JSS University, SS Nagar, Mysore- 570015, Karnataka, INDIA.

[2] Department of Studies in Biotechnology, University of Mysore, Manasagangotri, Mysore- 570 006, Karnataka, INDIA.

[3] Faculty of Life Sciences, JSS University, SS Nagar, Mysore- 570015, Karnataka, INDIA.

[4] Project Directorate on Animal Disease Monitoring and Surveillance (PD_ADMAS), ICAR, Hebbal, Bangalore- 560 024, Karnataka, INDIA.

Abstract
Leptospirosis is an emerging disease for which culture and identification are partly unresolved. In fact, 16S rRNA-based sequencing is the most widely used PCR methodology that can detect such uncultivable pathogens. However, this assay has some limitations linked to potential problems of contamination, which hampers diagnosis. To overcome this, we have a simple PCR strategy involving targeting of the gene encoding the RNA polymerase β subunit (*rpoB*), a highly conserved enzyme. The sequence of the *Leptospira rpoB* gene was determined and compared with the published sequence. Our findings have significant implications for the development of a new tool for the identification of spirochetes, especially if clinical samples are contaminated or when the infecting strain is uncultivable. The consistent use of PCR has improved the early diagnosis of Leptospirosis but the limitation is that it cannot provide information on the infecting *Leptospira* strain which provides important epidemiological data.

Keywords: histopathological studies, leptospirosis, phylogenetic analysis, polymerase chain reaction, *rpoB*.

1. Introduction

Leptospirosis is a worldwide zoonosis usually transmitted to humans through contaminated water or direct exposure to urine of infected animals. The genus *Leptospira* is an incredibly varied group of organisms containing hundreds of serovars and genetic types, which occupy diverse environments, habitats and life cycles [2]. Leptospires dwell in the renal tubules of their maintenance hosts and are excreted into the environment along with the urine. Humans may get infected indirectly from animals by contact with contaminated water, soil or mud under moist environment or by direct contact of urine [20].

The diagnosis of Leptospirosis has classically been described on the basis of serological data, an indirect biomarker, using the microscopic agglutination test (MAT). MAT results have provided epidemiologically important data allowing the identification of the infection sources or reservoirs and have largely contributed to the current knowledge of Leptospirosis epidemiology. However, MAT was shown to be a poor predictor of the infecting serovar [11].

Leptospira can be found in the blood during the first week of infective symptoms [19, 15], although culture provides valuable information and material, but it will be positive only in a minority of cases. PCR is more sensitive than culture for the detection of *Leptospira* in clinical samples [30].

Leptospirosis in dogs is caused primarily by *Leptospira interrogans* and *Leptospira kishumeri*. Thirty years back before the introduction of Leptospirosis vaccines the serovars that were believed to infect dogs were *Icterohaemorrhagiae* and *canicola.* Since the introduction of bivalent *Icterohaemorrhagiae* and *canicola* vaccines, more widespread involvement of additional serovars has been suspected, including that of *Grippotyphosa, pamona* and *automnalis* [31].

Leptospirosis results in illness severity of which ranges depending on the infecting serovar, geographical location, and host immune response. Some dogs display mild or no signs of diseases, whereas others develop severe illness or death, most often as a result of renal injury. Dogs may present with signs of hepatic failure, including manifestation of infection, which includes conjunctivitis [7, 22].

The incubation period for Leptospirosis can be as short as a few days, the organism replicating rapidly within the blood in a day after infecting and before invading tissues [29]. The incubation period taken in experimental studies is normally 7 days, but may vary depending on the infecting dose, strain, and host immune response [6]. Co-infection of *Leptospira* with other organism such as malarial parasites has been reported [3]. The *rpoB* gene encodes the β-subunit of DNA dependent RNA polymerase [16, 17], and rifampin resistance is related to mutation in a specific region of *rpoB*. Recently, *rpoB* sequences were used as an alternative tool either for determining the phylogeny or for identification of enteric bacteria [23], mycobacterium [14], and spirochetes including *Borrelia* [18, 26]. The *rpoB* gene has been shown to be useful and more discriminating alternative to the 16S rRNA gene for inferring phylogenetic relationship. It was earlier been demonstrated to be a suitable target to base species identification for the genera [25].

The polymerase chain reaction (PCR) is advantageous because of its ability to amplify within few hours, a specific sequence of pathogen present at low concentration. Since PCR detects nucleic acid of microbes irrespective of their capacity to cause infection [32]. This technique is found to be most simple, specific

and rapid method for the detection as well as differentiation of *Leptospira* as pathogenic or non-pathogenic.

Many other methods like the recombinant antigen coated latex beads could detect the specific antileptospiral antibodies in the acute phase of the illness have been proposed in recent years [4]. Several PCR based methods targeting various regions targeting different genes have been developed recently for the detection of *Leptospira* [27]. By use of real-time PCR, it is possible to quantify the amount of DNA/template. Many researchers have attempted to design PCR primers specifically for *Leptospira* sp. The sensitivity of PCR repeatedly precludes the need for isolation and culture, thus making it a rapid tool to detect the organism involved in acute infection [28].

While PCR-based protocols offer several advantages over standard culture techniques, the risk of cross-contamination is a major drawback. Detailed studies on diagnosis and epidemiology of Leptospirosis have not been done and as such no reports in India. Sensitive and specific confirmative diagnostic technique for *Leptospira* has not been developed. In the present study an attempt has been made to assess the usefulness of RNA polymerase β-subunit encoding gene (*rpoB*) sequence and used as an alternative tool for *Leptospira* detection and comparison of *rpoB* sequences with the other country isolates, which has been used for phylogenetic analyses. Recent work clearly illustrates that *rpoB* sequence analysis is a powerful tool for the identification of *Leptospira* species [2].

2. Material and Methods

2.1 Study population: One hundred and ten blood samples were collected from one hundred and ten dogs with clinical symptoms of high fever, chills, vomiting, bleeding gums, yellow urine from different places in and around Mysore, Karnataka, India, during the period- Sep 2011- Aug 2012. The study was approved by Institutional Animal Ethical Committee (IAEC) (Reg. No. MGZ/637/2011-12 Dt 12.07.2011).

2.2 Collection and testing of sera samples: Of the 110 sera samples, nineteen isolates received a confirmed diagnosis of Leptospirosis based on molecular diagnosis using PCR from field out breaks in canine farms in and around Mysore, Karnataka State, India. These isolates stored at room temperature in laboratory of Department of Studies in Biotechnology, University of Mysore and were used for the molecular diagnostics and genetic analysis. Details about the isolates are given in table (Table 1). After retrieval from liquid medium Ellinghausen McCullough Jensen and Haris (EMJH) culture the isolates were passaged through albino mice once to check the virulence.

2.3 DNA Isolation: DNA was extracted from the retrieved culture after confirming the culture is positive for *Leptospira* by using QIAgen DNA isolation kit (QIAgen, Germany). DNA extraction was carried out according to the manufacturer's instructions. The DNA was quantified using Nanodrop DNA quantifier and stored at -80° C until further processed.

2.4 Primers and PCR assay: PCR was performed on the obtained DNA template using reported primer pair (rpoB-F-CCTCATGGGTTCCAACATGCA and rpoB-R-CGCATCCTCRAAGTTGTAWCCTT) [2]. Briefly, PCR was performed by initial denaturation at 94°C for 3 min. Subsequent PCR amplification conducted with 40 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 1 min, extension at 72° C for 1 min followed by final extension for 20 min at 72° C. All the PCR products confirmed by digested with restriction enzyme *ApoI* (Fermentas, USA) and also the isolates positive for *Leptospira* in PCR assay were further confirmed by passaging into albino mice with the permission of Institutional Animal Ethical Committee. Five micro liter aliquot of each PCR product were mixed with 3µl of 6x loading dye (Fermentas, USA) and loaded on to the wells of 2% agarose gel in TAE buffer. It was run at 80v for about 45 min. DNA bands were visualized under UV Transilluminator and documented using gel documentation system with Quantity one software (Biorad, USA) and the PCR product was eluted using Eppendorf QIAquick gel extraction kit (QIAGEN, Germany).

2.5 Characterization of amplicons: The PCR products were subjected to restriction digestion using the enzyme *ApoI*. The products were incubated at 37°C in a water bath for 4 h. The reaction mixture consisted of 2.0 µl of
buffer, 1.0 µl of enzyme, 8.0 µl of DNA and 14.0 µl of nuclease free water. Fifteen microliter aliquot of each digested products were mixed with 3 µl of 6x loading dye (Fermentas, USA) and loaded on to the wells of 2% agarose gel in TAE buffer. It was run at 80v for about 45 min. DNA bands were visualized under UV-Transilluminator and documented using gel documentation system with quantity one software (Biorad, USA).

2.6 Transformation

Ligation mixture was prepared by adding 1µl of pGEMT Easy (50ng) vector and 1 µl of T4 DNA ligase (3U/ml) to 5 µl of 2x buffer. 5 µl of DNA (50ng/ml) were added to this mixture for ligation. Ligation was done for overnight at 4°C. Ligation mixture was addedto ice cold 200µl of compe tent cells and tapped gently. It was incubated on ice for 30 min ., heat shock was given at 42°C for 1 min. Immediately mixture was kept on ice for 2 min and 0.8 ml of LB broth was then added to it before incubating at 37°C for 90 min in an orbital shaker. It was centrifuged at 4000 rpm for 10 min and the pellet was plated on LB agar plate containing ampicillin (50 mg/mL) and 40 µl of 2% X-gal (5-bromo-4-chloro-3-indolyl-β-galactopyranoside) and 8 µl of 2% IPTG (Isopropyl-β-D-thiogalactoptranoside). The plates were then incubated over night at 37°C. Well isolated white colonies were picked and transferred to 5mL of LB broth containing 5µl of ampicillin. The tubes were incubated at 37°C in the shaker for overnight. Isolation and purification of plasmid DNA were done using QIAprep spin miniprep kit (Qiagen, Germany).

2.7 Characterization of Recombinants

The plasmids isolated from the white colonies were further characterized by the insert release after digestion with *EcoRI* (G AATTC) restriction enzyme. The enzyme *EcoRI* was used to cleave and release the insert in the plasmid. The reaction mixtures were prepared by taking 2.0 µl and subjected to digestion at 37°C in a water bath for 1h.

2.8 Experimental animal: In addition, the bacterial virulence expression was used as an approach to find out the expression of bacteria. Twenty albino mice of either sex weighing 30-40 g raised at the experimental animal production unit of Department of Zoology, University of Mysore,

Manasagangotri, Mysore, Karnataka, India, were used. The animal study was conducted taking all precautions to minimize the pain or discomfort to the animals after taking necessary approval from IAEC.

Leptospira isolates confirmed in the PCR assay were selected and the inoculum was prepared at the concentration of 10^5 organism/ml and mice were infected intraperitoneally with *Leptospira* along with appropriate controls. Infections were confirmed by subjecting the animals to blood tail vein puncture [24]. Blood samples were collected from mice and were tested for Leptospires by cultivating in liquid EMJH medium (Difco, USA) at 29º C and observed under dark field microscope (Zeiss, USA).

2.9 Histopathological studies: Histopathological analysis was done by fixing one kidney from each mouse in neutral buffered 10% formaline for ≥ 48 h followed by bisecting and embedding in paraffin. Section of 5 micron thickness was cut from each kidney and stained with haematoxylin and eosin. For histopathological analysis of liver, a piece of median lobe of liver was removed from each mouse and placed immediately in 10% neutral buffered formaline. Sections of 5 micron thickness were cut and stained with haematoxylin and eosin. Tissues were examined using a light microscope under different magnifications and images were captured [9].

2.91 Testing the PCR products by DNA sequencing: The amplified products in the samples positive for PCR were subjected to DNA sequencing. After the confirmation of the isolates all the products were purified and the products were sent for nucleotide sequencing of both strands to a commercial service (Europhins Pvt. Ltd, Bangalore, Karnataka, India). The products were sequenced using ABI-Prism (Applied Biosystems, USA) genetic analyzer that works based on the principle of Sangers' dideoxy termination method. The sequences were then analyzed by DNA star sequence alignment software and BLAST analysis was done to confirm the sequenced data with the standard strains and to determine the percent homology (www.ncbi.nlm.nih.gov/BLAST).

2.92 Phylogenetic analysis: Sequences were aligned with ClustalW on the EMBL-EBI web server (http://www.ebi.ac.uk/clustalW/). Phylogenetic tree was obtained from DNA sequences by the maximum likelihood method using the Mega 5.2v software (http://www.megasoftware.net). Evolutionary distances were estimated and the DNA sequences of all the isolates have been deposited in the GenBank database with the accession number as shown in table (Table 1). Branch supporting values were evaluated with 1000 bootstrap replications.

3. Results & Discussion

One hundred and ten samples from canines with septicemia were taken irrespective of age and sex for assessing Leptospirosis disease incidence. DNA extraction from the clinical specimens by QIAamp DNA isolation kit (Qiagen, Germany) yielded a substantial amount of DNA without any protein contamination. The primers had sufficient sensitivity to detect DNA concentration of 1 fg and it is specifically amplified Leptospiral DNA (Fig.1). Good amplifications were evident for PCR reaction carried out at the annealing temperature of 55° C for 1 min. The 600 bp amplicon was subjected to restriction digestion. The restriction enzyme pattern of this amplicon confirmed it to be the *rpoB* gene of *Leptospira* species. All those isolates, whose identity was confirmed by digesting with restriction enzyme *ApoI,* yielded two fragments of 188 bp and 412 bp size as shown in the figure (Fig. 2).

The PCR product of all the 19 isolates were then individually cloned into pGMET Easy TA cloning vector and transformed into JM109 *E.coli* competent cells and plated on LB-ampicillin plates with IPTG and X-gal (Fig. 3). The discrete colonies were then picked, cultures and plasmid was isolated. The Fig.4 illustrates the characterization of recombinants by releasing the insert using *EcoRI* restriction digestion enzyme.

Out of 110 samples of Leptospirosis suspected canine samples 60 samples were found positive under dark field microscopy, but only 19 isolates were positive for *Leptospira* and no bacteria were isolated from the remaining 91 samples. These PCR confirmed isolates were further confirmed by injecting the positive isolate to the experimental albino mice, in which all the mice with the experimental diet survived throughout the experimental period. The body weights of induced mice were reduced (data not shown).

The animals were sacrificed; liver and kidney of the deceased infected mice were taken out and processed for histopathological studies. Liver showed prominent changes including centriolobular necrosis, bile duct proliferation and disorganization of normal radiating pattern of the cell plate around central vein in mice induced with the positive samples, whereas kidney of the induced mice exhibited shrunken glomeruli and also mucopolysaccharides deposition (Fig. 5a and b). In our study we have used albino mice as animal model to find out the pathogenicity of bacterial isolates wherein Julie *et al*.2010, have used capybara rodent.

All PCR product of 600 bp correspondent to *rpoB* gene amplified and sequenced in order to perform molecular typing of the isolates. The BLAST analysis (www.ncbi.nlm.nih.gov/BLAST) demonstrates 100% identity with *rpoB* gene from *Leptospira interrogans* (data not shown). The deposited *rpoB* gene sequence for GenBank is shown in table (Table 1) and the sequence used for the study shown in table (Table 2).

Nineteen *Leptospira* spp. collected from the outbreaks in Karnataka state of India were nucleotide sequenced in the *rpoB* region. The *rpoB* gene encodes the β subunit of RNA polymerase, which produces RNA molecules in cells. Alignment of nucleotides of this region to those of 29 other strains, including 8 Indian isolates available in GenBank is given in Fig.6. Phylogenetic construction was performed from the above aligned sequence using ClustalW. We have constructed a phylogenetic tree using Maximum-Likelihood tree by Mega 5.2 software [10] based on *rpoB* gene sequence using many country isolates as reference strain with our sequences. This phylogenetic analysis could segregate the strains into four groups (Cluster I-IV) with the two major clusters (Cluster I and III).

On the phylogenetic tree, majority of Indian isolates have clustered in Cluster I. These Indian isolates including our isolated strains are very less divergent, on the phylogenetic tree the Indian isolates are placed separately from the other members of the cluster II, III and IV. In addition, the Indian isolates at cluster I are further subdivided into another branch cluster II indicating the minor genetic difference between France and the Indian isolates.

Interestingly, lone isolate (KC292643 DOB19) from our study belongs to subgroup 2.1 and has formed a major cluster with France and Brazil isolates. From the phylogenetic tree (Fig.6), it can be seen that this cluster shows higher divergence among them compared to another Indian cluster I. Comparison of the *rpoB* region and other regions of the genome from a large pool may be needed to determine the origin of the isolates. However, the isolate KC292643 DOB19, which was also collected from the same place differs from the other isolates of the same outbreak, there may be a *Leptospira* of two different ancestries. The sequence similarities in 600 bp segment of *rpoB* among the species, there was a formation of two major clusters (Cluster I and III) with two minor clusters (Cluster II and IV), the main branch of the tree was subdivided into two major groups (Fig. 6), the intergroup topology was different among the two major groups of clusters. The branches were more widely separated in the *rpoB* tree. However, the bootstrap value was > 95 %, p= 0.3 by chi square test. Nevertheless, the bootstrap value obtained using partial *rpoB* was significant. Using a partial *rpoB* gene sequence, we are able to distinguish accurately nineteen isolates of *L. interrogans.* This demonstrates the usefulness of *rpoB* sequence in identification of *Leptospira* from other species. Using the *rpoB* sequence analysis, it was possible to separate the 19 isolates and place them under different clusters/clades.

The highest similarities (>99%) was found between our *Leptospira* sequences KC292640, KC292639, KC282888, KC282887, KC28886, KC28885, KC28884, KC247150, KC247149, KC247147, KC192547, KC147204, KC192546, KC147148, KC192545, KC292642, KC29641 with Indian isolates JF718739, JF718729, JF718737, JN388652, JN388622, JN388653, JF718725, JN718738 and a small cluster was formed with in the same group contains two France originated isolates. *rpoB* strains across different geographical regions exhibit variability, therefore the analysis of *rpoB* gene based phylogeny helped us to distinguish the geographical origin. The result of phylogenetic tree dendrogram showed a diversity of polymorphism between *Leptospira* strains of different origin. All isolates were categorized in 2 major clusters (Cluster I and II), Cluster I comprises >50 % of all isolates. Our data demonstrates that the *rpoB* is a good reflection of the gene content of *Leptospira*.

Delay in the diagnosis of Leptospirosis may lead to outbreaks of abortion and stillborn fetuses in the herds. On the other hand, any diagnosis based on clinical symptoms is not always reliable; therefore, laboratory support is an important tool in the diagnosis of the disease.

PCR based methods targeting different locations of Leptospiral genome for specific amplification, which hold promise as molecular tool for the diagnosis of the disease. Since conventional culture techniques are cumbersome and potentially biohazardous, they are not routinely carried out, although serological tests are preferred, but they have lesser sensitivity. Molecular methods appear to provide definite diagnosis of this disease [33].

One of the main advantages of using this technique on various biological samples is the possibility to detect pathogenic Leptospires first by PCR assay and subsequently confirming it through restriction enzyme digestion thus improving the specificity of the test. Further sequence analysis may reveal the associated serovar causing the disease. To overcome the limitations of cultivation and serology, we have used PCR amplification of Leptospiral DNA for the diagnosis of Leptospirosis at an early stage of illness.

The PCR when tested directly without any culture techniques like MAT is more sensitive and thus suitable for screening samples in molecular epidemiological field studies. The advantage of the PCR and genetic typing is its ability to distinguish the pathogenic strain from the non-pathogenic one among *Leptospira* sp. Yet, for comprehensive species information, further typing method is needed to be applied. The 16S rRNA gene target could not differentiate pathogenic and nonpathogenic *Leptospira* [21, 5]. The PCR assay targeting partial sequence of *rpoB* gene, which showed good sensitivity and specificity with all the 19 samples tested. Hence, it may prove to be a valuable tool for early diagnosis of pathogenic Leptospires directly from biological samples with clinical suspicion of Leptospirosis.

Phylogenetic analysis of the sequences showed a great diversity of sequence typing and no clustering, due to the use of epidemiologically unrelated strains. Thus, when the two new loci sequences were concatenated to the original sequence typing observed the complete discrimination of the strains, although the adapted scheme remains limited to *L. interrogans* and *L. kirschneri* [8]. However, unlike 16S rRNA or any other rRNA whose primary structure is functionally critical, the *rpoB* gene seems to tolerate a more diverse sequence alteration without causing any changes in protein function. The tolerable sequence variation in *rpoB* gene becomes useful clue for species identification. Molecular typing approaches provide rapid typing as well as a highly perceptive assay useful for epidemiological studies of pathogenic bacteria [1].

Using *rpoB* sequence analysis, we were able to separate the *Leptospira* sp. tested in this study and place them under different clad in the phylogenetic analysis. Analysis of several strains of the most commonly encountered serovars may prove for defining the contribution of *rpoB* sequencing to the screening of *Leptospira* isolate by molecular studies and also the analysis of a segment of *rpoB* may be helpful as an initial screening test as mentioned in the present study.

This tool may lead to the identification of a new isolate of *Leptospira* using a system of similarity cut-off to define species. However, to use this in routine identification of *Leptospira,* these cut-offs will have to be validated on large collections of isolates, as reported with respect to *Corynebacterium* sp. [13].

Therefore, this study may be useful for the detection as well as identification of *Leptospira* in clinical samples. Infecting serovar may vary geographically among canine population depending on exposure to infected wild or domestic animals reservoir hosts. Complete information about the infecting serovars in the canine population is limited because published studies usually have not included isolation efforts. Based upon the phylogenetic analysis, DOB 19 isolate was found to be very unique and significant, as the isolate is very much closely related to the *L. autumnalis, L. canicola* and *L. pyrogens.* Therefore this can be considered as a new isolate which geographically differs when compared to other Indian isolates.

In conclusion, our results suggested valuable phylogenetic information that can be used to characterize relationship in *Leptospira* species. The level of genetic diversity observed among these strains indicates that a multilocus approach using more variable marker is required to differentiate between the closely related strains. The *rpoB* sequencing methodology was

validated from experiments performed with DNA from clinically suspected samples identified by routine methodology by field culturing and identification by dark field microscopy. The PCR products obtained were confirmed by digesting all the amplicons with *ApoI* for which the pattern was two bands of size ~188 and ~412 bp. As reported from the present study, the pGMET cloned product insert was also released by using *EcoRI* enzyme. However, this bacterial species was isolated from the blood of the canine, while there was no doubt, for the fact that the initial diagnosis was confirmed by PCR, but further confirmation was obtained by inoculating the *Leptospira* isolates to the experimental animal and finally the diagnosis was confirmed by sequencing of the *rpoB* amplicons. However, sequence analysis of the 600-bp region of the *rpoB* that we reported herein clearly revealed more extensive variation than expected leading to develop Leptospiral genetic diversity. Finally alignment of *Leptospira rpoB* sequence by clustal W method helped to construct the tree. Sequencing of such an important portion of the gene could be an alternative for the identification of *Leptospira* sp. in properly equipped molecular biology laboratories.

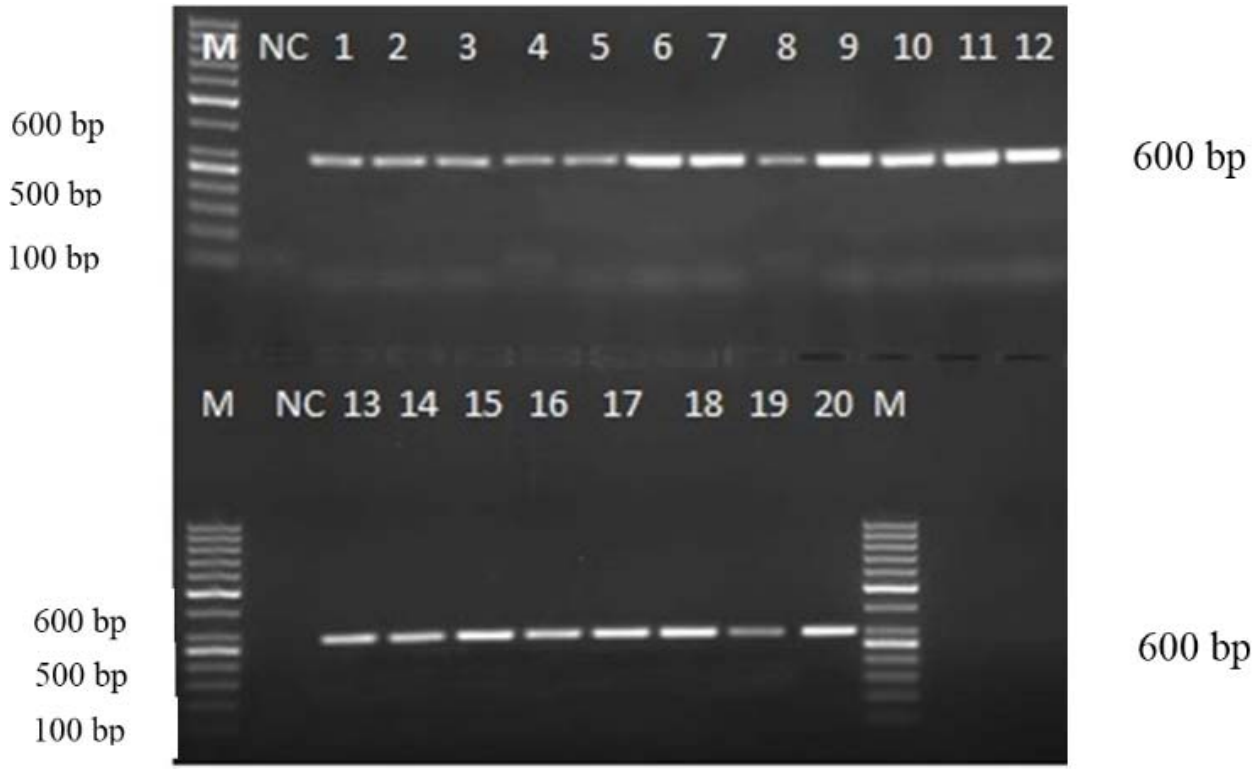

Fig 1: Electrophoresis in 2% agarose gel showing amplification for *rpoB* gene of *Leptospira* species. Analysis products amplified NC-Negative Control, Lanes 1-19- positive PCR product of *Leptospira* samples, Lane 20- positive control. M- 100bp Gene Ruler.

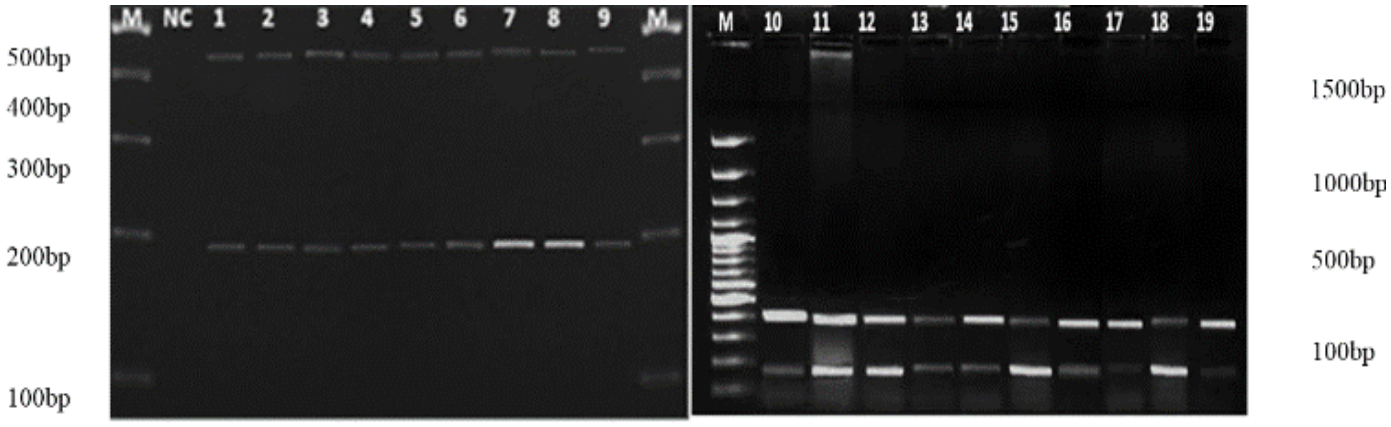

Fig 2: Agarose gel electrophoresis showing the result of restriction enzyme characterization of *rpoB* gene of *Leptospira.* Lane M- 100 bp Gene Ruler, NC- Negative control and Lane 1-19- PCR products digested with *ApoI,* two amplicons at ~188 bp and ~412 bp obtained.

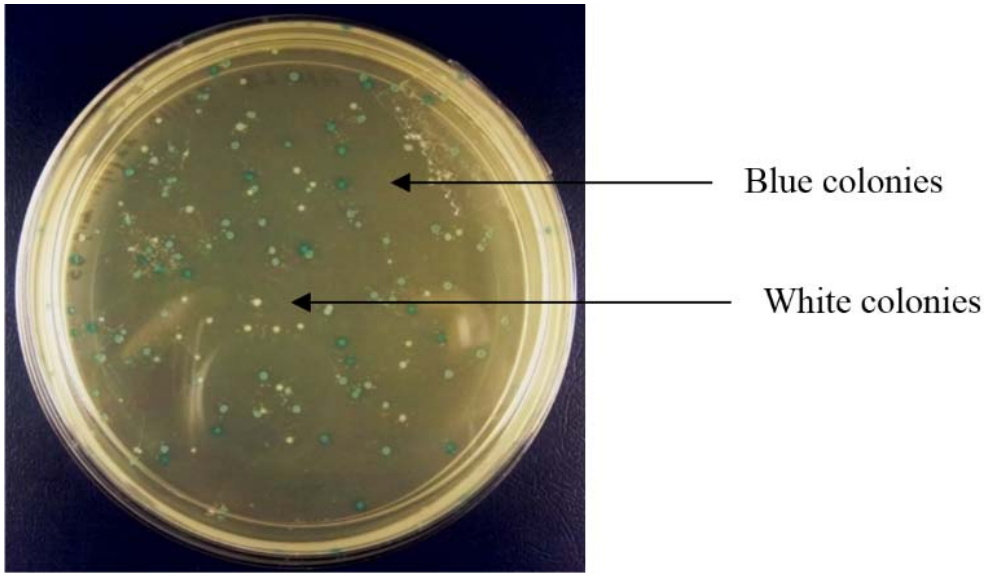

Fig 3: Typical recombinant colonies showing blue and white colonies. Blue: Colony with no vector; White: Colony with vector.

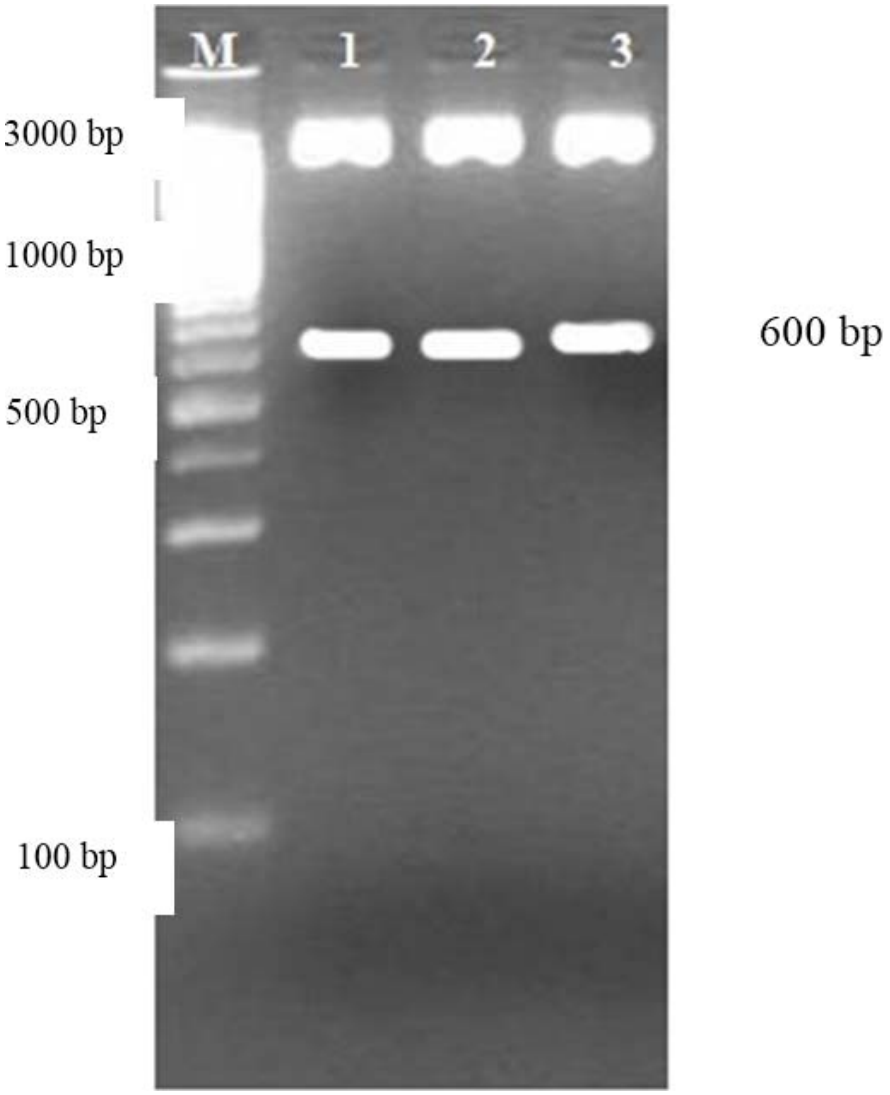

Fig 4: Agarose gel electrophoresis showing the result of insert release of *rpoB gene* from recombinant pGEMT vector. Lane M- 3kb Gene Ruler, Lane 1-3- Ligated products digested with *EcoRI*, two amplicons at ~600 bp and ~3000 bp obtained.

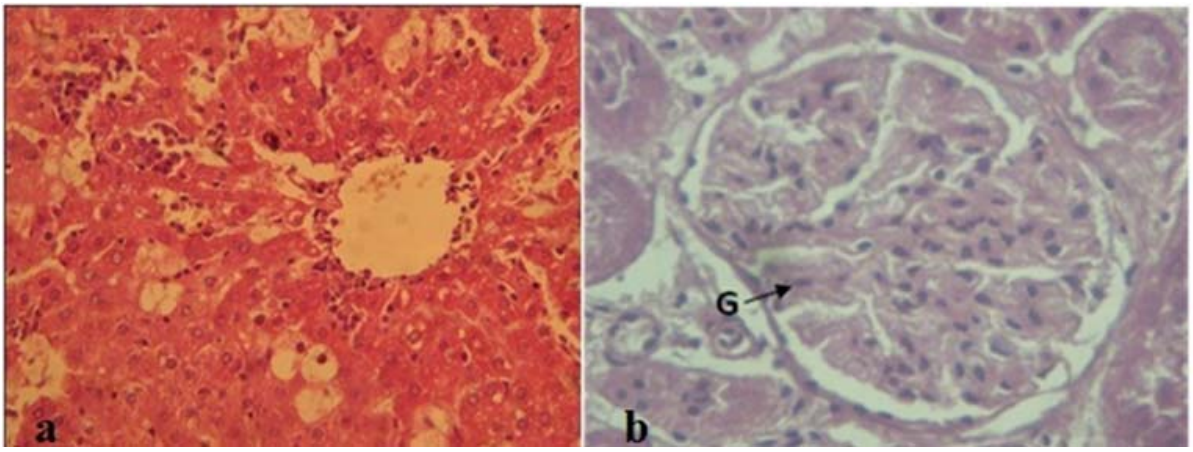

Fig 5a: Liver from mice induced with *Leptospira* species showing typical *Leptospira* infection b Shrunken glomerulus induced kidney of mice showing symptoms of Leptospiral infection. G=Glomerulus.

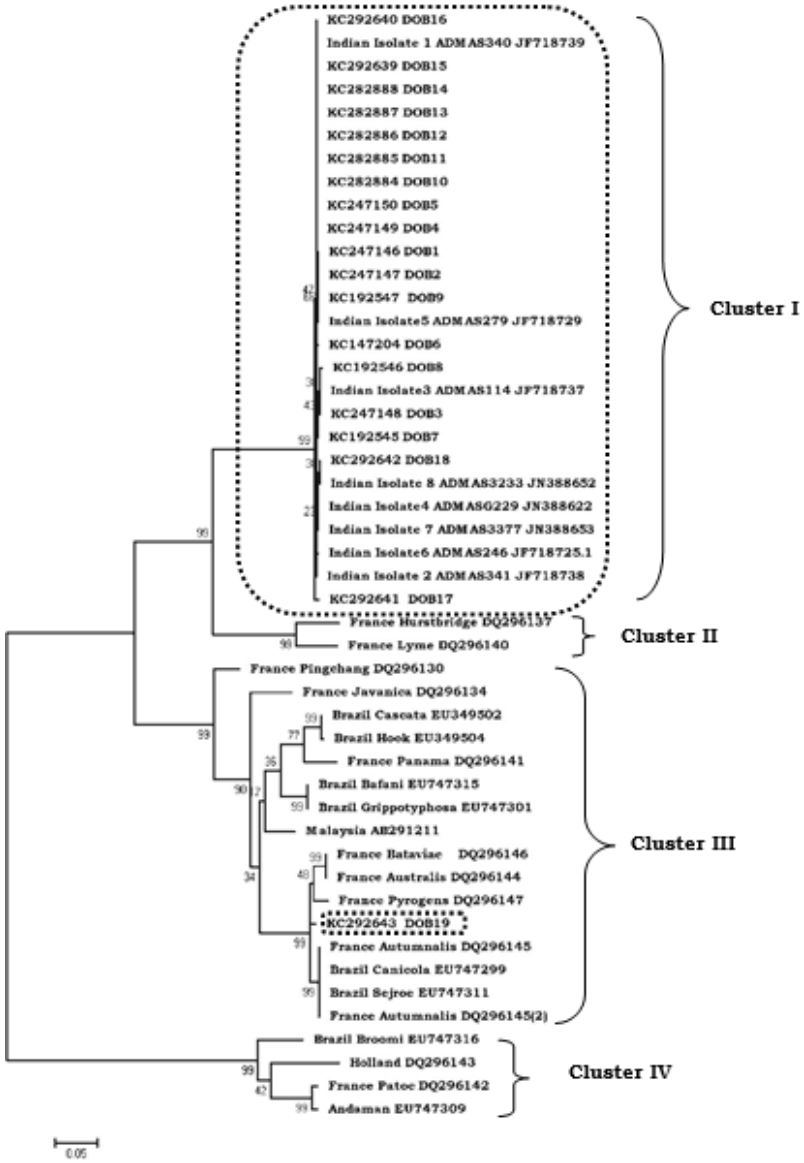

Fig 6: Maximum-likelihood phylogenetic tree of *Leptospira* isolates based on *rpoB* gene sequences. Percentages with cluster designations are *rpoB* gene sequence similarity. Bootstrap values obtained from 1,000 resampling of the dataset, are given at the nodes of the tree. Clusters based on *rpoB* gene sequence analysis are numbered consistently. GenBank accession numbers are given with the country name. Indian cluster on the phylogenetic tree is encircled. Only values greater than 90 % are shown at the branch points. Bars*:* 0.05 substitutions per nucleotide.

Table 1: Strains used in the study for partial *rpoB* gene sequencing and phylogenetic tree construction

Sl. No.	Isolate No.	Genbank Accession Code
1	Lepto/DOB 1	KC247146
2	Lepto/DOB 2	KC247147
3	Lepto/DOB 3	KC247148
4	Lepto/DOB 4	KC247149
5	Lepto/DOB 5	KC247150
6	Lepto/DOB 6	KC147204
7	Lepto/DOB 7	KC192545
8	Lepto/DOB 8	KC192546
9	Lepto/DOB 9	KC192547
10	Lepto/DOB 10	KC282884
11	Lepto/DOB 11	KC282885
12	Lepto/DOB 12	KC282886
13	Lepto/DOB 13	KC282887
14	Lepto/DOB 14	KC282888
15	Lepto/DOB 15	KC292639
16	Lepto/DOB 16	KC292640
17	Lepto/DOB 17	KC292641
18	Lepto/DOB 18	KC292642
19	Lepto/DOB 19	KC292643

The 19 sequences were deposited in the National Center for Biotechnology Information of US (NCBI) under the above mentioned accession codes with the details of isolate names and numbers.

Table 2: Reference strains used for partial *rpoB* gene sequencing and phylogenetic tree construction

Sl. No.	Species	Strain	*rpoB* Gene accession No.
1	*Leptospira genomospecies I*	80-142	DQ296130
2	*L. interrogans*	Bataviae	DQ296146
3	*L. interrogans*	Ballico	DQ296145
4	*L. noguchii*	Panama	DQ296141
5	*L. borgpetersenii*	Javanica	DQ296134
6	*Leptospira* species	ADMAS340	JF718739
7	*Leptospira* species	ADMAS341	JF718738
8	*Leptospira* species	ADMAS114	JF718737
9	*Leptospira* species	ADMASG229	JN388622
10	*Leptospira* species	ADMAS279	JF718729
11	*Leptospira* species	ADMAS246	JF718725
12	*Leptospira* species	ADMAS3377	JN388653
13	*Leptospira* species	ADMAS3233	JN388652
14	*L. interrogans*	Broomi	EU747316
15	*L. kirschneri*	Bafani	EU747315
16	*L.kischneri*	Grippotyphosa	EU747301
17	*L.noguchii*	Cascata	EU349502
18	*L. interrogans*	Canicola	EU747299
19	*L. borgpetersenii*	Sejroe	EU747311
20	*L. noguchii*	Hook	EU349504
21	*L. kmetyi*	Malaysia	AB291211
22	*Leptospira genomospecies* 3	Holland	DQ296143
23	*L.biflexa*	Patoc	DQ296142
24	*L. interrogans*	Autumnalis	DQ296145
25	*L. fainei*	Hurstbridge	DQ296137
26	*L. inadai*	Lyme	DQ296121
27	*L. interrogans*	Pyrogens	DQ296147
28	*L. interrogans*	Australis	DQ296144
29	*L.biflexa*	Andamana	EU747309

Sequences and GenBank accession numbers obtained from National Center for Biotechnology Information of US (NCBI) with the details of species and strain.

Conflict Of Interest Statement

There is no conflict of interest whatsoever among the authors

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