ORIGINAL RESEARCH ARTICLE

Development of PCR assay for targeting partial *lipL21* and *lipL41* gene of *leptospira*

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Abstract

Leptospirosis is a bacterial zoonotic disease caused by spirochetes of the genus *Leptospira* that affects human and a wide range of animals. The direct method of diagnosis of leptospirosis, has been so far by culture isolation but it is time consuming and potentially biohazardous. Another traditional method is the detection of antibodies (Serological tests) which is also a time consuming method and fails to identify the infecting serovar. To overcome these limitations associated with the cultivation and serology, we developed PCR assay targeting partial lipL21 gene and lipL41 gene of Leptospires using in-house designed P28/29 and P30/31 primers, with a product size of 385bp and 427bp. The amplicons were subjected to restriction enzyme digestion using RsaI, Pvu II and HindIII for product of P28/29 and ClaI, TaqI and RsaI were used for product of P30/31. The protocols were standardized and the assay targeting the partial lipL21 and lipL41 gene was found to be specific for eight pathogenic Leptospires out of nine leptospires tested. The products were then cloned in pGEMT Easy vector and sequenced to facilitate further studies. PCR could detect the target bacterial gene without any ambiguity and showed good efficiency in detection of targeted species in the sample. This simple, rapid and cost-effective method can be applicable in a prediction system to prevent disease outbreak by these Leptospira species and can be considered as an effective tool for disease diagnosis of Leptospira species.

Key words: PCR, lipL21, lipL41, Molecular Diagnostics, Transformation.

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Introduction

The economic importance of bovine leptospirosis includes loss due to abortion, loss of milk production and elevated veterinary costs and predominantly human infection (Levett *et al*., 2005). Leptospirosis also known as Weil's disease, canicola fever, canefield fever, nanukayami fever, 7-day fever and many more.

Leptospirosis recognized as one of the most common zoonoses. Leptospirosis is commonly transmitted to humans through contact of animal urine with unhealed breaks in the skin, eyes or with the mucous membranes. Outside tropical areas, leptospirosis cases have a relatively distinct seasonality with most of them occurring during August-September/February-March in year (Zhang *et al*., 1992).

The spectrum of human disease caused by leptospires is extremely wide, ranging from subclinical infection to a severe syndrome of multi organ infection resulting high mortality. This syndrome, icteric leptospirosis with renal failure, was first reported more than 100 years ago by Adolf Weil in Heidelberg. However, an apparently identical syndrome occurring in sewer workers was described several years earlier. Earlier descriptions of diseases that were probably leptospirosis were reviewed recently (Serres *et al.,* 1995).

Leptospira (from the Greek word *leptos* means fine or thin and Latin word *spira* means saprophytic species). *Leptospira* was first observed in 1907 in kidney tissue slices of a leptospirosis victim who was reported to die of "yellow fever" (Abdollahpour, 1990). The direct

method for diagnosis of leptospirosis, has been so far by culture isolation but it is time consuming and potentially hazardous. Another traditional method is the detection of antibodies (serological tests) which is also a time consuming method and fails to identify the infecting serovar. Classification and identification of *Leptospires* using serology is a difficult and tedious procedure. Recently molecular biology techniques have been introduced for detection. These techniques are predominantly used for facilitating the study of the epidemiology of leptospirosis. Genetic taxonomy involves DNA/DNA hybridisation and GC content the guanine-plus-cytosine mol percentages (G+C mol %) content of DNA. The genotypic classification based on DNA hybridization defined 21 genome species of *Leptospira* that include 29 serogroups and 269 serovars. Yasuda *et al.,* 1987 proposed a new classification of *Leptospira* based on DNA homology study on 46 pathogenic and non pathogenic serovars. Woodward and Redstone, 1994 developed a polymerase chain reaction (PCR) combined with restriction fragment length polymorphism which can be used for differentiation of *leptospira* serovars. It is suggested that the PCR combined with restriction fragment length polymorphism is useful tool for rapid detection and preliminary differentiation of *L*eptospires (Levett *et al.,* 2001).

Although over 269 serovars of *Leptospira* have been described, all members of the genus have similar morphology. *Leptospira* are spiral-shaped bacteria that are 6-20 µm long and 0.1 µm in diameter. One or both ends of the spirochete are usually hooked. Because they are so thin, live *Leptospira* are best observed by dark field microscopy. The bacteria have a number of freedom degrees; when ready to proliferate via binary fission, the bacterium noticeably bends in the place of the future split (Levett *et al., 2005*). *Leptospira* have a Gram-negative-like cell envelope consisting of a cytoplasmic and outer membrane. However, the peptidoglycan layer is associated with the cytoplasmic rather than the outer membrane, an arrangement that is unique to spirochetes. The two flagella of *Leptospira* extend from the cytoplasmic membrane at the ends of the bacteria into the periplasmic space and are necessary for the motility of *Leptospira*. The outer membrane contains a variety of lipoproteins and transmembrane outer membrane proteins. As expected, the protein composition of the outer membrane differs when comparing *Leptospira* growing in artificial medium, with *Leptospira* present in an infected animal. Several leptospiral outer membrane proteins have been shown to attach to the host extracellular matrix and to factor H, suggesting these proteins may be important for adhesion of *Leptospira* to host tissues and in resisting complement, respectively. The outer membrane of *Leptospira*, like those of most other Gram-negative bacteria, contains lipopolysaccharide (LPS). Differences in the highly immunogenic LPS structure account for the numerous serovars of *Leptospira*. Consequently, immunity is serovar specific; current leptospiral vaccines, which consist of one or several serovars of *Leptospira* endemic in the population to be immunized, protect only against the serovars contained in the vaccine preparation. Leptospiral LPS has low endotoxin activity. An unusual feature of leptospiral LPS is that it activates host cells via TLR2 rather than TLR4. The unique structure of the lipid A portion of the LPS molecule may account for this observation. Finally, the LPS O antigen content of *L. interrogans* differs in an acutely infected versus a chronically infected animal. The role of O antigen changes in the establishment or maintenance of acute or chronic infection, if any, is unknown (Zhang *et al,.* 1992*;* Bharti *et al.,* 2003). *Leptospires* enter into the body of a susceptible host through mucous membrane or abraded skin. After 4 to 10 days, the host becomes bacteraemic, this period lasting from hours to 7 days, and may be characterised by pyrexia and anorexia (Abdollahpour, 1990).

Materials and Methods

***Leptospira* Serovars.** A total of 9 *Leptospira* serovars namely, *L. icterohaemorrhagiae, L. canicola, L. pamona, L. autumnalis, L. javanica, L. pyrogenes, L. australis, L. hardjo and L. inadai* were procured from the repository of PD_ADMAS(Project Directorate on Animal Disease Monitoring and surveillance), UAS, Bangalore, India.

Cultivation of *Leptospira.* *Leptospira* serovars mentioned above were grown in Luria-Bertani (LB) agar (Difco Detroit, MI, USA) supplemented with 1-3% NaCl at 37^0C overnight.

DNA Preparation. Template Genomic DNA was extracted/isolated from pure culture of corresponding *Leptospires*, using QIAamp DNA minikit (QIAGEN, Germany) and other alternative method described by Hoshino *et al.,* 1998. For it freshly grown bacterial

colonies were suspended in 500 µl TE buffer or 50µl of mid-log phase bacterial culture was added to 450µl of TE buffer, then the cell suspension was boiled for 10 min followed by quick cooling on ice for 5 min, and centrifuged at 12,800 *g* for 5 min. The supernatant was collected and stored at -30^{0}C until use.

Primer Design. All available sequences of the target genes among *Leptospira* spp. were downloaded from the GenBank (http://www.ncbi.nlm.nih.gov/GenBank/GenBankSearch.html). PerlPrimer software by Marshall *et al.,* (2004) was used to design the primers. Sequence for LipL21 holding accession number AY688426 and *lipL41* sequence of *Leptospira borgpetersenii* serovar Hardjo-bovis holding accession number CP000348.1 was retrieved from NCBI-Genbank. It was then used to get conserved sequences. The conserved part of the nucleotide sequence was retrieved and used for primer designing. The criteria that was set for primer design was primer length is 20±2 Target temperature is 55±1° C. The overall theoretical specificities of the newly designed primers were checked using BLAST search. Details of the primers' sequences, their expected amplicons sizes after PCR, etc. are summarized in table (Table 1).

Standardization of polymerase chain reaction (PCR) protocol. In order to achieve the best sensitivity for the PCR using primer P28/29 several combinations of annealing temperatures from 58^{0}C to 68^{0}C were tested. The following conditions were chosen: PCR was performed in 25 ml reaction volume containing 10x Taq buffer with KCl (100mM Tris HCl, 50mM KCl, 0.8% Nonidet P40), 1.5mM $MgCl_2$, 2.5mM of each dNTPs, 2U of Taq polymerase, 25pmoles of each primer and 0.25ml of DNA (40ng/ml) extract from *L. icterohaemorrhagiae.* PCR chemicals were procured from Fermantas. The reactions were carried out in an Eppendorf thermocycler. Initially, PCR conditions were optimized by gradient PCR. The reaction started with an initial polymerase activating temperature of 95^{0}C for 5 min followed 35 cycles of denaturing at 95^{0}C for 1 min, annealing at 63^{0}C for 1 min at G ± 5^{0}C, elongation step at 72^{0}C for 1 min and a final elongation at 72^{0}C for 5 min. Finally annealing temperature was standardized to 60^{0}C for 1 min (Fig. 1a).

The PCR standardization using primers P30/31 was initiated in a 25 ml reaction volume containing 10x Taq buffer with KCl (100mM Tris HCl, 50mM KCl, 0.8% Nonidet P40), 1.5mM $MgCl_2$, 2.5mM of each dNTPs, 2U of Taq polymerase, 25pmoles of each primer and 0.25ml of DNA (40ng/ml) extract from *L. javanica.* The test was done for several combinations annealing temperatures from 55^{0}C to 65^{0}C. The reaction started with an initial denaturation temperature of 95^{0}C for 5 min followed 35 cycles of denaturing at 95^{0}C for 1 min, annealing at 60^{0}C for 1 min at G ± 5^{0}C, elongation step at 72^{0}C for 45 sec and a final elongation at 72^{0}C for 5 min. Finally annealing temperature was standardized to 60^{0}C for 1 min (Fig. 1b). Optimized annealing temperature details shown in table (Table 2).

Agarose gel electrophoresis. A 15 ml aliquot of each PCR product was mixed with 3µl of 6x loading dye (Fermentas) 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-Trans illuminator and documented using gel documentation system with Quantity one software (Biorad, U.S.A) and the PCR product was eluted using eppendorf QIAquick gel extraction kit (QIAGEN, Germany).

Table 2. Details of Standardized thermal cycling condition

	PCR reaction mixture(25ml)	
P28 /P29	10X buffer	2.50ml
	$MgCl_2$	1.50ml
	dNTPs	2.00ml
	Primer-Forward	0.50ml
P30/P31	Primer-Reverse	0.50ml
	Template	0.25ml
	Taq Pol	2.00ml
	Nuclease free water	15.75µl

Table 1. Details of the newly designed primers for specific identification

Primer	Target species/gene	Sequence(5'-3')	Amplicon Length	Reference (Accession no.)
P28/F	*Leptospira*/LipL21	CCAGCACTGACACCGGACAAA	385bp	AY688426
P29/R	*Leptospira*/LipL21	CCGGAACCAACCGCTTTACAT	385bp	AY688426
P30/F	*Leptospira*/LipL41	GACCTCAGTAAACGCGCCGATAT	427bp	CP000348.1
P31/R	*Leptospira*/LipL41	CAGCGGCTTCGTCCAATCCT	427bp	CP000348.1

Characterization of amplicons. The PCR product of primer P28/29 and primer P30/31 were subjected to RE digestion. The enzymes *Rsa*I(GT¯AC) and *Hind*III (A¯AGCTT) were used to characterize PCR product of P28/29 (Fig. 2a) and enzymes *Cla*I(AT¯CGAT), *Taq*I (T¯CGA) and *Rsa*I(GT¯AC) were used to characterize PCR product of P30/31 (Fig. 2b). The reactions were set as under and incubated at 37°C in a water bath for 4h. Restriction enzymes and its corresponding buffer were procured from Fermentas. *Cla*I and its corresponding buffer were procured from Promega. Details of the reaction mixture are summarized in Table 3.

A 15 ml aliquot of each digested products were mixed with 3µl of 6x loading dye (Fermentas) 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-Transi lluminator and documented using gel documentation system with Quantity one software (Biorad, U.S.A) (Fig. 2a and 2b).

Specificity the PCR Assay. The specificity of both the assay developed was tested by taking 10-20ng of each template DNA isolated from the different serovars and the PCR was carried out under the standardized conditions as shown in table (Table 4).

A 10ml aliquot of each PCR product mixed with 2ml of 6x loading dye (Fermentas) was run on a 2.5% agarose electrophoretic gel in TAE buffer and DNA bands were visualized under UV-Transilluminator and documented using gel documentation system with Quantity one software (Biorad, U.S.A) (Fig. 3a and 3b).

Transformation. Ligation mixture was prepared by adding 1ml of pGEMT Easy (50ng) vector at the rate of and 1ml of T4 DNA ligase (3U/ml) to 5ml of 2x buffer. 5ml of DNA (50ng/ml) was added to this mixture for ligation. Ligation was done at 4°C for overnight.

Ligation mixture was added to ice cold 200ml of competent cells and tapped gently, it was incubated on ice for 30 min., then Heat shock was given at 42°C for 1 min. Immediately mixture was kept over ice for 2min. 0.8ml of LB broth was then added to it and incubated at 37°C for 90 min in an orbital shaker. It was then centrifuged at 4000 rpm for 10 min and the pellet was plated on LB agar plate containing ampicillin (50mg/ml) and 40ml of 2% X-gal (5-bromo-4-chloro-3-indolyl-b-galactopyranoside) and 8ml of 2% IPTG (Isopropyl-b-D–thiogalactopyranoside). The plates were then incubated over night at 37°C.

Well-isolated white colonies (Fig. 4) were picked and transferred to 5ml of LB broth containing 5ml of ampicillin (50mg/ml). The tubes were then incubated at 37°C in the shaker for overnight. Plasmid DNA isolation and purification was done using QIAprep spin miniprep kit (Qiagen, Germany) and microcentrifuge.

Characterization of Recombinants. The plasmids isolated from the white colonies were further characterized by the insert release after digestion with *Eco*RI (G ¯AATTC) restriction enzyme. The enzyme *Eco*RI was used to cleave and release the insert in the plasmid (Fig. 5a and 5b). The reaction mixtures were prepared as per the table below and were subjected to digestion at 37°C in a water bath for 1h. Details of the reaction mixture are summarized in table (Table 5).

PCR for Confirmation. The PCR were carried out for confirmation of the recombinants using primer P28/29 and P30/31 under the standardized conditions as described previously using the plasmid DNA as template. It was performed in a 25 ml reaction volume containing 10x buffer (100mM Tris HCl, 50mM KCl, 0.8%Nonidet P40), 1.5mM $MgCl_2$, 2.5mM of each dNTPs, 2U of Taq polymerase, 25pmoles each primer and 1ml of template (1:10 diluted).

5ml aliquot of PCR products mixed with 1µl of 6x loading dye (Fermentas) and loaded on to the wells of 2.5% agarose gel. It was run at 80V for about 45 min. DNA bands were visualized under UV-Transilluminator and documented using Biorad gel documentation system with Quantity one software (Fig. 6a and 6b).

Reaction Mixture	LipL21		LipL41		
	RsaI	HindIII	ClaI	TaqI	RsaI
Buffer	2.0 ml	2.0 ml	2.0ml	2.0ml	2.0ml
Enzyme	1.0 ml	1.0 ml	1.0ml	1.0ml	1.0ml
DNA	8.0 ml	8.0 ml	5.0ml	5.0ml	5.0ml
Water	11.0 ml	11.0 ml	10.0ml	12.0ml	12.0ml
BSA	-	------	2.0ml	-	-

Table 3. Details of the Restriction Enzyme digestion reaction of LipL21 and LipL41

Results and Discussion

Specificity of *Leptospira* spp. Primers. Initially, PCR using P28/29 was standardized by carrying out reactions at various annealing temperatures ranging from 58°C to 68°C on *L. icterohaemorrhagiae* serovar which showed amplification at 58°C, 58.7°C and 60.8°C (Fig.1a)**.** A single band of approximately 385bp was observed at 60°C annealing temperature. A set of PCR reactions were done by varying the template concentration from 5ng to 60ngand could amplify the specific band at all the template concentrations. The PCR products were pooled and characterized by subjecting to RE digestion using *Rsa*I and *Hind*III restriction enzymes, both of which confirmed the product size of P28/29 to be 385bp (Fig.2a). The specificity of this primer was tested on nine different serovars namely *L. icterohaemorrhagiae, L. canicola, L. pamona, L. autumnalis, L. javanica, L. pyrogenes, L. australis, L. hardjo and L. inadai* under standardized condition. The assay targeting the partial sequence of *lipL21* was found to be specific for eight pathogenic *Leptospires* out of nine *Leptospires* tested (Fig. 3a)

For the PCR assay targeted to the partial sequence of *lipL41*, standardization was done initially by carrying out reactions at various annealing temperatures ranging from 58°C to 68°C on *L. icterohaemorrhagiae* serovar. This showed non-specific amplification at 58° C, 58.7°C, 60.8°C and 63.5°C. So PCR reaction using P30/31 was carried out on *L.javanica* template at two different $MgCl_2$ concentration i.e. **(**0.5, 1.0µl**).** No bands could be detected for PCR amplification at 0.5 µl $MgCl_2$ but specific band of 430bp was observed at 1.0 µl $MgCl_2$. Standardization was done by carrying out reactions at various annealing temperatures from 55° to 65°C for 1 min. Good amplification bands were observed for PCR reactions carried out at annealing temperatures 60.5°C, 61.8°C, 63.1°C, 64.2°C, 65.0°C and 65.5°C (Fig. 1b).

The 427bp amplicons thus amplified was subjected to RE. The restriction enzyme pattern of this amplicon confirms it to be a *lipL41* partial gene of *leptospira* genus (Fig. 2b).

The specificity of this primer was tested on nine different serovars under optimized conditions. The PCR assay showed nonspecific amplification along with the specific band. Primer degradation was suspected and the PCR reactions at the optimized conditions were repeated with fresh primer set P30/31. PCR reactions were again carried out on nine serovars at $MgCl_2$

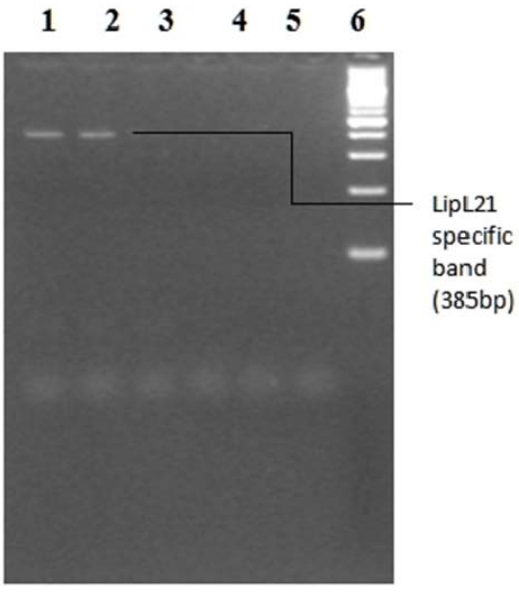

Figure 1. (a) Specificity and detection of annealing temperature of LipL21 gene. Annealing temperature was standardized to 60^0C for 1 min. Lane1-6, PCR products using DNA template of *L. icterohaemorrhagiae*. Lane 1-6 annealing temperature of each lane corresponding to 58^oC, 58.7^oC, 60.8^oC, 63.5^oC, 66.1^oC, 68^oC, respectively. Lane 7, 100bp DNA ladder (Fermentas). Gel run performed using 2% agarose.

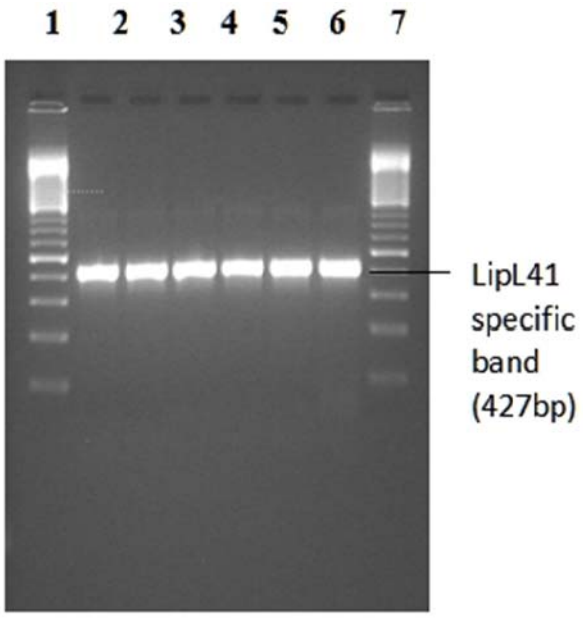

Figure 1. (b) Specificity and detection of annealing temperature of LipL41 gene. Annealing temperature was standardized to 60^0C for 1 min. Lane2-7, PCR products using DNA template of *L.* javanica. Lane 2-7 annealing temperature of each lane corresponding to 60.5^oC, 61.8^oC, 63.1^oC, 64.2^oC, 65.0^oC, 65.6^oC, respectively. Lane 1 and 8, 100bp DNA ladder (Fermentas). Gel run performed using 2% agarose.

Table 4. Details of the standardized conditions for PCR

	Organism	PCR reaction mixture (25ml)	
	L. javanica	10x buffer	2.50ml
	L. autumnalis	$MgCl_2$	1.50ml
P28 /29	*L. hardjo*	dNTP mix	2.00ml
	L. pyrogenes	Forward Primer	0.50ml
P30/31	*L. pamona*	Reverse Primer	0.50ml
	L. australis	Template	0.25ml
	L. icterohaemorrhagiae	Taq Pol	2.00ml
	L. inadai	Nuclease free water	15.75ml
	L. canicola		

Table 5. Details of the Restriction Enzyme digestion reaction for insert release

	LipL21	LipL41
Buffer (10x)	2.0 ml	2.0 ml
Enzyme	1.0 ml	2.0 ml
DNA	8.0 ml	5.0 ml
Water	11.0 ml	10.0 ml
Total	20.0 ml	20.0 ml

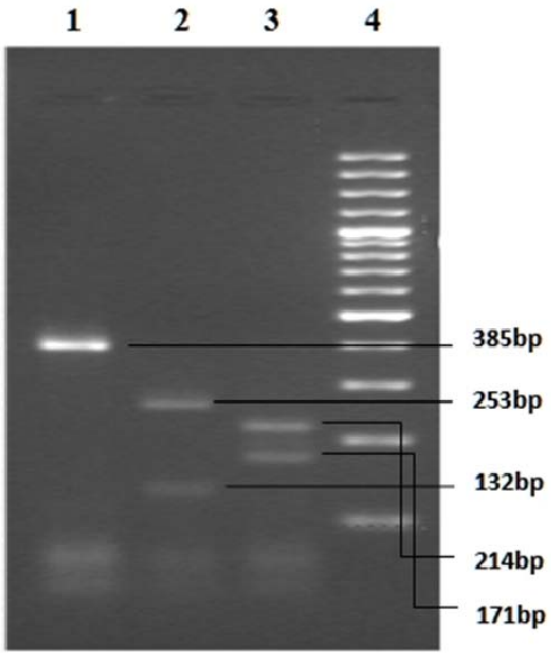

Figure 2. (a) Characterization of Amplicons using restriction enzymes RsaI in lane 2 and HindIII in lane 3 and lane DNA template without RE enzyme in lane 1, lane 4, 100bp Gene Ruler. Gel run performed using 2% agarose.

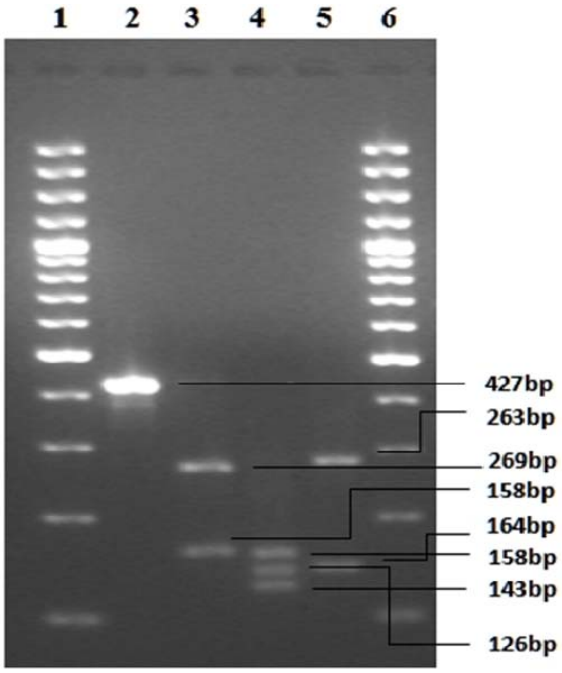

Figure 2. (b) Characterization of Amplicons using restriction enzymes ClaI in lane 3, TaqI in lane 3 and RsaI in lane 5, DNA template with out RE enzyme in lane 2, lane 1 and 6, 100bp Gene Ruler. Gel run performed using 2% agarose.

concentration of 1mM. This assay proved to be only specific to *L. javanica* serovar out of nine serovars tested (Fig. 3b).

The PCR product of both P28/29 and P30/31 of size 385bp and 427bp respectively were then individually cloned into pGEMT Easy TA cloning vector and transformed to JM109 E. *coli* competent cells and plated on LB ampicillin plates with IPTG and X-gal (Fig. 4). The discrete five white colonies and a blue colony were then picked, cultured and plasmid was isolated. The recombinants were then characterized by releasing the insert (Fig. 5a and 5b) using *EcoR*I restriction enzyme followed by PCR reaction (Fig. 6a and 6b) for confirmation. The cloned sequences were then sent for sequence analysis (MWG Biotech Pvt. Ltd., Bangalore) in order to facilitate further studies.

One of the main advantages of using this technique on various biological samples is possibility to detect pathogenic *Leptospires* first by PCR assay and subsequently confirming it through RE digestion thus improving the specificity of the test. Further sequence analysis may reveal the associated serovar causing the disease.

The direct method of diagnosis of leptospirosis is by

culture isolation, but it is time consuming and potentially biohazardous. Another traditional method is the detection of antibodies (serological tests) but it is also time consuming and fails to identify the infecting serovar. 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.

In a previous study by (Merien *et al*., 1992 and Gravekamp *et al,.* 1993), the 16S rRNA gene target could not differentiate pathogenic and nonpathogenic *Leptospira*. The development of PCR assay targeting partial sequence of *lipL21* and *lipL41* gene of pathogenic *Leptospires* using in-house designed P28/29 and P30/31 primers, with a product size of 385bp and 427bp, respectively. These primers were designed by using Gene Tool Lite software. The gene sequence coding for the respective surface protein was subjected to nucleotide blast to get conserved sequences. The most conserved sequence was then chosen and primers were designed.

The newly developed PCR using in-house designed primers produces expected amplicons sizes *i.e.* 385bp for *lipL21* and 427bp for *lipL41*. Afterwards when this PCR protocol was applied to examine all the test samples of this species also produces specific amplicons. No amplicons of specific size was observed in case of non-target *Leptospira,* or other bacterial

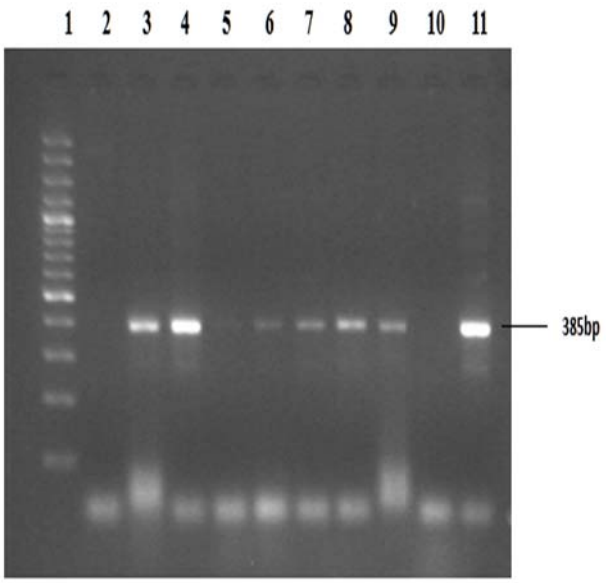

Figure 3. (a) The specificity of this primer tested on nine different serovars under optimized conditions for *lipL21*. Lane 3-11 different serovars, lane 2, negative control and lane 1, 100bp Gene Ruler. Gel run performed using 2.5% agarose.

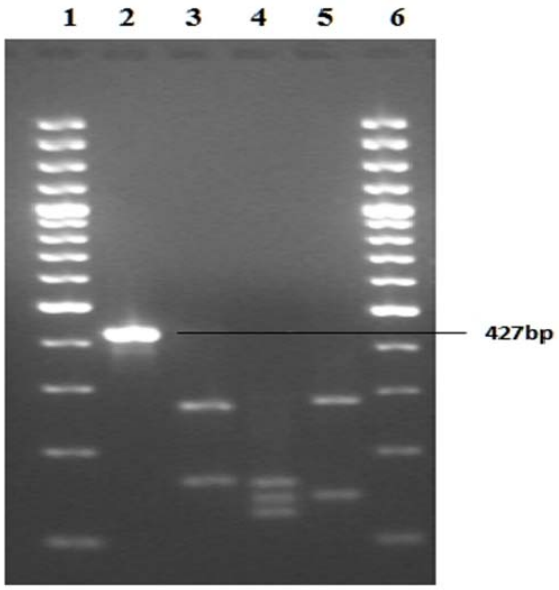

Figure 3. (b) The specificity of this primer tested on nine different serovars under optimized conditions for *lipL41*. Lane 2-5 different serovars and lane 1and 6, 100bp Gene Ruler. Gel run performed using 2.5% agarose.

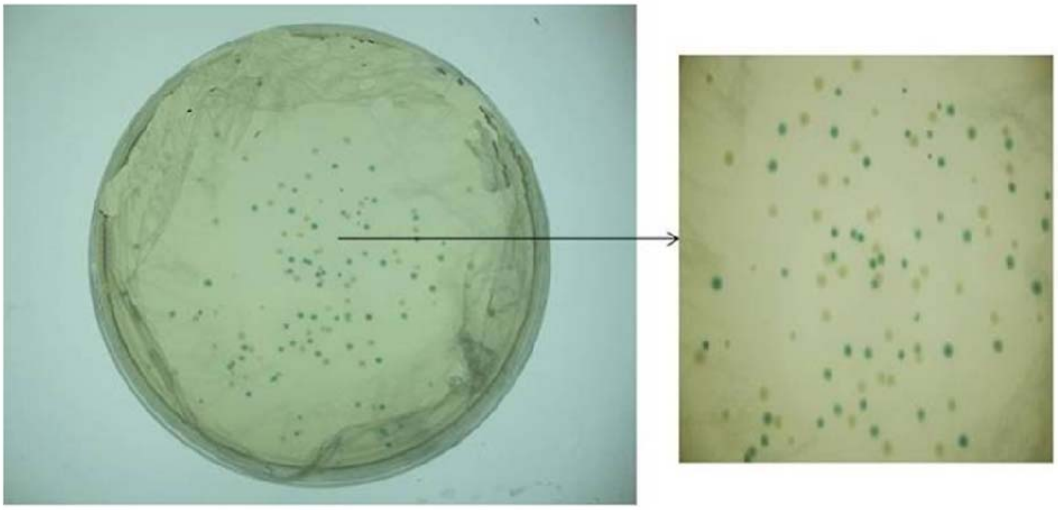

Figure 4. Plate showing recombinant colonies

species. Besides non-specific amplicon of different size did not appear in any case.

In conclusion, the assay targeting partial sequence of LipL41 gene showed high sensitivity and specificity for *L. javanica* out of 9 *Leptospires* tested. The PCR assay targeting partial sequence of *lipL21* gene, which showed good sensitivity and specificity with all the nine serovars tested and may also likely to detect other pathogenic serovars as the *lipL21* sequence targeted under the assay is conserved among all the pathogenic *Leptospires* hence may be valuable tool for early diagnosis of pathogenic *Leptospires* directly from biological samples with clinical suspicion of leptospirosis.

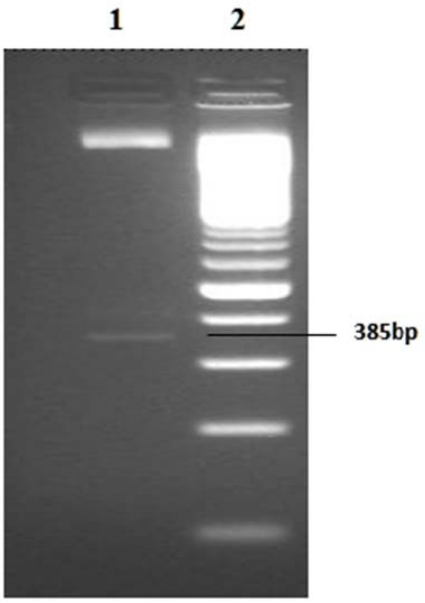

Figure 5. (a) Insert release of *lipL21*from recombinant pGEM-TE vector. Gel run performed using 2.5% agarose.

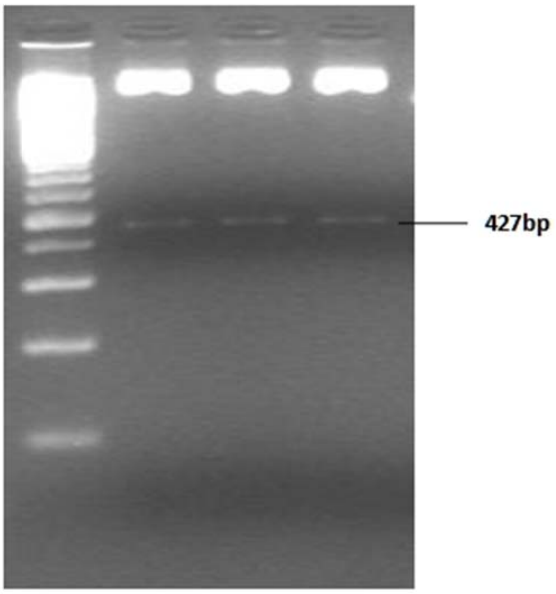

Figure 5. (b) Insert release of *lipL41* from recombinant pGEM-TE vector. Gel run performed using 2.5% agarose.

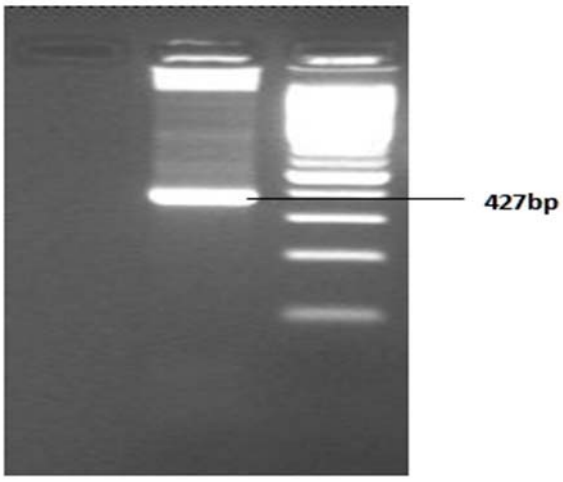

Figure 6. (a) PCR for *lipL21* gene for confirmation. Gel run performed using 2.5% agarose.

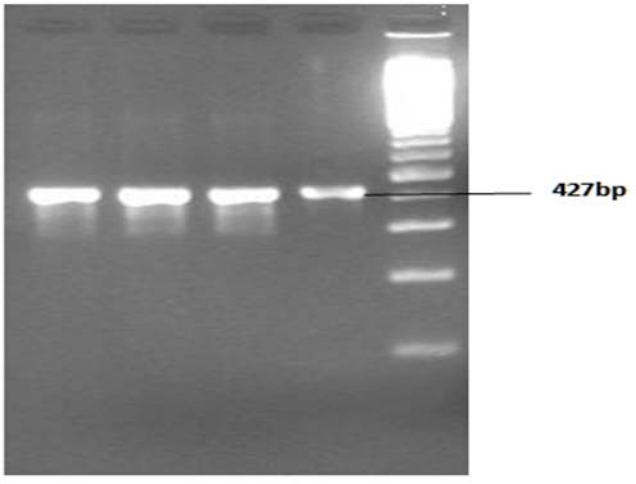

Figure 6. (b) PCR for *lipL41* gene for confirmation. Gel run performed using 2.5% agarose.

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