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COMPARISON OF DIFFERENT PCR ASSAYS BASED ON RIBOSOMAL AND OUTER MEMBRANE PROTEIN GENES FOR DIAGNOSIS OF LEPTOSPIROSIS

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ABSTRACT

Leptospirosis, a disease with protean manifestations, is caused by *Leptospira* species complex. A definite diagnosis of leptospirosis during the first week of the illness is a major challenge to investigating clinical laboratories. PCR is a rapid, sensitive and specific means of diagnosing *Leptospira* infections in the early phase of the disease. In this study, the applicability of routine PCR assays targeting ribosomal genes (16S rDNA and 23S rDNA) and outer membrane protein (omp) genes (*lipl*32, *lipl*21 and *ompl*1) were compared for diagnosis of leptospirosis in clinical settings. PCRs based on rDNA were found more robust, specific and detected more number of samples (20 of 310 blood samples of pyrexia of unknown origin cases of human and 5 of 10 urine samples of canine jaundice) than those of *omp* genes based PCRs. The sensitivity of rDNA PCRs was 10 leptospires/ml of EDTA blood and of *omp* gene based PCRs was 10^3 leptospires/ml. Further comparative analysis of these PCRs was undertaken with culture. All the samples found positive by rDNA PCRs yielded the growth of leptospires following inoculation in EMJH media. So, 16S and 23S rDNA PCRs as compare to *omp* gene based PCR were found to be the most suitable for diagnosis of leptospirosis.

KEYWORDS: Diagnosis, leptospirosis, PCR, 16S rDNA, 23S rDNA

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INTRODUCTION

Leptospirosis is a zoonosis of worldwide distribution[1], caused by infection with pathogenic spirochetes of the genus *Leptospira*. The disease is maintained in nature by chronic renal infection of carrier mammals, which excrete the organism in their urine[2]. Humans become infected through direct exposure to infected animals or their urine, or through indirect contact via contaminated water or soil[3]. Leptospirosis has been recognized as an emerging infectious disease, in part because of recent outbreaks[4-6]. Investigation of outbreaks would be greatly enhanced by the availability of rapid and sensitive diagnostic assays, which can confirm the diagnosis early in the clinical illness[7]. However, diagnosis based on the clinical picture of this disease is inaccurate, especially in the tropical regions where other similar acute, febrile illnesses are common. Leptospirosis may be confused with malaria, viral hepatitis, influenza, dengue fever, rickettsial infections, typhoid fever, melidiosis and others[8]. The available methods for diagnosis include Dark field microscopy, in- vitro culture, serology and molecular method, each one with its merits and de- merits. Serology usually does not contribute to early diagnosis of leptospirosis, because antibodies become detectable around the seventh day of the disease. Conventional methods such as dark-field microscopy or culture to detect leptospires in clinical samples are either unreliable or too slow to contribute to a rapid diagnosis[9-12]. Leptospires circulate in the blood of the patient until about the 10th day after the onset of symptoms. With the introduction of PCR, rapid detection of small numbers of leptospires in clinical samples has become practical due to specific amplification of leptospiral DNA[13-15]. To date, several primer pairs for PCR detection of leptospires have been described. These PCRs have shown various degrees of success in the diagnosis of leptospirosis. But to date, none has become widely available, that can be applied directly on clinical samples for detection of leptospires. In the present study reported primer pairs for ribosomal genes as well as outer membrane protein genes used in PCRs were evaluated on clinical samples of both human and animal sources for diagnosis of leptospirosis.

MATERIALS AND METHODS

(i) Leptospira reference strains

Pathogenic serovars of *Leptospira* species, viz, *L. interrogans* serovars Bataviae, Australis, Autumnalis, Canicola, Icterohaemorrhagiae copenhageni, Hebdomadis, Icterohaemorrhagiae haemorrhagiae and Djasiman; *L. kirschneri* serovars Cynopteri and Grippotyphosa; *L. santarosai* serovars Shermani and Celledoni; *L. weilii* serovar Sarmin and *L. borgpetersenii* serovars Ballum, Javanica and Tarassovi; and that of saprophytic species *Leptospira biflexa* serovar Patoc and *Leptospira meyeri* serovar Ranarum were obtained from the WHO Collaborating Center, Netherlands. Pathogenic serovars of *Leptospira* species *L. interrogans* serovar Pomona and Hardjo bovis; and the saprophytic *Leptospira* species *L. biflexa* serovar Andamana were obtained from the National Leptospirosis Reference Center, Regional Medical Research Center (ICMR), Port Blair, India. All the standard *Leptospira* serovars were maintained in Ellinghausen McCollugh Johnson Harris (EMJH) semi-solid media by regular sub-culturing after every 15 days at 28°C.

(ii) Other Bacteria

The standard culture of *Brucella abortus* strain 19, was procured from the Division of Biological Standardization, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh, India; *Yersinia enterocolitica* O: 9, *Escherichia coli* O157, and *Salmonella typhi* were obtained from Microbial Type Culture Collection, Chandigarh, India. The cultures were maintained by the regular passage using Brain heart infusion agar at 37°C after every one month.

(iii) Clinical samples

Three hundred and ten blood samples were collected from the patients having fever

(pyrexia) for more than 3 days of unknown origin (PUO) with or without jaundice from different regions of our country. Out of which, one hundred and ten samples were collected from Hossur (Tamil Nadu), 50 samples were collected from Dharwar (Karnataka) and 150 samples were collected from Gwalior (Madhya Pradesh). Two ml of blood from each suspected patient was collected in EDTA treated microtubes for the study. Ten urine samples in 20 ml volume were collected in sterile containers from dogs suffering from (PUO) with or without jaundice from CMVL, Meerut.

(iv) DNA extraction

For extraction of genomic DNA from standard cultures of *Leptospira* and other bacteria, exponentially growing cultures were pelleted at 12000g for 30 minutes 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. For preparation of genomic DNA from blood/ urine samples, 2 ml of blood or urine sample was centrifuged at 3000 rpm for 15 min for removal of RBCs from blood and debris from urine sample. Supernatant was collected and centrifuged at 13,000 rpm for 30 min. The pellet was processed for DNA preparation by commercially available kit (Qiagen, Germany) following manufacturer's instruction.

(v) PCR Amplification

The different sets of oligonucleotide primers that were employed in the study are shown in Table 1. Primers utilized for amplification of a genus specific region in 23S rDNA and pathogenic specific region in 16S rDNA of *Leptospira* sp were as described by Woo *et al*.,[16, 17] and Hookey[18] respectively. The primers targeting region between 270 and 692, encoding for *lipl*32 used were as described by Levett *et al*., [19]. The primers for other two genes, namely *ompl*1 and *lipl*21 were designed indigenously with the help of Gene Runner software (Hastings Software Inc., USA). All the sets of primers were synthesized from M/s Genetix, New Delhi.

Table 1
Primer Sequences with Tm used in Different PCRs

Gene	Specificity	Primer Sequence	Size (in mer)	Tm (in °C)
16S rRNA	Pathogenic F	5'-CGCTGGCGGCGCGTCTTAAA-3'	20	66
	Pathogenic R	3'-AAGGTCCACATCGCCACTT-5'	19	60
23S rRNA	Genus F	5'-GACCCGAAGCCTGTCGAG-3'	18	60
	Genus R	3'-GCCATGCTTAGTCCCGATTAC-5'	21	53
*lipl*32	Pathogenic F	5'-CGCTGAAATGGGAGTTCG TATGATT- 3'	25	59.9
	Pathogenic R	5'-CCAACAGATGCAACGAAAG ATCCTTT- 3'	26	60
*ompl*1	Pathogenic F	5' -GGATTTGGGTTACAGTTA-3'	18	49
	Pathogenic R	5'-TCTGCTGATTTGCCCACC - 3'	18	56
*lipl*21	Pathogenic F	5' – GACGCAACTACTGTAG - 3'	16	49.2
	Pathogenic R	5' – ACGTTCTTCCCAGTTGT - 3'	17	50.4

PCR was performed as per the method of Mullis and Faloona[20] and Saiki *et al*.,[21]. The PCRs were first standardized on all the standard serovars of *Leptospira* sp mentioned above. Briefly, each 25 µl reaction mixture contained approximately 50 ng of DNA, 2.5U of Taq polymerase, 200 mM dNTPs, varying concentrations of $MgCl_2$, and primers as outlined in Table 2. Amplification was performed on an icycler thermocycler (BioRad) using the PCR conditions as described in Table 3. Each 5 µl PCR product was electrophoresed on a 1.2% agarose gel for 30 min at 85 V. Amplification products were observed following ethidium bromide staining. Standardized PCRs conditions were then applied on 310 blood samples of human PUO and 10 urine samples of canine jaundice. Table 2

Reaction mix used for PCR

Component	Volume	Concentration
10x PCR buffer	2.5 µl	1x
25 mM $MgCl_2$	Variable	Variable
dNTP mix (2 mM each)	2.0 µl	200 mM of each dNTP
Primer A	Variable	0.1-1.0 µM
Primer B	Variable	0.1-1.0 µM
Taq DNA polymerase	0.3 µl	1 unit / reaction
Distilled water	Variable	-----
Template DNA	Variable	~250 ng
Total volume	25 µl	-----

Table 3
PCR Conditions applied

Step	Time	Temperature
Initial Denaturation	4 minutes	95°C
3 step cycling		
Denaturation	1 minutes	95°C
Annealing	1 minutes	Variable
Extension	2 minutes	72°C
Repeat cycles 30 times		
Final extension	10 minutes	72°C

vi) Isolation of Leptospira from clinical samples

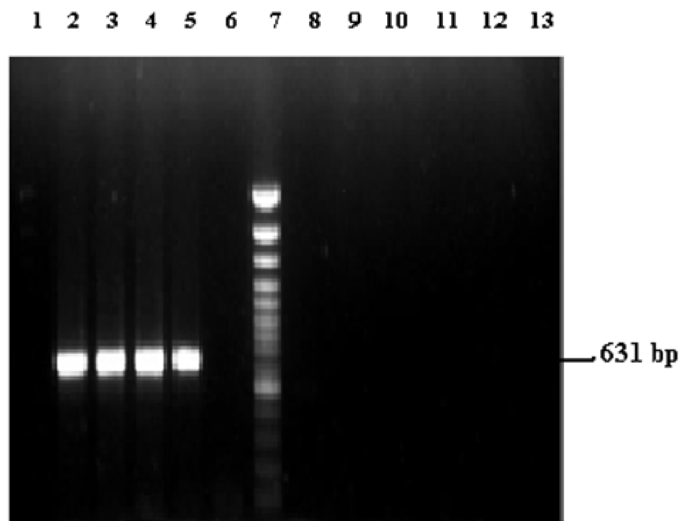
Clinical samples, both blood and urine were inoculated in semi-solid EMJH media supplemented with 200 µg/ml of 5-fluorouracil and 10 percent pooled rabbit sera for isolation of *Leptospira*. 0.1 ml of each blood sample was inoculated separately in media tubes and then incubated at 28°C for 30-45 days. Urine samples were first centrifuged at 3000 rpm to remove debris, followed by centrifugation of supernatant at 13000 rpm for 30 min. Pellet were inoculated in media tubes and incubated at 28°C for 30-45 days. Tubes were observed weekly to check presence any growth.

RESULTS

1. Ribosomal gene based PCRs

The ribosomal gene based PCRs namely 16S rDNA and 23S rDNA were standardized on all the standard serovars of *Leptospira* sp undertaken for the study. Both PCRs were standardized with varying concentrations of $MgCl_2$, primers and annealing temperatures. 16S rDNA PCR yielded optimal amplification of 631 bp with all the 15 standard pathogenic serovars of *Leptospira* at 2 mM $MgCl_2$, 0.5 µM primers and annealing temperature of 68°C. No amplification was seen with the three saprophytic serovars and other bacteria viz. *E. coli*, *Salmonella typhi*, *Brucella abortus* and *Yersinia enterocolitica* (Figure 1).

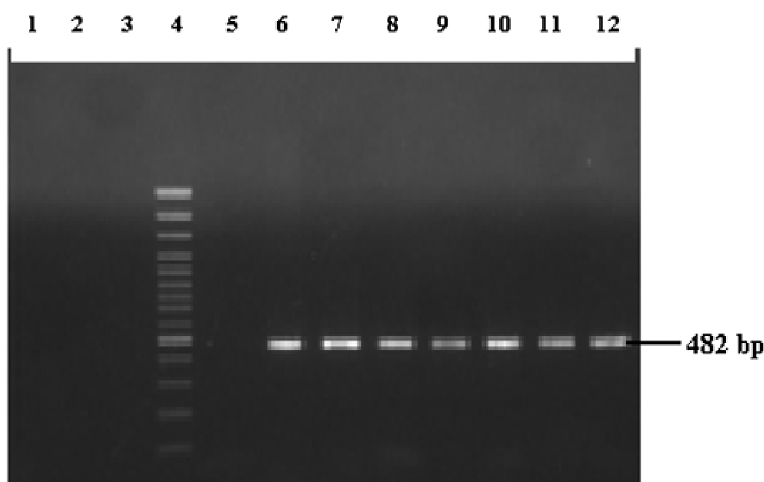
Figure 1
The 16S rRNA PCR on Standard Serovars of Leptospira species and *species belonging to enterobacteriaceae*



***Lane 1: Andamana. Lane 2: Australis, Lane 3: Autumnalis, Lane 4: Grippotyphosa, Lane 5: Icterohamaerrhagie hamaerrhagie, Lane 6: Patoc, Lane 7: 100 bp Marker, Lane 8: Ranarum, Lane 9: E. coli, Lane 10: Salmonella typhi, Lane 11: Brucella abortus, Lane 12: Yersinia enterocolitica, Lane 13: Nuclease free water as negative control*.**

The 23S rDNA PCR showed optimum amplification of 482 bp with all the standard pathogenic and saprophytic serovars of *Leptospira* undertaken for the study at 1.5 mM $MgCl_2$, 0.5 µM primers and annealing temperature of 50° C (Figure 2). No amplification was seen with other bacteria viz. *E. coli*, *Salmonella typhi*, *Brucella abortus* and *Yersinia enterocolitica*.

Figure 2
The 23S rRNA PCR on Standard Serovars of Leptospira species and species belonging to enterobacteriaceae

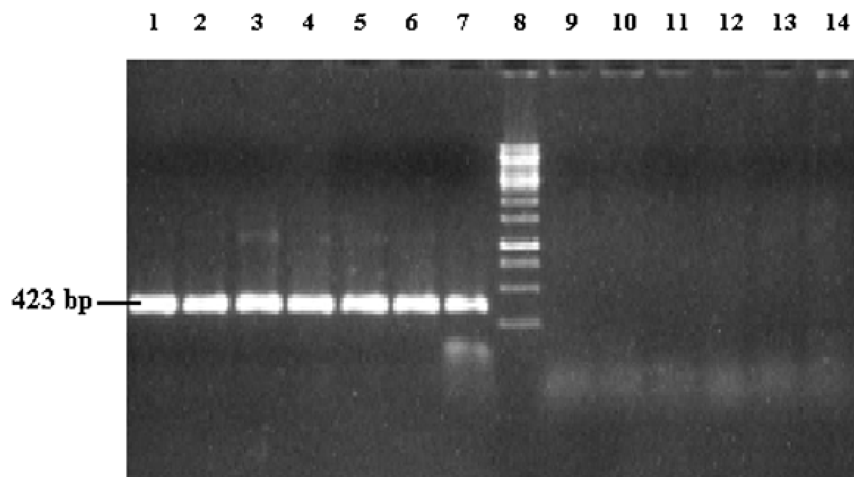


Lane 1: E. coli, Lane 2: Salmonella typhi, Lane 3: Brucella abortus, Lane 4: 100 bp Marker, Lane 5: Yersinia enterocolitica, Lane 6: Andamana. Lane 7: Australis, Lane 8: Autumnalis, Lane 9: Grippotyphosa, Lane 10: Icterohamaerrhagie hamaerrhagie, Lane 11: Patoc, Lane 12: Ranarum.

2. Outer membrane protein gene PCRs

The outer membrane protein genes *lipl*32, *lipl*21 and *ompl*1 based PCRs were standardized on all the standard serovars of *Leptospira*. All the three PCRs were standardized with varying concentrations of $MgCl_2$, primers and annealing temperatures. The optimum amplifications of 423 bp of *lipl*32 were obtained at 2.5 mM $MgCl_2$, 1.0 µM primers and annealing temperature of 60°C, 400 bp of *lipl*21 at 1.5 mM $MgCl_2$, 1.0 µM primers and annealing temperature of 49°C and 800 bp of *ompl*1 at 2 mM $MgCl_2$, 1.0 µM primers and annealing temperature of 50°C (Figures 3-5). No amplifications of these genes were observed in the three saprophytic serovars and other bacteria viz; *E.coli*, *Salmonella typhi*, *Brucella abortus* and *Yersinia enterocolitica*.

Figure 3
lipl32 PCR on Standard Serovars of Leptospira species and species belonging to enterobacteriaceae

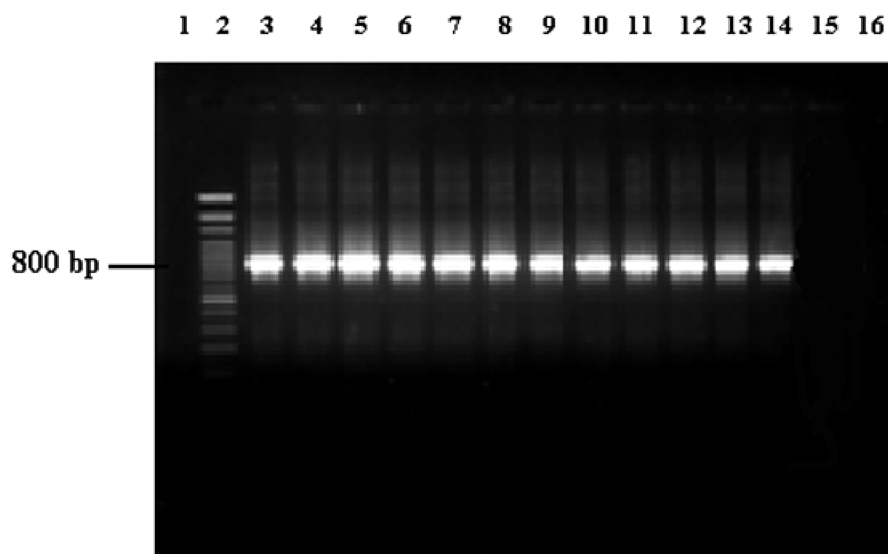


Lane 1: Australis, Lane 2: Autumnalis, Lane 3: Grippotyphosa, Lane 4: Icterohamaerrhagie hamaerrhagie, Lane 5: Canicola, Lane 6: Hebdomadis, Lane 7: Pomona, Lane 8: 1 Kb Marker, Lane 9: Patoc, Lane 10: Ranarum, Lane 11: E. coli, Lane 12: Salmonella typhi, Lane 13: Brucella abortus, Lane 14: Yersinia enterocolitica.

Figure 4
lipl21 PCR on Standard Serovars of Leptospira species and species belonging to enterobacteriaceae

Lane 1: 1 Kb Marker, Lane 2: Australis, Lane 3: Autumnalis, Lane 4: Grippotyphosa, Lane 5: Icterohamaerrhagie hamaerrhagie, Lane 6: Canicola, Lane 7: Hebdomadis, Lane 8: Patoc, Lane 9: E. coli, Lane 10: Salmonella typhi

Figure 5
ompl1 PCR on Standard Serovars of Leptospira species and species belonging to enterobacteriaceae



Lane 1: Patoc, Lane 2: 100Kb Marker, Lane 2: Australis, Lane 3: Autumnalis, Lane 4: Grippotyphosa, Lane 5: Icterohamaerrhagie hamaerrhagie, Lane 6: Canicola, Lane 7: Hebdomadis, Lane 8: Pomona, Lane 9: Hardjo, Lane 10: Ballum, Lane 11: Bataviae, Lane 12: Sarmin, Lane 13: Shermani, Lane 14: Cynopteri, Lane 15: E. coli, Lane 16: Salmonella typhi.

3. Sensitivity of different PCRs

The ribosomal gene based (16S and 23S rDNA) PCRs reproducibly showed the sensitivity of 10 leptospires/ml and *omp* gene based (*lipl*32, *lipl*21 and *ompl*1) PCRs showed10^3 leptospires/ml of blood sample.

4. Evaluation of different PCRs in clinical samples and comparison with culture method

All the PCRs as standardized above were then applied on 310 blood samples of human PUO and 10 urine samples of canine jaundice. Table 4 shows the comparison of all the PCRs. The 16S rDNA and 23S rDNA based PCRs were found to be more sensitive than outer membrane gene based PCRs. They could detect leptospiral DNA in 20/310 blood and 5/10 urine samples of human and canine subjects respectively. The *Lipl*32 gene PCR detected leptospiral DNA in 13 blood and four urine samples of human and canine respectively. PCRs based on *lipl*21 and *ompl*1 genes could detect the *Leptospira* specific DNA in 7 human blood samples and 2 canine urine samples. The results of 16S rDNA and 23S rDNA PCRs were compared with conventional culture method. All the blood and urine samples found positive by these PCRs yielded the growth of leptospires following inoculation in EMJH media.

Table 4
Evaluation of different PCRs on clinical samples

Type of samples	Source of samples	No. of samples	Samples positive by PCRs				
			16S rDNA	23S rDNA	*lipl*32	*lipl*21	*ompl*1
Blood (Human)	Hossur (T. N.)	110	10	10	8	4	4
	Dharwar (Karnataka)	50	5	5	3	2	2
	Gwalior (M.P)	150	5	5	2	1	1
Urine (Canine)	Meerut (U.P.)	10	5	5	4	2	2

DISCUSSION

Leptospirosis has been under reported and under diagnosed from India due to a lack of awareness of the disease and lack of appropriate diagnostics facilities in most parts of the country[22]. The protean manifestations of this disease may present a confusing picture to the clinicians. The disease cannot be diagnosed on clinical grounds alone because its clinical presentations are diverse, ranging from undifferentiated fever to fulminant disease with death occurring in 5–25% of severe cases. It may be confused with malaria, viral hepatitis, influenza, dengue fever, rickettsial infections, typhoid fever, melidiosis and others[23]. Detection of leptospires by culture constitutes the definitive diagnosis; however, it is hampered by slow growth rates of *Leptospira* strains and the long incubation periods before an isolate is established in culture[2, 3]. For this reason, culture is not considered useful as a routine test for diagnosis of individual patients, but remains important for epidemiological purposes. Therefore, diagnosis of leptospirosis is usually accomplished retrospectively by serological tests such as detection of specific antibodies by microscopic agglutination test (MAT), by indirect hemagglutination assay (IHA) or by immuno-enzymatic assays (ELISA).[24] The MAT is the most widely used diagnosis test. However, the MAT may also present problems because of the requirement for live cultures of different *Leptospira* serovars prevalent in a particular geographical area. PCR is more sensitive and rapid method than culture and MAT for the definitive diagnosis of Leptospirosis. A number of PCR assays for leptospiral DNA in tissues and body fluids of animals have been described.[25,26] Results could be obtained in about 1 or 2 days post-infection in comparison to serological or culture methods that may yield the results 7-30 days post-infection. In this study, the applicability of different PCRs targeting genes like 16S rDNA, 23S rDNA, *lipl*32, *lipl*21 and *ompl*1[16-19] were evaluated for diagnosis of leptospirosis in clinical settings. Both 16S and 23S rDNA PCRs were found to be the most suitable for diagnosis of leptospirosis. These PCRs could detect leptospiral DNA in blood and urine samples of human and canine subjects with high sensitivity and specificity.

CONCLUSION

So, 16S and 23S rDNA PCRs as compare to *omp* gene based PCR were found to be the most suitable for diagnosis of leptospirosis**.** These PCRs have advantages over cumbersome and time taking MAT and culture methods and can rapidly confirm the diagnosis in the early phase of the disease when leptospires may be present and before antibody titer is in detectable levels.

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COMPETING INTERESTS

The authors have no financial or non-financial competing interests.

AUTHOR CONTRIBUTIONS

All the authors contributed equally in conceiving, designing and performing the experiments.

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