

Development of a Multiplex PCR Assay for Detection and Discrimination of Pathogenic and Saprophytic *Leptospira* in Water

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Abstract

Leptospirosis is a zoonosis prevalent in tropical countries and affects animals and humans alike. *Leptospira interrogans*, the causative organism for this waterborne infection, spreads through the urine of infected animals. There is a direct link between contaminated water and *Leptospira* outbreaks. This study reports a rapid assay to detect and differentiate pathogenic *Leptospira* from non-pathogenic in environmental water using multiplex PCR. The assay uses primers targeting the *LipI32* and *LipI21* gene. The multiplex PCR has been standardized using 11 pathogenic and one saprophytic serovar of *Leptospira*. The analytical sensitivity of the developed method was evaluated with different concentrations of template DNA. This method was used to screen water samples collected from 20 different sources from Chengalpattu town in Kancheepuram District, Tamil Nadu, India. Of the 20 water samples screened, 13 samples tested positive for pathogenic *Leptospira*, and seven samples tested negative. Four water samples were found to carry both pathogenic and saprophytic species. The developed multiplex PCR assay is highly useful for detecting and distinguishing pathogenic and saprophytic leptospires in water.

Keywords: Leptospirosis, waterborne infection, water, pathogenic serovars, multiplex PCR

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INTRODUCTION

Leptospirosis is a zoonosis having a global presence with a higher prevalence in tropical regions. Heavy rainfall and flooding result in large epidemics causing deaths in developed and developing countries¹⁻³. Humans are infected via exposure to the urine of infected animals or through contaminated soil or water⁴. *Leptospira* species display different affinities and adaptations in different hosts and exhibit clinical symptoms of varying degrees⁵. Rodents are reservoirs and carriers of *Leptospira* but rarely show symptoms of the disease.

Meanwhile, other animals like livestock, cattle, and pets exhibit severe symptoms, including multiple organ injury and abortion upon infection^{6,7}. Similarly, in infected humans, the disease's clinical manifestations range from mild, flu-like symptoms to severe conditions like jaundice, renal impairment, and hemorrhage to life-threatening conditions⁵. A direct correlation between *Leptospira* epidemics and direct contact with contaminated environmental water has been reported. A damaged skin barrier like a cut or an open wound is the most common route of pathogen entry^{2,8,9}.

Based on 16S rRNA phylogeny, the genus *Leptospira* is arranged into three large subgroups and includes at least 21 species. Pathogenic (Group I), Intermediate pathogenic (Group II), and saprophytes are the three subgroups. The infectious groups I and II include 14 species, and the non-infectious group III comprises 7 species referred to as saprophytes and is not known to cause infections¹⁰. Group I pathogens like *L. kirschneri*, *L. noguchii*, and *L. interrogans* have 250 distinct serotypes. They cause disease whose symptoms range from mild infections to severe disease and death. Intermediate pathogens mostly cause mild illnesses that are easily treatable and without fatal complications. Saprophytic *Leptospira* belongs to group III. They are free-living environmental microorganisms and are morphologically similar to pathogenic *Leptospira*, making it hard to discriminate between them by microscopic observation alone¹¹.

Currently, the microscopic agglutination test (MAT) is used to detect leptospirosis. Though this is deemed the gold standard, it lacks sensitivity and cannot be used for early diagnosis.

Culture-based methods are time-consuming and hence unsuitable for rapid diagnosis. Several molecular methods, including conventional PCR, duplex PCR, nested PCR, real-time PCR, and loop-mediated isothermal amplification (LAMP), have been used to detect pathogenic *Leptospira* in clinical samples¹²⁻¹⁶. These PCR based target virulent genes like *LipI32*, *lfb*, *ligA*, and *ligB2*¹⁷⁻¹⁹. Sequence variations in the housekeeping genes, including *gyrB*, *rrs*, and 16S rRNA, have also been exploited to distinguish between pathogenic and saprophytic *Leptospira* in clinical samples^{19,20}.

This study is designed to develop a multiplex PCR assay for rapid detection and differentiation of *Leptospira* strains in environmental water. In order to achieve this, a combination of primers that target a portion of the *LipI21* gene unique to saprophytic *Leptospira* and pathogen-specific *LipI32* gene were used. *LipI32* codes for a surface lipoprotein with a molecular weight of 32kDa and is present only in pathogenic *Leptospira*. Meanwhile, *LipI21* encodes another lipoprotein with a molecular weight of 21kDa. The study was performed using 12 *Leptospira* strains and was successfully used to detect and differentiate between the *Leptospira* strains in environmental water samples.

MATERIALS AND METHODS

Leptospira Strains

A total of 12 *Leptospira* strains obtained from Dr. Natarajasrinivasan, Department of Microbiology, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India, were used in this study (Table 1). The cultures were maintained in a liquid medium (*Leptospira* Medium Base M1009, Himedia) supplemented with 5-fluorouracil and *Leptospira* Enrichment (Himedia). All cultures were incubated at 30°C.

Primers Used

Oligonucleotide primers used in this study (Table 2) were synthesized by Priority Life Science, Coimbatore, Tamil Nadu, India. These primers were used to amplify *LipI21* (582 bp) and *LipI32* (719 bp) regions from *L. meyeri* serovar Semarang and *L. interrogans* serovar Icterohaemorrhagiae respectively. The 582 bp region amplified by *LipI21* FP and *LipI21* RP is specific to saprophytic *Leptospira*²¹.

DNA Extraction

All genomic DNA used in the study was isolated using DNeasy Blood and Tissue kit by Qiagen (Germany). 100 ng of genomic DNA from the different pathogens was used for subsequent PCR reactions.

Sample collection

Twenty different water samples were collected from in and around Chengalpattu town in Kancheepuram District, Tamil Nadu, India. The collected water samples were stored in a sterile glass container at -20°C. The water samples were concentrated for 45 minutes using a vacuum concentrator (Eppendorf, India) and used as templates for multiplex PCR.

Multiplex PCR reaction

A 30 µL PCR mix contains 100 ng of genomic DNA from respective pathogens, 10 pM/L of each primer, 10 mM/L KCl, 20 mM/L Tris-HCl, 8 mM/L MgSO₄, 10 mM/L (NH₄)₂SO₄, 1U of Taq DNA polymerase. The amplicons were visualized on a 1.5% agarose gel stained with ethidium bromide. *LipI21* and *LipI32* amplicons were cloned into the pGEM[®]T-Easy vector (Promega, USA) and

used as positive template controls in the study. The multiplex PCR used an initial denaturation temperature of 95°C for 5 minutes, 35 iterations of denaturation (94°C - 30 seconds), annealing (55°C - 45 seconds) and extension (72°C - 45 seconds), followed by a final extension at 72°C for 5 minutes. Analytical sensitivity of the multiplex PCR was evaluated with varying template DNA concentrations (50 ng/µL, 12.5 ng/µL, 125 ng/µL, 780 pg/µL, 190 pg/µL, 47.5 pg/µL). All experiments were performed in triplicates.

RESULTS

All 12 cultures used in this study were screened for the presence of their respective marker gene (i.e., *LipI32* in the case of pathogenic species and a stretch of DNA sequences in *LipI21* that is found explicitly in non-pathogens). A multiplex PCR technique was developed using recombinant plasmids containing genes *LipI21* and *LipI32*. The amplification products of 582 bp (*LipI21*) and 719 bp (*LipI32*) were observed in a single PCR reaction. The products were confirmed by comparing the uniplex PCR products of *LipI21*

Table 1. List of *Leptospira* strains used in this study

No.	<i>Leptospira</i> strains	<i>LipI21</i>	<i>LipI32</i>
1.	<i>L. interrogans</i> serovar Icterohaemorrhagiae	-	+
2.	<i>L. interrogans</i> serovar Hebdomadis	-	+
3.	<i>L. interrogans</i> serovar Pomona	-	+
4.	<i>L. interrogans</i> serovar Pyrogens	-	+
5.	<i>L. interrogans</i> serovar Sejroe	-	+
6.	<i>L. interrogans</i> serovar Bataviae	-	+
7.	<i>L. interrogans</i> serovar Autamnalis	-	+
8.	<i>L. interrogans</i> serovar Canicola	-	+
9.	<i>L. interrogans</i> serovar Australis	-	+
10.	<i>L. kirschneri</i> serovar Grippotyphosa	-	+
11.	<i>L. borgpetersenii</i> serovar Ballum	-	+
12.	<i>L. meyeri</i> serovar Semarang	+	-

"-": absent "+" : present

Table 2. List of primers used in this study

Target Region	Primer	Sequence (5'-3')	Ref.
<i>LipI32</i>	<i>LipI32</i> FP	CCCCATGAAAAAAGCTTTTCGATTTTGGC	21
	<i>LipI32</i> RP	CCCCTGGAATACCTGGTGGGAAAA	
<i>LipI21</i>	<i>LipI21</i> FP	ATGAAGAAATCACTTATCG	
	<i>LipI21</i> RP	TTACTGAACAGCAGTTGC	

and *Lipl32* (Fig. 1). The multiplex PCR's analytical sensitivity was found to be 190 pg/ μ L (Fig. 2). All the pathogenic cultures used in this study amplified the *Lipl32* region except the saprophytic *Leptospira* serovar Semarang, which tested positive for *Lipl21*. None of the cultures showed amplification products of both genes (Fig. 3). These

outcomes indicate the specificity of the developed method.

In this study, the water samples were concentrated and used as a template for a multiplex PCR. Thirteen water samples tested positive for pathogenic *Leptospira*, and seven samples showed negative amplification for

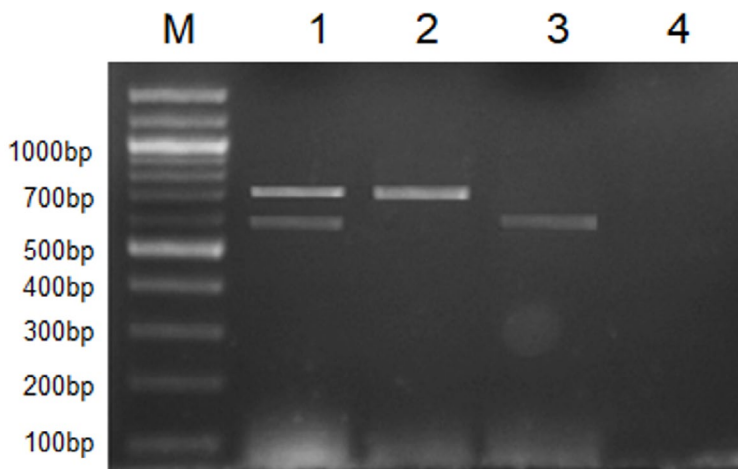


Fig. 1. Multiplex PCR for pathogenic and saprophytic *Leptospira*. M: 100 bp marker, 1: *Lipl32* and *Lipl21*, 2: *Lipl32* from *L. interrogans* ser. Icterohaemorrhagiae, 3: *Lipl21* from *L. meyeri* ser. Semarang, 4: Negative control.

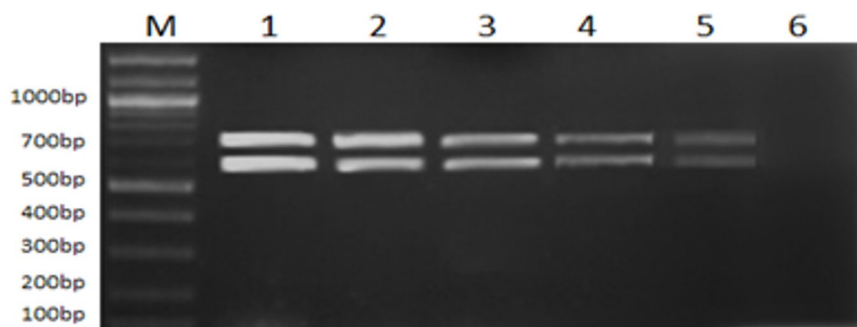


Fig. 2. Sensitivity analysis of Multiplex PCR with different template concentrations of *Lipl32* and *Lipl21*. Lane M: 100bp Marker, 1: 50ng/ μ L, 2: 12.5ng/ μ L, 3: 3.125ng/ μ L, 4: 780pg/ μ L, 5: 190pg/ μ L, 6: 47.5pg/ μ L.

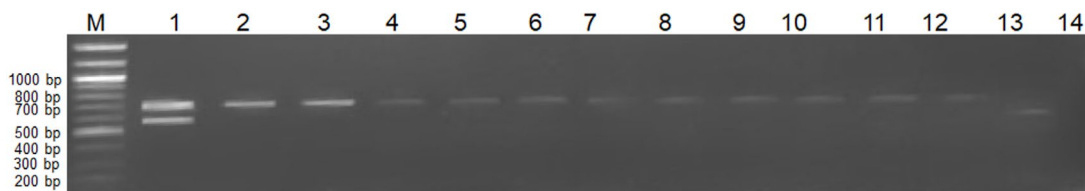


Fig. 3. Multiplex PCR with pathogenic and saprophytic cultures
Lane M: 100bp Marker, 1: *Lipl32* and *Lipl21* plasmid (positive control). 2: *L. interrogans* ser. Icterohaemorrhagiae, 3: *L. interrogans* ser. Hebdomadis, 4: *L. interrogans* ser. Pyrogenes, 5: *L. interrogans* ser. Sejroe, 6: *L. kirschneri* ser. *S. Grippotyphosa*, 7: *L.interrogans* ser. Bataviae, 8: *L. interrogans* ser. Canicola, 9: *L. borgpetersenii* ser. Ballum, 10: *L.interrogans* ser. Pomona, 11: *L. interrogans* ser. Autamnalis, 12: *L interrogans* ser. Australis, 13: *L.meyeri* ser. Semarang, 14: Negative control.

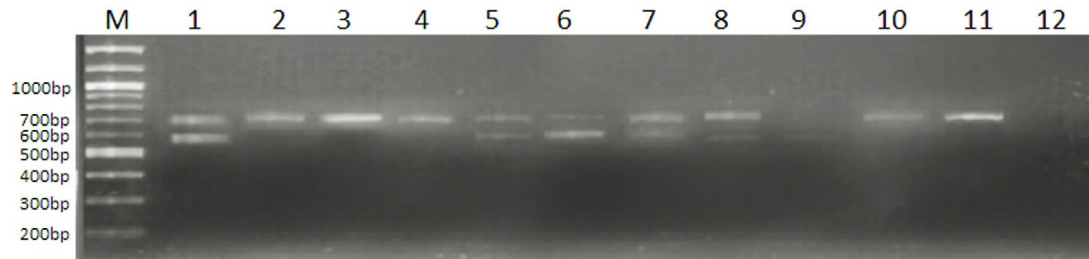


Fig. 4. Multiplex PCR for environmental water samples. Lane M: 100 bp Marker, Lane 1: control plasmid *LipI21* and *LipI32*, Lane 2 to 11: water samples, Lane 12: Negative control

pathogenic and saprophytic *Leptospira*. Four water samples were found to carry both pathogenic and saprophytic *Leptospira*. Few amplification products of negative and positive reactions are shown in Fig. 4.

DISCUSSION

The misdiagnosis of leptospirosis can be attributed to the similarity between the clinical symptoms of leptospirosis and other febrile diseases like dengue and malaria. This, in turn, has led to the underestimation of the prevalence of the disease²². Long incubation periods required for the growth of *Leptospira* make culturing difficult for diagnosis, and observation using dark-field microscopy is unreliable. The serological test MAT, which is considered the benchmark technique for diagnosing this disease, has several drawbacks as it is arduous, slow, and requires maintaining an elaborate collection of reference strains^{23,24}.

The extensive use of molecular techniques has upgraded the speed, sensitivity, and specificity of leptospirosis diagnosis. Many PCR-based methods have been reported for *Leptospira* detection, but each technique comes with limitations¹⁶. The sensitivity and specificity of the PCR significantly depend on choosing reliable target genes and primer optimization. The marker gene *LipI32* used in this study is specific to pathogenic *Leptospira* species. The *LipI21* gene is present in both pathogenic and non-pathogenic *Leptospira*, but sequence variations exist between the *LipI21* sequence of these two *Leptospira* groups. We exploited this sequence variation and developed a multiplex PCR targeting *LipI32* and *LipI21* genes to screen for pathogenic and saprophytic *Leptospira* species. When performed using genomic DNA from pathogenic and non-pathogenic cultures,

the assay showed an analytical specificity of 100%. Water samples from multiple sources were tested using this multiplex PCR method to determine the assay's effectiveness in the field. It was observed that the multiplex PCR assay successfully detected & differentiated between the two groups, even in water samples that contained both pathogenic and non-pathogenic *Leptospira*. These results indicate the usefulness of this method for screening environmental samples. This will help take necessary measures to prevent exposure to *Leptospira*-contaminated water.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

FUNDING

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DATA AVAILABILITY

Details of the isolates and primer sequences used are included in the form of tables. Results of all experiments performed are included as figures in the manuscript.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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