

Simultaneous detection of *LipL32* and *LipL21* genes of pathogenic leptospira from serum samples of bovines by multiplex PCR

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Abstract

Leptospirosis is a worldwide zoonotic disease of cattle associated with pathogenic leptospiral infection. This study focuses in the use of a molecular tool to detect pathogenic leptospiral infection in bovines by targeting the outer membrane proteins *LipL32* and *LipL21* simultaneously in a multiplex PCR. Sixteen pathogenic reference strains and 10 bovine serum samples were analyzed for simultaneous detection of both genes at appropriate annealing conditions. These findings are suggestive of the fact that multiplex PCR can be used to detect major outer membrane proteins of pathogenic leptospira from serum samples. Further it aided in the differentiation of pathogenic and non-pathogenic species of leptospira too. This study will definitely serve as a valuable tool, as it suggests the importance of *LipL32* genes as potential candidates for vaccine development to control animal Leptospirosis.

Introduction

Leptospirosis is one of the world's most important zoonotic disease. It is primarily an infectious disease of ruminants like cattle, sheep and other animal species like dogs and pigs. Man gets infected by contact with infected animals or contaminated animal products.¹ *Leptospira* organisms can be found virtually in all tropical and temperate regions of the world and a large number of serovars distinguishable into broad categories of pathogenic and non-pathogenic groups have been reported. Leptospirosis causes significant economic losses in the cattle industry worldwide due to abortion, reduced milk yield and infertility.² *Leptospira interrogans* serovar hardjo is the

primary causative agent of bovine Leptospirosis throughout the world and responsible for most of the losses attributable to the disease.³ The incidence of *Leptospira* is especially highest during the seasons of heavy rains and floods.⁴

In the present scenario there is difficulty in isolating pathogenic *Leptospira* from serum samples. Molecular tools based on PCR have been proven to be valuable in overcoming these limitations. This methodology can be used to specifically detect the pathogen as it can target specific genes, especially those related to the surface macromolecules that are not present in the saprophytic group.⁵ The present paper focuses on the use of a multiplex PCR assay to simultaneously detect major outer membrane protein Lipopolysaccharide 32 (*LipL32*) and Lipopolysaccharide 21 (*LipL21*) in bovine serum samples from Pudukkottai district of Tamil Nadu, India.

Materials and Methods

Leptospira reference strains

Seventeen reference strains of pathogenic *Leptospira* species cultured and maintained in our laboratory were used in the present study (Provided by ICMR, Port Blair, WHO reference Centre Andaman) (Table 1). The leptospiral strains were cultured in Ellinghausen Mc Cullough Johnson Harris (EMJH) (Difco) media supplemented with 10% Enrichment (Difco) media at 29°C for 7 days, Patoc was the non-pathogenic serovar of *Leptospira* used in the present study.

Serum samples

Serum samples collected from bovines in Pudukkottai district of Tamil Nadu, India was used in the present study.

DNA extraction

DNA was extracted from the reference strains and serum samples by modified Boiling method. Briefly, 200 µL of samples was centrifuged at 10,000 rpm for 10 min. The supernatant was discarded and the pellet was washed twice with phosphate buffered saline (PBS). The final pellet was resuspended in 100 µL of nuclease free water and boiled for 15 min and immediately snap chilled in ice for 10 min. Centrifuged at 10,000 rpm for 5 min and the supernatant containing DNA was quantitated and 1 µg of DNA was used in the Multiplex PCR assay.

Multiplex PCR

Amplification of *LipL32* and *LipL21* genes, which encodes the outer membrane lipoprotein of pathogenic leptospira, was carried out

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using gene specific primers (Table 2).

The two sets of primers are gene specific only for pathogenic strains of leptospira. Multiplex PCR amplification was performed in a final reaction volume of 25 µL. All reactions contained 200 µM dNTPs, 1 mM MgCl₂, 10 pM of each primer, 1.0 U of Taq polymerase and Magnesium free PCR buffer. PCR amplification was performed using the following conditions: Initial denaturation for one cycle at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min with a final elongation time of 72°C for 10 min. The amplified product was electrophoresed on 1.3% agarose gels at 100 volts and stained with ethidium bromide. The *LipL32* gene produced an amplified product of 756bp and *LipL21* gene produced a product of 561bp, and was compared with standard molecular weight DNA ladder.

These products were not detected in the non-pathogenic serovar.

Results and Discussion

Seventeen reference strains of pathogenic leptospira and 1 strain of non-pathogenic leptospira and 10 bovine serum samples were tested for the simultaneous detection of both *LipL32* and *LipL21* genes. The multiplex PCR was used to amplify specific leptospiral outer membrane protein (OMP) sequences of *LipL32* and *LipL21* genes. The *LipL32* gene detected a product of 756bp and the *LipL21* gene could detect a product of 561 bp simulta-

neously in 16 reference strains and all 10 serum samples used in the present study (Figures 1 and 2). *LipL21* gene was not detected in serovar ballum, but *LipL32* could be detected in this serovar also. This is suggestive of how conserved *LipL32* gene is in detecting pathogenic serovars, and explains its role of how valuable it serves for being used as a potential vaccine candidate and as a diagnostic tool.

Three classes of leptospiral OMPs have been identified. The most abundant class comprises the outer membrane lipoproteins and includes the major OMP and immunodom-

inant protein antigen LipL32 and LipL21 the *in vivo*-down-regulated protein LipL36 LipL48 and the surface-exposed protein LipL41.⁶ The present methodology followed in the extraction of DNA was good enough to amplify the target genes of OMP *LipL32* and *LipL21*. The results confirm that both these genes amplify the target gene sequences in pathogenic leptospira species. The bovine serum samples suspected for leptospirosis was sent during monsoon season. It can be well correlated that the monsoon period plays a crucial role in the dissemination of this pathogen from the urine of infected animals.⁷ Detection of both the

genes in serum samples of bovines is suggestive of a clinical leptospiral infection as it very well correlates with the amplified gene regions of the pathogenic reference strains. These findings show that *LipL21* and *LipL32* are surface-exposed, abundant outer membrane lipoprotein that is expressed during infection and conserved among pathogenic *Leptospira* species.⁸

The positivity of all the bovine serum samples analysed and detected by multiplex PCR for two OMP genes is clearly suggestive of the animals having picked up the infection from contaminated water sources or from rodents and other domestic animals which might have been chronic carriers to the infection and would have disseminated the organism through their urine into the soil. Multiplex PCR targeting of the two OMP genes used in the present study aids in the detection of pathogenic leptospira and will be an identifiable marker during infections and outbreaks of leptospirosis. *LipL32* and *LipL21* are the major outer membrane protein found on the surface of pathogenic leptospira and have been reported to be highly conserved in pathogenic species of *Leptospira*.⁹ Pathogenic strains can be detected rapidly for diagnostic purposes. Further, the multiplex PCR takes care as well of the pathogenic and saprophytic group designation.

PCR based fingerprinting facilitates characterization of pathogenic leptospiral isolates at serovar level aiding in defining the genomic pattern of leptospira of a specific area. Since multiplex PCR using two sets of primers gives a specific amplification, pathogenic strains can be detected rapidly and is of immense value in early diagnosis of leptospirosis with enhanced sensitivity and reproducibility. It is well recognized that rainy weather plays a crucial role in the environmental dissemination of this pathogen from the urine of infected animals. Pathogenic leptospira are usually maintained by animal carriers and are rarely isolated from surface water. Our data is in agreement with other authors in that it suggests that these carrier animals are one of the important factors in determining the risk of leptospirosis transmission.¹⁰

Table 1. List of reference serogroups / serovars / strains of *Leptospira*.

Code	Serogroup	Serovar	Strain
AUS	Australis	Australis	Ballico
AUT	Autumnalis	Bangkinang	Bangkinang I
BAL	Ballum	Ballum	Mus127
BAT	Bataviae	Bataviae	Swart
CAN	Canicola	Canicola	H.Utrecht IV
CYN	Cynopteri	Cynopteri	3522 C
DJA	Djasiman	Djasiman	Djasiman
GRI	Grippotyphosa	Grippotyphosa	Moskva V
SEG	Sejroe	Hardjo	Hardjopraj
HEB	Hebdomadis	Hebdomadis	Hebdomadis
ICT	Icterohaemorrhagiae	Icterohaemorrhagiae	RGa
JAV	Javanica	Poi	Poi
LOU	Louisiana	Louisiana	LSU 1945
POM	Pomona	Pomona	Pomona
PAN	Panama	Panama	CZ 214 K
PYR	Pyrogenes	Pyrogenes	Salinem
RAN	Ranarum	Ranarum	ICF

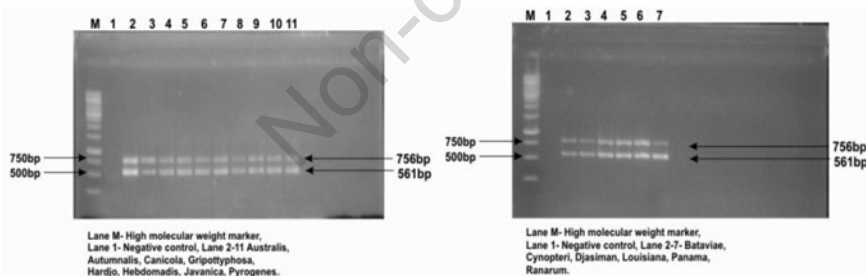


Figure 1. Multiplex PCR on *Leptospira* reference strains. Detection of outer membrane protein genes *LipL32* and *LipL21* in pathogenic strains of leptospira by Multiplex PCR.

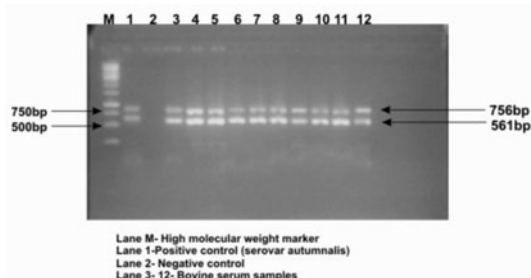


Figure 2. Multiplex PCR on bovine serum samples. Detection of outer membrane proteins *LipL32* and *LipL21* genes of pathogenic leptospira in bovine serum samples by multiplex PCR.

Table 2. Gene specific primers used for multiplex PCR.

Target gene	Primer sequence
<i>LipL32</i>	FP - CATATGGGTCTGCCAAGCCTAAA
	RP - CTCGAGTTACTTAGTCGCGTCAGAA
<i>LipL21</i>	FP - CATATGAAAGACGCAACTACTGTAG
	RP - CTCGAGACGTTCTCCAGTTGT

Conclusions

Outer membrane proteins of pathogenic leptospira are valuable tools for being used as vaccine candidates. In the present study LipL32 and LipL21 OMP conserved genes of pathogenic leptospira were used simultaneously in a multiplex PCR for detecting leptospiral infection in bovines. Though LipL21 is found conserved among pathogenic forms, in the present study gene targets for LipL21 was not detected in serovar ballum of *Leptospira borgpeterseni* genomospecies. This probably could be due to variations in the relative abundances of leptospiral OMPs between strains and serovars. On the other hand LipL32 gene targets could be detected in this genomospecies also and is clearly indicative and is suggestive of the value of LipL32 gene as a potent diagnostic gene for pathogenic leptospira.

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