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Research Article

THE IMPORTANCE OF VIRULENCE ASSOCIATED GENE SEGMENTS OF LEPTOSPIRA IN DIAGNOSIS OF CANINE LEPTOSPIROSIS

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ABSTRACT

Leptospirosis in canines is a health issue inspite of appropriate vaccination strategies being followed. Present vaccines include only a few serogroups and hence infection is picked up by pets from other circulating serogroups of pathogenic leptospira, that does not find a place in the immunizing vaccines. Under such conditions, appropriate diagnosis becomes the need of the hour. Polymerase chain reaction (PCR) is a sensitive diagnostic test to detect past and present infections caused by virulence proteins. The present study remarks on the importance of the gene of virulence Loa22 in the diagnosis of canine Leptospirosis.

INTRODUCTION

Leptospirosis is a worldwide zoonotic disease (Bharti *et al.*2003), caused by pathogenic spirochetes of the genus Leptospira. There are about 250 serovars of leptospira grouped into 29 serogroups. The prevalence of canine leptospirosis varies by region and season, and is considered an emerging infectious disease in dogs. Many dogs with mild leptospiral infection never show any sign at all. When clinical signs do occur they usually appear between 4 and 12 days after exposure to the bacteria, and can include fever, muscle pain, vomiting, diarrhea, loss of appetite, lethargy, dehydration,depression, jaundice, painful inflammation within the eyes and blood in the urine. (Adler *et al*. 2010).

Loa22 is a putative determinant of leptospira, that play a vital role in virulence and is the first genetically defined virulence factor in leptospira species (Meenambigai *et al.,* 2015). The only gene that till date fulfils the molecular koch's postulates for a virulence factor is Loa22. Loa 22 is exposed on the bacterial surface and its expression is up-regulated in an acute model of infection (Nally *et al*., 2007). Loa22, a novel lipoprotein is highly conserved among pathogenic leptospires (Koizumi *et al* 2003).

Canine leptospirosis is under diagnosed due to its wide spectrum of clinical presentations and the lack of a rapid and sensitive test for the accurate diagnosis of acute and chronic infections. ELISA antigens do not support detection of all infecting serovars (Tappero *et al.,* 2000). Recombinant antigens such as LipL32, ompL1, LipL41, LipL36 and heat-shock protein (Hsp58) of leptospira have also been recently developed for evaluation as diagnostic reagents (Flannery *et al* 2001). The immunoreactive proteins, previously described were LipL41, LipL32, LipL21 (Nally *et al* 2007). In the present study the virulence gene Loa22 of leptospira has been targeted for diagnosis of canine Leptospirosis as it is highly conserved among all pathogenic serogroups of leptospira.

MATERIALS AND METHODS

Culturing pathogenic leptospira

Pathogenic leptospira serogroup autumnalis was cultured in Ellinghausen Mc culloughs Johnson Harris (EMJH) medium with 10% enrichment for supplementation of long chain fatty acids and carbon as a source of nutrients for the growth of leptospires. The bacterial strains were grown for 5 - 7 days to reach a density of 2 x 10^8 organisms/ml.

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Nucleic acid extraction

Genomic DNA was extracted from the reference strains utilizing the Quiagen column Kit. Briefly, the samples were vortexed and centrifuged at 10,000 rpm (300xg) for 5 minutes. The pellet was resuspended in PBS and made upto 200 µl. Proteinase K (20µl) was added followed by the addition of 200µl of AL buffer, vortexed and incubated at 56˚C for 10 minutes.The samples were centrifuged at 5,000 rpm for 1 minute.200µl of Ethanol was added and mixed by pulse-vortexing for 1 minute.The content was added into the QIAamp Spin Column (in a 2ml collection tube) and centrifuge at 8,000 rpm (6000xg) for 1 minute.The tube containing the filtrate was discarded.500µl of Buffer AW1 was added and centrifuge at 8,000 rpm (6000xg) for 1 minute.500µl of Buffer AW2 was added and centrifuge at 14,000 rpm (20,000xg) for 3 minutes and the tube containing the filtrate was discarded. QIAamp spin column was transferred into the fresh 2ml collection tube. 200µl of Buffer AE was added, incubated at room temperature for 10minutes and centrifuged at 8000rpm (6000xg) for 5 minutes. The tube containing the filtrate was collected and stored at -20°C to be used for amplification by PCR.

The genomic DNA of clinical serum samples of canines suspected for leptospirosis was extracted by CTAB method

Polymerase Chain Reaction

Designed Primer Sequence for Virulence Gene Loa 22

F 5'-CG**GGATCC**TAAATTCGCAACAGC -3'
R 5'-CCGG**GAATTC**TACAAAAGCAGAC
PCR reaction mix was prepared as follows…

Mastermix	**12.5 µl**
Loa22 Forward primer (20 pmol)	1 µl
Loa22 Reverse primer (20pmol)	1 µl
Template DNA	5 µl
Nuclease-free water	5.5 µl

Total volume 25µl

PCR reaction condition is as follows…

Initial Denaturation	**94 °C**	**5 mins**	
Denaturation	94 °C	30 sec	35 cycles
Annealing	60 °C	30 sec	
Extension	72 °C	30 sec	
Final Extension	72 °C	10 mins	

The PCR products were analyzed on a 1.5 % agarose gel.

RESULTS AND DISCUSSION

The DNA extracted from 12 reference serogroups of pathogenic Leptospira was subjected to amplification of the partial length gene of Loa22 with the designed primers. The amplified gene fragment were electrophoresed on 1.5% agarose gel and an amplicon size of 250 bp was obtained. Various methods are being followed for extraction of genomic DNA. Zhang *et al*., in 2010 extracted genomic DNA from L. *interrogans* serovar Lai using a genomic extraction kit for bacteria (Takara Biotechnology, Dalian, China). (Oliveria *et al*., 2011) carried out genomic DNA isolation from leptospira cultures by guanidine detergent lysis method using DNAzol Reagent (Invitrogen). In the present study, genomic DNA from 12 Leptospira reference serogroups were extracted by Qiagen DNA mini kit method and canine serum samples was extracted by CTAB method (Saad Masri *et al*.,1997)).

In this study all the 12 reference strains of leptospira amplified the loa22 gene with the designed primers yielding a product of 250 bp. Meenambigai *et al* (2015) reported Loa 22 as a putative determinant of leptospira, that plays a vital role in virulence and is the first genetically defined virulence factor in leptospira species. In this study we report the upregulation of Loa 22 during clinical leptospiral infections in canines, in comparison to other outer membrane proteins. A total of 70 canine blood samples referred for clinical leptospirosis was screened by Polymerase chain reaction (PCR) targeting conserved Loa 22.Fifteen (15) samples showed upregulation of Loa 22 gene targets during early infection, with a prevalence rate of 21.4 %. Similar work was done using Leptospira interrogans serovar Lai responsible for promoting inflammatory response in cultured renal cells using rLoa22 by amplifying the gene with pair of primers containing *EcoR1* and *BamH1* recognition site near 5' ends for cloning the gene (Zhang *et al*., 2010). The present study holds relevance and shows that the putative virulence gene Loa22 can be used as an appropriate diagnostic tool for detection of canine Leptospirosis.

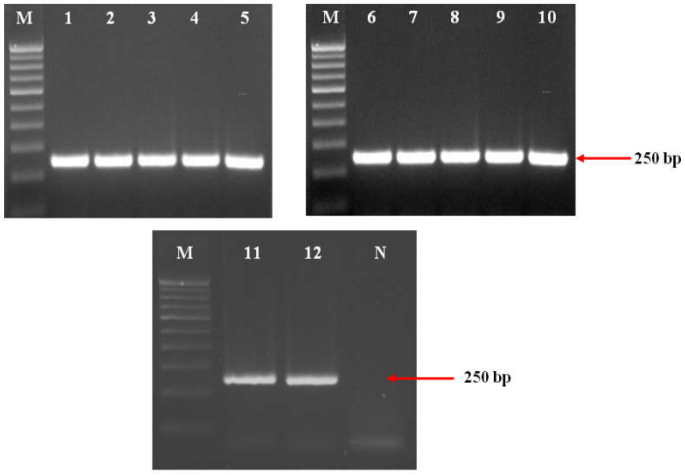

Fig 1 Amplification of Loa22 gene for 12 pathogenic serogroups of Leptospira by PCR

Legend: Lane M - DNA marker 100 - 1000 bp range, Lane 1-12 : 250 bp amplicon of Loa 22 gene from 12 reference serogroups viz., Australis, Autumnalis, Ballum, Canicola, Grippotyphosa, Sejro, Hebdomadis, Icterrohaemorrhagiae,Javanica, Pomona, Pyrogenes and TarassoviLane N - Negative control

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