Virulence Gene Loa22 – The Molecular Diagnostic Beacon of Canine Leptospirosis

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Abstract---Leptospirosis is the most geographically widespread zoonoses of public health significance. Loa 22 is 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 Dark field microscopy (DFM),Microscopic agglutination test (MAT) and Polymerase chain reaction (PCR) targeting conserved Loa 22, LipL32 and Lig B genes of pathogenic leptospira. Fifteen (15) samples showed upregulation of Loa 22 gene targets during early infection, with a prevalence rate of 21.4 % .The targets for LipL32 and Lig B was not detected. Sequencing, blasting and phylogenetic analysis revealed the close association of the clinically significant samples with Leptospira interrogans strain Lai. These results are suggestive of the fact that the virulence gene loa 22 is the holy grail of leptospira diagnostics, as it abides the molecular koch's postulates to be used as a diagnostic marker and is also a prospective vaccine candidate.

Keywords - Leptospira, Loa22, OMP, Phylogeny

I.INTRODUCTION

LEPTOSPIROSIS, a zoonotic disease of worldwide significance, is caused by spirochetes of the genus Leptospira The disease occurs mostly in developing countries and is reemerging in developed countries. The transmission of the infection has been associated with rodent reservoirs, which contaminate the environment through their urine[1]. Leptospirosis has been thought to most commonly affect young-adult, large breed, outdoor dogs; however, small dogs in urban areas can also contract the disease. It is characterized by abortion,still birth, infertility, and death in domestic animals. The prevalence of canine leptospirosis varies by region and season, and is considered an emerging infectious disease in humans as well as dogs [2],[3].

As the course of leptospirosis varies from mild to rapidly fatal forms, the laboratory based techniques are important for the definitive diagnosis. Culturing of the organism is the most demonstrative approach, but it is very laborious and cumbersome. Polymerase Chain Reaction (PCR) based molecular technique can be potentially rapid and specific means of diagnosis of leptospirosis especially in cases of outbreaks [4],[5]. Moieties expressed on the surface of leptospires, are believed to be determinants in the pathogen's interaction with the host and its ability to cause virulence. Few proteins have been experimentally shown to be present on the leptospiral surface [6]. Together, about twelve proteins have been identified as outer membrane proteins and includes LipL32 , LigB and Loa22[7].

The only gene that till date fulfils the molecular Koch's postulates for a virulence factor is loa22. The C-terminal of Loa22 consists of an OmpA domain, which contains a predicted peptidoglycan-binding motif. Disruption of loa22 by Himar1 insertion in L. interrogans led to a complete loss of virulence in guinea pig models [6]. Loa22 is exposed on the bacterial surface and its expression is up-regulated in an acute model of infection [8].

In the present study, the upregulation of loa22 gene transcripts in comparison to other ourer membrane proteins Lipl32 and LigB during clinical leptospiral infections in canines is reported. These genes were targeted as they are conserved only among pathogenic serogroups of Leptospira.

II. MATERIALS AND METHODS

1.*Culturing Bacterial Strains*

Pathogenic leptospires were cultured in Elling hausen Mc culloughs Johnsons harris (EMJH) medium with 10% enrichment to supplement long chain fatty acids and carbon as a source of nutrients for their growth. The cultures were incubated at 28-30°C and routinely subcultured at 7-10 day intervals. Stock cultures were maintained in semi-solid media with 0.2% agar [9].

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2. *Sera Samples*

Sera samples (n=70) were collected from canines, with clinical signs of pyrexia, hematuria,and elevated biochemical parameters. All the suspect sera was screened under Dark field microscopy (DFM) [10].

3.*Microscopic Agglutination Test (Mat).*

The MAT was used as the reference method to determine serum titers, using a battery of live pathogenic leptospires encompassing 3 genomospecies (Table- I). Quantitative MAT was performed with 12 reference serogroups [11] .

4. DNA Isolation

The genomic DNA from Leptospira reference serogroups were extracted by Qiagen DNA mini kit method as per manufacturers instructions.and that of canine serum samples was extracted by CTAB method [12]. This was used as template in PCR assay with primers targeting Loa 22 , Lig B and LipL32 genes

5. *PCR Assay*

Oligonucloetides were designed for genes targeting Loa22, LipL32 and LigB (Table - II). PCR was performed with 12 reference serogroups and 70 canine sera samples. The reaction components consisted of 12.5 µL of 2X red dye master mix (Genei) , 1µL (25 pmol) each of forward and reverse primers and 100 ng of template DNA. in a 25 µL reacton mixture. Specific PCR was performed with the following conditions: Initial denaturation at 94 °C for 5 min, followed by 30 cycles if denaturation at 94 °C for 1 min, annealing for Loa 22 and Lig B at 60 ºC for 1 min and 58^0 C for LipL32, extension at 72 °C for 1min and a final extension at 72 °C for 7 min. The products were checked for amplification by electrophoresis on a 1.5% agarose gel. The amplicons were documented.

6. *DNA Sequencing And Phylogenetic Analysis*

The 245 bp amplicons of Loa 22 gene transcripts amplified from serum samples (n=15) were gel purified (Qiagen quick gel extraction kit) and quantitated (Biophotometer, eppendorf) prior to sequencing.. DNA sequencing was performed at Eurofins, Bangalore, India. Homology of Loa 22 gene was ascertained by blasting the sequence with pathogenic leptospira sequences deposited with NCBI. Phylogenetic tree was constructed on Loa 22 gene transcripts with MEGA6 version software tools.

III. RESULTS

DFM and MAT

Leptospires were not detected in any of the samples screened by DFM.. Among the 70 samples screened by MAT, 24 samples showed low titres (<1:200) and 12 samples showed high titres (>1:800) .

PCR

DNA from 12 serogroups of leptospira when subjected to PCR amplification, amplicons of 245, 380 and 721 bp were observed for Loa 22 in all 12 serogroups, Lig B in eight serogroups and LipL32 in 11 serogroups respectively (Table-III).The amplification of DNA from reference leptospiral serogroups achieved with Loa 22, Lig B and LipL32 has been shown (fig.1 – fig 3).

PCR ON CANINE SAMPLES

Loa 22 gene targets were amplified in 15 out of 70 serum samples tested. None of the samples could detect amplicons for LigB and LipL32 gene targets. (Fig-4 – Fig 6)

SEQUENCING AND PHYLOGENY

Evolutionary relationships of taxanomy was inferred using the Neighbor-Joining method. Divergence times for all branching points in the topology were calculated with the RelTime method using the branch lengths contained in the inferred tree. Evolutionary analyses were conducted with MEGA 6 software programme. All the 15 sequenced canine samples showed its close association with Leptospira interrogans strain Lai .(Fig - 7)

TABLE I
12 PATHOGENIC LEPTOSPIRAL SEROGROUPS USED IN MAT

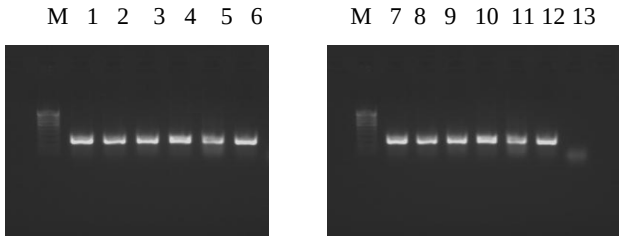
Sl.No	Serogroup	Serovar	Strain
1.	Australis	australis	Ballico
2.	Autumnalis	rachmati	Rachmati
3.	Ballum	ballum	Mus127
4.	Canicola	canicola	Hond Urecht IV
5.	Grippotyphosa	grippotyphosa	Moskva
6.	Sejroe	hardjo	Hardjopraj
7.	Hebdomadis	hebdomadis	Hebdomadis
8.	Icterro haemorrhaegiae	Icterro haemorrhaegiae	RGA
9.	Javanica	poi	Poi
10.	Pomona	pomona	Pomona
11.	Pyrogenes	pyrogenes	Salinem
12.	Tarassovi	tarassovi	peripellicin

TABLE II
PRIMER SEQUENCES OF CONSERVED GENES OF LEPTOSPIRA

S.No	Gene	Primer sequence	Size
1.	Loa22	F5' GAACTCGAGAGATA GTTACGCTCTTGAA-3 R5'CGGGAATTCGCAAAA CGAAAAGTGACT-3'	245 bp
2.	LigB	F5'ATCCGAAGTGGCATAA CTCTCCTCAT-3' R5'ATTTTCAAGATTTGTTC TCCAGATTT-3'	380 bp
3.	LipL32	F5' TGGTCTCTCGAGCCTA AAAAGCTCTT-3' R5'TTTCAAGAATTCAGCA GCGATAGC-3'	721 bp

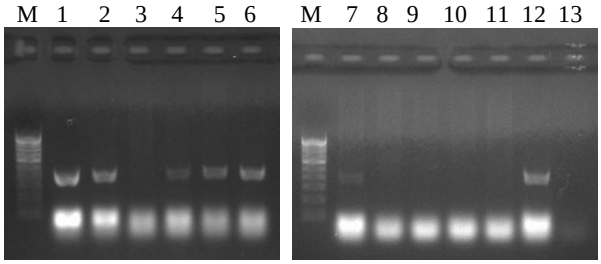
TABLE III
DETECTION OF VIRULENT GENE TARGETS BY PATHOGENIC SEROGROUPS OF LEPTOSPIRA

Gene	1	2	3	4	5	6	7	8	9	10	11	12
Loa22	+	+	+	+	+	+	+	+	+	+	+	+
LigB	+	+	-	+	+	+	+	-	-	-	-	+
LipL32	+	+	-	+	+	+	+	+	+	+	+	+



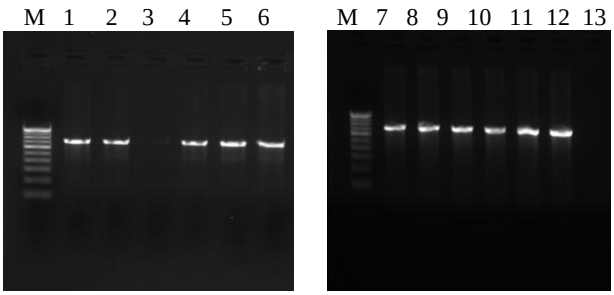
Legend : M - DNA marker 100bp range, Lane 1-12 245bp amplicon of 12 reference serogroups , lane 13 – Negative control

Fig. 1 PCR amplification of Loa 22 gene targets among 12 pathogenic serogroups of leptospira



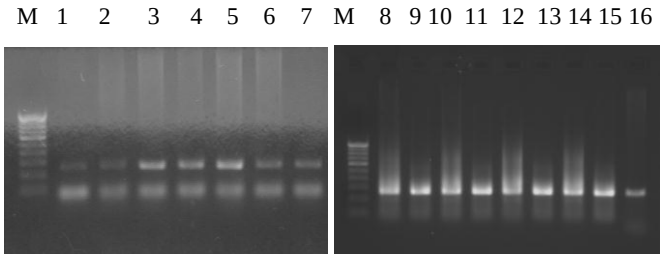
Legend : M - DNA marker 100bp range, Lane 1-12 380bp amplicon of 12 reference serogroups , lane 13– Negative control

Fig 2 Amplification of Lig B gene targets among 12 pathogenic serogroups of Leptospira



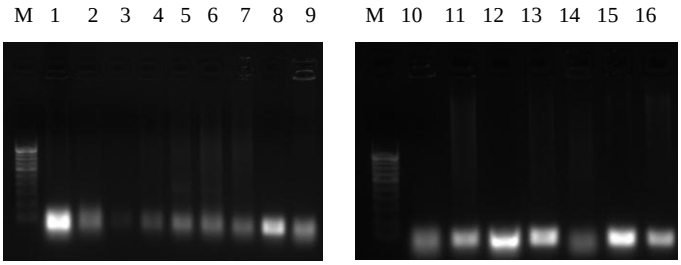
Legend : M - DNA marker 100bp range, Lane 1-12 721 bp amplicons of 12 reference serogroups , lane 13 Negative control

Fig 3 Amplification of LipL32 gene targets among 12 pathogenic serogroups of Leptospira



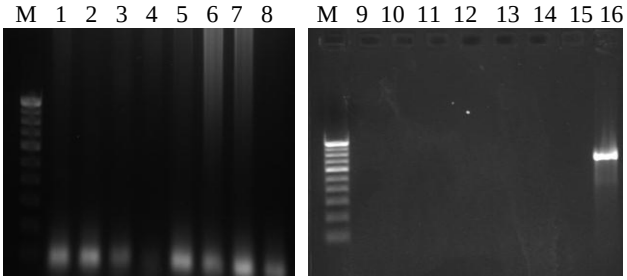
Legend : M - DNA marker 100bp range, Lane 1-15 245 bp amplicons of serum samples , lane 16 – Positive control

Fig 4 PCR amplification of Loa 22 virulent gene target from canine serum samples



Legend : M- DNA marker 100bp range, Lane 1-15 shows negative for serum samples , lane15– Negative control

Fig 5 : Amplification of Lig B gene from serum samples of canines



Legend : M - DNA marker 100bp range, Lane 1-15 shows negative for LipL32 gene for serum sample , lane16– positive control.

Fig 6 : PCR amplification of LipL32 gene from serum samples of canines

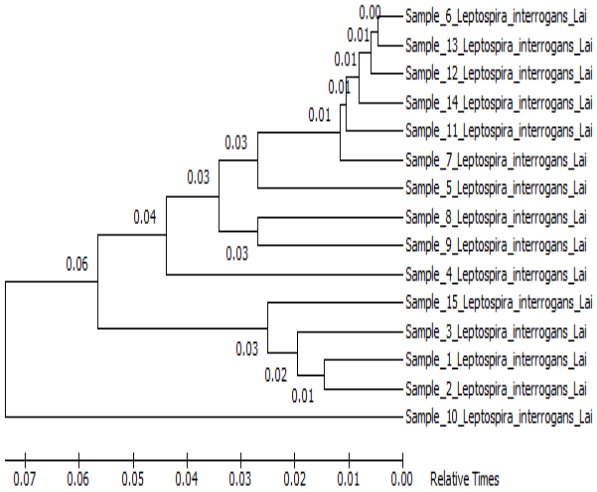


Fig 7 Evolutionary relationships of taxanomy

IV. Discussion

Pathogenic species of leptospira causes leptospirosis globally. Clinical symptoms of this disease do not always allow a precise diagnosis because these are highly non specific and may be mistaken with those produced by several pathogens. This makes the clinical diagnosis alone a non appropriate method that must be accompanied by laboratory tests in order to achieve accurate diagnostic results which may contribute to design of appropriate disease control strategies [9].

In this study, 70 suspect canine sera were examined for 12 reference Leptospira serogroups by the microscopic agglutination test (MAT), which is the gold standard reference serologic test for diagnosing leptospirosis. Totally 34 samples

showed varying titres in MAT with low titres (_< 1 in 200) in 24 sand high titres (> 1in 800) in 12 out of 70 sera samples screened. The MAT cannot provide an early diagnosis since it relies on antibodies to leptospiral antigens and cannot detect the infection until 5–7 days post-exposure.

There was a MAT negative sample that was positive in our study. A specific diagnosis of leptospirosis can be obtained by serological and molecular methods. On the other hand, microbiological culture that is currently considered the most reliable technique for the diagnosis of Leptospira is an expensive time consuming test that may last up to 8 weeks for results [13].

Lipopolysaccharide (LPS) is the main lipid component of the outer leaflet of the outer membrane of Gram negative bacteria. LPS is responsible for the antigenic diversity of pathogenic Leptospira.Based on the isolation techniques, three classes of leptospiral outer membrane proteins have been described as transmembrane, lipoprotein and peripheral membrane proteins. Haake et al. and his group [15] have made major contributions to the analysis of the leptospiral membrane proteins.

LipL32 is surface exposed and accounts for 75% of the outer membrane proteome. LipL32 was long believed to be a putative virulence factor. However this protein is not a prerequisite for virulence since mutant LipL 32 deficient strain still retains its virulence in experimental animals [16]. The role of this major outer membrane protein in pathogenesis remains unclear and is a matter for debate.

Lig proteins are considered a putative virulence factor since members of the bacterial Ig-like superfamily mediate pathogen-host cell interactions, such as invasion and host cell attachment, in other bacteria.LigB mutation in L. interrogans, does not affect the ability of the bacterium to cause acute leptospirosis or persistent renal colonisation in hamsters and rats, respectively [17].

The only gene to date that fulfils Koch's molecular postulates for a virulence factor gene is loa22. Disruption of loa22 by Himar1 insertion in L. interrogans led to a complete loss of virulence in the guinea pig model [15]. Application of mutagenesis has shown that Loa22 was required for full virulence [7],[16]. It was reported that the outer membranes proteins LipL32 and LigB are not essential for Leptospira to cause either acute disease or renal colonization [16],[17].

The goal of this study was to determine the suitable molecular tool for detecting clinically significant canine leptospirosis. Among 70 canine samples, upregulation of Loa22 gene transcripts could be detected in 15 sera and transcripts for LigB and LipL32 gene could not be detected in any of the sera samples screened. This shows the specificity and sensitivity of the Loa 22 virulence gene in detecting early leptospiral infections among canines. Further, the phylogenetic analysis reveals the close association of the field samples with Leptospira *interrogans* strain Lai, which indirectly correlates its association with the detection of the gene of virulence from the same strain on whole genome sequencing.

The discovery of genetic tools to transform leptospires has circumvented a major barrier to elucidating pathogen-related determinants of virulence and has led to the identification of Loa22 as the first virulence factor in Leptospira [6]. Furthermore, Loa 22 is conserved among pathogenic Leptospira and abides the molecular koch's postulates to be used as a diagnostic marker [18], suggesting that it plays a vital role in disease pathogenesis. The results in this study suggest that the virulence gene loa 22 can be used as a diagnostic marker for canine leptospirosis and is also a prospective vaccine candidate.

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