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Differentiation of Infected from Vaccinated Dogs by Latex Agglutination Test Using Genus Specific Recombinant Letospiral Immunoglobulin like B Protein of Leptospira

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Abstract: Leptospirosis is considered the most widespread zoonotic disease in the world. The present study evaluated the efficacy of recombinant leptospiral lipoprotein LigB (rLigB) as a DIVA strategy tool when employed in LAT to differentiate between dogs vaccinated against leptospirosis and those that are naturally infected. A total of 100 sera collected from dogs vaccinated against leptospirosis with microscopic agglutination titre ranging between 100 to 1600 and 25 sera samples from dogs suspected of having leptospirosis were subjected to rLigB based LAT. The sera from MAT positive vaccinated dogs (88%) were found to be negative in rLigB based LAT, whereas all the 22 MAT positive sera of infected animals gave positive results. Thus, the rLigB based LAT could be a useful, rapid, screening test to differentiate between vaccinated and naturally infected canine sera. Present study also indicates that the sensitivity and specificity of recombinant LigB protein based Latex Agglutination Test was comparable to MAT which is universally considered the Gold Standard Test for diagnosis of leptospirosis since LAT test result for the naturally infected canine sera was in concordance with MAT. The study conducted using MAT for suspected field sera revealed *L. Icterohaemorrhagiae* and *L. grippotyphosa* as the most prevalent serovars in canines in Bareilly region. In conclusion, the rLigB based LAT having sensitivity and specificity and also possessing the additional advantage of being a DIVA strategy tool, it can serve as a pen site diagnostic test.

Key words: Leptospirosis・MAT・rLigB Based LAT・DIVA

INTRODUCTION

Leptospirosis of animal and man is considered as an important re-emerging infectious disease worldwide. Leptospirosis in dogs is recognized as a risk factor for human Leptospirosis [1-3]. Increased in rainfall is associated with a rise in the prevalence of leptospirosis in dogs [4]. In case of dogs, the symptoms include fever, vomiting, dehydration, hepatitis, nephritis and death [5]. Infection can lead to pulmonary haemorrhage, renal and hepatic failure but sometimes it leads to multi-organ failure and even death [6]. An infected dog can also act as an asymptomatic carrier and shed infectious organisms in the urine for its entire lifetime [7, 8]. The diagnosis of leptospirosis is very difficult in early stages of the disease because initial symptoms are often indistinguishable from that of other febrile illness [9, 10]. Considering the problems in the isolation or demonstration of leptospires in clinical samples, emphasis has been largely laid on serodiagnosis. Microscopic agglutination test (MAT) is considered as the gold standard test for detecting leptospirosis has been the test of choice for the diagnosis of leptospirosis. This test is highly sensitive when performed on paired sera (acute and convalescent) and is serovar/serogroup specific. MAT is useful for epidemiological purpose since it gives clear idea regarding the circulating serovars in an endemic region. Ideally MAT should be performed on paired sera collected during acute and convalescent stage of the disease to find out sero-conversion or four-fold rise in

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titre, which is the evidence of current or recent infection [11]. Since collection of convalescent serum sample is difficult in routine practice, several disease investigators usually consider a titre of 1 in 100 as a significant titre of diagnosis without considering the endemicity or baseline titres in the community.

However MAT requires the use of several leptospiral serovars in their active growth phase whose maintenance is difficult, expensive and tedious and time consuming [12-14]. The danger of acquiring the infection to lab technicians while handling the live leptospiral antigens, the cumbersome mechanisms of recording the results and the need for paired sera samples to confirm the disease which delays disease diagnosis has frustrated many researchers to adopt it as a routine test. Moreover MAT can give false negative test result when sera is collected from a patient in the early stages of the disease or when the patient is infected with a serovar which is not included in the test. MAT has also been reported to give false positive test result in canines affected with Lyme disease and hence low MAT titres need to be judged with some degree of caution. MAT cannot distinguish between IgM antibodies indicative of current infection and IgG antibodies indicative of past infection. Further, MAT cannot differentiate between vaccinated and naturally infected animal sera and gives positive test result in both the cases which suggests that this test is not DIVA (Differentiating Infected from Vaccinated Animals) strategy based.Therefore, more widely acceptable approaches of serodiagnosis like ELISA, Lepto Dipstick, Lepto dri-dot, Lepto lateral-flow, Latex Agglutination test have been developed, which take the advantage of presence of genus specific proteins present in the outer membrane of the pathogenic leptospiral organisms [15-18].

Three classes of these leptospiral proteins i.e. outer membrane proteins (OMPs) have been identified. The most abundant class comprises the outer membrane lipoproteins which includes immune-dominant protein antigen Lip L32 [19], the in vivo down regulated protein LipL 36, LipL 48 [20], surface exposed LipL41 [21] and Bacterial immunoglobulin like domain proteins Lig A and Lig B [22].

The antigens which are well characterised for their diagnostic potential so far, are Lip L32 and Lip L41 [23]. Apart from these two lipoproteins, Lig A and Lig B proteins are drawing much attention. Anti Lig B IgM antibodies were found in sera of 92 % patients during acute phase leptospirosis [24]. The research workers opined that the use of this marker may aid the prompt and timely diagnosis required to reduce the high mortality associated with severe forms of the disease. Moreover, recent studies indicate the use of N-terminal, conserved region of Lig B protein in differentiating between naturally infected and vaccinated canine sera [25, 26]. So, this study investigated the potential of using genus specific rLigB of leptospira based LAT as a diagnostic as well as a DIVA strategy tool. Thus the aim of the study were (1) standardisation of diagnostic test like LAT employing recombinant LigB protein with sera samples from field, (2) comparison of the efficacy of LAT with MAT (3) differentiation of vaccinated from naturally infected sera and (4) efficacy of vaccine in immunized dogs.

MATERIALS AND METHODS

Recombinant Lig B Protein Expression and Coating on Latex Beads: In order to express the N terminal conserved region of Lig B gene, a set of primers has been designed targeting a 1200 bp long region of LigB genes using Integrated DNA Technology software, NEB cutter and Editseq of DNAstar. PCR was performed to amplify the 1200 bp long conserved N terminal region of LigB gene. The amplicon was extracted from gel using the instructions given by the manufacturer and using the PCR product as template, Nested PCR was performed using a set of internal primers which gave rise to a product of 600 bp length. The 1200bp amplicon was then inserted into TA cloning vector pTZ57R using DNA Ligase enzyme. This recombinant vector was used to transform competent DH5á cells treated with chilled 0.1 M CaCl2 and the recombinants obtained were plated on LB Ampicillin plates. In order to confirm the recombinant clones, Colony PCR and RE digestion of the vector using SacI and HindIII enzymes were done for confirmation. The insert released from pTZ57R vector was ligated on to pQE 30 vector treated with the same set of RE enzymes and was transformed into competent M15 cells of *E coli* and the recombinants obtained were plated on LB Ampicillin plates containing Kanamycin. Colony PCR and RE digestion of the pQE vector using SacI and HindIII enzymes were also done for confirmation. The confirmed recombinant cells were used for expression of Lig B gene by inducing the cells with 1mM IPTG during the log phase of growth and SDS-PAGE was then performed to check for the expression of the recombinant protein. LigB protein appeared as a thick band at the expected size of 46 KDa. Later the minimum concentration required for the induction of LigB protein was determined to be 0.1mM

IPTG. The time kinetics for the recombinant protein was also studied and it was found that detectable amount of recombinant protein was present in SDS PAGE starting from two hours after induction and after eight hours maximum amount of LigB protein was detected in SDS PAGE. The bulk production of the recombinant LigB protein was done by inoculating 0.5ml of bacterial culture in 100 ml of LB broth containing Ampicillin and Kanamycin. The bacterial cells were harvested by centrifugation and the bacterial pellet obtained was dissolved in lysis buffer (pH 8.0) and kept in a rocking platform for 2 hours. The lysis buffer containing lysed bacterial cells was then centrifuged twice at 10,000 rpm for 20 minutes. The clear supernatant obtained was passed through Ni-NTA agarose chromatography column. The 6X Histidine present in the N terminal portion of the Lig B protein helps the protein to bind to Ni present in the chromatography column. The wash buffer (pH 6.3) was used to wash away any unbound protein. The bound Lig B protein was eluted using elution buffer (pH 4.5). The presence of the purified protein was then determined using SDS-PAGE and the elution fractions E4-E6 (0.5 ml each) contained the maximum concentration of the protein. This fraction were used for dialysis against PBS to remove urea and the dialyzed protein used to coat the latex beads which had been used for performing LAT against field sera. These sensitized latex beads were stored at 4^0C and used for performing LAT against field sera suspected for Leptospirosis by detection of anti-LigB antibodies. Dot Blot and Western blot analysis were also done to test the immunoreactivity of rLigB Protein using known positive and negative canine sera samples.

Screening of Vaccinated and Naturally Infected Canine Sera by rLigB based Latex Agglutination Test: A total of 100 canine sera samples from healthy dogs immunized against leptospirosis using tetravalent vaccine giving protection against *L. Pomona*, *L. Grippotyphosa, L. Icterohaemorrhagiae* and *L Canicola*. brought to the Veterinary Polyclinic, Indian Veterinary Research Institute were collected. Serum samples from 25 dogs showing clinical signs suspected of leptospirosis and further supported by biochemical test parameters such as liver function (Elevated AST and ALT levels) and Kidney function tests (Elevated levels of BUN and Creatinine) suggestive of leptospirosis were also collected. All the vaccinated canine sera samples were tested by MAT as described by Cole, *et al.* [27] with the whole cell live antigens of the following serovars: Pomona, Grippotyphosa, Icterohaemorrhagiae and Canicola. These samples were further tested by r LigB LAT. The field sera samples suspected for leptospirosis were tested by MAT against a battery of 12 serovars available with the laboratory viz. Australis, Autumnalis, Ballam, Canicola, Grippotyphosa,Hardjo prajitno, Hebdomadis, Icterohaemorrhagiae, Javanica, Pomona, Pyrogenes and Tarassovi. All the field sera were then tested by rLigB based LAT and the test results of both the tests were compared.

RESULTS

PCR Amplification, rLig B Protein Expression and Dot ELISA/Western Blot: The result of PCR (1200 bp amplicon) confirming the N-terminal conserved region of LigB gene and nested PCR (600 bp amplicon) for confirming the PCR product were shown in Fig. 1 and 2 respectively. The clones CL1, CL2, CL3, CL5, CL6 and CL8 expressed rLigB protein upon IPTG induction (Fig. 3). The minimum concentration required for the induction of LigB protein was determined to be 0.1mM IPTG (Fig. 4). The time kinetics for the recombinant LigB protein expression showed that detectable amount of recombinant protein was present in SDS PAGE starting from two hours after induction and after eight hours maximum amount of LigB protein was detected in SDS PAGE (Fig 5). SDS PAGE of purified rLigB protein was showed in Fig 6. The elution fractions E4-E6 (0.5 ml each) containing the maximum concentration of the protein obtained after purification of His-tagged LigB protein by Ni-NTA affinity chromatography is shown in Fig. 7. Dot Blot (Fig. 8) as well as Western blot analysis (Fig. 9) of canine sera confirmed rLigB protein as an immunodominant protein against which antibodies are produced in host during active infection and further proved that anti LigB antibodies are present in sera of infected animals in sufficient quantity. *Microscopic agglutination test*.

Among 100 sera from immunized dogs subjected to MAT, 88 samples gave positive reaction with titres ranging from 100 to 1600. Out of the four strains tested (*L. Icterohaemorrhagiae*, *L. canicola*, *L. grippotyphosa* and L. pomona), all 88 samples showed good seropositivity to *L. icterohaemorrhagiae*, *L. grippotyphosa* and L. pomona. However only 32 of the 88 MAT positive vaccinated sera showed seropositivity to Hond Utrecht IV strain of *L. Canicola* available with the laboratory. Of the 100 sera sample, 12 samples did not give any reaction. 22 out of 25 sera samples from leptospira suspected dogs gave seropositivity by MAT with titres ranging from 100 to 800. *L. icterohaemorrhagiae* and *L. grippotyphosa* were the serovars prevalent.

Fig. 1: PCR amplification of Lig B gene
M: molecular marker 100bp
L1: Lig B gene amplicon (1200bp)

Fig. 2: Nested PCR for confirmation of Lig B gene
M: Marker
L1: Nested PCR product

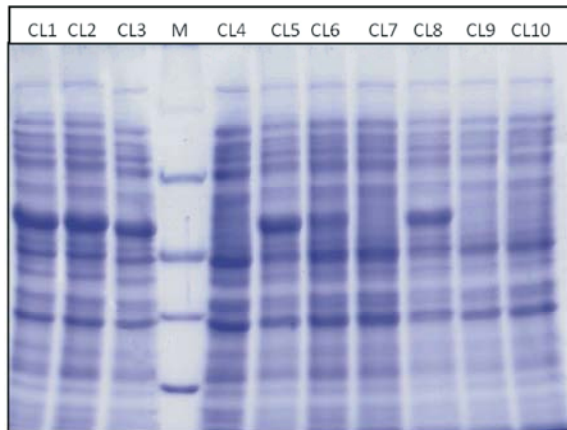

Fig. 3: Clones Expressing rLigB

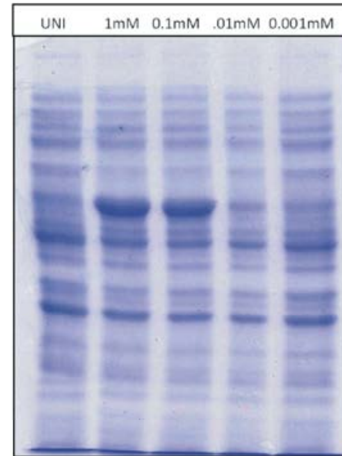

Fig. 4: Expression kinetics showing minimum concentration of IPTG required for recombinant Lig B protein induction

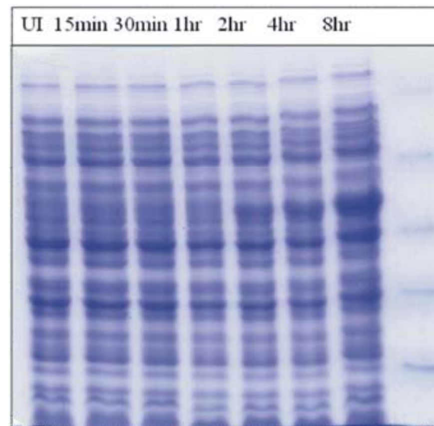

Fig. 5: Expression kinetics of recombinant Lig B protein at various time intervals

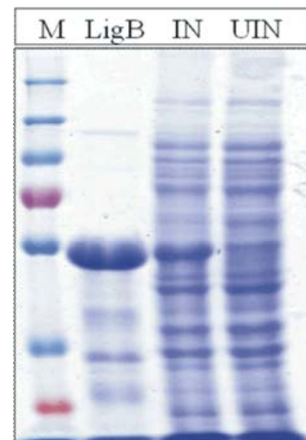

Fig. 6: SDS-PAGE showing lysate of uninduced and induced M15 cells along with purified Lig B protein

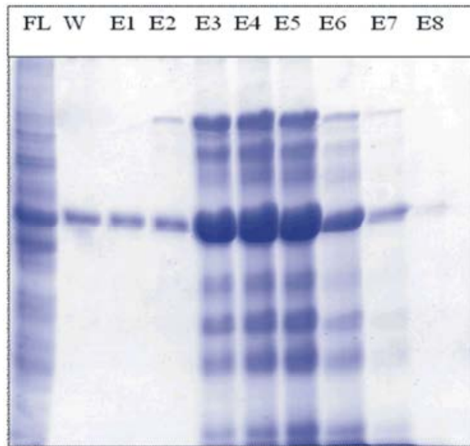

Fig. 7: SDS-PAGE showing purified recombinant Lig B protein in various elusion fractions

Fig. 8: Dot blot showing immunoreactivity of rLigB protein against Canine sera

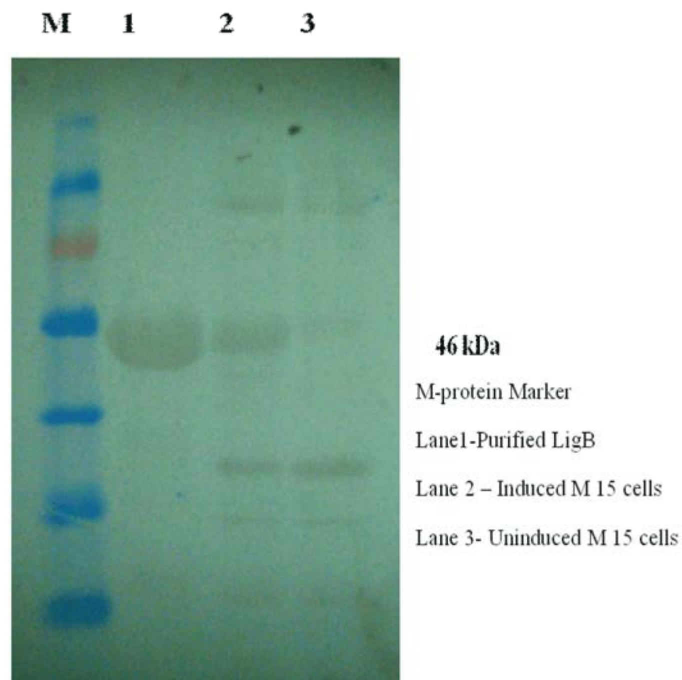

Fig. 9: Western blot Analysis of LigB Protein using Canine sera

Fig. 10: Seroreactivity of rLigB coated microspheres with canine sera

Recombinant Lig B based Latex Agglutination Test: All the 88 samples which showed positive reaction by MAT were also tested by LAT. 81 sera samples gave negative reaction, whereas 7 samples showed positive reaction. Among the immunized dogs, 12 dogs did not react both by MAT and LAT. All the 22 MAT positive clinically suspected dogs gave positive reaction by LAT (Fig. 10) indicating that there is concordance in the results obtained by both the tests.

DISCUSSION

Latex Agglutination Test employing recombinant LigB protein would serve as a simple, sensitive pen site diagnostic test which can give rapid and reliable diagnosis of Leptospirosis under field conditions. This diagnostic test would not require the use of highly skilled labour and the test results would be very easy to interpret. Thus LAT can be recommended for field studies in which speed and simplicity are crucial [27]. When the field sera is mixed with rLigB coated latex beads, anti-LigB antibodies present in the sera of dogs naturally infected by leptospirosis interact with the antigen leading to the formation of fine and clearly visible granular agglutination. The intensity of the agglutination depends on concentration of the antibodies in a sera sample. Clearly visible granular agglutination indicates the presence of specific antibodies to leptospires. In stronger reactions due to sera of high antibody titre, fine granular clumps tend to settle at the edge of the circle and the reaction time to form fine granular clumps is lesser than 60 seconds. Agglutination that occurs beyond 2-3 minutes may be due to evaporation and should be treated as doubtful cases. When Latex beads are sensitised with rLigB protein which are cross reactive lipoproteins found on the outer membrane of most pathogenic leptospiral serovars, this test would be able to detect infection caused by any pathogenic leptospiral serovars. Unlike MAT which employs live whole Leptospiral antigens which pose danger to the lab technician performing the test, LAT employing rLigB protein is completely safe and non-hazardous. The coated latex particles are stable for long periods at 4°C and this long shelf life and cost effectiveness of this test makes this test a very desirable diagnostic tool for detecting leptospirosis. Other potential advantages of rLigB based LAT include its portability, limited amount of generated biomedical waste. The test finds high level of application in developing countries such as India where it can be used in resource-poor settings, by investigators with only limited training [28].

The present study indicates that the sensitivity and specificity of recombinant LigB protein based Latex Agglutination Test was comparable to MAT which is universally considered the Gold Standard Test for diagnosis of leptospirosis. LAT test result was in concordance with MAT since all the 22 MAT positive sera gave positive reaction with rLigB based LAT. It was also observed that a quantitative relation existed between both the tests. Strongly reacting field sera showing titre of 1:800 or above by MAT invariably gave +++/ ++++ test result with rLigB based LAT while weakly and moderately reactive field sera showing titre of 1:100 and 1:200 respectively by MAT gave + and ++ test result with rLigB based LAT. The present study indicates *L. icterohaemorrhagiae* and *L. grippotyphosa* were the serovars prevalent in canines naturally infected by leptospirosis in Bareilly region of Uttar Pradesh, India.

Currently available serological tests cannot discriminate between vaccine-induced leptospiral antibodies and those due to infection. Microscopic Agglutination Test (MAT) is not a DIVA strategy based tool and hence it gives positive test reaction with both vaccinated and naturally infected sera. Few researchers evaluated the efficacy of recombinant leptospiral lipoprotein recombinant LigB (rLigB) as an antigen in ELISA to differentiate between dogs vaccinated against

leptospirosis and those that are naturally infected. In this present study, rLig B based LAT gave negative reaction in sera of vaccinated dogs whereas naturally infected animal sera gave positive reaction, suggesting that rLigB protein based LAT has a potential for being a DIVA strategy tool. LigB protein is a virulence determinant expressed by pathogenic Leptospira during infection. Hence Anti-Lig B antibodies are produced in naturally infected animals & rLigB protein based LAT gives a +ve test result with naturally infected animal sera. In routine Leptospira vaccination, killed whole cell bacterin is used, hence there is no production of Anti-Lig B antibodies & hence with vaccinated sera, rLigB protein based LAT gives a negative test result.

Vaccinated dogs may develop MAT titres up to 1600 or higher to various serovars [29-31].The vaccine that combined Grippotyphosa/Pomona induced MAT titre between 1: 100 and 1: 400 within 7 weeks of administration and showed reactivity to the whole-cell proteins of leptospires by Western blot analysis [32]. Based on our result, the tetravalent vaccine containing *L. Icterohaemorrhagiae*, Pomona, Grippotyphosa and canicola exhibited a MAT titre ranging from 1:100 to 1:1600. The sera samples from immunized dogs which did not react both by MAT and LAT may be due to vaccination failure. Out of 88 sera samples from immunized dogs, 7 gave positive reaction both by MAT and LAT which may be due to natural leptospira infection following vaccination in these dogs. Dogs vaccinated against leptospirosis are susceptible to natural infection with virulent leptospiral serovars and can shed the organisms through urine even though these animals do not develop any signs or symptoms suggestive of leptospirosis. These dogs can give positive reaction by both MAT and rLigB based LAT. Therefore, the development of diagnostic reagents based on antigens that are only expressed during infection would be a valuable tool to identify animals that contract leptospirosis despite vaccination.

CONCLUSIONS

In Conclusion, the rLigB based LAT having sensitivity and specificity comparable to MAT and also possessing the additional advantage of being a DIVA strategy tool can serve as a pen site diagnostic test in well established Veterinary hospitals as well as in mobile veterinary clinics operating in remote, resource-poor settings.

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