Evaluation of Recombinant LigB Based In-house Latex Agglutination Assay for Sero-surveillance of Canine Leptospirosis

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ABSTRACT

Leptospirosis is an important zoonosis with broad host range. Dogs are one of the important maintenance hosts where the disease ranges from in-apparent to clinically overt. Globally, leptospirosis is misdiagnosed or under diagnosed in companion animals like dogs in developing countries such as India due to lack of rapid, affordable and user friendly diagnostic aids. Hence, it is high time to develop rapid, inexpensive and reliable pen-side diagnostic tests with high sensitivity, specificity and accuracy. In the present study, recombinant LigB protein based latex agglutination test has been evaluated for serodiagnosis of canine leptospirosis with results obtained within two minutes having proven sensitivity (83.47%), specificity (99.06%) and high degree of accuracy (94.56%). It pitches for its use as a rapid spot test following further validation in peripheral levels of animal health care system.

Key words: Leptospirosis, recombinant LigB, dog

Introduction

Leptospirosis is a neglected zoonotic disease prevalent globally in both developing and developed nations (Li et al., 2013; Murray, 2013). Dogs affected with leptospirosis show varying clinical features starting from inapparent infection to hepto-renal insufficiency and death (Quinn et al., 2002). Pathogenic serovars infecting dogs include Icterohaemorrhagiae, Canicola, Pomona, Bratislava and Grippotyphosa (Faine et al., 1999). The fact that leptospirosis is a major health problem in canine population along with their role as a potential reservoir for human infection makes early identification and management of this disease of paramount importance in these animals (Goldstein, 2010). The disease has often been overlooked by clinicians mainly due to its overlapping clinical features which mimic several diseases in dogs and the non availability of rapid, simple and low cost diagnostic aids (Senthil kumar et al., 2007). Therefore, the need of the hour is development of pen-side diagnostics which can aid in diagnosing the disease at field level itself without resorting for time consuming laboratory test results (Behera et al., 2014).

MAT is considered as the “gold standard” for serodiagnosis of leptospirosis and has been approved by the World Organization for Animal Health for various animal species (OIE, 2008). This is due to unsurpassed diagnostic (serovar/serogroup) specificity in comparison with other currently available tests (Faine et al., 1999). However, the inherent pitfalls of MAT such as the use of live culture which pose serious health risk to laboratory personnel (Sykes et al., 2011), the need for paired serum samples for disease confirmation (Faine et al., 1999), cumbersome test procedure of reacting serum with different panels of live leptospira antigens (Rajapakse et al., 2015) and the need for verifying serovar identity regularly to ensure accurate results (Cerqueira et al., 2010) have forced disease investigators to search for alternative field oriented tests. The major reasons for shifting of focus towards outer membrane proteins (OMPs) as a target for serodiagnosis is the conserved nature of these antigens which are found ubiquitously in most pathogenic leptospiiral serovars (Raja and Natarajaseenivasan et al., 2015). The advent of recombinant DNA technology enabled the use of OMPs found ubiquitously in the outer membrane of pathogenic leptospires such as OMPL1 (Natarajaseenivasan et al., 2008; Subathra et al., 2013), LipL32 (Hartleben et al., 2013), LipL21 (Joseph et al., 2012), LipL41 (Mariya et al., 2006) and LigB (Deneke et al., 2014) to be employed in
molecular diagnostic assays. Diagnostic kit based on such recombinant proteins would not give false positive test result in canines infected with saprophytic leptospires and other spirochetes as these proteins are expressed only in pathogenic leptospires (Dey et al., 2007; Deneke et al., 2014).

Bacterial immunoglobulin like domain proteins Lig A and Lig B are expressed during host infection and appear to induce strong antibody responses in patients and infected animals (Matsunaga et al., 2003; Palaniappan et al., 2004). The use of recombinant LigB is well characterised for its diagnostic potential (Matsunaga et al., 2003; Sankar et al., 2010). Nonetheless, its usage for the detection of anti-leptospiral antibodies in livestock and companion animals has been limited (Sankar et al., 2010; Deneke et al., 2014). In this study, the diagnostic potential of recombinant LigB (rLigB) based latex agglutination test (LAT) is compared with gold standard MAT by screening 423 canine sera samples collected from different parts of India.

Materials and Methods

Microscopic agglutination test (MAT)

A total of 423 sera samples collected from dogs were tested by MAT for detecting antibodies using 16 leptospiral serovars viz., Australis, Autumnalis, Ballum, Bataviae, Canicola, Cyanopteri, Dejasiman, Grippotyphosa, Hardjo, Hebdomadis, Icterohaemorrhagiae, Louisiana, Pomona, Pyrogenes, Javanica and Tarassovi grown in Ellinghausen,McCullough, Johnson and Harris (EMJH) liquid media (Difco laboratories, USA) at 30°C. Microscopic Agglutination Test (MAT) was performed as per the standard protocol described by Cole et al. (1972) with dilution of sera done serially from 1:50 to 1:3200 in phosphate buffered saline (PBS, pH 7.2) and sera were incubated at 30°C for 3 hr with suspension of live Leptospira serovars having concentration 2×10⁸ cells/ml. The positive reaction was characterized by agglutination of at least 50% of Leptospira cells.

Production and purification of recombinant LigB protein

Recombinant LigB protein was expressed, purified and IgG based latex agglutination test (LAT) was performed as per the method described earlier (Deneke et al., 2014; Smits et al., 2000). The recombinant plasmid pQE30 (Qiagen, U.S.A.) containing LigB gene available with Genetic engineering lab in Bacteriology and Mycology Division, IVRI, was used to transform competent M15 strains of E. coli cells treated with chilled 0.1 M CaCl₂. The transformants which appeared on LB Ampicillin plate were grown in bulk in LB broth contain Ampicillin and kanamycin and then induced with 1 mM IPTG during early log phase of growth and after overnight incubation were centrifuged. The cell pellet obtained was treated with lysis buffer (pH 8.0) and then centrifuged twice. The supernatant obtained was passed through a nickel-nitritolriacetic acid affinity chromatography column. The column was later washed with wash buffer (pH 6.3) to remove any unbound protein in the column. Only 6x tagged LigB protein remained in the column which was eluted on addition of elution buffer (pH 4.5). The eluted protein was then dialysed with 1x PBS to remove urea. The dialysed LigB protein was then buffered with glycine buffered saline (pH 8.2) and this protein was then used for performing LAT.

Recombinant LigB based latex agglutination test (LAT)

Latex beads were sensitized with recombinant LigB antigen according to the method of Lengyel et al. (1993) with some modifications. A 2.6% suspension of blue dyed latex particles (0.8 mm diameter, Sigma, USA) was washed twice with glycine buffered saline (Glycine 0.1 M, NaCl 0.17 M; pH 8.2) by centrifugation at 8000 rpm for 3 min each time. Finally, the latex beads were made into a 2% suspension with glycine buffered saline which was later mixed with an equal volume of recombinant LigB antigen (100 µg/ml) diluted in the same buffer. The mixture was incubated at 37°C for 6 h with constant mixing. The sensitized beads were further blocked with BSA (5 µg/ml) and incubated overnight. Latex beads were centrifuged and the pellet was finally resuspended in glycine buffered saline as a 2% suspension containing 0.02% sodium azide. The sensitized latex beads were stored at 4°C until use. The LAT was performed on glass slides by mixing equal volume of serum (20 µl) and sensitized beads (20 µl). The slide was rotated gently in order to mix the reagent and the serum samples. The result was read within 2 min. Samples were considered positive when there is formation of agglutination. The sera samples which gave only homogenous suspension with sensitized latex beads even after 2 minutes were deemed negative.

Statistical analysis

The relative sensitivity, specificity, +ve, -ve predictive value and concordance value of the recombinant LigB antigen based LAT for the detection of anti-leptospiral antibodies in human sera were compared with MAT as described below (Senthilkumar et al., 2008).

Sensitivity (%) = [a/(a+c)] ×100, where “a” is the number of serum samples positive by the LAT and MAT, “c” is the number of serum samples positive by MAT but negative by the LAT.

Specificity (%) = [d/(b+d)] ×100 where “d” is the number of serum samples negative by the LAT and MAT, “b” is
the number of serum samples negative by MAT but positive by the LAT Concordance= \[\frac{(a+d)}{(a+b+c+d)} \times 100\]

**Results and Discussion**

In the present study, recombinant LigB protein expression was confirmed by SDS-PAGE analysis in which a 46 kDa protein was produced after induction with 1mM IPTG during the log phase of growth (Figure 1). The gene expression level was high and the yield of LigB protein was approximately 20 mg purified protein per liter of induced culture.

Out of 423 sera samples tested, 121 sera (28.6%) were found positive for antibodies in MAT and 104 sera (24.58%) were found positive in rLigB based ELISA. The predominant serovars reported in the present study was Icterohamorrhagiae 102 (35.41%), followed by Grippotyphosa 52 (23.95%), Pyrogens 35 (13.19%), Javanica 27 (9.9%), Canicola 12 (4.16%), Pomona 11 (3.81%), Australis 08 (2.0%), Autumnalis 6 (2.08%), Dejasiman 07 (2.43%), Cyanopteri 06 (2.08%) and Tarassovi 02 (0.69%). Table 1 provides information regarding the sensitivity, specificity and accuracy of rLigB based LAT in comparison with MAT. rLigB based LAT showed sensitivity, specificity and accuracy of 83.47%, 99.06% and 94.56%, respectively. LAT based on rLigB antigen showed high agreement (kappa value 0.860) with the gold standard test, MAT. Our results are in agreement with findings of other researchers where LAT using different recombinant proteins have shown high sensitivity and specificity (Dey et al., 2007; Deneke et al., 2014). Our study clearly suggests that LAT would serve as a platform for early testing for effective case management and early case finding for epidemiological investigation. The intensity of the agglutination depends on amount of the antibodies present in a serum sample. In this study, sera samples which agglutinated within 30s were treated as 3+, which is due to presence of high antibody titre in the respective serum. Agglutination reaction beyond 2 min was considered negative (Fig. 2).

In the present study, a truncated recombinant LigB protein of 46 kDa, which represented the N terminal portion of the gene was used as antigen for sensitizing latex beads. The main advantage of LigB based LAT is that this protein is ubiquitously found on the outer membrane of most of the pathogenic leptospiral serovars and therefore enable detection of antibodies present in sera samples of dogs infected by any pathogenic leptospiral serovar. Meanwhile, MAT can give false negative test result when sera are collected from an animal in the early stages of the disease and due to non inclusion of the relevant serovars of a particular geographical region as live antigen. In this study, overall seroprevalence of leptospirosis in dogs was found to be 28.6%, by adopting gold standard test MAT. All the sera

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<tr>
<td></td>
<td>Positive</td>
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<tr>
<td>rLigB LAT</td>
<td>101 (a)</td>
<td>03 (b)</td>
</tr>
<tr>
<td>Positive</td>
<td>20 (c)</td>
<td>299 (d)</td>
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<td>Total</td>
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Sensitivity=101/121×100=83.47%; Specificity=299/302×100=99.06%; Accuracy=400/423×100=94.56%

**Table 1: Comparison of Recombinant LigB LAT with Microscopic agglutination test (MAT)**

![SDS PAGE depicting purified LigB Protein](image1)

![Latex Agglutination Test showing positive and negative canine sera samples for leptospirosis](image2)
showing a MAT titre value of $\geq 1:100$ were taken as positive. Ictero-haemmorhagiae was found to be the predominant serovar in present study which is in concordance with earlier studies (Srivastava, 2008).

In companion animals such as dogs, early diagnosis and treatment play a pivotal role in effective control of leptospirosis in human host (Bharti et al., 2003; Goldstein, 2010). However, the lack of adequate and ready to use diagnostic aid largely contributes to the under reporting of the disease in animals in general and dogs in particular (Adler and Moctezuma, 2010; Senthilkumar et al., 2008). Currently available diagnostic methods require sophisticated laboratories and technical expertise. Hence, the focus of the present study is to develop an in-house latex agglutination test that can be performed with minimal training and limited equipments with results in minutes, particularly in the resource poor settings of the developing world like India. In diagnostic tests such as MAT where whole cell antigenic preparation of leptospira are being used, cross reacting antibodies from patients known to suffer from syphilis, relapsing fever, lyme disease, enteric fever, dengue and malaria have been reported to give a titre of 80 or 100. However, recombinant protein based serological tests such as LigB based LAT give minimal false positive reactions due to absence of such cross reacting antibodies. (Flannery et al., 2001).

Recombinant protein based latex agglutination test for diagnosis of leptospirosis is ideally suited for emerging economies where several disease investigators have reported leptospirosis in both humans and animals (Favero et al., 2002; Srivastava et al., 2008; Li et al., 2013). The need for specialized laboratories equipped with skilled lab personnel, the esoteric nature of interpreting MAT test results and the use of live culture which pose serious health risk to laboratory technician who performs the test have restricted the accessibility of MAT to a select few in urban parts of India. However, LigB based LAT which has the potential to circumvent several inherent defects associated with MAT, can be performed and interpreted by semi-skilled animal health personnel in resource poor settings such as veterinary dispensaries situated at remote areas which makes this rapid screening test ideal even at the peripheral level of a animal health care system. Other noteworthy advantages of rLigB based LAT include its long shelf life, portability, limited amount of generated biomedical waste and cost effectiveness.

The present study clearly suggest the use of recombinant antigen based spot test as an alternate test to MAT in resource poor settings of developing countries such as India.

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References


