

Evaluation of recombinant leptospiral antigen LipL41 in enzyme-linked immunosorbent assay and latex agglutination test for serodiagnosis of canine leptospirosis

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ABSTRACT

Leptospirosis is an important global veterinary health problem caused by spirochetes belonging to the genus *Leptospira*. Leptospire are ubiquitous in nature, and therefore able to adapt to both the ambient environment and the renal tubules of chronically infected reservoir hosts. Microscopic agglutination test (MAT) remains a specialized test, which is not generally performed in the routine diagnostic laboratories. In an effort to develop new diagnostic tests to achieve high sensitivity, the focus is mainly on the outer membrane proteins. LipL41 is one among the immunogenic outer membrane proteins that is highly conserved among pathogenic species of *Leptospira*. The recombinant based outer membrane protein antigen LipL41 was evaluated for use in the diagnosis of canine leptospirosis by enzyme-linked immunosorbent assay (IgG-ELISA) and latex agglutination test (LAT). The sensitivity and specificity of the developed assays were analyzed with the microscopic agglutination test (MAT) using 221 canine serum samples. The sensitivity and specificity of the ELISA were 83.33% and 93.07%, and the sensitivity and specificity of the latex agglutination test were 95.83% and 96.04% respectively. The assays proved to be as sensitive, specific and accurate as the standard microscopic agglutination test (MAT).

Key words: enzyme-linked immunosorbent assay, latex agglutination test, leptospirosis, dogs, rLipL41

Introduction

Leptospirosis is a zoonotic infection with a worldwide distribution affecting wild and domestic vertebrates, the incidence of the disease being higher in tropical climates

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(PLANK and DEAN, 2000). Leptospirosis had been suspected in India since the early part of the 20th century (CHOWDHARY, 1903; BARKER, 1926). This disease remains underdiagnosed largely due to the broad spectrum of signs and symptoms attributable to this spirochetal pathogen. Investigation of large outbreaks would be greatly enhanced by the availability of rapid and sensitive diagnostic assays that could confirm the diagnosis in the early stage of the clinical illness.

Diagnosis of leptospirosis is usually accomplished retrospectively by serology, because the culture requires both special media and incubation of several weeks (LEVETT, 2003). The most commonly used serological test for diagnosis of leptospirosis is the microscopic agglutination test (MAT). However, antibodies are undetectable before 8-10 days after the onset of disease. Furthermore, MAT requires a large number of *Leptospira* strains to be maintained live as a source of antigens, and paired serum samples are needed for the correct interpretation of the results. In addition, it is laborious and potentially hazardous to the laboratory staff (ELLIS, 1984). Enzyme-linked immunosorbent assays (ELISAs) (TERPSTRA et al., 1985; WINSLOW et al., 1997; CUMBERLAND et al., 1999) and other rapid serologic tests based on whole-cell leptospiral antigen preparations have been developed for use as an alternative method to screen for leptospiral infection. RAMADASS et al. (1999) standardized a rapid, semi-quantitative latex agglutination test (LAT) using a whole cell antigen for the detection of leptospiral antibodies in the serum samples of human and animals. The rapidity, simplicity and economics of the LAT were found to fulfill the requirements of a rapid screening test for leptospiral antibodies.

There is an urgent need to develop a rapid, sensitive and appropriate diagnostic test that could be used in a routine diagnostic laboratory to detect antibodies against leptospires. LipL41, one of the major leptospiral outer membrane proteins (OMPs) has been identified as a serodiagnostic marker for screening leptospiral infection (HAAKE et al., 1999). Results of the surface immunoprecipitation studies also suggest that LipL41 is surface exposed and is expressed during infection of the mammalian host (SHANG et al., 1996). The outer membrane proteins LipL32, LipL41, OmpL1 and LipL21 are expressed only in the pathogenic leptospira species and conserved among more than 200 serovars of *Leptospira*. In the present study, the recombinant LipL41 (rLipL41) protein from *Leptospira* was expressed as a fusion protein and evaluated for rapid serodiagnosis of leptospirosis in dogs.

Materials and methods

Bacterial strains and media. The reference leptospiral strains maintained throughout the study were obtained from Koninklijk Instituut voor de Tropen (KIT), Amsterdam, The Netherlands, and National Leptospirosis Reference Center, Port Blair, Andaman and Nicobar Islands, India. The *L. interrogans* serovars Australis, Autumnalis, Canicola,

Javanica, Pomona, Icterohaemorrhagiae, Grippotyphosa, and Pyrogenes were grown in EMJH medium at 29 °C to 30 °C and their growth was assessed by dark field microscopy. The bacterial host cell *Escherichia coli* DH5 α (*supE44* Δ *lac U169* [ϕ 80 Δ *lacZ M15*] *hsd R17 recA1 end A1 gyr A96 thi-1 rel 41*) maintained in LB agar and LB broth was used for the expression of recombinant antigen.

Serum samples from dogs suspected of leptospirosis and control samples. Two hundred and twenty one canine serum samples included in the study were collected from suspected cases with pyrexia and other illness irrespective of breed, sex and age, at Madras Veterinary College Hospital. Samples from apparently healthy individuals with vaccination details were also collected to serve as a means of negative control.

Antisera. Rabbits of 6-8 weeks old were immunized with sonicated whole cell *Leptospira interrogans* serovar Icterohaemorrhagiae ($\sim 1 \times 10^8$ cells/mL) mixed with 1 mL of Freund's complete adjuvant. Two boosters were given every 2 weeks with Freund's incomplete adjuvant. Hyperimmune serum was tested and collected one week after the final booster injection.

Microscopic agglutination test (MAT). The microscopic agglutination test was carried out as per the method of COLE et al. (1973), using a panel of 8 reference *Leptospira interrogans* serovars: Australis, Autumnalis, Canicola, Pomona, Grippotyphosa, Icterohaemorrhagiae, Javanica, and Pyrogenes. Reciprocal agglutination titres of greater than or equal to 100 were considered as positive reactions.

Recombinant leptospiral antigen LipL41. The reference *Leptospira interrogans* serovar Icterohaemorrhagiae (strain RGA) was utilized for preparing rLipL41. The PCR primers used to amplify part of leptospiral outer membrane protein gene LipL41 (1077 bp) were: forward oligonucleotide primer with an *NcoI* restriction endonuclease site (underlined): 5'-TG TTA CCC ATG GGG AGA AAA TTA TCT TCT CT -3' and reverse oligonucleotide primer with an *XhoI* restriction endonuclease site (underlined): 5' AAA GGA CTC GAC TTA CTT TGC GTT GCT TTC -3', which were selected based on the LipL41 primer sequences of *L. kirschneri* species by HAAKE et al. (1999). Then, the amplicon (Genbank accession no. [DQ132992](#)) was cloned into an expression vector pPRO EX HT 'b' (pPRO-LipL41-140). The expression was induced by adding Isopropyl β -D thiogalactoside (IPTG) at 1 mM final concentration to the log-phase culture of pPRO-LipL41-140. The expressed fusion protein was purified by ultra centrifugation (SHAJI DANIEL, 2000). Briefly, the culture pellet was harvested from 500 mL of culture and resuspended in ice with 7.5 mL of Buffer A (50 mM Tris HCl pH 8.0, 7.5% glycerol, 0.1 mM EDTA, 1 mM DTT, 50 mM NaCl) and 0.75 mL of Buffer B (50 mM EDTA, 10% Triton X-100, 2.5 mg/mL lysozyme, 2 mM phenylmethyl sulfonyl fluoride (PMSF) or β -mercaptoethanol). After 1 hr incubation, 5 mM MgCl₂ was added and the cells sonicated for 2 periods of 30 sec at constant pulse. The lysate was clarified at 18,000 g for 30 min

in a refrigerated centrifuge. The supernatant was layered over Buffer C (30% glycerol containing 50 mM Tris-HCl, pH 8.0, 150 mM potassium acetate, 5 mM MgCl₂, 0.5 M NaCl, 1 mM β-mercaptoethanol) and centrifuged at 1, 43, 700 g for 2 hrs. The pellet was resuspended in Buffer C and stored in aliquots at -80 °C. The purified protein was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the specificity was analysed by immunoblotting using rabbit hyperimmune serum.

IgG-enzyme-linked immunosorbent assay (IgG-ELISA). The optimum concentration of the purified rLipL41 recombinant antigen was determined by the checkerboard titration (DEY et al., 2004). Flat-bottom polystyrene microtitre plates (MaxiSorp, Nunc) were coated with 100 µL volume of purified antigen in 0.06 M carbonate-bicarbonate buffer, pH 9.6, at the concentration of 10 ng/well for screening the dog serum samples. The plates were washed thrice with PBST (Phosphate buffered saline, pH 7.2 with 0.05% Tween 20) and blocked with 2% Bovine Serum Albumin Fraction V (BSA) (Sigma, USA) for 1 hr at 37 °C. The plates were again washed thrice with PBST and incubated with 100 µL of two-fold serially diluted serum samples in PBST from 1:100 to 1:3,200 dilutions for 1 hr. After three washes with PBST, wells were added with 1:15,000 dilution of rabbit anti-dog IgG HRP conjugate (Bangalore Genei, India) and incubated for 1 hr at 37 °C. The substrate solution ABTS (2,2'-Azino di-ethyl benz-thiozoline-6-sulphonic acid) (Sigma, USA) was added to the wells at 100 µL volume and allowed to react for 10 min. When the colour developed, the absorbance at 405 nm was measured in an ELISA reader (Biotek instruments Inc., USA). The cut-off point for the ELISA was calculated as the double of the mean value of absorbance obtained with three negative control serum samples from healthy dogs.

Latex agglutination test (LAT). A 10% suspension of dyed latex particles (0.8 µm dia, Sigma, USA) was coated with rLipL 41 antigen (25 µg/mL) using 0.06 M carbonate-bicarbonate buffer, pH 9.6, and kept at 37 °C for 6 hrs with constant shaking (SOHINI DEY, 2003). The sensitized beads were centrifuged at 6,800 g for 3 min and the pellet resuspended as a 1% suspension in phosphate buffered saline (PBS) containing 5 mg/mL of bovine serum albumin (BSA). The latex beads were left at 37 °C overnight with constant shaking. Latex beads were centrifuged as before and the pellet resuspended in PBS containing 0.5 mg/mL of BSA and 0.1% sodium azide as 0.25% suspension. 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. The slide was rotated briefly in order to mix the reagent and the serum samples. The result was read within 2 min. The test score was positive if agglutination occurred, indicated by the formation of fine granular particles, which tend to settle at the edge of the droplet. If the suspension remained homogenous, the test was scored negative.

Statistical analysis. The relative sensitivity, specificity and accuracy (in percent) of the ELISA and latex agglutination test for the detection of leptospiral antibodies in dog serum samples were determined in comparison to the MAT as described below:

Sensitivity = $a/(a+c) \times 100$, where 'a' is the number of serum samples positive by the test and MAT, 'c' the number of serum samples positive by MAT but negative by test.

Specificity = $d/(b+d) \times 100$ where 'd' is the number of serum samples negative by test and MAT, 'b' the number of serum samples negative by MAT but positive by test.

Accuracy = $a+d/(a+b+c+d) \times 100$

An intuitive method for calculating predictive values (in percent) for positive and negative test results was done as below:

$PV+ = a/(a + b) \times 100$

$PV- = d/(c + d) \times 100$ (JACOBSON, 1998).

Results

IgG-enzyme-linked immunosorbent assay. Leptospiral antigen rLipL41 expressed in the expected size of 39 kDa was purified by ultracentrifugation. Prior to being used in ELISA, the rLipL41 antigen was tested for specificity in immunoblots with hyper immune serum raised in rabbits. The optimum concentration of the purified antigen was determined by the checkerboard titration as 10 ng/well for screening canine serum samples. Among 221 canine serum samples 107 (48.4%) were positive. The sensitivity, specificity and accuracy of the assay in relation to the standard MAT are shown in Table 1. The positive and negative predictive values of the developed IgG-ELISA for canine serum samples were 93.46% and 82.46% respectively.

Table 1. Comparison of IgG-ELISA and microscopic agglutination test

		MAT		Total
		Positive	Negative	
ELISA	Positive	100 (a)	7 (b)	107
	Negative	20 (c)	94 (d)	114
Total		120	101	221

$\chi^2 = 128.18^{**}$, $K = 0.76$, Sensitivity: 83.33%, Specificity: 93.07%, Accuracy: 87.78%, ****Highly significant $P \leq 0.01$**

Table 2. Comparison of latex agglutination test and microscopic agglutination test

		MAT		Total
		Positive	Negative	
LAT	Positive	115 (a)	4 (b)	119
	Negative	5 (c)	97 (d)	102
Total		120	101	221

$\chi^2 = 186.26^{**}$, $K = 0.92$, Sensitivity: 95.83%, Specificity: 96.04%, Accuracy: 95.93%, ****Highly significant** $P \leq 0.01$

Latex agglutination test. Purified rLipL41 antigen at the concentration of 25 $\mu\text{g/mL}$ was coated onto 0.8 μm diameter latex beads. When equal volume of serum samples and antigen coated beads were mixed, clear agglutination was observed in positive serum samples. In 221 dog serum samples, 119 (53.84%) were positive with LAT. The sensitivity, specificity and accuracy of the assay in relation to the standard MAT are shown in Table 2. The positive and negative predictive values of the latex agglutination test for dog serum samples were 96.64% and 95.09%, respectively.

Discussion

Leptospirosis may be diagnosed in the laboratory either by isolating the causal organism (or) by demonstrating a rise in specific serum antibody. Though the culture is of undoubted epidemiological importance, the time needed to culture and identify the infective organism permits only a retrospective diagnosis. For detection of specific antibody, the microscopic agglutination test (MAT) is the standard reference test for leptospiral diagnosis. Dependence upon the MAT results delays the establishment of the cause of outbreaks, as seen in several investigations (ANONYMOUS, 1997). So far, the efforts to develop new diagnostic tests aimed at achieving high sensitivity in the acute phase have focused primarily on detecting IgM that binds to the whole cell antigen preparations, and appears to be a broadly reactive antigen (TERPSTRA et al., 1985; FAINE et al., 1999) which is a disaccharide epitope present in nonpathogenic leptospires, as well as in a diverse group of non-leptospiral species (MATSUO et al., 2000). Enzyme-linked immunosorbent assays (ELISAs) and other rapid serologic tests based on whole-cell leptospiral antigen preparations (SMITS et al., 1999; SMITS et al., 2000) have been developed for use as an alternative method to screen for leptospiral infection, although the MAT is still required for case confirmation (FAINE et al., 1999).

Highly conserved OMPs are of special significance in serodiagnosis and vaccine development for leptospirosis. The leptospiral OMPs expressed during mammalian

infection may have potential immunoprotective capabilities. However, the lack of an effective, widely available laboratory tool remains a major problem and the standard serological tests for case confirmation need to be optimized. Recombinant protein-based serologic tests may achieve high sensitivity and specificity because of the high concentration of immunoreactive antigens, which could be used in the assays, and the lack of non-specific moieties present in whole cell preparations (FLANNERY et al., 2001). An ideal test will need to discriminate between leptospirosis and a broad spectrum of diseases that cause acute febrile illness and have overlapping clinical presentations. Because the burden of leptospirosis is greatest in developing countries, there is a need to develop a test that can both be carried out at a low cost and can easily be standardized for use in field settings.

In the present work we report the evaluation of recombinant leptospiral antigen LipL41 in IgG-ELISA and LAT with serum samples from dogs clinically suspected of leptospirosis. Hence, the developed IgG-ELISA had lesser sensitivity when compared to MAT, where the reaction is mainly due to IgM response. The sensitivity and specificity of the latex agglutination test were comparable to that of MAT, indicating that these agglutination assays were mainly based on IgM response in the initial phase of clinical illness. LAT has been standardized and used for detection of antibodies to many infectious diseases such as infectious bursal disease in poultry (NAKAMURA et al., 1993). For leptospirosis antibody screening, an initially heat stable antigen was tried for conducting LAT, and the overall sensitivity and specificity was reported as 82.3% and 94.6% (SMITS et al., 2000). They concluded that the assay is easy to perform and did not require special skills or equipment. The reagents had a long shelf life, even at tropical temperatures. VERMA and RAMADASS (2005) used whole cell, sonicated antigen in LAT and found it to give better results than the heat stable antigen, attributing the results to the high protein content of the sonicated antigen. In the present study, the rLipL41 latex agglutination test showed 95.83% of sensitivity in canine serum samples when compared with standard MAT, and the kappa value ($K = 0.92$) also showed perfect agreement between the tests. IgG-ELISA with the recombinant LipL41 antigen developed in the study had a sensitivity of 83.33% relative to the MAT and the kappa value ($K = 0.76$) showed substantial agreement when compared to IgG-ELISA with MAT. The sensitivity found in this study was higher than that reported by FLANNERY et al. (2001) who evaluated the same recombinant antigen (LipL41) for the diagnosis of human leptospirosis. In conclusion, these findings suggest that rLipL41 antigen is a specific, sensitive and practical test for the detection of antibodies against canine leptospiral infection. The latex agglutination test could be used as a rapid screening test even at the peripheral level of a health care system.

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SAŽETAK

Leptospiroza je uzrokovana spirohetama roda *Leptospira* i predstavlja važan zdravstveni problem za veterinarsku medicinu. Leptospire su posvudašnje bakterije pa se mogu prilagoditi i okolišnim uvjetima i uvjetima u bubrežnim tubulima kronično zaraženog domaćina. Mikroskopska aglutinacija i nadalje ostaje standardni test koji se općenito ne provodi u svakidašnjoj laboratorijskoj dijagnostici. U nastojanju da se razviju novi dijagnostički testovi veće osjetljivosti, istraživanja su usmjerena na proteine vanjske membrane. LipL41 je jedan od imunogenih proteina membrane koji je visoko konzerviran među patogenim vrstama leptospira. Rekombinantni protein vanjske membrane LipL41 vrednovan je kao antigen za uporabu u imunoenzimnom testu i lateks aglutinaciji za dijagnosticiranje leptospiroze u pasa. Pretragom 221 uzorka pasjeg seruma, analizirana je osjetljivost i specifičnost spomenutih testova. Osjetljivost imunoenzimnog testa u odnosu na mikroskopsku aglutinaciju iznosila je 83,33%, a specifičnost 93,07%. Osjetljivost lateks aglutinacije bila je 95,83%, a specifičnost 96,04%. Dokazano je da su razvijeni testovi osjetljivi, specifični i točni kao i standardni test mikroskopske aglutinacije.

Cljučne riječi: leptospiroza, pas, imunoenzimni test, lateks aglutinacija, rLipL41
