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Latex agglutination test for the detection of canine leptospiral antibodies using recombinant OmpL1 antigen

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ABSTRACT

The recombinant leptospiral transmembrane protein OmpL1 (rOmpL1) was evaluated for use in the diagnosis of canine leptospirosis by latex agglutination test (LAT). The microscopic agglutination test (MAT) of 513 serum samples from dogs suspected of leptospirosis showed that 446 (83.68%) samples had positive reciprocal agglutination titers, which ranged from 100 to 1600. In the rOmpL1 based LAT, the sensitivity and specificity were observed as 93.05% and 91.95% respectively in comparison with MAT. The results were graded from +1 to +3 to discover the visual degree of agglutination. The sensitized latex beads could be stored at 4°C for two months without showing loss of activity. LAT is a simple, inexpensive and rapid serodiagnostic test for leptospirosis without expertise.

Key words: canine leptospirosis, Latex agglutination test, recombinant OmpL1 protein, serodiagnosis

Introduction

Leptospirosis is considered as one of the most widespread zoonoses worldwide (LEVETT, 2001). The disease is caused by spirochetes of the genus *Leptospira*. The genus *Leptospira* is classified serologically into two species, the pathogenic species *L. interrogans* and the saprophytic species *L. biflexa*. There are more than 200 serovars of *L. interrogans* and more than 60 serovars of *L. biflexa*. Most of these serovars can infect different animal species, but there is a primary host reservoir for each serovar, which ensures the survival and dissemination of the organisms (BIRNBAUM et al., 1998). For example, the dog is the maintenance host for *L. interrogans* serovar Canicola and is considered as an incidental host for a variety of other serovars (RENTKO et al., 1992).

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Definitive laboratory diagnosis of leptospirosis requires detection of the organisms in a clinical specimen or a fourfold or greater rise in microscopic agglutination test (MAT) titer in the setting of an appropriate clinical syndrome.

The most frequently used diagnostic approach for leptospirosis has been that of serology. The MAT is the serological test used in reference laboratories, because of its high degree of sensitivity and specificity (COLE et al., 1973). However, MAT is a complex test that requires a large coverage of the antigenic diversity represented in a given testing area. Moreover, antibodies usually appear within 5-7 days after the development of symptoms; they usually peak by the fourth week, but detectable titers may persist for years (ADLER and FAINE, 1978; CUMBERLAND et al., 2001). Hence, interpretation of the results is difficult without paired specimens collected at the appropriate times; therefore, results are also usually not available quickly.

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 in dogs. Outer membrane proteins (OMPs) seem to play an important role in pathogenicity of bacteria. OmpL1 is the 31 kDa transmembrane protein characterized in 1993 (HAAKE et al., 1993) and expressed only in pathogenic *Leptospira*. A recombinant protein OmpL1 ELISA was described for the detection of canine leptospirosis (OKUDA et al., 2005). In the present study, recombinant OmpL1 (rOmpL1) protein has been evaluated as an antigen in Latex agglutination test (LAT) for rapid serodiagnosis of canine leptospirosis.

Materials and methods

Bacterial strains and media. A panel of eight Leptospira interrogans serovars viz., Australis, Autumnalis, Canicola, Javanica, Pomona, Icterohaemorrhagiae, Grippotyphosa and Pyrogenes was used for MAT. They were grown in EMJH liquid and semisolid media (Difco, USA) at 29-30 °C and the growth was assessed by dark field microscopy regularly. These reference leptospiral strains were obtained from Koninklijk Institute voor de Tropen (KIT), Amsterdam, The Netherlands and National Leptospirosis Reference center, Port Blair, Andaman and Nicobar Islands, India. Escherichia coli BL21 (DE3) (F^- ompT $hsdS_B$ ($r_B^-m_B^-$) gal dcm (DE3)) (Invitrogen, USA) was used as host cells for the expression of recombinant antigen and was grown and maintained in the LB broth and agar (Invitrogen, USA).

Canine sera. Five hundred and thirteen canine blood samples suspected for leptospirosis were collected from Leptospirosis Research Laboratory, Center for Animal Health Studies, Madhavaram, Chennai-600051 and Small Animal Clinics of Madras Veterinary College, Chennai-600 007 and stored at -20°C until used. Twenty sera samples were also collected from apparently healthy dogs with vaccination details to serve as negative controls.

Microscopic agglutination test. The gold standard serodiagnostic test for leptospirosis is MAT, which was performed as per the method of COLE et al. (1973). Briefly, the sera from the dogs were serially diluted from 1:50 to 1:3200 in phosphate buffered saline (PBS), pH 7.2 and allowed to react with live antigen suspensions of the panel of 8 reference leptospiral serovars. After 2 hours incubation at 37 °C, the serum-antigen mixtures were examined by dark field microscopy for the presence of agglutination/ clearance of the organisms and the titers were determined. Reciprocal agglutination titers of greater than or equal to 100 were considered as positive reactions.

Antisera. Rabbits of 6-8 weeks old were immunized with sonicated whole cell *L. interrogans* serovar Icterohaemorrhagiae (~$1x10^8$cells/mL) mixed with 1 mL of Freund's complete adjuvant. Two boosters were given every 2 weeks with Freund's incomplete adjuvant. Hyper immune serum was tested by agar gel immunodiffusion (AGID) and latex agglutination test, and collected one week after the final booster injection (RAMADASS et al., 1999).

Production of rOmpL1 antigen. The primers were designed with built-in restriction enzyme sites (Table 1) from the previously reported gene sequences of *L. kirschneri* serovar Australis strain Ballico (OKUDA et al., 2005). The gene encoding OmpL1 (906 bp, Genbank accession no: EU 046341) was amplified by PCR from the genomic DNA of *Leptospira interrogans* serovar Australis and cloned into pRSET'a' expression vector (Invitrogen, USA) and transformed into *Escherichia coli* BL21 (DE3) (Invitrogen, USA) cells. The recombinant clones were induced with 1mM final concentration of Isopropyl β-D thio galactoside (IPTG). The polyhistidine (6X-His) tagged fusion proteins were purified under denaturing conditions by Nickel-NTA affinity chromatography. The recombinant proteins were dialyzed in PBS, pH 7.2 and the concentration was determined by Lowry's method (Bangalore Genei, India)

Table 1. Primer design for amplification of leptospiral transmembrane proteingene OmpL1 by PCR

	Primer sequence (5'→3')	Restriction enzyme sites
Forward	AAA GGA CTC GAG GTA GCA CTA TCT TCG	Xho I
Reverse	TGT TAC CCA TGG AGA TTT GCC CAC CGA CAA C	Nco I

Latex agglutination test (LAT). A 10% suspension of the latex particles (0.8 µm dia, Sigma, USA) was coated with rOmpL1 antigen (25 µg/mL) using 0.06 M carbonate-bicarbonate buffer (Na_2CO_3 1.59 g and $NaHCO_3$ 2.93 g in one litre of distilled water, pH 9.6), and kept at 37 °C for 6 hours with constant shaking (RAMADASS et al., 1999). The

sensitized beads were centrifuged at 6,800 g for 3 min and the pellet resuspended as a 1% suspension in PBS containing 5 mg/mL of bovine serum albumin (BSA). The latex beads were incubated 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 used. The LAT was performed on glass slides by placing equal volumes of serum and sensitized beads (20 μL of each). The slide was rotated briefly for mixing the sensitized beads 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. The stability of the latex beads was routinely checked by keeping the beads at 4 °C and also at room temperature.

Evaluation of LAT and MAT. The relative sensitivity, specificity and concordance (in percent) of the Latex agglutination test for the detection of leptospiral antibodies were determined in comparison to the MAT as described below.

Sensitivity = a/(a+c) x 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) x 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.

Concordance = (a+d)/(a+b+c+d) x 100

Results

A 10% latex suspension was sensitized with 25 μg/mL of the recombinant OmpL1 antigen. The positive results of the latex agglutination test were graded to evaluate the visual degree of agglutination as +1 to +3 depending on the extent of agglutination and time taken for development of agglutination (Table 2). The serum samples were considered as negative if no agglutination was observed after 2 min. The stability of the antigen-coated beads stored for 2 months at 4 °C showed no loss of activity for both weak and strong positive samples. The relative sensitivity, specificity and concordance of the LAT as compared to MAT are presented in Table 3.

Table 2. Latex agglutination test showing the degree of agglutination withpositive dog serum samples

Total positive serum samples	Grading of LAT results		
	+1	+2	+3
422 (79.17%)	161 (38.15%)	108 (25.59%)	153 (36.25%)

Table 3. Comparison of rOmpL1 based latex agglutination test with MAT in serodiagnosis of canine leptospirosis

	MAT		
	+	-	Total
LAT			
+	415 (a)	7 (b)	422
-	31 (c)	80 (d)	111
Total	446	87	533

Sensitivity: 93.05%; Specificity: 91.95%; Concordance: 92.87%; $\chi 2 = 319.02$** (**Highly significant); K = 0.77

Discussion

Laboratory diagnosis of leptospirosis is an area poorly understood by many of the workers involved in leptospirosis diagnosis and surveillance. Selection of the right specimens and tests and the correct interpretation of test results are important in order to provide better treatment at the earliest opportunity. Conventional bacteriological techniques such as isolation of the organisms from clinical specimens or serology using MAT are laborious, time-consuming and require well-established laboratory facilities and skilled manpower (VIJAYACHARI and SEHGAL, 2006). Most institutes or hospitals in developing countries may not have such facilities for serodiagnosis of leptospirosis. Hence, there is a need to develop an improved diagnostic kit for the detection of leptospiral infection in the initial phase of the disease (DEY et al., 2007). Several diagnostic kits have been developed based on whole cell antigen preparations, which showed non-specific moieties and impurity of the immunodominant antigen. Recombinant antigen based serological tests may achieve higher sensitivity and specificity than other tests.

In leptospirosis, antibodies usually appear within 5-7 days after the onset of symptoms and antibodies persist in detectable quantities for many months (FAINE, 1982; FARR, 1995). From a clinical point of view, early detection of disease is very important. The fact that the specific immunological reactions are characterized by their increased production of antibodies that permits the development of sensitive early detection assays forms the basis for these assays. The sensitivity of our Latex agglutination test was equal to or slightly higher than that of MAT. The overall sensitivity and specificity of this test were in agreement with the MAT for the diagnosis of acute human leptospirosis as described by SMITS et al. (2000) whose overall test sensitivity and specificity were 82.3% and 94.6% respectively. Similar results have been reported when rLipL32 antigen was used for the detection of leptospiral antibodies (DEY et al., 2007).

In the present study, recombinant OmpL1 based latex agglutination test developed for the detection of leptospiral antibodies was proved to be a very useful rapid test for immunodiagnosis. Among 533 serum samples, 446 samples (83.68%) had positive reciprocal MAT titers, which ranged from 100 to 1600. MAT results indicated that the prevalent serovars were Canicola, Australis, and Grippotyphosa. A total of 422 canine serum samples were positive by the latex agglutination test. The sensitivity and specificity were very high and showed more than 90% with MAT. The kappa value of 0.77 showed perfect agreement between MAT and LAT. From this result we conclude that the LAT is an extremely simple and inexpensive test that does not require expertise or sophisticated equipments and could also be used for the detection of leptospiral antibodies in place of the microscopic agglutination test (MAT), which requires live leptospiral cultures, expertise, time and is also more expensive.

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SAŽETAK

Procijenjena je vrijednost rekombinantnog leptospiralnog transmembranskog proteina OmpL1 (rOmpL1) u testu lateks aglutinacije za dijagnostiku leptospiroze u pasa. Od 513 pretraženih uzoraka seruma pasa sumnjivih na leptospirozu, mikroskopskom aglutinacijom ustanovljeno je 446 (83,68%) pozitivnih uzoraka u aglutinacijskom titru od 1:100 do 1:1600. Osjetljivost lateks aglutinacije uporabom rOmpL1 iznosila je 93,05%, a specifičnost 91,95% u odnosu na mikroskopsku aglutinaciju. Jačina aglutinacije prikazana je od +1 do +3. Senzibilizirane lateks kuglice mogu se pohraniti pri 4 °C tijekom dva mjeseca bez gubitka aktivnosti. Lateks aglutinacija je jednostavna za izvedbu, jeftina i brza te se može rabiti za serološku dijagnostiku leptospiroze.

Ključne riječi: leptospiroza, pas, lateks aglutinacija, rekombinantni protein OmpL1, serološka dijagnostika