A pooled recombinant antigen-based enzyme-linked immunosorbent assay for serodiagnosis of canine leptospirosis

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ABSTRACT

A pooled recombinant antigen-based enzyme-linked immunosorbent assay (ELISA) was developed to measure the presence of antibodies in sera of 200 dogs suspected to be suffering with leptospirosis. The microscopic agglutination test (MAT) revealed that 52 (26%) samples had positive agglutination titers. With pooled LipL32 and LipL41 recombinant antigen ELISA, 62 (31%) samples were found positive. The ELISA showed a sensitivity of 100% and a specificity of 93.2%. The pooled recombinant antigen ELISA could be an alternative to the MAT for a reliable diagnosis of leptospiral infection in dogs.

Keywords: Canine leptospirosis, ELISA, MAT, recombinant antigen

Introduction

Leptospirosis is an important zoonotic problem caused by pathogenic spirochetes of the genus Leptospira and traditionally considered as an occupational risk among persons exposed to contaminated water or infected animal urine (Faine et al., 1999; Farr, 1995). Leptospira interrogans serovars Canicola and Icterohaemorrhagiae are mainly responsible for causing canine leptospirosis (Forrest et al., 1998); however, it may also be associated with other serovars, viz., Pomona, Bratislava and Grippotyphosa (Prescott et al., 1996; Birnbaum et al., 1998). Laboratory diagnosis of leptospirosis is predominantly achieved either by isolation of the causative organism or by serological tests. Bacteriological methods for the isolation of pathogenic Leptospira serovars are slow, labor intensive and often unsuccessful. The most commonly used serological test for the diagnosis of leptospirosis is the microscopic agglutination test (MAT). The test is laborious, time-consuming (Champagne et al., 1991) and requires live leptospires as antigens, causing problems in antigen standardization and posing a considerable risk to laboratory personnel. Enzyme-linked immunosorbent assays (ELISAs) (Hartman et al., 1984; Ribotta et al., 2000) and other rapid serological tests based on whole-cell leptospiral antigen preparations (Ramadass et al., 1999) have been developed for use as an alternative method to screen leptospiral infections.

Recombinant protein based ELISA is a suitable and safe procedure for the examination of a large number of sera as it involves use of an immunodominant antigen and lacks the non-specific moieties present in whole-cell preparations (Flannery et al., 2001). The recombinant leptospiral lipoproteins so far utilized for diagnosis in ELISA have been LipL32 and LipL41 (Bomfim et al., 2005; Maria Rosa et al., 2005; Mariya et al., 2006 Senthil

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Kumar et al., 2007). In the present study, recombinant LipL32 and LipL41 antigens were used individually in ELISA to screen serum samples from dog suspected of having leptospirosis. A few serum samples showed some variability in results with respect to the two antigens used. Hence, the present study was undertaken to evaluate the diagnostic potential of a pooled LipL32 and LipL41 recombinant antigen for the serodiagnosis of canine leptospirosis.

Materials and Methods

Leptospira strains

Leptospira serovars used in the study were maintained in semi-solid and liquid EMJH media (Ellinghausen and McCullogh, 1965) with serial passages.

Serum samples

Blood samples were obtained from 200 pet dogs suspected for leptospirosis from different parts of India. Samples were centrifuged at 2000 rpm for 20 min and the serum samples were stored at -20 °C until use.

Microscopic agglutination test (MAT)

The MAT was performed as described by Cole et al. (1973) in microtitre plates using reference strains of 11 Leptospira interrogans serovars namely Canicola, Pomona, Autumnalis, Australis, Pyrogenes, Patoc, Javanica, Hebdomadis, Icterohaemorrhagiae, Grippotyphosa and Hardjoprajitno. Reciprocal agglutination titres of greater than or equal to 100 were considered as positive reactions.

Enzyme-linked immunosorbent assay (ELISA)

The ELISA was performed as described by Engvall and Perlmann (1971) with some modifications. Recombinant LipL32 (rLipL32) and LipL41 (rLipL41) were purified as described by Maria et al. (2006) from Escherichia coli cells transformed with Leptospira interrogans serovar Canicola LipL32 and LipL41 gene inserts in pProEXHTb vector. The optimum concentration of the pooled recombinant antigen for conducting ELISA was determined by checker board analysis. Flat-bottomed polystyrene microtitre plates (Nulge, Nunc Int., Nunc) were coated with 100 μl of the pooled antigen (rLipL32 50ng + rLipL41 50 ng), suspended in 0.05 mM sodium carbonate buffer (ph 9.6), and incubated at 4°C overnight. Then 4 washings with PBS containing 0.5% Tween-20 (PBS-T) were given out and 100 μl of PBS with 5% (w/v) skim milk powder was added to the wells as a blocking agent. The plates were incubated at 37°C for 1 h and again washed as earlier. Now 100 μl of 1:100 dilution of the canine serum was added to the wells. The plates were incubated at 37°C for 1 h after which they were again washed 4 times with PBS-T, followed by addition of 100 μl of the diluted (1:3000) IgG anti-dog conjugate (Sigma) and further incubation at 37°C for 1 h. The plates were again washed and 100 μl of ortho phenylene diamine (Fasta, Sigma) solution was added ([20 mg OPD dissolved in 50 ml of substrate buffer containing 0.03% (v/v) hydrogen peroxide, 25 mM citric acid, 50 mM disodium hydrogen phosphate (pH 5.0)], and further incubated at room temperature for 10 min in dark for colour development. Thereafter, reaction was stopped using

Table 1: Evaluation of pooled recombinant antigen ELISA in comparison with standard test MAT

<table>
<thead>
<tr>
<th>MAT</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>52 (a)</td>
<td>10 (b)</td>
<td>62</td>
</tr>
<tr>
<td>Negative</td>
<td>- (c)</td>
<td>138 (d)</td>
<td>138</td>
</tr>
<tr>
<td>Total</td>
<td>52 (a+c)</td>
<td>148 (b+d)</td>
<td>200</td>
</tr>
</tbody>
</table>

Sensitivity=100%; Specificity=93.2%; Accuracy=95%; $x^2=155.97; k=0.99$
100 μl stopping solution (1M H₂SO₄) added to the wells. The optical density (OD) of the plates was measured at 492 nm for colour development using ELISA reader (Multiscan EX, Lab system).

Initial assays were performed to determine ideal serum dilution for conducting ELISA using 52 MAT positive canine serum samples. Sera were diluted in serial two-fold dilutions from 1:10 to 1:1280 and the mean of the O.D. of duplicate samples was calculated for analysis. The best results were obtained with serum samples diluted to 1:100. The serum that showed the highest value of absorbance at this dilution was chosen as a positive control. The cut-off value for interpretation of ELISA was determined by calculating the mean OD obtained with the MAT negative sera. The mean OD value plus three times the standard deviation was taken as the cut-off point.

Statistical analysis

The relative sensitivity, specificity and accuracy of the pooled recombinant antigen ELISA for the detection of anti-leptospiral antibodies in dog sera were obtained in comparison to the MAT as described below.

Sensitivity= \( \frac{a}{a+c} \times 100 \) where ‘a’ is the number of sera positive by MAT and ELISA, ‘c’ the number of sera positive by MAT but negative by ELISA.

Specificity= \( \frac{d}{b+d} \times 100 \), where ‘d’ was the number of sera negative by ELISA and MAT, ‘b’ the number of sera negative by MAT but positive by ELISA.

Accuracy= \( \frac{(a+d)}{(a+b+c+d)} \times 100 \)

Results and Discussion

Although MAT is the test of choice, however, it is fraught with several weaknesses such as it is tedious, time consuming and highly serovar specific. Recombinant antigen-based serologic tests may have higher sensitivity and specificity as the target antigen is immunodominant and devoid of any non-specific moieties as present in whole-cell preparations. The recombinant leptospiral antigens utilized in the diagnostic assay in the present study are constitutively expressed by all pathogenic serovars of Leptospira, hence, are of significant importance for the diagnosis of leptospirosis.

Out of 200 serum samples tested with MAT, 52 (26%) were found positive against various Leptospira serovars viz., Canicola, Pomona, Autumnalis, Australis, Pyrogenes, Patoc, Javanica, Hebdomadis, Icterohaemorrhagiae, Grippotyphosa and Hardjo.

A total of 57 sera samples were found positive in recombinant LipL32 ELISA and 53 in recombinant LipL41 ELISA. Using a pool of the antigens, 62 were found positive. Thus, the pooled antigen was superior to the individual antigens. The efficacy of the pooled antigen ELISA was, therefore, evaluated in comparison with the standard MAT. The sensitivity, specificity and accuracy of pooled recombinant antigen ELISA were evaluated in comparison with standard MAT and the results are depicted in Table 1. The sensitivity was found to be 100% since all MAT positive sera came positive in ELISA. The specificity in comparison with MAT was 93.2%, since ten MAT negative sera came positive in ELISA. This may be due to the reason that only 11 Leptospira serovars were used for conducting MAT. Moreover, the antibody titres in these sera may be lower than the level detectable by MAT. ELISA, being broader in seroreactivity as well as more sensitive in nature, detected all such MAT negative sera. The pooled ELISA showed kappa value 0.99 suggesting perfect agreement.

In conclusion, the pooled recombinant antigen-based ELISA developed for the detection of anti-leptospiral antibodies in canine was demonstrated to be sensitive, specific and accurate when compared to the standard serologic test, MAT. Thus, the use of the pooled LipL32 and LipL41 recombinant antigen in ELISA has the potential to become a useful tool for serodiagnosis of canine leptospiral infection.

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References


