Diagnosis of leptospirosis by recombinant antigen based single serum dilution ELISA

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Background & objectives: Leptospirosis, a zoonosis with a worldwide distribution is an acute febrile illness caused by spirochaetes of the pathogenic Leptospira interrogans. Microscopic agglutination test (MAT), the reference method for diagnosis was successively done to evaluate the modified ELISA which was developed with the recombinant LipL32 antigen for the detection of anti-leptospiral antibodies in human serum samples.

Methods: The recombinant LipL32 antigen was developed from the serovar Pomona strain Pomona of the pathogenic L. interrogans species. The predicted titre at a single working dilution was plotted against the observed antiserum titre. Subsequently, predicted antibody activity titres were determined directly from the standard curve by solving the regression line equation. The relative sensitivity, specificity and accuracy of the single dilution ELISA for the detection of anti-leptospiral antibodies were determined in comparison to the MAT.

Results: A linear relationship was found between the predicted antibody titres at a single working dilution of 1:250 and the corresponding observed serum titres by the standard serial-dilution method. Regression analysis was used to determine a standard curve from which an equation was derived that allowed demonstration of the mentioned correlation. The equation was then used to convert the corrected absorbance readings of the single working dilution directly into the predicted ELISA antibody titres. A high level of sensitivity of 96 per cent and specificity of 91 per cent between ELISA and MAT titres was found. The kappa value was almost 1.0 indicating perfect agreement.

Interpretation & conclusions: The r LipL32 ELISA was proved to be sensitive, specific and accurate as compared to the standard MAT and the test could be efficiently utilized as a screening test for a large number of human serum samples for the detection of leptospiral antibodies.

Key words Human - leptospirosis - recombinant antigen - single serum dilution ELISA

Leptospirosis, a zoonosis with a worldwide distribution, is an acute febrile illness caused by spirochaetes of the pathogen Leptospira interrogans affecting a wide range of animal and human hosts1. Humans are accidental hosts and become infected through contact with an environment contaminated by the urine of a shedder host, such as rodents. The incidence is significantly higher in tropical countries than in temperate regions2,3. In 1988, during the peak of the monsoon season, serum and urine samples from 40 patients revealed 82.5
per cent had specific leptospiral antibodies, with titres ranging from 1:160 to 1:6400 by microscopic agglutination test (MAT) against different serovars, and 71 per cent seropositive cases were diagnosed by MAT with clinical suspicion of leptospirosis in 1996. In India an outbreak has been reported during the post-cyclone investigation in Orissa in late nineteenth.

Leptospirosis is often misdiagnosed as influenza, aseptic meningitis, encephalitis, dengue fever, hepatitis or gastroenteritis. Timely diagnosis is essential because prompt, specific treatment as early in the illness as possible is important to ensure a favourable clinical outcome. MAT the standard reference test for the detection of leptospiral-specific antibodies, has a high sensitivity only when it is performed on paired samples. MAT on acute samples has very low sensitivity but can give presumptive information on the serogroup of leptospira. But the application of the test requires a detailed knowledge of the locally occurring strains, as the predominant serovars have to be selected for use as antigens. Dependence upon the MAT often results in delay in establishing the cause of outbreaks. Because of the complexity of the MAT, rapid screening tests for leptospiral antibodies in acute infection have been developed viz., complement fixation (CF), immunoglobulin M (IgM) enzyme linked immunosorbent assay (ELISA), latex agglutination test (LAT). MAT can also detect IgM antibodies. The reason for possible higher sensitivity of ELISA during the acute stage could be the genus specific broadly reactive antigens rather than serogroup-specific antigens as used in MAT.

This study was carried out to use a recombinant LipL32 based ELISA for the rapid measurement of specific antibody activity in a large number of human serum samples by analyzing a single serum dilution. The conventional serial dilution ELISA has been modified to a recombinant antigen based single serum dilution ELISA to reduce the reagent costs and increase the number of the samples to be screened as described earlier for canine leptospirosis.

Material & Methods

A PCR- based approach was used to clone and express a portion of LipL32 gene encoding the major leptospiral outer membrane protein from *Leptospira interrogans* serovar Pomona strain Pomona. The nucleotide sequence of the PCR-amplified LipL32 gene was submitted to GenBank under the accession number AY223718. The recombinant LipL32 histidine tagged fusion protein expressed from pProEXHTa expression vector (Life Technologies, USA) was purified by affinity chromatography and dialyzed overnight at 4°C against phosphate buffered saline (PBS) containing 10 per cent glycerol and 0.025 per cent sodium azide.

Flat-bottom polystyrene microtitre plates (Corning, USA) were coated with recombinant LipL32 antigen and were stored at 4°C overnight. By checkerboard titration, the optimal antigen concentration (0.5 ng to 1.5 μg/well) was determined. Plates were incubated with blocking solution (PBST with 2% w/v bovine serum albumin) for 1 h at 37°C, and after three washes with PBST, stored at -20°C until use.

**Serum samples:** A total of 136 human serum samples were screened. The blood samples from the patients suffering from febrile conditions were kindly provided by the Department of Microbiology, Madras Medical College, Chennai during the period of 2003-2004. The blood samples were centrifuged at 1500 x g for 20 min for the separation of serum. Serum samples were stored at -20°C until use. Out of 51 samples which were negative by both MAT and single dilution ELISA, 19 samples were collected from patients suffering from other febrile illness like typhoid, acute jaundice and hepatitis. The serum samples were diluted 1:125 in PBST and 100 μl were serially diluted (1:125 to 1:16,000) in duplicates. Following incubation at 37°C for 1 h, the wells were washed with PBS supplemented with 0.05 per cent tween 20.

**Control sera:** Two positive and two negative control wells were included in duplicate in each plate. The positive controls were MAT-positive serum serovar Pomona 1:400 and lenterohaemorrhagiae 1:200 from individuals suffering from leptospirosis with an optical density at 405 nm of approximately 0.6 after 5 min of substrate development. The negative controls were MAT-negative serum, which resulted in an optical density at 405 nm of approximately 0.05 after 5 min of substrate development.

A volume of 100 μl of peroxidase-conjugated IgG fraction of rabbit anti-human conjugate (Banglore Genei, India) diluted 1: 4000, was added to each test as well as the control wells.

Substrate 2, 2'- azino di-ethyl benz- thiazoline- 6 sulphonic acid (ABTS) (Sigma, USA), 5.5 mg was added to 25 ml of sodium citrate buffer (pH 4.2) and 25 μl of 35 per cent hydrogen peroxide. The substrate, 100 μl/ well was immediately dispensed into the wells and
incubated until the colour reaction developed. The plates were read at 405 nm in an ELISA reader (Bio Tek Instruments Inc., USA). The mean absorbance of three negative control wells was calculated. Any serum sample showing an OD above the mean + (3 × standard deviation) of the negative wells was considered positive.

**IgM ELISA**: A commercially available IgM ELISA kit (Pan Bio Pty Ltd., Brisbane, Australia) obtained from Lister Laboratory, Chennai was used to determine IgM antibodies in the serum samples with recombinant LipL32 as antigen. The method was followed as per the manufacturer’s protocol. As described in the ELISA procedure, the serum samples were tested on the plate coated with the recombinant LipL32 antigen. A volume of 100 µl of peroxidase-conjugated IgM fraction of rabbit anti-human conjugate (provided with the kit) was added to each test as well as the control wells. The plates were read at 405 nm following addition of the substrate. The Pan Bio units were calculated and interpreted as instructed: a positive sample was defined as having a sample calibrator absorbance ratio of > 1.0, and a negative sample was defined as having a sample calibrator absorbance ratio of < 1.0.

**Microscopic agglutination test (MAT)**: The MAT was performed as described in microtitre plates. A result of 50 per cent agglutination of leptospires at 1:100 dilutions was considered significant, i.e., the serum samples having titres ≥ 100 were considered positive. A total of 56 serum samples with negative titres at a dilution of 1:100 against serovars Pomona, Icterohaemorrhagia, Canicola, Grippotyphosa, Autumnalis, Javanica and Hebdomidis were used to determine the relative specificity of the ELISA. For determination of the relative sensitivity of the ELISA, 80 sera showing MAT titres > 100 to different serovars were employed.

**Determination of ELISA antibody titres**: ELISA antibody titres were determined by standard serial dilution, and end-points were calculated by a subtraction method as described below. First, a total of 15 ELISAs were run with different negative serum pools to provide absorbance data for the construction of a positive-negative threshold (PNT) baseline, which was used to determine the observed ELISA antibody titres. A mean absorbance and three standard deviation units above the mean absorbance were calculated from duplicates of all trials at each negative serum dilution. The resulting three standard-deviation baseline unit values were then plotted on log-log paper and used as PNT baseline for calculation of the observed antibody titres. Next, the absorbance of these test sample dilutions was corrected by subtracting the appropriate average absorbance of internal negative control serum dilutions from the same trial. The ELISA values of the sample sera were expressed as sample to positive ratio ([S/P ratio = (OD of sample - OD of negative control) / (OD of positive control - OD of negative control)]).

Finally, the corrected average absorbance of the test samples was calculated and the observed antibody end point titres were defined as the point at which the plotted lines intersect the PNT. The positive sera samples were titrated serially using the ELISA and corrected as described above.

The coefficient of correlation (r) between the log_{10} end titre and the S/P ratio (at serum dilutions of 1:125 to 1: 16,000) showing the maximum value of ‘r’ was chosen for the derivation of the regression equation (y = a + bx, where y = the log_{10} S/P ratio of the test serum, x = log_{10} antibody titre of the test serum at the chosen dilution, a = intercept of the regression equation, b = slope of the line) for predicting log_{10} end titre by single dilution ELISA. After regression analysis, a standard curve was constructed with absorbance and titre values obtained from this antiserum. The predicted titre at a single working dilution was plotted against the observed antiserum titre. Subsequently, predicted antibody activity titres were determined directly from the standard curve by solving the regression line equation.

**Sensitivity, specificity and per cent agreement**: The sensitivity, specificity and per cent agreement of the single dilution ELISA were calculated considering MAT at a titre of 1 in 100 as gold standard. The per cent agreement of ELISA and MAT with a titre of 1 in 100 was also calculated.

**Results**

The optimum concentration of the purified recombinant LipL32 antigen which showed maximum difference between the positive and negative sera was determined to be 50 ng/well. Observed titres of serum samples were calculated by plotting the average of triplicate, corrected absorbance readings at serial dilutions until the PNT baseline was intersected (Fig. 1).

A linear relationship existed between the predicted titres of the positive sera at a single working dilution (1:250) and the corresponding observed titres as determined by the serial dilution. Regression analysis yielded a regression line with a correlation coefficient of
0.864 (Fig. 2). The average absorbance at 1:250 was corrected by the subtraction method and the predicted titres were determined using the regression line equation, \( \log_{10} (S/P \text{ ratio}) = \text{intercept} + \text{slope} (\log_{10} \text{ titre}) \) or rewritten as, \( \log_{10} (S/P \text{ ratio}) = -3.3 + 0.849 (\log_{10} \text{ titre}) \).

The sensitivity, specificity and accuracy of the assay relative to the reference method MAT are shown in the Table. The serum samples collected from the patients suffering from other febrile illnesses were negative by this assay. The cut-off value was determined at a dilution of 1:1000 (\( \log_{10} \text{ value} =3.0 \)) and above where the absorbance of the negative sera plateau whereas the positive sera continued to show a high absorbance value (Fig. 1). This was a qualitative comparison using MAT titres 100 as positive and the predicted single dilution ELISA titre greater than or equal to 1000 as positive.

Forty five serum samples were tested for IgM. The positive to sample calibrator ratio was 0.8. Those samples which were tested positive for IgM antibodies using the coated plates provided in the kit were also positive with the recombinant LipL32 antigen.

**Discussion**

Leptospirosis is now identified as one of the emerging infectious diseases, exemplified by large outbreaks in Nicaragua, Brazil, India, Southeast Asia, the United States and Malaysia\(^8\). A rapid, accurate method for the diagnosis of leptospirosis is important to both the clinician and the patient. The MAT however, despite its wide-spread usage and international recognition, has a number of limitations. These include the need to use hazardous live bacteria, expertise to test each serum sample against multiple serovars of this organism and is time consuming. MAT is inadequate for rapid case identification since it can only be performed in a few reference laboratories and requires analysis of paired sera to achieve sufficient sensitivity\(^9\). In this study, the conserved nature and high level of expression of LipL32 only among the pathogenic *Leptospira* species suggested that the recombinant LipL32 (rLipL32) ELISA may exhibit similar performance to MAT regardless of the locally predominant serovar. The serovar Pomona strain Pomona of *L. interrogans* species was chosen for cloning and expression of the recombinant outer membrane protein. The serovar Pomona antigen reacts broadly to many species as it is prevalent in canine leptospirosis\(^10\), bovine leptospirosis\(^11\) and human leptospirosis\(^12\).

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<th>MAT</th>
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**Table.** Relative sensitivity, specificity and accuracy values of the developed ELISA to detect pathogenic leptospiral antibodies using MAT as a reference standard

Sensitivity = 96.25% (95% CI - 88.7, 99.0)
Specificity = 91.07% (95% CI - 79.6, 96.7)
Accuracy = 94.12% (95% CI - 88.4, 97.2)
Kappa value = 0.987
This study described the single serum dilution ELISA by establishing a nearly linear relationship between the corrected titre of antisera at a single working dilution of 1:250 and the corresponding observed antibody titre as reported for the detection of antibodies against the Newcastle disease virus and canine leptospirosis. The use of single serum dilution reduces the reagent costs and technical time significantly, and may reduce error inherent in serial dilutions. In this method the PNT baseline was arbitrarily set at an absorbance by plotting the average of triplicate, corrected absorbance readings at serial dilutions of the observed titres for positive and negative sera. In human samples, which indicate that at a dilution of 1:1000 (log 

value = 3) and above, the absorbance of the negative sera plateaued whereas, the positive sera continued to show a high absorbance value. Thus the results were interpreted as; human serum samples with the antibody titres > 1000 were considered positive, between 500 - 1000 as suspected and should be retested, and below 500 as negative. The S/P ratio is used instead of the OD values at a particular dilution for standardization of single dilution ELISA since a great variation in OD values for a particular sample was observed depending upon the assay conditions, time of incubation, etc. However, the S/P ratio for a particular serum sample at the same dilution remained relatively constant under varying test conditions. The serum samples of known identity were deliberately selected from a previous study for the standardization of serial and single dilution ELISA to increase the confidence of the regression equation so that unknown sera with different antibody titres could be evaluated perfectly in this system. This was in conformity with earlier studies where a similar strategy was adopted while standardizing single dilution ELISA for detecting antibodies to other pathogens.

A relatively high sensitivity and specificity between ELISA and MAT titres indicated that both tests measured similar trends in exposure to leptospirosis. The kappa value was almost 1.0 indicating perfect agreement. The ELISA had a specificity of 91.07 per cent relative to the MAT. This relative specificity value, which was obtained with sera defined by the MAT, may contain non-agglutinating leptospiral antibodies that are detectable by ELISA but not by the MAT, which can only detect agglutinating antibodies as reported. A few samples collected from MAT confirmed leptospirosis cases that had reciprocal MAT titres ≥ 100 had negative recombinant LipL32 ELISA result. The low background reactivity may be due, in part, to the restricted expression of LipL32 in pathogenic leptospires and not saprophytic forms that are ubiquitous in the environment.

Performance of the rLipL32 antigen with the commercially available PanBio IgM ELISA revealed that the rLipL32 detected IgM response. This finding is contradictory to the findings reported by Flannery et al. The predominant humoral response during acute phase infection is believed to be due to IgM antibodies, which are directed primarily against carbohydrate epitopes. The IgG response to rLipL32 and other recombinant antigens were found to have kinetics comparable to that of the IgM response to whole antigen preparations. It was further explained that the rapid rise in IgG antibody may have been due to a memory response in individuals with prior exposure to leptospires. Alternatively, this could represent a rapid IgM to IgG class switch phenomenon. These findings suggest that early host immune response to Leptospira infection is characterized by both IgM and IgG antibodies specific for different moieties, as observed in the early response to Borrelia. Moreover, in our study a few human serum samples showed high IgM as well as IgG titres, similar to that by Terpstra et al. where characteristically high IgM, IgG and agglutinating titres were found only in patients with current leptospirosis.

The recombinant LipL32 ELISA, in this study also differentiated patients with leptospirosis from those with other important causes of acute jaundice and febrile illnesses, such as typhoid, since the serum samples positive for typhoid and hepatitis did not show any reaction with the recombinant protein, which corroborates the findings of Flannery et al. In conclusion, rLipL32 single serum dilution ELISA with its excellent diagnostic specificity and sensitivity, convenient technical features, its potential for automation, may be efficiently utilized as a screening test for a large number of serum samples for the detection of leptospiral antibodies.

References


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