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Title: Development of an In-House ELISA as an alternative method for the serodiagnosis of leptospirosis

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Highlights

- Confirmatory laboratory diagnosis of leptospirosis is a major challenge
- In-House ELISAs established to detect anti-leptospiral antibodies
- Factors responsible for the manifestation of severe leptospirosis forms are unclear
- High anti-leptospiral IgG in severe leptospirosis patients compared to mild patients
- Anti-leptospiral IgG could be used as a biomarker to predict disease severity

Abstract

Background: Leptospirosis is most often clinically diagnosed and a laboratory test with high diagnostic accuracies is required.

Methodology: IgM and IgG-ELISAs using *Leptospira* antigens were established and evaluated in relation to the Microscopic Agglutination Test (MAT). Antigen preparation consisted either saprophytic *Leptospira biflexa* to detect genus specific antibodies (genus-specific ELISA) or a pool of five most prevalent *Leptospira interrogans* serovars in Sri Lanka to detect serovar specific antibodies (serovar-specific ELISA). IgM and IgG immune responses in severe and mild leptospirosis patients (n=100 in each group) were studied.

Results: ELISAs showed high repeatability and reproducibility. Serovar-specific IgM-ELISA showed sensitivity of 80.2% and specificity of 89%; genus-specific IgM-ELISA showed sensitivity of 83.3% and specificity of 91%. Serovar and genus-specific IgG-ELISA showed sensitivities of 73.3% and 81.7%, and specificities of 83.33%. Commercial IgM-ELISA showed sensitivity and specificity of 79.2% and 93% respectively. Commercial IgG-ELISA showed sensitivity, specificity of 50% and 96.7% respectively.

IgM levels observed in mild and severe leptospirosis (ML & SL) patients were significantly higher than healthy control (HC) group, having absorbance mean of 0.770, 0.778 and 0.163 respectively. In contrast, SL patients had significantly higher mean anti-leptospiral IgG levels

compared to both ML and HC groups (0.643, 0.358 and 0.116 respectively; ANOVA, $P < 0.001$). Presence of anti-leptospiral IgG above OD 0.643 optical density (OD) at 1:100 could predict high risk of severe disease.

Conclusion: Serovar-specific In-House ELISAs could be used for the laboratory diagnosis of leptospirosis in endemic settings. Observed high levels of anti-leptospiral IgG suggest its value as a predictor for disease severity.

Key words: Leptospirosis, IgM and IgG antibodies, Sero-diagnosis, In-House ELISA

Background

Leptospirosis known as the most widespread zoonosis worldwide is one of the major threat to public health and cause economic loss in the farming industry in tropical and subtropical regions (Bharti et al, 2003). It has an incidence greater in warm-climate and endemic in areas with high rainfall, close human contact with livestock, poor sanitation with workplace exposure to the organism. According to worldwide surveys, an estimated count of 550,000 cases are recorded annually, with around 55000 deaths (Viyachari et al, 2008; Costa et al 2015). Sri Lanka experienced large outbreaks in 2008 and 2011 with the incidence of 35.7 and 65.5 per 100,000 populations and since then the rate of incidence remains high (Agampodi et al, 2011; Warnasekara et al, 2019).

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The variety of clinical symptoms makes the leptospirosis clinical diagnosis a challenge. Therefore, rapid and appropriate laboratory diagnostic tests are required for the confirmatory diagnosis for patient management. The definitive laboratory diagnosis by isolating the

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pathogenic *Leptospira* from blood, urine or tissues require precise culture media and prolonged incubation time makes it impractical for routine diagnosis. Reference serological test i.e.; *m*icroscopic *a*gglutination *t*est (MAT) is time consuming and expensive, since it requires a large number of species and serovars of *Leptospira* to be maintained in culture, professional expertise and paired sera for confirmation. Therefore, MAT is available only at reference laboratories. Several alternative immunodiagnostic assays have been developed in recent years to accommodate the requirement of easy laboratory tests (Sehgal et al, 1999; Panwala et al, 2011; Niloofa et al, 2015).

In Sri Lanka, MAT is the main test for laboratory confirmation of leptospirosis. However, it is conducted only at one central reference laboratory at the Medical Research Institute (MRI) in Colombo and not widely available at peripheral hospitals. Although MAT is a functional laboratory assay that is considered as one of the reference standards, MAT based detection has its own limitation since agglutinating antibodies are detectable after 8-10 days of illness. MAT is recommended by Leptospirosis Burden Epidemiology Reference Group (LERG) to be used after eight days of fever and generally requires paired sera for detection of antibody rise or sero-conversion.

Clinical features of leptospirosis vary and may progress to multi-organ failure and death. Initial clinical symptoms and laboratory test results associated with severe forms remain unclear. Severe manifestations such as Weil’s syndrome occur in 5-15% of human leptospirosis which has a 10-15% case fatality rate and severe pulmonary hemorrhage syndrome (SPHS) is associated with a case fatality rate of >50% (Vijayachari et al, 2008; Gouveia et al, 2008).

Prompt triage of high-risk patients is essential and critical due to complications require intensive care, hemodialysis and monitoring. Factors responsible for the manifestation of severe forms are not clearly established. Pathogen and host related factors are believed to play an important role in development of severe leptospirosis. Signs and symptoms of renal and hepatic involvement appear in the immune phase when specific antibodies begin to be detected. Thrombocytopenia has shown to be present in severe leptospirosis patients and has been attributed to immune mechanisms (Devanport et al, 1989; De Silva et al, 2014). Anti-leptospiral IgG and IgA antibodies have shown to be present in higher amounts in patients with severe leptospirosis (Abdulkadar et al, 2002).

In this study, a conventional microplate ELISA was developed to detect IgM and IgG antibodies against the antigens prepared from saprophytic serovar and five predominantly circulating pathogenic serovars (during the year 2012) in Sri Lanka. This ELISA was evaluated against the serological reference test MAT and also compared with a commercial IgM and IgG ELISAs. Using these In-House ELISAs anti-leptospiral IgM and IgG was measured to determine possible association with severity of the disease.

Methods

Preparation of antigens

In-House ELISA was established using saprophytic serovar (Patoc) and the five most prevalent pathogenic *Leptospira interrogans* serovars (Pyrogenes, Australis, Icterohaemorrhagiae, Canicola and Hardjo) in Sri Lanka (Agampode et al 2008; Agampodi et al 2013; Koizumi et al 2009). *Leptospira biflexa* serovar Patoc strain Patoc-I, *Leptospira interrogans* serovar Hardjoe strain Hardopraj in Sejroe serogroup, serovar Pyrogenes strain Salinem in Pyrogenes serogroup,

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serovar Australis strain Ballico in Australis serogroup, serovar Icterohaemorrhagiae strain RGA in Icterohaemorrhagiae and serovar Canicola strain H Uterecht in serogroup Canicola were cultured in EMJH (Ellinghausen-McCullough-Johnson-Harris) medium (Difco) supplemented with 10% rabbit serum. Fresh culture containing 10^7 cells/mL and incubated in dark at 30 ºC with mild shaking for 14 days to yield a cell density of 10^8 cells/mL. The culture was killed using 0.5 mg/L sodium azide and sonicated to prepare antigens and re-suspended in phosphate buffered saline (PBS) to 25% of the original volume (Tansuphasiri et al, 2005). Protein content of antigen preparations was determined according to Bradford method (Bradford 1976). Equal protein content (w/v antigen) from each antigen preparation was mixed to obtain a preparation of pooled antigen. Antigen preparation from *Leptospira biflexa* serovar Patoc strain Patoc I was used separately for ELISA. Antigen preparation was made into small aliquots and stored at -20 ºC until use.

Establishment, optimization and validation of in-house ELISA for detection of human anti-leptospiral antibodies

ELISA was optimized in a flat-bottomed polystyrene microplate (polysorb, Nunc, USA) by varying concentrations of IgM/IgG-HRP conjugate (1:4000 & 1:8000) and using two different buffers (Carbonate buffer (CB), pH 9.6 and PBS, pH 7.2) for coating the antigen. Optimization was performed using six serum samples serially two-fold diluted at 1:100 – 1:3200. Based on repeat experiments, the optimum criteria were selected and used (Supplementary Table 1 and 2). Plates were coated with 100 µl of 2 µg/mL antigenic proteins in 0.05M carbonate buffer and incubated at 37 ºC for 2 hours in a moist chamber and at 4 ºC for 12 hours. Plate was blocked with 300 µl of PBS-0.05% Tween-20 (PBST) containing 5% skimmed milk after being washed

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three times with PBST. After six washes 100 µl of each serum dilution (serially diluted with PBS containing 5% BSA) treated with rheumatoid factor were according to the designed plate plan, and incubated at 37 ºC for 1 hour in a moist chamber. After washing six times, 100 µL of goat anti-human IgM/IgG (µ, γ chain specific respectively) horseradish peroxidise (HRP) conjugate (MyBioSource, USA) diluted to 1:8000 in PBS containing 5% skimmed milk was added. Plate was washed six times after incubation for an hour at 37 ºC in a moist chamber. Substrate solution containing 100 µL of 3'3'5'5'-tetramethyl benzidine dissolved in dimethyl sulfoxide and phosphate citrate buffer (pH 5.0) and H_2O_2 was added and incubated in dark at room temperature for 20 minutes. The reaction was stopped by adding 50 µL of 1M HCl. Optical density (OD) at 450 nm was read using an ELISA reader (ELx 800 – Universal Microplate reader, Bio-Tek Instruments INC, Canada). Mean+2SD of OD_{405} of healthy control was used as the cut-off value. Samples were tested in duplicates with overlapping samples from the previous experiments to maintain the minimal inter and intra assay variation.

IgM ELISA (Institut Virion\Serion GmbH, Warburg, Germany) was performed according to manufacturer's instructions (http://www.virion-serion.de/fileadmin/templates/tpl1/global/download/flyer/Flyer_ELISA_classic_Leptospira_EN.pdf). Each kit was performed with a negative control, positive control and cut-off calibrator in duplicate. Absorbance reading of the above in a test obeying the specifications of the Serion ELISA indicates that the test is valid. Results were obtained using the evaluation table provided along with the kit. Interpretation of results for Serion-ELISA classic *Leptospira* IgM was as follows: anti-leptospiral IgM <15 IU/ml gives a negative result suggesting no evidence of recent infection, 15-20 IU/ml gives a borderline

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result suggesting that may be a recent infection and >20 IU/ml gives a positive result which is interpreted as a recent or current infection.

Serum sample selection

Samples for In-House ELISA development

A total of 302 acute serum samples, were randomly selected from a bank of samples from cases identified during ongoing study from 2012-2014. All samples were previously tested with MAT and commercially available IgM and IgG ELISA (Niloofa et al, 2015). A total of 202 samples were confirmed for leptospirosis, while 100 samples were negative for leptospirosis. For the development of IgG ELISA, out of these 202 MAT positive and 100 MAT negative samples, a subsample of 92 acute samples (laboratory confirmed MAT positive = 62 and MAT negative 30) were selected. Samples were collected at a mean day of 7.2 (range from 2 to 16) after the onset of symptoms.

Samples from severe and mild leptospirosis patients

Severe leptospirosis was defined as described previously based on the following: evidence of major organ dysfunction (liver, kidney, lung or heart), admission to the intensive care unit, hospital stay for longer than 10 days, or death. The involvement of organs was defined according to Rajapakse *et al* (Rajapakse et al 2015; Kalugalage et al, 2013). Sera from laboratory confirmed (MAT positive), severe and mild leptospirosis patients and healthy controls (n=100 each) were randomly selected from the study group. Serum samples were tested for IgM and IgG, using the In-House ELISA (serovar-specific) detecting antibodies against the pooled pathogenic antigen preparation.

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Statistical analysis

Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) version 17.0 (IBM, Armonk, NY, USA). The data are represented as mean and standard error of mean (SEM) or standard deviation (SD). The significant values are set at $P \leq 0.05$. Independent sample t-test for two independent variables was used to test the difference between groups, at a 95% interval. Pearson correlation coefficients were calculated to determine the correlations. Receiver Operator Curve (ROC) analysis was performed to identify a suitable cut-off value for established In-House ELISAs. The sensitivity and specificity of microplate IgM and IgG ELISA were determined by using the results of MAT as gold standard. Kappa test was used to find the degree of agreement between the tests. Repeatability and reproducibility were calculated using one-way ANOVA.

Results

Establishment, optimization of anti-leptospiral IgM and IgG In-House ELISA

Sera samples representing MAT titers >100, 200, 400, 800, 1600 and 3200 were tested in both dilutions of anti-human IgM/IgG HRP conjugate (1:4000 and 1:8000) and in the antigen coating buffers 0.05M CB and 0.13M PBS. Mean OD values of both conjugate dilutions 1:4000 and 1:8000 were not greatly differing from each other. Results are shown in Supplementary Table 01. As majority of the samples did not show significantly different OD levels in either concentration of conjugate levels, 1:8000 was selected as optimum concentration of conjugates, taking into account the cost of conjugate.

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Sera samples representing MAT titers >100, 200, 400, 800, 1600 and 3200 were tested in both antigen coating buffers 0.05M CB and 0.13M PBS using conjugate of 1:8000 dilutions. OD values for CB were greater than that of PBS in both ELISAs at 1:100, 1:200, 1:400, 1:800 and 1:1600 serum dilutions (Supplementary Table 02). CB IgM-ELISA mean OD values showed decreasing concave follow through with the reciprocal of serum dilutions and at low dilutions it shows clear mean OD value separation between positive and negative sera. As obtaining a clear OD is critical for the result in samples which have low amount of antibody is present, 0.05M carbonate buffer was selected for subsequent use.

In-House anti-leptospiral IgM-ELISA

For the IgM ELISA developed with pooled antigens of pathogenic serovars, OD value of 0.286 was determined as the cutoff value from ROC analysis with an area of 0.905 ($p<0.001$) under the curve. Of 202 MAT positive samples, 161 (79.7%) samples showed an OD value higher than the cutoff. The maximum OD was 1.892 and the minimum OD was 0.032. Of 100 MAT negative samples, 9 (9%) had higher OD values than the cutoff. The healthy control subjects showed a mean OD value of 0.182 and standard deviation of 0.084. This ELISA showed a sensitivity of 80.2% and a specificity of 89% and a good agreement (κ=0.639) against MAT. Results from saprophytic IgM In-House ELISA, pathogenic IgM In-House ELISA and commercially available ELISA are given in Table 1.

For the IgM-ELISA developed with saprophytic antigens, OD value of 0.305 was determined as the cutoff value from ROC analysis with an area of 0.871 ($p<0.001$) under the curve. Of 202 MAT positive samples, 168 (83.16%) samples showed an OD value higher than the cutoff. The

maximum OD was 1.550 and the minimum OD was 0.052. Of 100 MAT negative samples, 11 (11%) had higher OD values than the cutoff. The healthy control subjects showed a mean OD value of 0.164 and standard deviation of 0.083. This ELISA showed a sensitivity of 83.3% and a specificity of 91% against MAT and a good agreement with MAT (κ=0.698). The commercial IgM-ELISA showed sensitivity and specificity of 79.2% and 93% respectively and a good agreement of κ=0.663.

In-House anti-leptospiral IgG-ELISA

When pathogenic leptospiral antigens were used, OD value of 0.264 was determined as the cutoff from ROC analysis with an area of 0.868 ($p<0.001$) under the curve. Of 62 MAT positive samples, 44 (70.9%) samples showed an OD value higher than the cutoff. The maximum OD was 1.334 and the minimum OD was 0.123. Of 30 MAT negative samples, 05 (10%) had higher OD values than the cutoff. The maximum OD was 0.548 and the minimum was 0.084. The healthy control subjects showed a mean OD value of 0.231 and standard deviation of 0.097. This ELISA showed a sensitivity of 73.3% and a specificity of 83.3% against MAT and a fair agreement with MAT (κ=0.328). Table 2 shows the diagnostic performances of the In-House IgG ELISAs developed and commercially available IgG ELISA.

When saprophytic antigen from *Leptospira bilfexa* serovar Patoc strain Patoc-1 was used, OD value of 0.338 was determined as the cutoff from ROC analysis with an area of 0.833 ($p<0.001$) under the curve. Of 62 MAT positive samples, 49 (79%) samples showed an OD value higher than the cutoff. The maximum OD was 1.013 and the minimum OD was 0.136. Of 30 MAT negative samples, 05 (16.7%) had higher OD values than the cutoff. Healthy control subjects showed a

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mean OD value of 0.280 and standard deviation of 0.125. This ELISA showed a sensitivity of 81.7% and a specificity of 83.3% against MAT and a good agreement between In-House IgM-ELISA (κ=0.328). Whereas the commercial IgG-ELISA showed a sensitivity and specificity of 50% and 96.9% respectively with an agreement of κ=0.371.

Anti-leptospiral IgM and IgG levels among mild and severe leptospirosis patients

Similar levels of IgM in severe and mild patients were observed whereas significantly higher IgG levels were detected in patients with severe leptospirosis. Total anti-leptospiral IgM antibody was detected in 96% of both mild (Median day 05) and severe (Median day 07) patients, whereas, anti-leptospiral IgG was detected 35% (Median day 05) and 76% (Median day 07) respectively. The mean anti-leptospiral IgM level of mild and severe patients was significantly higher than that of control group (Mean OD_{450} value at 1:100 dilutions were 0.778, 0.770 and 0.163 respectively; ANOVA, $p<0.001$). However, the mean anti-leptospiral IgG levels of severe patients were significantly higher than that of mild patients and control group (0.643, 0.358 and 0.116 respectively; ANOVA, $p<0.001$) (Fig 1). Further, IgG levels of mild sera were significantly higher compared to control group (ANOVA, $p<0.001$). Same pattern was observed when IgM and IgG titers were analyzed. Multiple regression analysis was run to predict anti-leptospiral IgM & IgG against pooled pathogenic antigens from gender, age and day of sampling. These variables statistically did not show any significant affect on the anti-leptospiral IgM and IgG antibodies $F(3,194) = 3.259$, $p>0.0005$, $R^2=0.048$ and $F(3,194) = 1.809$, $p>0.0005$, $R^2=0.027$ respectively. Summary of the results and characteristics of the study groups are shown in Supplementary Table 03.

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Discussion

Diagnosis of human leptospirosis is a serious medical and public health issue of tropical countries. Simple, rapid and appropriate diagnostic tests are required to aid clinical case identification and facilitate outbreak investigations. Although MAT is considered serological standard test, studies have shown the inherent problems in using it for diagnosis of acute leptospirosis due to factors such as low sensitivity on the acute sample, requiring a paired testing and technical difficulties such need of high level of technical expertise, and the maintenance of a large panel of live pathogenic *Leptospira* standard cultures (Limmathurotsakul et al, 2012). The use of live organisms also creates a risk of laboratory-acquired infection to the technicians. MAT detects both IgM and IgG agglutinating antibodies. However, MAT also produces a large number of false negative results in the early-course of infection, as the agglutinins detectable by MAT appear after day 8 of the illness, reach the peak by the fourth week and may persist for several months (Cumberland et al, 2001). MAT requires testing paired sera collected at appropriate time intervals for an accurate interpretation of results. Thus, while it is advantageous for epidemiological purposes, there are limitations in its value in the acute clinical setting. Due to the laboriousness of the assay, MAT is routinely available only in the central laboratories; in Sri Lanka MAT is only available at the National Leptospirosis Reference Laboratory, Medical Research Institute, Colombo. Thus, there is a clear need for reliable and valid rapid diagnostic tests for leptospirosis which can be made available to the treating team, in order to diagnose and treat leptospirosis early.

The ideal test for leptospirosis are expected to have specific characteristics; high sensitivity and specificity at acute phase, availability at reasonable cost and its ability to produce reliable results at the earliest. Several other immunodiagnostics have been evaluated as alternatives to MAT

with IgM-ELISA being the most discussed assay. ELISA detectable antibodies are shown to appear earlier than MAT detectable agglutinating antibodies. Ability of ELISA to detect and measure specific antibody levels compared to MAT has proved to be advantageous.

Anti-leptospiral IgM detection by ELISA is much sensitive than MAT and gives a positive result in early acute stage of the infection (Cumberland et al, 1999). Since IgM antibodies are detectable during the first week of illness, it is helpful for the diagnosis of the infection and initiation of treatment. Although there are several anti-leptospiral IgM detecting test kits which are commercially available for diagnosing leptospirosis, most of them use antigens prepared from non-pathogenic *Leptospira biflexa* serovar Patoc strain Patoc I. These tests have a varying sensitivity and specificity values and they also depend on the *Leptospira* serovars used and the procedure of antigen extraction. Therefore, antigen was prepared from five prevalent serovars of *Leptospira* in Sri Lanka. This approach takes advantage of cross-reactive antigens in crude extract, which are shared among diverse pathogenic leptospiral serovars. These broadly reactive antigens include outer membrane protein antigens and leptospiral lipopolysaccharide (LPS). Not only the serovars used, but also the procedures for antigen extraction, may affect the sensitivity and specificity of an ELISA. Previous studies have used whole-cell crude antigen or partially purified extract from surface antigenic fractions from saprophytic and pathogenic *Leptospira* of different serovars rather than purified antigen. Here we used whole cell crude antigen because it was simple and inexpensive for preparation. This approach also takes the advantage of cross-reactive antigens in crude extract, which are shared among the diverse leptospiral serovars. Use of sonicated antigen avoids the risk of infection to laboratory technicians and requires no maintenance of live, hazardous stock cultures. There is no commercial ELISA with pathogenic leptospiral antigens

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widely available. Hence, development of In-House ELISA with locally prevalent pathogenic serovars are highly applicable to leptospirosis endemic countries. Another added advantage is that, this test could be reviewed and the antigen combination could be changed according to the circulating *Leptospira* serovar of the region.

The developed serovar-specific ELISA showed high diagnostic accuracies suggesting that it could be used for the diagnosis of leptospirosis. These findings have showed to be consistent with similar anti-leptospiral ELISA studies (Tansuphasari et al, 2005; Cumberland et al, 1999). Diagnostic accuracies of the developed assays showed to be somewhat similar to the commercially available test kits. However, considering the facts of In-House ELISAs could be used to detect the specific circulating serovar and the assay itself could be modified when there are out breaks appear it can be said that In-House ELISA could be more advantageous.

In our study, findings have shown the presence of high level and prevalence of IgG antibodies in severe leptospirosis patients compared to mild leptospirosis patients. This suggest that the detection of anti-leptospiral IgG antibodies in high levels above OD at 1:100 of 0.643 could be used as a predictive marker to identify the tendency for disease progression. Similar findings were shown in a recent study conducted in Brazil, using 61 patient samples (Lessa-Aquino et al, 2017). Here in our study, a larger group of patients were tested and therefore the importance of IgG antibodies in predicting severity is showed with significance.

Conclusions

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IgM-ELISA developed using locally most prevalent leptospiral antigens offered good sensitivity, specificity and yielded results comparable to the reference MAT. The assay is comparatively easy to perform, can easily accommodate a large number of samples and provides less subjective results than MAT. Thus, it could be used for diagnosis of acute leptospirosis instead of MAT. Patients with severe leptospirosis showed significantly higher levels anti-leptospiral IgG which suggest that the levels anti-leptospiral IgG may be used as a predictor of disease severity.

Declarations

Ethical statement

The Ethics Review Committee of the Faculty of Medicine, University of Colombo (EC-12-056), approved study protocol. Patients and control subjects were enrolled in the study after obtaining informed written consent.

Conflicts of interest

The authors have no conflicts of interest to declare that are relevant to the content of this article.

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Contribution

Roshan Niloofa: Study design, Sample collection, Perform experiments, Data analysis, Writing the first draft, Editing and proofreading

Lilani Karunanayake: Perform experiments, Editing and proofreading

H Janaka de Silva: Study design, Editing and proofreading

Sunil Premawansa: Study design, Editing and proofreading

Senaka Rajapaksee: Study design, providing patients, Editing & proofreading

Shiroma Handunnetti: Study design, Data analysis, Editing & proofreading

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References

Abdulkader RC, Daher EF, Camargo ED, Spinosa C, da Silva MV. Leptospirosis severity may be associated with the intensity of humoral immune response. Rev Inst Med Trop Sao Paulo. 2002;44(2):79-83. https://doi.org/10.1590/S0036-46652002000200005

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Agampodi SB, Peacock SJ, Thevanesam V, Nugegoda DB, Smythe L, Thaipadungpanit J, et al. Leptospirosis outbreak in Sri Lanka in 2008: lessons for assessing the global burden of disease. The Am J Trop Med Hyg. 2011;85(3):471-8. https://doi.org/10.4269/ajtmh.2011.11-0276

Agampodi SB, Thevanesam V, Wimalarathna H, Senarathna T, Wijedasa MH. A preliminary study on prevalent serovars of leptospirosis among patients admitted to teaching hospital, Kandy, Sri Lanka. Indian J Med Microbiol. 2008;26(4):405-6. https://doi.org/10.4103/0255-0857.43557

Agampodi SB, Moreno AC, Vinetz JM, Matthias MA. Utility and limitations of direct multi-locus sequence typing on qPCR-positive blood to determine infecting Leptospira strain. The Am J Trop Med Hyg. 2013;88(1):184-5. https://doi.org/10.4269/ajtmh.2012.12-0526

Bharti AR, Nally JE, Ricaldi JN, Matthias MA, Diaz MM, Lovett MA et al. Peru-United States Leptospirosis Consortium. Leptospirosis: a zoonotic disease of global importance. Lancet Infect Dis. 2003;3(12):757-71. https://doi.org/10.1016/s1473-3099(03)00830-2

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;7;72:248-54.

Journal Pre-proof

Costa F, Hagan JE, Calcagno J, Kane M, Torgerson P, Martinez-Silveira MS, et al. Global Morbidity and Mortality of Leptospirosis: A Systematic Review. PLOS Negl Trop Dis.2015; 9(9):e0003898. https://doi.org/10.1371/journal.pntd.0003898

Cumberland P, Everard CO, Levett PN. Assessment of the efficacy of an IgM-ELISA and microscopic agglutination test (MAT) in the diagnosis of acute leptospirosis. Am J Trop Med Hyg. 1999;61(5):731-4. https://doi.org/10.4269/ajtmh.1999.61.731

Cumberland P, Everard CO, Wheeler JG, Levett PN. Persistence of anti-leptospiral IgM, IgG and agglutinating antibodies in patients presenting with acute febrile illness in Barbados 1979-1989. Eur J Epidemiol. 2001;17(7):601-8.

Davenport A, Rugman FP, Desmond MJ, Ganta R. Is thrombocytopenia seen in patients with leptospirosis immunologically mediated? J Clin Pathol. 1989;42(4):439-40. https://doi.org/10.1136/jcp.42.4.439

De Silva NL, Niloofa M, Fernando N, Karunanayake L, Rodrigo C, De Silva HJ, et al. Changes in full blood count parameters in leptospirosis: a prospective study. Int Arch Med. 2014;7:31. https://doi.org/10.1186/1755-7682-7-31

Journal Pre-proof

Journal Pre-proof

Gouveia EL, Metcalfe J, de Carvalho AL, Aires TS, Villasboas-Bisneto JC, Queirroz A, et al. Leptospirosis-associated severe pulmonary hemorrhagic syndrome, Salvador, Brazil. Emerg Infect Dis. 2008;14(3):505-8. https://doi.org/10.3201/eid1403.071064

Kalugalage T, Rodrigo C, Vithanage T, Somaratne P, De Silva HJ, Handunnetti S, et al. Low serum total nitrite and nitrate levels in severe leptospirosis. BMC Infect Dis. 2013;13:206. https://doi.org/10.1186/1471-2334-13-206.

Koizumi N, Gamage CD, Muto M, Kularatne SA, Budagoda BD, Rajapakse RP, et al. Serological and genetic analysis of leptospirosis in patients with acute febrile illness in kandy, Sri Lanka. Jpn J Infect Dis. 2009;62(6):474-5.

Lessa-Aquino C, Lindow JC, Randall A, Wunder E, Pablo J, Nakajima R, et al. Distinct antibody responses of patients with mild and severe leptospirosis determined by whole proteome microarray analysis. PLOS Negl Tro Dis. 2017;11(1):e0005349. doi:10.1371/journal.pntd.0005349 doi: 10.1371/journal.pntd.0005349

Limmathurotsakul D, Turner EL, Wuthiekanun V, Thaipadungpanit J, Suputtamongkol Y, Chierakul W, et al. Fool's gold: Why imperfect reference tests are undermining the evaluation of novel diagnostics: a reevaluation of 5 diagnostic tests for leptospirosis. Clin infect dis. 2012;55(3):322-31. doi: 10.1093/cid/cis403.

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Niloofa R, Fernando N, de Silva NL, Karunanayake L, Wickramasinghe H, Dikmadugoda N, et al. Diagnosis of Leptospirosis: Comparison between Microscopic Agglutination Test, IgM-ELISA and IgM Rapid Immunochromatography Test. PloS ONE. 2015;5;10(6):e0129236. doi: 10.1371/journal.pone.0129236

Panwala T MS, Patel P. Seroprevalance of leptospirosis in south gujarat region by eveluating the two rapid commercial diagnostic kits against the MAT test for detection of antibodies to leptospira interrogans. National J Com Med.2011; 2(1):64-70.

Rajapakse S, Weeratunga P, Niloofa MJ, Fernando N, Rodrigo C, Maduranga S, et al. Clinical and laboratory associations of severity in a Sri Lankan cohort of patients with serologically confirmed leptospirosis: a prospective study. Trans R Soc Trop Med Hyg. 2015;109(11):710-6. doi: 10.1093/trstmh/trv079

Sehgal SC, Vijayachari P, Sharma S, Sugunan AP. LEPTO Dipstick: a rapid and simple method for serodiagnosis of acute leptospirosis. Trans R Soc Trop Med Hyg. 1999;93(2):161-4. doi: 10.1016/s0035-9203(99)90293-6

Tansuphasiri U, Deepradit S, Phulsuksombati D, Tangkanakul W. Two simple immunoassays using endemic leptospiral antigens for serodiagnosis of human leptospirosis. The Southeast Asian J Trop Med Public Health. 2005;36(2):302-11.

Journal Pre-proof

Journal Pre-proof

Vijayachari P, Sugunan AP, Shriram AN. Leptospirosis: an emerging global public health problem. J. Biosci.2008; 33(4):557-69.

Vinetz JM. Leptospirosis. Curr Opin Infect Dis. 2001;14(5):527-38.

Virion/Serion. Serion ELISA classic Leptospira IgM (quantitatitive) instructions. Available from: http://www.virion-serion.de/fileadmin/templates/tpl1/global/download/flyer/Flyer_ELISA_classic_Leptospira__EN.pdf
Accessed 17th September 2013

Warnasekara, J., Koralegedara, I. & Agampodi, S. Estimating the burden of leptospirosis in Sri Lanka; a systematic review. *BMC Infect Dis.* 2019; **19,** 119. https://doi.org/10.1186/s12879-018-3655-y https://doi.org/10.1186/s12879-018-3655-y

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Fig 01 Anti-leptospiral IgM and IgG antibody levels in severe and mild leptospirosis patients. Bars represents the mean ±SEM of OD 450 of 1:100 serum dilutions in healthy controls, mild and severe leptospirosis patients.

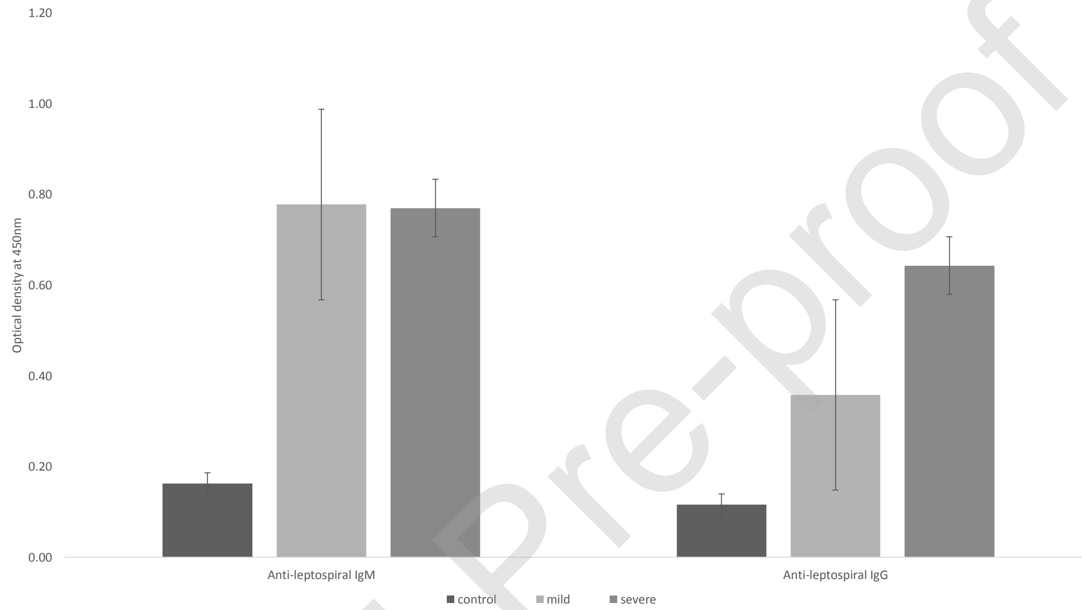

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Table 1. Performance of In-House and commercially available IgM-ELISAs in diagnosis of acute leptospirosis

Criteria	In-House ELISA		Commercially available ELISA
	Genus-specific (Saprophytic)	Serovar-specific (Pathogenic)	Genus-Specific (Saprophytic)
Leptospirosis Sample:			
Positive (n)	168	161	160
Negative (n)	34	41	42
Healthy Control:			
Positive (n)	9	11	7
Negative (n)	91	89	93
Diagnostic Sensitivity (%)	83.3	80.2	79.2
Diagnostic Specificity (%)	91.0	89.0	93.0
Positive Predictive Value (%)	94.9	94.7	63.2
Negative Predictive Value (%)	72.8	68.4	69.9
Accuracy (%)	85.8	82.8	83.8
Agreement (κ)	0.698	0.639	0.663

- Cut-off levels for In-House ELISAs were OD_{450nm} 0.305 and 0.286 for genus-specific (saprophytic) and serovar-specific (pathogenic) respectively; 20IU/ml for the commercially available ELISA

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Table 2. Performance of In-House and commercially available IgG ELISAs in measurement of anti-leptospiral antibodies

Criteria	In-House ELISA		Commercially available ELISA
	Genus-specific (Saprophytic)	Serovar-specific (Pathogenic)	Genus-Specific (Saprophytic)
Leptospirosis Sample:			
Positive (n)	49	44	31
Negative (n)	13	18	31
Healthy Control:			
Positive (n)	5	5	1
Negative (n)	25	25	29
Diagnostic Sensitivity (%)	81.7	73.3	50
Diagnostic Specificity (%)	83.3	83.3	96.7
Positive Predictive Value (%)	90.7	89.8	51.7
Negative Predictive Value (%)	69.4	60.9	48.3
Accuracy (%)	69.56	69.56	33.7
Agreement (κ)	0.328	0.328	0.371

- Cut-off levels for In-House ELISAs were OD_{450nm} 0.338 and 0.264 for saprophytic and pathogenic respectively; 15IU/ml for the commercially available ELISA

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