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Development of in-house ELISAs as an alternative method for the serodiagnosis of leptospirosis

Roshan Niloofa[a,*], Lilani Karunanayake[b], H. Janaka de Silva[c], Sunil Premawansa[d], Senaka Rajapakse[e], Shiroma Handunnetti[a]

[a] Institute of Biochemistry, Molecular Biology and Biotechnology, University of Colombo, Colombo, Sri Lanka
[b] Department of Bacteriology, Medical Research Institute, Colombo, Sri Lanka
[c] Faculty of Medicine, University of Kelaniya, Kelaniya, Sri Lanka
[d] Faculty of Science, University of Colombo, Colombo, Sri Lanka
[e] Tropical Medicine Research Unit, Faculty of Medicine, University of Colombo, Colombo, Sri Lanka

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ABSTRACT

Background: Leptospirosis is most often diagnosed clinically, and a laboratory test with high diagnostic accuracy is required.
Methods: IgM and IgG ELISAs using *Leptospira* antigens were established and evaluated in relation to the microscopic agglutination test (MAT). Antigen preparation consisted of saprophytic *Leptospira biflexa* to detect genus-specific antibodies (genus-specific ELISA) and a pool of the five most prevalent *Leptospira interrogans* serovars in Sri Lanka to detect serovar-specific antibodies (serovar-specific ELISA). IgM and IgG immune responses were studied in severe and mild leptospirosis patients (n = 100 in each group).
Results: The ELISAs showed high repeatability and reproducibility. The serovar-specific IgM-ELISA showed a sensitivity of 80.2% and specificity of 89%; the genus-specific IgM-ELISA showed a sensitivity of 83.3% and specificity of 91%. The serovar- and genus-specific IgG-ELISAs showed sensitivities of 73.3% and 81.7%, respectively, and specificities of 83.3% and 83.3%, respectively. The commercial IgM-ELISA showed a sensitivity of 79.2% and specificity of 93%. The commercial IgG-ELISA showed a sensitivity of 50% and specificity of 96.7%. IgM levels observed in mild and severe leptospirosis patients were significantly higher than in the healthy control group, with mean absorbance values of 0.770, 0.778, and 0.163, respectively. Severe leptospirosis patients had significantly higher mean anti-leptospiral IgG levels compared to both mild leptospirosis patients and healthy control group subjects (0.643, 0.358, and 0.116, respectively; ANOVA, $p < 0.001$). The presence of anti-leptospiral IgG above an optical density of 0.643 at 1:100 could predict a high risk of severe disease.
Conclusion: The serovar-specific in-house ELISA could be used for the laboratory diagnosis of leptospirosis in endemic settings. The high levels of anti-leptospiral IgG observed suggest its value as a predictor of disease severity.

Introduction

Leptospirosis, known as the most widespread zoonosis worldwide, is one of the major threats to public health and causes economic losses in the farming industry in tropical and subtropical regions (Bharti et al., 2003). The incidence of leptospirosis is greater in warm climates and it is endemic in areas with high rainfall, close human contact with livestock, poor sanitation, and workplace exposure to the organism. According to worldwide surveys, an estimated 550,000 cases are recorded annually, with around 55,000 deaths (Vijayachari et al., 2008; Costa et al., 2015). Sri Lanka experienced large outbreaks of leptospirosis in 2008 and 2011, with incidence rates of 35.7 and 65.5 per 100,000 population, and the incidence rate has remained high since (Agampodi et al., 2011; Warnasekara et al., 2019).

The wide range of clinical symptoms makes the clinical diagnosis of leptospirosis a challenge. Therefore, rapid and appropriate laboratory diagnostic tests are required for the confirmatory diagnosis for patient management. The definitive laboratory diagnosis made via the isolation of the pathogenic *Leptospira* from blood, urine, or tissues requires precise culture

* Corresponding author at: Department of Zoology and Environment Sciences, Faculty of Science, University of Colombo, Colombo 03, Sri Lanka.
E-mail address: roshan.niloo@zoology.cmb.ac.lk (R. Niloofa).

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media and a prolonged incubation time, making this method impractical for routine diagnosis. The reference serological test, i.e., the microscopic agglutination test (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, the MAT is available only at reference laboratories. Several alternative immunodiagnostic assays have been developed in recent years to accommodate the requirement for an easy laboratory test (Sehgal et al., 1999; Panwala and Patel, 2011; Niloofa et al., 2015).

In Sri Lanka, the MAT is the main test for the laboratory confirmation of leptospirosis. However, this test is conducted only at one central reference laboratory at the Medical Research Institute (MRI) in Colombo, hence it is not widely available at peripheral hospitals. Although the MAT is a functional laboratory assay that is considered as one of the reference standards, MAT-based detection has its own limitations, since agglutinating antibodies are detectable only after 8–10 days of illness. The Leptospirosis Burden Epidemiology Reference Group (LERG) recommend that MAT is performed at >8 days of fever, and paired sera are generally required for the detection of an antibody rise or seroconversion.

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, which has a 10–15% case fatality rate, occur in 5–15% of human leptospirosis cases, and leptospirosis-associated 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, as 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 important roles in the 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 been shown to be present in severe leptospirosis patients and has been attributed to immune mechanisms (Davenport et al., 1989; De Silva et al., 2014). Anti-leptospiral IgG and IgA antibodies have been shown to be present in higher amounts in patients with severe leptospirosis (Abdulkader et al., 2002).

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

Methods

Preparation of antigens

The in-house ELISAs were established using a saprophytic serovar (Patoc) and the five most prevalent pathogenic *Leptospira interrogans* serovars in Sri Lanka: Pyrogenes, Australis, Icterohaemorrhagiae, Canicola, and Hardjo (Agampodi et al., 2008, 2013; Koizumi et al., 2009). *Leptospira biflexa* serovar Patoc strain Patoc 1, *Leptospira interrogans* serovar Hardjo strain Hardopraj in the Sejroe serogroup, serovar Pyrogenes strain Salinem in the Pyrogenes serogroup, serovar Australis strain Ballico in the Australis serogroup, serovar Icterohaemorrhagiae strain RGA in the Icterohaemorrhagiae serogroup, and serovar Canicola strain H Utrecht in the Canicola serogroup were cultured in Ellinghausen–McCullough–Johnson–Harris medium (EMJH) (Difco) supplemented with 10% rabbit serum. A fresh culture containing 10^7 cells/ml was incubated in the dark at 30 °C 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; it was then resuspended in phosphate buffered saline (PBS) to 25% of the original volume (Tansuphasiri et al., 2005). The protein content of the antigen preparations was determined according to the Bradford method (Bradford, 1976). An equal protein content (w/v antigen) from each antigen preparation was mixed to obtain a preparation of pooled antigen. The antigen preparation from *Leptospira biflexa* serovar Patoc strain Patoc 1 was used separately for ELISA. The antigen preparation was made into small aliquots and stored at −20 °C until use.

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

The ELISA was optimized in a flat-bottomed polystyrene microplate (Polysorb; Nunc, USA) by varying the concentrations of IgM/IgG–horse radish peroxidase (HRP) conjugate (1:4000 and 1:8000) and using two different buffers for coating the antigen: carbonate buffer (CB; pH 9.6) and PBS (pH 7.2). Optimization was performed using two-fold serial dilutions of six serum samples at 1:100–1:3200. The optimum criteria were selected and used based on repeat experiments (Supplementary Material Tables S1 and S2). The plates were coated with 100 μl of 2 μg/ml antigenic proteins in 0.05 M CB and incubated at 37 °C for 2 h in a moist chamber and at 4 °C for 12 h. The plate was blocked with 300 μl of PBS–0.05% Tween-20 (PBST) containing 5% skimmed milk after being washed three times with PBST. After six washes, 100 μl of each serum dilution (serially diluted with PBS containing 5% bovine serum albumin (BSA)) treated with rheumatoid factor absorbent were added according to the designed plate plan, and incubated at 37 °C for 1 h in a moist chamber. After washing six times, 100 μl of goat anti-human IgM/IgG (μ and γ chain-specific, respectively)–HRP conjugate (MyBioSource, USA), diluted to 1:8000 in PBS containing 5% skimmed milk, was added. The plate was washed six times after incubation for 1 h at 37 °C in a moist chamber. Substrate solution containing 100 μl of 3′,3′,5′,5′-tetramethylbenzidine dissolved in dimethyl sulfoxide and phosphate citrate buffer (pH 5.0) and H_2O_2 was added, and this was then incubated in the dark at room temperature for 20 min. The reaction was stopped by adding 50 μl of 1 M HCl. The optical density (OD) at 450 nm was read using an ELISA reader (ELx 800 Universal Microplate Reader; Bio-Tek Instruments Inc., Canada). The mean + 2 standard deviations OD_{405} value of healthy control subjects was used as the cut-off value. Samples were tested in duplicate with overlapping samples from the previous experiments to maintain the minimum inter- and intra-assay variation.

The commercial IgM-ELISA (Institut VirionSerion GmbH, Warburg, Germany) was performed according to the manufacturer's instructions (http://www.virion-serion.de/fileadmin/templates/tpl1/global/download/flyer/Flyer_ELISA_classic_Leptospira_EN.pdf) Virion/Serion (2021). Each assay 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. The results were obtained using the evaluation table provided with the kit. Interpretation of the results for the Serion-ELISA classic *Leptospira* IgM was as follows: anti-leptospiral IgM <15 IU/ml represents a negative result suggesting no evidence of recent infection, IgM 15–20 IU/ml represents a borderline result suggesting that there may have been a recent infection, and IgM

>20 IU/ml represents 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 an ongoing study from 2012 to 2014. All samples had previously been tested with a MAT and commercially available IgM and IgG ELISAs (Niloofa et al., 2015). A total of 202 samples were confirmed for leptospirosis, while 100 samples were negative for leptospirosis. For the development of the 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. The samples were collected at a mean 7.2 days (range 2–16 days) 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. Organ involvement was defined according to Rajapakse et al. (2015) (Kalugalage et al., 2013). Serum samples 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) to detect antibodies against the pooled pathogenic antigen preparation.

Statistical analysis

The statistical analyses were performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). The data are presented as the mean and standard error of the mean (SEM) or standard deviation (SD) values. The level of significance was set at $p \leq 0.05$. The independent sample t-test for two independent variables was used to test the difference between groups, at a 95% confidence interval. Pearson correlation coefficients were calculated to determine the correlations. Receiver operating characteristic (ROC) curve analysis was performed to identify a suitable cut-off value for the established in-house ELISAs. The sensitivity and specificity of the microplate IgM and IgG ELISAs were determined using the results of the MAT as the gold standard. The kappa test was used to determine the degree of agreement between the tests. Repeatability and reproducibility were calculated using one-way analysis of variance (ANOVA).

Results

Establishment and optimization of the anti-leptospiral IgM and IgG in-house ELISAs

Serum 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, 0.05 M CB and 0.13 M PBS. Mean OD values of the two conjugate dilutions, 1:4000 and 1:8000, did not differ greatly from each other. The results are shown in Supplementary Material Table S1. As a majority of the samples did not show significantly different OD levels in either concentration of conjugate preparation, 1:8000 was selected as the optimum concentration of conjugates, taking into account the cost of the conjugate.

Serum samples representing MAT titers >100, 200, 400, 800, 1600, and 3200 were tested in both antigen coating buffers, 0.05 M CB and 0.13 M PBS, using 1:8000 conjugate dilutions. OD values for CB were greater than those for PBS in both ELISAs at 1:100, 1:200, 1:400, 1:800, and 1:1600 serum dilutions (Supplementary Material Table S2). CB IgM-ELISA mean OD values showed decreasing concave follow-through with the reciprocal of serum dilutions, and at low dilutions it showed clear mean OD value separation between positive and negative sera. As obtaining a clear OD is critical for the result in samples that have a low amount of antibody present, 0.05 M CB was selected for subsequent use.

In-house anti-leptospiral IgM-ELISA

For the IgM-ELISA developed with pooled antigens of pathogenic serovars, an OD value of 0.286 was determined as the cut-off value from the ROC analysis, with an area under the curve (AUC) of 0.905 ($p < 0.001$). Of 202 MAT-positive samples, 161 (79.7%) showed an OD value higher than the cut-off. The maximum OD was 1.892 and the minimum OD was 0.032. Of 100 MAT-negative samples, nine (9%) had an OD value higher than the cut-off. 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%, specificity of 89%, and a good agreement ($\kappa = 0.639$) against the MAT. Results from the saprophytic IgM in-house ELISA, pathogenic IgM in-house ELISA, and commercially available ELISA are given in Table 1.

Table 1
Performance of in-house and commercially available IgM-ELISAs in the diagnosis of acute leptospirosis.

Criteria	In-house ELISA		Commercially available ELISA
	Genus-specific (Saprophytic)	Serovar-specific (Pathogenic)	Genus-specific (Saprophytic)
Leptospirosis sample (n)			
Positive	168	161	160
Negative	34	41	42
Healthy control sample (n)			
Positive	9	11	7
Negative	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

The cut-off level for the in-house ELISA was OD_{450} 0.305 for genus-specific (saprophytic) and OD_{450} 0.286 for serovar-specific (pathogenic). The cut-off for the commercially available ELISA was 20 IU/ml.

For the IgM-ELISA developed with saprophytic antigens, an OD value of 0.305 was determined as the cut-off value from the ROC analysis, with an AUC of 0.871 ($p < 0.001$). Of 202 MAT-positive samples, 168 (83.16%) showed an OD value higher than the cut-off. The maximum OD was 1.550 and the minimum OD was 0.052. Of 100 MAT-negative samples, 11 (11%) had an OD value higher than the cut-off. 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 specificity of 91% against the MAT, and a good agreement with the MAT ($\kappa = 0.698$). The commercial IgM-ELISA showed a sensitivity of 79.2%, specificity of 93%, and a good agreement of $\kappa = 0.663$.

In-house anti-leptospiral IgG-ELISA

When pathogenic leptospiral antigens were used, an OD value of 0.264 was determined as the cut-off from the ROC analysis, with an AUC of 0.868 ($p < 0.001$). Of 62 MAT-positive samples, 44 (70.9%) showed an OD value higher than the cut-off. The maximum OD was 1.334 and the minimum OD was 0.123. Of 30 MAT-negative samples, five (16.7%) had an OD value higher than the cut-off. The maximum OD was 0.548 and the minimum OD 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 specificity of 83.3% against the MAT, and a fair agreement with the MAT ($\kappa = 0.328$). Table 2 shows the diagnostic performance of the in-house IgG-ELISAs and the commercially available IgG-ELISA.

When saprophytic antigen from *Leptospira biflexa* serovar Patoc strain Patoc 1 was used, an OD value of 0.338 was determined as the cut-off from the ROC analysis, with an AUC of 0.833 ($p < 0.001$). Of 62 MAT-positive samples, 49 (79%) showed an OD value higher than the cut-off. The maximum OD was 1.013 and the minimum OD was 0.136. Of 30 MAT-negative samples, five (16.7%) had an OD value higher than the cut-off. Healthy control subjects showed a mean OD value of 0.280 and standard deviation of 0.125. This ELISA showed a sensitivity of 81.7% and specificity of 83.3% against the MAT, and a fair agreement between in-house IgG-ELISA and the MAT ($\kappa = 0.328$). The commercial IgG-ELISA showed a sensitivity and specificity of 50% and 96.9%, respectively, with an agreement of $\kappa = 0.371$.

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

Similar levels of IgM were observed in severe and mild patients, 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 5) and severe (median day 7) patients, whereas anti-leptospiral IgG was detected in 35% of mild patients (median day 5) and 76% of severe patients (median day 7). The mean anti-leptospiral IgM level of mild and severe patients was significantly higher than that of the control group (mean OD_{450} values at 1:100 dilution 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 those of mild patients and the control group (0.643, 0.358, and 0.116, respectively; ANOVA, $p < 0.001$) (Figure 1). Furthermore, IgG levels in serum from mild patients were significantly higher when compared to the levels in the control group (ANOVA, $p < 0.001$). The same pattern was observed when IgM and IgG titers were analyzed.

Multiple regression analysis was run to predict anti-leptospiral IgM and IgG against pooled pathogenic antigens from sex, age, and day of sampling. These variables did not show any statistically significant effect on the anti-leptospiral IgM or IgG antibodies: $F(3,194) = 3.259$, $p > 0.0005$, $R^2 = 0.048$ and $F(3,194) = 1.809$, $p > 0.05$, $R^2 = 0.027$, respectively. A summary of the results and characteristics of the study groups is given in Supplementary Material Table S3.

Discussion

The diagnosis of human leptospirosis is a serious medical and public health issue in tropical countries. Simple, rapid, and appropriate diagnostic tests are required to aid clinical case identification and facilitate outbreak investigations. Although MAT is considered the standard serological test, studies have shown the inherent problems in using this test for the diagnosis of acute leptospirosis, due to factors such as low sensitivity on the acute sample, therefore requiring paired testing, and technical difficulties, such as the need for a 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 for the technicians.

The MAT detects both IgM and IgG agglutinating antibodies. However, the 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 illness, reach a peak by the fourth week, and may persist for several months (Cumberland et al., 2001). The MAT requires testing paired serum samples collected at appropriate time intervals for an accurate interpretation of the

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

Criteria	In-house ELISA		Commercially available ELISA
	Genus-specific (Saprophytic)	Serovar-specific (Pathogenic)	Genus-specific (Saprophytic)
Leptospirosis sample (*n*)			
Positive	49	44	31
Negative	13	18	31
Healthy control sample (*n*)			
Positive	5	5	1
Negative	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

The cut-off level for the in-house ELISA was OD_{450} 0.338 for genus-specific (saprophytic) and OD_{450} 0.264 for serovar-specific (pathogenic). The cut-off for the commercially available ELISA was 15 IU/ml.

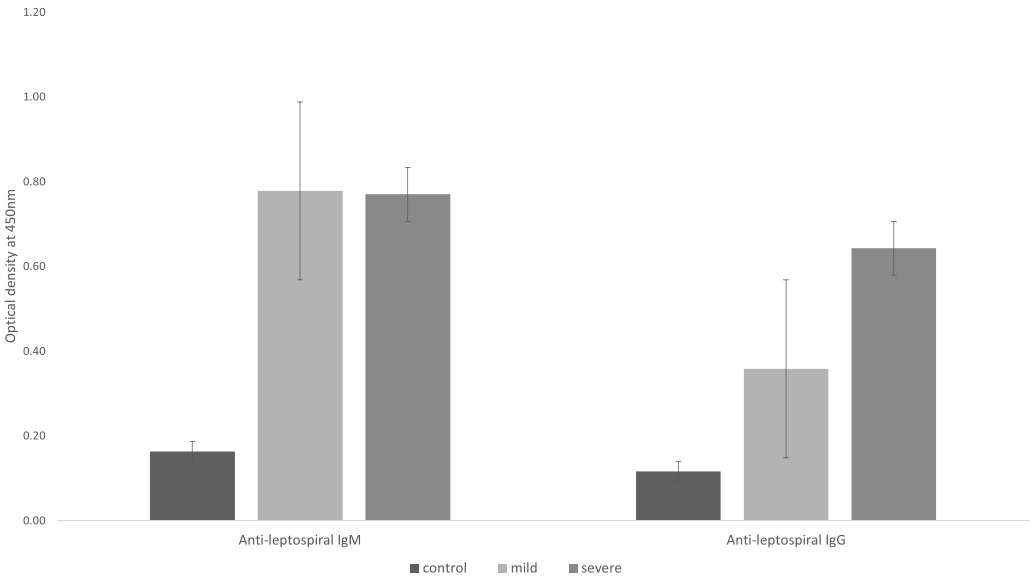

Figure 1. Anti-leptospiral IgM and IgG antibody levels in severe and mild leptospirosis patients. Bars represents the mean ± SEM OD_{450} values of 1:100 serum dilutions in healthy controls, mild and severe leptospirosis patients.

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, the MAT is often routinely available only in central laboratories. In Sri Lanka, the MAT is only available at the National Leptospirosis Reference Laboratory, MRI, Colombo. Thus, there is a clear need for reliable and valid rapid diagnostic tests for leptospirosis that can be made available to the treating team, in order to diagnose and treat leptospirosis early.

The ideal test for leptospirosis is expected to have specific characteristics: high sensitivity and specificity in the acute phase, availability at a reasonable cost, and the ability to produce reliable early results. Several other immunodiagnostics have been evaluated as alternatives to the MAT, with IgM-ELISA being the most discussed assay. ELISA-detectable antibodies are shown to appear earlier than MAT-detectable agglutinating antibodies. The ability of ELISA to detect and measure specific antibody levels compared to the MAT has proven to be advantageous.

Anti-leptospiral IgM detection by ELISA is much more sensitive than the MAT and gives a positive result in the early acute stage of the infection (Cumberland et al., 1999). Since IgM antibodies are detectable during the first week of illness, IgM is helpful for the diagnosis of the infection and initiation of treatment. Although there are several commercially available anti-leptospiral IgM detecting test kits for diagnosing leptospirosis, most of them use antigens prepared from non-pathogenic *Leptospira biflexa* serovar Patoc strain Patoc 1. These tests have varying sensitivity and specificity values and they also depend on the *Leptospira* serovars used and the procedure of antigen extraction. Therefore, in this study, antigen was prepared from five serovars of *Leptospira* that are prevalent in Sri Lanka. This approach takes advantage of cross-reactive antigens in crude extract that are shared among diverse pathogenic leptospiral serovars. These broadly reactive antigens include outer membrane protein antigens and leptospiral lipopolysaccharide (LPS).

The sensitivity and specificity of an ELISA is affected not only by the serovars used, but also by the procedures used for antigen extraction. 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. In the present study, whole cell crude antigen was used, because this was simple and inexpensive to prepare. This approach also takes advantage of cross-reactive antigens in the crude extract that 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 widely available commercial ELISA with pathogenic leptospiral antigens. Hence, the development of in-house ELISAs with locally prevalent pathogenic serovars is highly applicable to leptospirosis endemic countries. Another added advantage is that this test can be reviewed and the antigen combination can be changed according to the circulating *Leptospira* serovar in the region.

The serovar-specific ELISA developed here showed high diagnostic accuracy, suggesting that it could be used for the diagnosis of leptospirosis. The results were found to be consistent with those of similar anti-leptospiral ELISA studies (Tansuphasiri et al., 2005; Cumberland et al., 1999). The diagnostic accuracies of the developed assays were observed to be somewhat similar to those of the commercially available test kits. However, considering the fact that these in-house ELISAs could be used to detect the specific circulating serovars and that the assay itself could be modified when there are outbreaks, the in-house ELISAs would appear to be more advantageous.

The study findings showed the presence of a high level and prevalence of IgG antibodies in severe leptospirosis patients compared to mild leptospirosis patients. This suggests that the detection of anti-leptospiral IgG antibodies at high levels above an 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). In the present study, a larger group of patients was tested and therefore the importance of IgG antibodies in predicting severity showed significance.

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

leptospiral IgG, which suggests that the levels of anti-leptospiral IgG may be used as a predictor of disease severity.

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Ethics statement

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

Conflict of interest

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

Author contributions

Roshan Niloofa: study design, sample collection, performed experiments, data analysis, writing the first draft, editing and proofreading. Lilani Karunanayake: performed experiments, editing and proofreading. H. Janaka de Silva: study design, editing and proofreading. Sunil Premawansa: study design, editing and proofreading. Senaka Rajapakse: study design, providing patients, editing and proofreading. Shiroma Handunnetti: study design, data analysis, editing and proofreading.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijid.2021.01.074.

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, doi:http://dx.doi.org/10.1590/S0036-46652002000200005.

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, doi:http://dx.doi.org/10.4103/0255-0857.43557.

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, doi:http://dx.doi.org/10.4269/ajtmh.2011.11-0276.

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, doi:http://dx.doi.org/10.4269/ajtmh.2012.12-0526.

Bharti AR, Nally JE, Ricaldi JN, Matthias MA, Diaz MM, Lovett MA, et al. Leptospirosis: a zoonotic disease of global importance. Lancet Infect Dis 2003;3(12):757–71, doi:http://dx.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.

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, doi:http://dx.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, doi:http://dx.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, doi:http://dx.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, doi:http://dx.doi.org/10.1186/1755-7682-7-31.

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, doi:http://dx.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, doi:http://dx.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:http://dx.doi.org/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:http://dx.doi.org/10.1093/cid/cis403.

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;10(6)e0129236, doi:http://dx.doi.org/10.1371/journal.pone.0129236.

Panwala TMS, 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:http://dx.doi.org/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:http://dx.doi.org/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.

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

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 17 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, doi:http://dx.doi.org/10.1186/s12879-018-3655-y.