An Improved Enzyme-Linked Immunoassay for the Detection of *Leptospira*-Specific Antibodies

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Abstract. Leptospirosis is a neglected zoonotic disease with worldwide endemicity and continues to be a significant public health burden on resource-limited populations. Previously, we produced three highly purified recombinant antigens (rLipL32, rLipL41, and rLigA-Rep) and evaluated their performance of detecting *Leptospira*-specific antibodies in enzyme-linked immunosorbent assay (ELISA) as compared with the microscopic agglutination test (MAT). The overall sensitivity of this assay approached 90%. Recently, another recombinant antigen (rLigB-Rep) was prepared. We tested each individual antigen and a 1:1:1:1 mixture of these four antigens for the detection of *Leptospira*-specific antibodies in ELISA. The performance of these recombinant antigens was evaluated with a much larger febrile patient panel (337 MAT-confirmed positive sera and 92 MAT-negative sera from febrile patients). Combining the detection results of immunoglobulin M and immunoglobulin G from these four individual antigens, the overall sensitivity was close to 90% but the specificity was only 66%, based on the MAT reference method. The overall sensitivity and specificity of the four-antigen mixture were 82% and 86%, respectively. The mixture of four antigens also exhibited a broader reactivity with MAT-positive samples of 18 serovars from six major pathogenic *Leptospira* species. Given the limitations of MAT, the data were further analyzed by Bayesian latent class model, showing that ELISA using a 1:1:1:1 mixture still maintained high sensitivity (79%) and specificity (88%) as compared with the sensitivity (90%) and specificity (83%) of MAT. Therefore, ELISA using a mixture of these four antigens could be a very useful test for seroprevalence studies.

INTRODUCTION

Leptospirosis is a widespread zoonosis caused by pathogenic spirochetes belonging to the genus *Leptospira*.^{1,2} More than one million cases and about 60,000 deaths due to leptospirosis are reported each year worldwide.³ The disease is most common in developing countries, particularly in South and Southeast Asia, the Caribbean, Latin America, and Oceania.^{1,4–12} Humans are usually infected by *Leptospira* through direct or indirect exposure to the urine of infected wild or domestic animals.^{13,14} Because clinical features of leptospirosis are nonspecific and very similar to other febrile illnesses, many leptospirosis patients remain unrecognized or are misdiagnosed as dengue, malaria, or other conditions.^{15,16} Leptospirosis has emerged as an important cause of pulmonary hemorrhage syndrome and acute kidney injury in many endemic regions.^{17–19} Case fatality rates for pulmonary hemorrhage syndrome and Weil's disease (icteric leptospirosis) are reported at 10% and 70%, respectively.¹³ The lack of a reliable diagnostic test also contributes to the underreporting of cases and the delay of antibiotic treatment, leading to higher morbidity and mortality.^{20,21}

Microscopic agglutination test (MAT) is considered the standard serological test for the diagnosis of leptospirosis. The presence of *Leptospira*-specific antibodies in patient serum is determined via incubation with serogroup-specific live leptospires and observing for the presence of microscopic agglutination of the antigen.²² The major drawbacks of MAT include a complex and time-consuming bacterial culturing procedure, skill in interpreting the result, and potential hazards to laboratory staff because of the risk of exposure to live bacteria. Although molecular assays have been demonstrated to be useful for the detection of pathogen in acute

samples, they are limited by the transient nature of bacteremia in the early phase of infection and the requirement of sophisticated instrument and extensive end user training.²³ Therefore, serology tests are still the choice in many developing countries.

Recombinant surface proteins or lipoproteins such as LipL32, LipL41, OmpL1, Loa22, and immunoglobulin-like proteins of *Leptospira* have been used as antigens in ELISA to detect *Leptospira*-specific antibodies.^{23–28} In general, the antigens used for ELISA may not cover the full diversity of circulating strains, resulting in the limited sensitivity of these tests.^{2,13}

Previously, we produced three highly purified recombinant antigens rLipL32, rLipL41, and rLigA-Rep for the detection of *Leptospira*-specific antibodies in ELISA. The overall sensitivity was close to 90% based on the MAT results for 85 samples from four serovars when combining the results of antibody detection from these three antigens.²⁷ In this study, we cloned a part of the repeat region in LigB antigen gene and prepared the expressed recombinant protein (rLigB-Rep) with high purity. A panel of 429 human sera (337 MAT positive and 92 MAT negative) from Peru with MAT titers greater than 100 against one or more of the 18 serovars maintained at Naval Medical Research Unit 6 were used to evaluate the sensitivity and specificity of these four antigens individually and mixed together in a 1:1:1:1 ratio in ELISAs.

MATERIALS AND METHODS

Bacterial strains and vectors. The genomic DNA of *Leptospira interrogans* serovar Copenhageni strain Fiocruz L1-130 (ATCC, Manassas, VA) was used as the template for the cloning of recombinant protein as described before.²⁷ *Escherichia coli* Top10 (Life Technologies, Grand Island, NY) was used for cloning. The cloned gene was inserted into pET28a (EMD Millipore, Billerica, MA) for the expression of recombinant protein in *E. coli* BL21 (DE3) (Life Technologies) under the control of phage T7 *lac* promoter.²⁹

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Recombinant antigen preparation. Cloning of the gene coding for repeat region of LigB protein into the expression vector pET28a. A synthetic gene for the coding region of amino acids 630–931 of LigB was cloned into SmaI-digested pBluescript II SK (Bioclone, San Diego, CA). pBluescript II SK-LigB plasmid was digested with NdeI and XhoI and the LigB gene fragment was ligated into the expression vector pET28a. The resulting plasmids contained an insert coding His tag at both N- and C-terminus of the selected repeat region of LigB. Top10-competent cells were transformed with the ligation mixture and colonies were screened for the presence of inserts with the correct size. The final sequences were confirmed by DNA sequencing of the resulting plasmid.

Expression and purification of the recombinant LipL32, LipL41, LigA, and LigB proteins. The rLipL32, rLipL41, and rLigA were prepared as previously described.³⁰ The recombinant LigB (rLigB-Rep) proteins were prepared similarly. *Escherichia coli* BL21 (DE3) was transformed with plasmids carrying the LigB insert. The recombinant *E. coli* colony with high expression levels of the desired protein was cultured overnight in Overnight Express medium TB (EMD Millipore, Billerica, MA) in the presence of kanamycin at 37°C with shaking at 200 rpm. Cell pellets from 500 mL cultures were resuspended in 20 mL of buffer A (20 mM Tris-HCl, pH 8.0, 0.5 M NaCl). Cells in the suspension were ruptured by passing through EmulsiFlex-B15 (Avestin, Ottawa, Canada) twice. The cell lysate was centrifuged at 10,000 × *g* for 30 minutes at 4°C in a Thermo centrifuge (model IEC MultiRF; Thermo Scientific, Waltham, MA). The rLigB-Rep was expressed as an inclusion body. The pellets of the inclusion body were resuspended in Hisbind buffer containing 8 M urea by vortexing, placed on a shaker at room temperature for an additional 10 minutes, and centrifuged for 30 minutes at 10,000 × *g*. The supernatant was applied onto a 3-mL nickel column (Ni-NTA) equilibrated with the same buffer containing 8 M urea. The column was washed extensively twice with 30 mL of Hisbind buffer containing 8 M urea. The rLigB-Rep was eluted from the column by a step gradient of 3 mL of 25, 50, 100, 200, 400, 600, and 1,000 mM imidazole in Hisbind buffer with the presence of 8 M urea. Peak fractions were pooled for refolding as described for rLipL41.²⁷ The refolded rLigB-Rep was stored at –20°C.

ELISA. ELISA procedures were the same as previously described to detect immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies against rLipL32, rLipL41, rLigA-Rep, and rLigB-Rep individually and against a 1:1:1:1 mixture of these four recombinant antigens.²⁷ Microtiter plates (96 well) were coated for 40 hours at 4°C with antigen(s) diluted in phosphate-buffered saline (PBS) and subsequently blocked with 10% skim milk in PBS for 1 hour at room temperature. For single antigen ELISA, each well was coated with 300 ng of protein. For the 1:1:1:1 mixture, each protein was 75 ng. Different amounts of 1:1:1:1 mixture (300, 450, and 600 ng) were tried to determine the optimum amount for coating. The amount 300 ng/well was chosen because a higher amount did not increase the ELISA signal more than the background signal.

Human sera. A total of 429 sera samples collected from febrile patients (337 MAT positive with titers greater than 100 against 18 different *Leptospira* serovars (Australis, Autumnalis, Bataviae, Borincana, Bratislava, Canicola, Celledoni, Copenhageni, Cynopteri, Djasiman, Grippotyphosa, Harjo,

Icterohaemorrhagiae, Panama, Pyrogenes, Tarassovi, Varillal, and Wolffi) from six major pathogenic *Leptospira* species (*L. interrogans*, *Leptospira kirschneri*, *Leptospira borgpetersenii*, *Leptospira santarosai*, *Leptospira weilii*, and *Leptospira licerasiae*) and 92 MAT negative) from the Iquitos area of Peru were received from Naval Medical Research Unit 6, Lima, Peru. The 92 negative sera were used as local negative control to calculate cutoff values for the ELISA. The sample collection date after the onset of fever is listed in Table 1.

Ethics. The study was approved by the Naval Medical Research Center Institutional Review Board (case number PJT.15.02 and Protocol NMRC.2000.0006) in compliance with all applicable federal regulations governing the protection of human subjects. Informed consent was obtained from all study participants.

Data analysis. The raw data was analyzed using the average of duplicates. The cutoff value for both IgM and IgG was determined using the formula described below to calculate the proper cutoff value in immunoassay studies.³¹

$$\text{Cutoff} = X + \text{SD} t(1 + (1/n))^{1/2}$$

Based on a critical *t*-value of 1.6618 for a 95.0% confidence level for a one-tailed *t*-distribution of 92 control samples (*N* = 92), the cutoff for this study was determined to be 1.6708 standard deviations plus the average absorbance (*X*) of an ELISA test (i.e., IgG or IgM) from the MAT-negative samples. A sample with an optical density reading higher than the calculated cutoff value was determined to be positive. Combining the results of IgM and IgG, a sample was considered positive if either IgM or IgG was classified as a positive.

We divided our data analysis into two separate parts: first, we considered MAT as a standard reference, then we calculated the sensitivity and specificity for each ELISA (individual antigen or 1:1:1:1 mixture) in Tables 2–7 accordingly. Second, recognizing that MAT can be an imperfect standard, we further applied Bayesian Latent Class Analysis (LCA) to both MAT and ELISA jointly to determine the true sensitivity and specificity of each test and the prevalence of disease. Given the fact that it is more practical to use the ELISA data from 1:1:1:1 mixture of rLipL32, rLipL41, rLigA-Rep, and rLigB-Rep than to use ELISA data from individual proteins, we concentrated on the comparison of the ELISA using 1:1:1:1 mixture to the MAT test.

Bayesian LCA for two conditional independent diagnostic tests was considered by Joseph et al.³² In this method, the prevalence of the disease, sensitivity and specificity of MAT, and sensitivity and specificity of ELISA are considered as unknown parameters. They first derive the likelihood function which relates the frequency of the observed data as a function of the five latent parameters. Then they specify a prior

TABLE 1
Summary of the sample collection date after onset of fever

Group	Leptospirosis	Local control
Number of sera	337	92
Number of sera with day after onset of fever	309	88
Range (days)	0–63	0–53
Median (days)	13	3
Number of sera between 0 and 7 days	136	80
Number of sera between 8 and 20 days	82	3
Number of sera > 20 days	91	5

TABLE 2

Summary of the immunoglobulin M ELISA results against rLipL32, rLipL41, rLigA-Rep, rLigB-Rep, and four antigen mixture in 337 MAT-positive and 92 MAT-negative samples

Antigen	MAT		ROC fitted area
	Positive (337)	Negative (92)	
	No. of positive (% of sensitivity)	No. of positive (% of specificity)	
rLipL32	80 (24)	3 (97)	0.730
rLipL41	76 (23)	5 (95)	0.721
rLigA-Rep	138 (41)	4 (96)	0.786
rLigB-Rep	119 (35)	4 (96)	0.753
1:1:1:1 mixture*	152 (45)	8 (91)	0.792

MAT = microscopic agglutination test.

* 1:1:1:1 ratio of rLipL32, rLipL41, rLigA-Rep, and rLigB-Rep.

distribution for the five parameters to incorporate their knowledge about the parameters before collecting the data. The prior distribution can be obtained from past data or expert knowledge or both. The posterior distribution which is obtained from the Bayes rule is intractable in this case, so they propose a Gibbs sampler which is a Markov chain Monte Carlo simulation method to generate samples from the posterior distribution of the five parameters. The mean, median, or mode of the posterior distribution are usually used to estimate the unknown parameters. However, the conditional independent assumption between MAT and ELISA is too restricted and is not appropriate for our data, so we applied the fixed effects model³³ where the two tests are assumed to be conditionally dependent. We observed the frequency counts in a 2×2 contingency table classified by the MAT (test 1) and ELISA (test 2) binary results. We modeled the counts of this table by a multinomial distribution with parameters n (the total sample size) and probability vector (p_{11} , p_{12} , p_{21} , and p_{22}), where each probability was modeled by a two-component mixture distribution for the joint probability of the MAT and ELISA outcomes mixed by the latent disease state. In total, we needed the following seven parameters to describe the four cell probabilities: prevalence of the disease, π ; sensitivity of test 1, S_1 ; sensitivity of test 2, S_2 ; specificity of test 1, C_1 ; specificity of test 2, C_2 ; the covariance between the two tests within the diseased population, covs; and that for the non-diseased population, covc. For each test, $j = 1$ or 2 ; let $T_j = 1$, if the test was positive; $T_j = 0$ otherwise. Let D denote the disease state, so $D = 1$ denotes that the subject is diseased, $D = 0$ otherwise. Therefore, $S_j = P(T_j = 1 | D = 1)$ and $C_j = P(T_j = 0 | D = 0)$. So, following Vacek et al.³⁴ and Dendukuri and Joseph,³³

TABLE 3

Summary of the immunoglobulin G ELISA results against rLipL32, rLipL41, rLigA-Rep, rLigB-Rep, and four antigen mixture in 337 MAT-positive and 92 MAT-negative samples

Antigen	MAT		ROC fitted area
	Positive (337)	Negative (92)	
	No. of positive (% of sensitivity)	No. of positive (% of specificity)	
rLipL32	137 (41)	7 (92)	0.767
rLipL41	153 (45)	4 (96)	0.809
rLigA-Rep	163 (48)	4 (96)	0.827
rLigB-Rep	201 (60)	5 (95)	0.845
1:1:1:1 mixture*	238 (71)	6 (93)	0.874

MAT = microscopic agglutination test.

* 1:1:1:1 ratio of rLipL32, rLipL41, rLigA-Rep, and rLigB-Rep.

TABLE 4

Summary of the ELISA results (detectable IgM or IgG) against rLipL32, rLipL41, rLigA-Rep, and rLigB-Rep in 337 MAT-positive and 92 MAT-negative samples

Antigen	MAT	
	Positive (337)	Negative (92)
	No. of positive* (% of sensitivity)	No. of positive* (% of specificity)
rLipL32	167 (50)	10 (89)
rLipL41	189 (56)	9 (90)
rLigA-Rep	221 (66)	8 (91)
rLigB-Rep	239 (71)	9 (90)

IgG = immunoglobulin G; IgM = immunoglobulin M; MAT = microscopic agglutination test.

* IgM or IgG above cutoff value.

we modeled the eight conditional probabilities of the possible outcomes by

$$\begin{aligned}
 P(T_1 = 1, T_2 = 1 | D = 1) &= S_1 S_2 + \text{covs}, \\
 P(T_1 = 1, T_2 = 0 | D = 1) &= S_1 (1 - S_2) - \text{covs}, \\
 P(T_1 = 0, T_2 = 1 | D = 1) &= (1 - S_1) S_2 - \text{covs}, \\
 P(T_1 = 0, T_2 = 0 | D = 1) &= (1 - S_1)(1 - S_2) + \text{covs}, \\
 P(T_1 = 1, T_2 = 1 | D = 0) &= (1 - C_1)(1 - C_2) + \text{cove}, \\
 P(T_1 = 1, T_2 = 0 | D = 0) &= (1 - C_1) C_2 - \text{cove}, \\
 P(T_1 = 0, T_2 = 1 | D = 0) &= C_1 (1 - C_2) - \text{cove}, \\
 P(T_1 = 0, T_2 = 0 | D = 0) &= C_1 C_2 + \text{cove}.
 \end{aligned}$$

The specific probability expressions for each of the four outcomes are given in the Supplemental Appendix as a mixture of two distributions. When we collected data for all subjects, we had essentially three degrees of freedom, because the sum of the frequency counts of this table was fixed by the total sample size (429). We resolved to Bayesian LCA to make use of the prior distribution to restrict the values of the unknown parameters to a feasible region and to circumvent the unidentifiability issue. We used openBUGS³⁵ to simulate samples from the posterior distribution and to obtain the Bayes estimates of these parameters with their 95% credible intervals. On the prior choices for the parameters, it was natural to assume independent beta distributions for the first five parameters^{33,35}: $\pi \sim \text{beta}(\alpha_\pi, \beta_\pi)$, $S_j \sim \text{beta}(\alpha_{S_j}, \beta_{S_j})$, $C_j \sim \text{beta}(\alpha_{C_j}, \beta_{C_j})$, $j = 1, 2$, where the hyperparameters could be chosen to match the prior mode and confidence level. Suppose $\pi \sim \text{beta}(a, b)$, then mode = $(a-1)/(a+b-2)$, $0.95 = \Pr(\pi > c)$. So by specifying the model of π and the cutoff value c , we can determine the two

TABLE 5

Analysis of ELISA data (detectable IgM or IgG) from using different combinations of rLipL32, rLipL41, rLigA-Rep, and rLigB-Rep individually and in a mixture

Antigen	MAT	
	Positive (337)	Negative (92)
	No. of positive* (% of sensitivity)	No. of positive* (% of specificity)
rLipL32 + rLipL41 + rLigA-Rep†	283 (84)‡	23 (75)‡
rLipL32 + rLipL41 + rLigB-Rep†	291 (86)‡	24 (74)‡
rLipL32 + rLigA-Rep+rLigB-Rep†	289 (86)‡	23 (75)‡
rLipL41 + rLigA-Rep+rLigB-Rep†	300 (89)‡	24 (74)‡
rLipL32 + rLipL41 + rLigA-Rep+ rLigB-Rep†	303 (90)‡	31 (66)‡
1:1:1:1 mixture	276 (82)	13 (86)

IgG = immunoglobulin G; IgM = immunoglobulin M; MAT = microscopic agglutination test.

* IgM or IgG above cutoff value.

† Combinations of ELISA data using individual protein as the antigen.

‡ Sera that have antibody levels above cutoff value against at least one antigen.

TABLE 6

Antibody responses against 1:1:1:1 mixture of rLipL32, rLipL41, rLigA-Rep, and rLigB-Rep according to days after onset of fever in leptospirosis patient sera

Range of days after onset of fever	No. of sera	No. (%) positive against		
		IgM	IgG	IgM + IgG*
0–7	136	54 (40)	82 (60)	107 (79)
8–20	82	41 (50)	57 (70)	68 (83)
21–63	91	42 (46)	67 (74)	78 (86)

IgG = immunoglobulin G; IgM = immunoglobulin M. Only 309 of 337 leptospirosis patient sera have the information for days after onset of fever.

* IgM or IgG above cutoff value.

hyperparameters a and b . Similarly, we can choose the hyperparameters of the sensitivity and specificity of each test by specifying their modes and cutoff values. In our case, we chose the two hyperparameters to be the same for tests 1 and 2 to reflect our impartial prior judgment for the two tests. It is usually harder to obtain prior information on the covariance terms, so we selected a uniform prior distribution over the feasible region for each covariance term as in Dendukuri and Joseph.³³ We use $Covs$ to denote the covariance between the two tests, given the disease (positive) population. Let $Ls = (S_1 - 1)(1 - S_2)$, and $Us = \min(S_1, S_2) - S_1 S_2$. The feasible range of $Covs$ could be shown to be (Ls, Us) , so we selected $Covs \sim \text{Unif}(Ls, Us)$. Similarly, we selected a uniform prior $\text{Unif}(Lc, Uc)$ for $Covc$, the covariance term of the non-disease population, where $Lc = (C_1 - 1)(1 - C_2)$, and $Uc = \min(C_1, C_2) - C_1 C_2$. Then, we evaluated the conditional correlation between the two tests for the disease population $\rho_p = Covs / \sqrt{S_1[1 - S_1]S_2[1 - S_2]}$ and for the non-diseased population $\rho_n = Covc / \sqrt{C_1[1 - C_1]C_2[1 - C_2]}$.

RESULTS

Production of rLigB-Rep. The leptospiral immunoglobulin-like (Lig) protein family has three members (LigA, LigB, and LigC).^{30,36,37} These Lig proteins have 12–13 tandem bacterial

immunoglobulin-like repeat domains.³⁸ LigA and LigB proteins of *L. interrogans* have 100% sequence identity in the region of amino acid 312–630 and only 43% sequence identity in the region of 630–931, respectively. Previously, the rLigA-Rep consisting of repeat domains three, four, five, and part of six (amino acid 312–630) was used in ELISA. In this study, rLigB-Rep consists of repeat domains seven, eight, nine, and part of domain six and 10 (amino acids 630–931) (Figure 1), was prepared in a similar way. Because it is anticipated that rLigB contains additional epitopes than in rLigA, the combination of rLigA and rLigB will most likely have a higher sensitivity than individual rLigA or rLigB.

Data analysis using MAT as the standard reference and IgM and IgG ELISA results using individual antigen. Purified rLipL32, rLipL41, rLigA-Rep, and rLigB-Rep were used as antigens to detect the presence of IgM and IgG specific for *Leptospira* in an ELISA. A total of 337 MAT-positive samples and 92 MAT-negative samples were tested for specific IgM and IgG antibodies against each recombinant antigen. Tables 2 and 3 list the sensitivity, specificity, and receiver operating characteristic (ROC) curve fitted area for the IgM and IgG ELISA results of each antigen (ROC curves in Supplemental Figures 1 and 2). There were 80 samples (24%) and 137 samples (41%) that showed detectable IgM and IgG against rLipL32, respectively; 76 samples (23%) and 153 samples (45%) against rLipL41, respectively; 138 samples (41%) and 163 samples (48%) against rLigA-Rep, respectively; and 119 samples (35%) and 201 samples (60%) against rLigB-Rep. ROC curve analysis, which evaluates sensitivity and specificity along a curve, produced fitted areas of 0.730 and 0.767 for the detection of IgM and IgG against rLipL32, respectively; 0.721 and 0.809 for the detection of IgM and IgG against rLipL41, respectively; 0.786 and 0.827 for the detection of IgM and IgG against rLigA-Rep, respectively; and 0.753 and 0.845 for the detection of IgM and IgG against rLigB-Rep, respectively.

Combining IgM and IgG results, the sensitivity of the ELISA using one single antigen ranged from 50% to 71% (Table 4,

TABLE 7

Comparison of the IgM and IgG responses against 1:1:1:1 mixture of rLipL32, rLipL41, rLigA-Rep, and rLigB-Rep in 337 leptospirosis patient sera with primary infecting serovar

Species	Serovar*	No. of sera	No. (%) positive		
			IgM	IgG	IgM + IgG†
<i>Leptospira interrogans</i>	Australis	1	1 (100)	1 (100)	1 (100)
	Autumnalis	6	1 (17)	5 (83)	5 (83)
	Bataviae	36	14 (39)	26 (72)	31 (86)
	Bratislava	157	65 (41)	119 (76)	136 (87)
	Canicola	13	5 (38)	12 (92)	12 (92)
	Copenhageni	3	1 (33)	3 (100)	3 (100)
	Djasiman	12	8 (67)	9 (75)	11 (92)
	Grippotyphosa	10	3 (30)	8 (80)	8 (80)
	Harjo	6	4 (67)	5 (83)	5 (83)
	Icterohaemorrhagiae	138	67 (49)	104 (75)	120 (87)
	Panama	38	15 (39)	28 (74)	32 (84)
	Pyrogenes	4	2 (50)	4 (100)	4 (100)
	Wolffi	3	3 (100)	3 (100)	3 (100)
	Tarassovi	3	2 (67)	3 (100)	3 (100)
<i>Leptospira borgepeterseni</i> ‡	Cynopteri	10	3 (30)	1 (10)	3 (30)
<i>Leptospira santarosai</i>	Borincana	4	4 (100)	3 (75)	4 (100)
<i>Leptospira wilei</i>	Celledoni	1	1 (100)	1 (100)	1 (100)
<i>Leptospira licerasiae</i>	Varillal	44	21 (48)	23 (52)	30 (68)

IgG = immunoglobulin G; IgM = immunoglobulin M; MAT = microscopic agglutination test.

* If samples had the same highest MAT titer against multiple serovars, they were listed in each respective serovar.

† IgM or IgG above cutoff value.

‡ *Leptospira borgepeterseni*.

FIGURE 1. LigA and LigB proteins of *Leptospira interrogans* from amino acids 301–960. Repeats three to nine are indicated with a dotted line; asterisks indicate identical amino acids; colons indicate semi-conserved amino acids; and periods indicate similar amino acids. Amino acid 312, 630, and 931 are highlighted in gray.

Among the 337 MAT-positive samples, the presence of IgM or IgG of some samples can only be detected by one specific antigen but not by any of the other antigens. Each protein had a set of samples for which it was uniquely detected for either IgM or IgG. Three (1%) samples had detectable antibodies against rLipL32 only, 14 (4%) samples against rLipL41, 12 (4%) samples against rLipA-Rep. and 20 (6%) samples

IgM and IgG ELISA results with antigen mixture. A 1:1:1:1 mixture of the recombinant proteins rLipL32, rLipL41, rLigA-Rep, and rLigB-Rep was used to detect the presence of IgM

and IgG antibodies specific for *Leptospira*. The ELISA using the mixture of recombinant antigens identified 152 (45%) and 238 (71%) samples as positive for IgM and IgG, respectively. ROC analysis demonstrated a fitted area under the curve of 0.792 and 0.874 for the detection of IgM and IgG, respectively (Tables 2 and 3, Supplemental Figures 1 and 2). By combining IgM and IgG results, the ELISA using 1:1:1:1 antigen mixture had an overall sensitivity of 82% and specificity of 86% (Table 5).

Among the 337 MAT-positive sera, 309 had known collection dates after fever onset. Of the 136 MAT-positive samples collected between 0 and 7 days after the onset of fever, 54 (40%) and 82 (60%) samples showed detectable IgM and IgG, respectively. Of the 82 MAT-positive samples collected between 8 and 20 days after the onset of fever, 41 (50%) and 57 (70%) samples showed detectable IgM and IgG, respectively. Of the 91 MAT-positive samples collected more than 20 days after the onset of fever, 42 (46%) and 67 (74%) samples showed detectable IgM and IgG, respectively. The overall sensitivity was 79%, 83%, and 86% for samples collected during day 0–7, day 8–20, and day > 20 after the onset of fever (Table 6).

The 337 patient sera used in this study had MAT titers greater than 100 against one or more of 18 serovars. The highest agglutinating titer serovar is considered to be the primary infecting serovar for each patient. The numbers of sera in all 18 primary infecting serovars, the serovars that exhibited the highest titers, were listed in Table 7. Samples from all 18 primary infecting serovars had detectable IgM and IgG antibodies to the mixture of rLipL32, rLipL41, rLigA-Rep and rLigB-Rep (Table 7). Among these 18 primary infecting serovars, Bratislava and Icterohaemorrhagiae were the most prevalent serovars circulating in the study population, followed by Bataviae, Panama, and Varillal. There were more than 40% of the serum samples that exhibited the highest MAT titer against Bratislava or Icterohaemorrhagiae and much fewer number of samples (11–13%) against Bataviae, Panama, or Varillal. There were only 1–13 patient sera that exhibited the highest MAT titer against the other 13 serovars, suggesting these serovars may be less prevalent in Peru. In general, more sera had detectable IgG than IgM, except those with the highest MAT titer against Cynopteri and Borincana. Excluding those samples with the highest MAT titer against Cynopteri and Varillal, more than 83% of the remaining sera were seropositive by combining the results of IgM or IgG.

Results from Bayesian LCA for both MAT and ELISA using the 1:1:1:1 mixture of the four recombinant proteins. In this section, we evaluated the two serological tests, MAT and ELISA, by relaxing the assumption that MAT is 100% correct in both the positive and negative detection of *Leptospira*-specific antibodies, as performed in the previous section. Because the 1:1:1:1 mixture has the best performance (considering both sensitivity and specificity) as compared with individual protein and different combinations (Tables 4 and 5) based on MAT results, this 1:1:1:1 mixture ELISA was chosen to be analyzed along with MAT. Our data consisted of 278, 59, 13, and 79, respectively, for the frequency counts for 1) both tests were positive, 2) MAT positive and ELISA negative, 3) MAT negative and ELISA positive, and 4) both tests were negative. It was straightforward to show that the odds ratio of the 2×2 table was 28.63 with 95% confidence interval (CI) to be (14.88, 54.60) via the normal distribution assumption on the log odds ratio.³⁹ Therefore, this

suggested that the two tests were dependent, and our conditionally dependent model described previously was more appropriate than any independent models.

We selected $\pi \sim \text{beta}(13.322, 6.281)$ to reflect our belief on the mode of π to be 0.70 and the probability of 95%, sure that π is at least 0.5; $S_j \sim \text{beta}(9.628, 3.876)$, $j = 1, 2$, to reflect our prior belief on the mode of each sensitivity was 0.75 and with strength bigger than 95% sure of the belief that each sensitivity is at least 0.5; $C_j \sim \text{beta}(15.034, 2.559)$, $j = 1, 2$, to reflect our belief on the mode of the each specificity to be 0.9 and the probability of 95% sure of the belief that each specificity is at least 0.7. We used openBUGS to implement the Markov chain Monte Carlo method for Bayesian computation. Our results were based on a single chain with 1,000 iterations as burn-in and 100,000 more iterations for inference. Table 8 summarizes the parameter estimates of the prevalence of the *Leptospira*-specific antibodies, sensitivity, and specificity of each test, the correlation between the tests with the disease and that without the disease, and with their standard errors and their 95% credible intervals. The results from our analysis showed that the prevalence of the *Leptospira*-specific antibodies was 82% in this set of fever patient sera, the sensitivity and specificity of the MAT were estimated to be 90% and 83%, and that of ELISA were estimated to be 79% and 88%, with satisfactory standard errors reported (Table 8). Moreover, the correlation coefficient between the two tests without disease was estimated to be 0.27, with (−0.155, 0.826) to be its 95% CI. With disease the correlation was estimated to be 0.420, with a CI of 0.026–0.653. Note, this latter interval was strictly positive, not containing zero, pointing to the positive correlation between the two tests in the disease state. Thus, explaining the need for modeling conditional dependence between the two tests as performed in Dendukuri and Joseph.³²

DISCUSSION

Microscopic agglutination test is considered the reference method for serological detection of leptospirosis. The agglutination of leptospires is thought to be mediated by lipopolysaccharide (LPS)-specific antibodies.⁴⁰ However, this test requires the maintenance of a large panel of live pathogenic cultures. The use of live *Leptospira* organisms also creates a risk of laboratory-acquired infection to the laboratory technicians.⁴¹ As an alternative, whole cell-based serological assays that use antigens from nonpathogenic *Leptospira biflexa* serovar Patoc have been developed with field evaluations of these assays suggesting low sensitivity (39–72%) during the

TABLE 8
Bayesian latent class Analysis of MAT and ELISA data from using rLipL32, rLipL41, rLigA-Rep, and rLigB-Rep in a 1:1:1:1 mixture

	Mean	SD	MC_error	Md	95% credible interval
π	0.821	0.0454	2.011E-4	0.8208	(0.730, 0.908)
S_1	0.902	0.0361	1.800E-4	0.9043	(0.828, 0.965)
S_2	0.789	0.0415	1.846E-4	0.788	(0.710, 0.872)
C_1	0.819	0.0935	3.229E-4	0.8308	(0.609, 0.963)
C_2	0.868	0.0671	2.164E-4	0.8761	(0.717, 0.973)
ρ_p	0.397	0.1594	7.246E-4	0.4197	(0.026, 0.653)
ρ_n	0.289	0.2706	8.49E-4	0.2725	(−0.155, 0.826)

IgM = immunoglobulin M; IgG = immunoglobulin G; MAT = microscopic agglutination test; MC_error = Monte Carlo error; Md = median; SD = standard deviations. π is prevalence of the disease; S_1 is the sensitivity of MAT; S_2 is the sensitivity of ELISA; C_1 is the specificity of MAT; C_2 is the specificity of ELISA; ρ_p is the correlation between the two tests with the disease population; ρ_n is correlation between the two tests with the non-disease population.

early phase of infection.^{42–50} To overcome this limitation, several recombinant protein-based serological tests have been developed using outer membrane proteins from pathogenic species.^{24–28}

Previously, we produced three immunogenic antigens (rLipL32, rLipL41, and rLigA-Rep) and demonstrated that they can be used for the detection of *Leptospira*-specific antibodies by using ELISA with a small panel of 85 MAT-confirmed febrile patient sera.²⁷ Lessa-Aquino et al.⁵¹ showed that LipL32 and the repeats 7–13 of immunoglobulin-like repeat domain of Lig proteins were the most reactive targets in a protein microarray assay. Although LigB is present in all pathogenic strains but the sequence of repeats 6–10 of LigB has low homology (43%) with the sequence of LigA,⁵² we decided to produce rLigB-Rep (LigB repeats 6–10) and evaluated the feasibility of including rLigB-Rep to further improve the performance of our ELISA using a much larger sample size (429 MAT-confirmed febrile patient sera). All four individual antigens had very similar ROC fitted area for the detection of IgM (0.721–0.786, Table 2) and slightly higher fitted area for the detection of IgG (0.767–0.845, Table 3). All four antigens showed more robust IgG interaction to the MAT-positive sera than IgM interaction. The overall sensitivity for individual antigen ranged from 50% to 71% and specificity were all close to 90% (Table 4). This is very similar to what we observed earlier with a much smaller sample size (sensitivity around 60% and specificity around 90%).²⁷

In this study, we also performed ELISA with a 1:1:1:1 mixture of the four recombinant proteins. The ROC fitted area for the detection of IgM (0.792) and IgG (0.874) using the protein mixture were higher than individual antigens alone, indicating that the performance of the antigen mixture is better than individual antigens. Combining the IgM and IgG results, the antigen mixture showed an 82% sensitivity with a much better specificity of 86% (Table 5). Table 6 showed that as the days after onset of fever increased, the percentage of patients with detectable antibodies against those proteins also increased. Our recombinant proteins were all derived from the *L. interrogans* serovar Copenhageni strain Fiocruz L1-130. These antigens are highly conserved among a broad range of pathogenic *Leptospira* species (99% amino acid sequence homology for LipL32 and LigA, 95% for LipL41, and greater than 90% for LigB).^{38,53} In our panel, 13 out of 18 serovars belong to *L. interrogans* and the sensitivity of ELISA were all above 80% (Table 7). Interestingly, we did observe lower sensitivity in ELISA with samples that had the highest agglutination titers against Cynopteri (*Leptospira kirschneri*) and Varillal (*L. licerasiae*). Serovar Cynopteri was first isolated from the short-headed fruit bat of Indonesia,⁵⁴ and serovar Varillal was identified in Peru as antigenically distinct from all known serogroups of *Leptospira*.⁵⁵ The alignment of LipL32, LipL41, and Lig proteins of serovar Cynopteri and Varilla showed low sequence homology to serovar Copenhageni. This may explain the low sensitivity in ELISA using antigens derived from *L. interrogans* serovar Copenhageni strain Fiocruz L1-130.

Previous studies in Peru showed that the seroprevalence of leptospirosis was 28% in Belen (urban slum in the city of Iquitos), 17% in rural villages around Iquitos, and 0.7% in a desert shantytown near Lima, and observed an age-dependent increase in leptospiral seropositivity which may result from continuous exposure throughout life.⁵⁶ Jo et al. also found high seroprevalence (64.6% by MAT or 15% by IgM

ELISA) of leptospirosis among rice farmers.⁵⁷ Unfortunately, the age, profession, and detailed collecting site information was not available for our samples in this study. Because all our patient samples were collected from the Iquitos area, it is possible many of them could have previous *Leptospira* infections. This is consistent with the ELISA results that showed robust IgG and weak IgM interactions to all four recombinant antigens.

Microscopic agglutination test is a subjective test, even for clinical leptospirosis cases, with questionable validity for its use as an immunological gold standard for evaluation of other serological rapid diagnostics.^{58,59} Recently, Niloofa et al.⁶⁰ suggested that MAT is an imperfect gold standard for early detection of leptospirosis because agglutination antibodies, mainly against LPS, take 1–2 days longer to appear than *Leptospira* genus-specific IgM antibodies. Nonetheless their study showed that MAT is useful as a confirmatory test for epidemiological studies.⁶⁰

Microscopic agglutination test requires the continuous maintenance of a large panel of leptospiral cultures. It is a complex and time-consuming method. It cannot detect IgM and IgG separately. The results are read under a dark field microscope for agglutination with no precise interpretation. A well-trained technician can only perform MAT testing on about 60 samples in a single day. ELISA, in contrast, is much easier to perform, can test a large number of samples at the same time, can detect both IgM and IgG separately, is less subjective, and shows more accurate and precise results. One technician can easily test 1,000 samples per day by using ELISA. The recombinant protein-based ELISA is thought to give a higher sensitivity and specificity because of the higher concentrations of immunogenic antigens and specific antigenic moieties.⁶¹ Our data showed that no single antigen can detect antibodies in all samples from different serovar infections, even with the use of highly conserved proteins (such as LipL32) in ELISA. Therefore, four antigens were included in our ELISA to increase the assay's sensitivity. To further simplify the overall ELISA procedures, we tested the possibility of mixing four antigens together in ELISA format. Thus, instead of performing a total of eight ELISAs (IgM and IgG for four different antigens individually), we were able to achieve relatively high sensitivity (82%) and specificity (86%) by performing only two ELISAs, one IgM and one IgG, with a mixture of four recombinant antigens.

Between 8 and 10 false positives were found among the MAT-negative samples against rLipL32, rLipL41, rLigA-Rep, and rLigB-Rep. These may come from *E. coli* impurities that present in the recombinant protein preparations or the cross-reactive epitopes on the protein. The cross-reactive epitopes may be identified by protein sequence analysis and pepscan and be removed from future antigen preparations to improve the ELISA's specificity.

Although the highest agglutinating titers against a particular serovar may not be the best way to predict the infecting serovar, the fact that these recombinant antigens were able to detect antibodies in samples with the highest agglutinating titers against 18 different serovars from six major pathogenic *Leptospira* species, strongly indicates its broadness in detection of serovar-specific antibodies.

Understanding that MAT is an imperfect gold standard, we also applied an appropriate Bayesian latent class model to the

outcomes of MAT and ELISA jointly that incorporates the conditional dependence between the two tests, the true sensitivity and specificity of each test, and the prevalence of the disease. The Bayesian estimates of the sensitivity and specificity of MAT were 90% and 83%, and those of ELISA were 79% and 88%. Although the ELISA sensitivity is lower than that MAT, the specificity is higher. In many resource-limited areas, including most of the leptospirosis endemic areas, laboratory capabilities to culture and detect pathogenic microorganisms are often inadequate. Our results strongly support the suitability of using this four-recombinant protein mixture in ELISA for seroprevalence studies in areas where a large numbers of samples will be tested and where culturing and maintaining the multiple live *Leptospira* species is not feasible.

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