INTRODUCTION

Leptospirosis is caused by spirochaetes of the genus Leptospira. It is considered to be the most widespread bacterial zoonotic disease in the world and recognized as an emerging infectious disease. More than 1.7 million cases of severe zoonotic disease in the world and recognized as an emerg-

Leptospira was reported.37 It is considered to be the most widespread bacte-

rial zoonotic disease in the world and recognized as an emerging infectious disease.1,2 More than 1.7 million cases of severe leptospirosis are reported each year, with case mortality rate about 10%.3 It is most common in developing countries, particularly in the Caribbean, Latin America, the Indian subcontinent, Southeast Asia, and Oceania, although locally acquired cases in industrialized countries are well described.1,4–13 In recent years, a new trend in human leptospirosis outbreaks related to outdoor recreational activities or natural disasters has been observed.14–22 Symptoms of leptospirosis are non-specific and may be easily confused with other febrile illnesses, such as dengue or malaria, which require different treatment regimens. Therefore, timely diagnosis is essential, because antibiotic therapy provides the greatest benefit when initiated early in the course of illness.23,24

Currently, the microscopic agglutination test (MAT) is the standard serological method for the diagnosis of leptospirosis, in which whole cell antigens representing different serogroups of leptospirosis are mixed with serum samples and examined by dark-field microscopy for agglutination.25 It is technically complex and time consuming, because the method requires many serovars of Leptospira to be cultured.26 Culturing Leptospira to obtain the whole cell antigen is particularly labor intensive and requires special precautions to prevent infection of laboratory staff. Because MAT relies on the detection of antibodies to leptospiral antigens, it is limited by the low sensitivity when acute serum samples were tested.27 Commercial assays based on whole cell antigen of leptospira were microscopic agglutination test (MAT)-confirmed positive sera from febrile patients in Peru, 22 sera were indigenous MAT-negative febrile patient sera, and 36 sera were from patients with other febrile diseases from Southeast Asia, where leptospirosis is also endemic. Combining the results of immunoglobulin M (IgM) and IgG detection from these three antigens, the overall sensitivity is close to 90% based on the MAT. These results suggest that an ELISA using multiple recombinant antigens may be used as an alternative method for the detection of Leptospira-specific antibodies.

Detection of Leptospira-Specific Antibodies Using a Recombinant Antigen-Based Enzyme-Linked Immunosorbent Assay

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Abstract. We produced three highly purified recombinant antigens rLipL32, rLipL41, and rLigA-Rep (leptospiral immunoglobulin-like A repeat region) for the detection of Leptospira-specific antibodies in an enzyme-linked immunosorbent assay (ELISA). The performance of these recombinant antigens was evaluated using 121 human sera. Among them, 63 sera were MAT-negative febrile patient sera, and 36 sera were from patients with other febrile diseases from Southeast Asia, where leptospirosis is also endemic. Combining the results of immunoglobulin M (IgM) and IgG detection from these three antigens, the overall sensitivity is close to 90% based on the MAT. These results suggest that an ELISA using multiple recombinant antigens may be used as an alternative method for the detection of Leptospira-specific antibodies.

MATERIALS AND METHODS

Bacterial strains and vectors. The genomic DNA of L. interrogans serovar Copenhagheni strain Fiocruz L1-130 (ATCC, Manassas, VA) was used as the template for cloning of all recombinant proteins. Escherichia coli Top10 (Life Technologies, Grand Island, NY) was used for general cloning. The cloned genes were inserted into pET28a (EMD Millipore, Billerica, MA) for the expression of recombinant proteins in E. coli BL21 (DE3) (Life Technologies, Grand Island, NY) under the control of phage T7 lac promoter.39

Recombinant antigen preparation. Cloning of the gene coding for LipL32, LipL41, and the repeat region of LigA proteins into the expression vector pET28a. A primer pair (LipL32f [5'-GGTGTTCTATGGTCTGGCAAGCCTA AAAAGC-3'] and LipL32r [5'-CCGCTCGAGCTTAGTCG CTCAGAAGCAGC-3']) was designed by using the nucleotide sequence of the open reading frame for LipL32 from strain L1-130 (GenBank accession no. AF245281.1). The coding region of full length protein minus predicted signal peptide for LipL32 (amino acids 25–272) was amplified by polymerase chain reaction (PCR) using genomic DNA isolated from L. interrogans strain L1-130 as the template. The primer pair for

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LipL41 (LipL41f [5'-GGTGGTCATATGGCTACAGTGA TGTAGAATATCC-3'] and LipL41r [5'-CGCCTCGAGCTT TGCGTGTGTTCATCAACG-3']) was designed for the coding region of full length protein minus predicted signal peptide for LipL41 (amino acids 22–355), and primer pair for LigA (LigAf [5'-AAGAATCATATGGCAGCCTTATTT CTATTTCTGT-3'] and LigAr [5'-CGCCCTCGAGAATAAT CCGTATTAGGGAAATCC-3']) was designed for the coding region of amino acids 312–630. Each PCR product was digested with NdeI and XhoI and ligated into the expression vector pET28a. The resulting plasmids contained a sequence coding His tag at both N and C termini of LipL32, LipL41, and LigA 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 (50 mg/L) at 37°C with shaking at 200 rpm. Cell pellets from 500 mL cultures were resuspended in 20 mL buffer A of 20 mM Tris·HCl, pH 8.0, and 0.5 M NaCl after centrifugation. Cells were ruptured by sonication (Ultrasonic Liquid Processor Model VirSonnic 475; VIRTIS Company, Gardiner, NY) five times at setting 3 for 10 seconds each time, with cooling on ice for 1 minute between each sonication. Cell extract was centrifuged at 10,000 × g for 30 minutes at 4°C in a Thermo centrifuge (model IEC MultiRF; Thermo Scientific, Waltham, MA). The recombinant LipL32 (rLipL32) and recombinant LigA (rLigA-Rep) were expressed in soluble form, but recombinant LipL41 (rLipL41) was expressed as an inclusion body. For the purification of rLipL32 or rLigA-Rep, the cell lysate supernatant was applied onto a 3 mL nickel column (Ni-NTA) equilibrated with 20 mM Tris·HCl, pH 8.0, 0.5 M NaCl, and 10 mM imidazole (Hisbind buffer). The column was washed extensively with 30 mL Hisbind buffer containing 1 mM (ethylenedinitrilo) tetraacetic acid (EDTA) and 1 mM dithiothreitol (DTT) and finally, buffer A containing 1 mM EDTA and 1 mM DTT without urea. Before dialysis, the protein concentration of the eluates from the nickel column was adjusted to less than 1 mg/mL and dialyzed against 10 volumes of 6 M urea in buffer A for 60 minutes at 4°C. The same procedure was repeated with 4, 2, and 1 M urea in buffer A. The final dialysis was in 10 volumes of the eluates of buffer A without urea with one initial change of buffer at 60 minutes and finally, overnight at 4°C. The refolded rLipL41 was stored at −20°C. 

**ELISA.** ELISA was used in the detection of immunoglobulin M (IgM) and IgG antibodies against rLipL32, rLipL41, and rLigA-Rep. Different amounts (0.15, 0.3, 0.45, and 0.6 μg/well) of each antigen were used to coat the ELISA plate to determine the optimum amount for coating. The optimal amount was determined to be 0.3 μg/well, because 0.45 and 0.6 μg did not increase the signal. Microtiter plates (96 well) were coated for 40 hours at 4°C with recombinant antigen diluted in phosphate buffered saline (PBS) and blocked with 10% skim milk in PBS for 1 hour. Patient sera diluted 1:100 in PBS with 5% skim milk were then added to the plate, incubated for 1 hour at room temperature, and washed three times for 10 minutes each with 0.1% Tween-20 in PBS. Peroxidase-conjugated rabbit anti-human IgG (Santa Cruz Biotechnology, Dallas, TX) at 1:4,000-dilution or anti-human IgM (Dako, Carpinteria, CA) at 1:1,000 dilution was added. After 1 hour of incubation at room temperature, the plates were washed as previously described before the addition of the 2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonate) (ABTS) substrate (Kirkegaard & Perry, Gaithersburg, MD). Optical density at 405 nm (OD405) was measured after 30 minutes of incubation at room temperature in a plate reader (Molecular Devices, Sunnyvale, CA).

**Human sera.** In total, 85 sera collected from individual febrile patients (63 MAT positive sera with titers greater than 100 against different *Leptospira* serovars [Bataviae, Bratslavia, Icterohaemorrhagiae, and Varillal] and 22 MAT negative sera) from the Iquitos area of Peru were received from Naval Medical Research Unit 6, Lima, Peru. The 22 negative sera were used as local negative controls to receive cutoff values for the ELISA. The sample collection date after onset of fever is listed in Table 1; 36 archived sera from patients residing in the leptospirosis-endemic area of Southeast Asia with other febrile illness (9 patients with scrub typhus, 9 patients with murine typhus, 9 patients with spotted fever-type rickettsioses, and 9 patients with Q fever) were used as other control specimens.

**Ethics.** The study was approved by the Naval Medical Research Center Institutional Review Board (Case Number PJJ61 and Protocol NMIMCD.2000.0006) in compliance with all applicable federal regulations governing the protection of human subjects. Informed consent was obtained from all study participants.

**Table 1**

<table>
<thead>
<tr>
<th>Summary of the sample collection date after onset of fever</th>
<th>Group</th>
<th>Leptospirosis</th>
<th>Local control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of sera</td>
<td>63</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Number of sera with day after onset of fever</td>
<td>60</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Range (days)</td>
<td>0–44</td>
<td>0–71</td>
<td></td>
</tr>
<tr>
<td>Median (days)</td>
<td>16</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Number of sera between 0 and 7 days</td>
<td>22</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Number of sera between 8 and 20 days</td>
<td>17</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Number of sera &gt; 21 days</td>
<td>21</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>
RESULTS

Production of rLipL32, rLipL41, and rLigA-Rep. LipL32 and LipL41 are highly conserved immunodominant cell surface proteins among pathogenic leptospiral serovars. The Lig protein family has three members, LigA, -B, and -C, all with high molecular mass. These Lig proteins have 12–13 tandem bacterial Ig-like repeat domains. The first six domains from the amino terminus are highly conserved among different strains. The coding region of amino acids 312–630 of LigA consists of repeat domains three, four, five, and part of six. It is 98.5% homologous to the same domains from LigB within the same strain. The percentage of DNA sequence identity of LigB among different strains of leptospira ranges from 67.9% to 97.1%. The rLipL32 and rLigA-Rep were purified under the native condition, and the rLipL41 was purified under the denatured condition. Fractions collected during different steps of the purification procedure were analyzed by SDS-PAGE (Figure 1A). The rLipL32, rLipL41, and rLigA-Rep were highly purified using nickel column (Figure 1B).

IgM and IgG ELISA results for patient sera. The purified rLipL32 and rLigA-Rep and refolded rLipL41 were used as antigens to detect the presence of IgM and IgG specific for Leptospira in an ELISA. The IgM ELISA results for 63 leptospirosis serum samples against rLipL32, rLipL41, and rLigA-Rep are shown in Figure 2A–C, respectively, and the IgG ELISA results for 63 leptospirosis serum samples against rLipL32, rLipL41, and rLigA-Rep are shown in Figure 3A–C, respectively. Table 2 lists the results of patient samples that had specific IgM and IgG antibodies against each recombinant antigen. There were 7 (11%) and 39 (62%) samples that showed detectable IgM and IgG against rLipL32, respectively; 8 (13%) and 33 (52%) samples that showed detectable IgM and IgG against rLipL41, respectively; and 15 (24%) and 33 (52%) samples that showed detectable IgM and IgG against rLigA-Rep, respectively. Patient samples that had specific antibodies (IgG or IgM) from different combinations of either rLipL32 or rLipL41, rLipL32 or rLigA-Rep, rLipL41 or rLigA-Rep, and rLipL32,
or rLipL41, or rLigA-Rep were 53 (84%), 52 (83%), 45 (71%), and 57 (90%), respectively (Table 3). Of 63 MAT positive sera, 60 sera have the known collection date after onset of fever, and of 22 local controls, 20 controls have that information (Table 1). There is a wide range of collection date for the MAT negative local controls, but more than one-half of them were collected in the first 7 days after onset of fever (Table 1). The MAT-positive samples were collected evenly throughout the 44 day period. The IgM responses were only detected in five (23%) samples among those samples collected in the first 7 days after onset of fever. The percentage increased to 53% among the samples collected between days 8 and 20 and dropped back to 43% for the samples collected between 21 and 44 days after onset of fever (Table 4). The IgG responses were detected in 68%, 71%, and 95% samples among those samples collected during days 0–7, 8–20, and 21–44 after onset of fever, respectively (Figures 2 and 3 and Table 4).

In 63 MAT-confirmed patient sera used in this study, the sera with the highest agglutinating titers against the serovars Bataviae, Bratislava, Icterohaemorrhagiae, and Varillal were 10, 29, 12, and 12, respectively (Table 5). Among 10 patient sera with the highest serovar Bataviae titers, more samples had IgG antibodies to rLipL32 than rLipL41 or rLigA-Rep. Among 29 patient sera with the highest serovar Bratislava titers, the rLigA-Rep IgG ELISA had the highest number of positive followed by rLipL32 and rLipL41. Among 12 patient sera with the highest serovar Icterohaemorrhagiae titers, 75% of sera had IgG antibodies to rLipL32 or rLipL41, and 58% of sera had IgG antibodies to rLipL41 above the cutoff value. Among 12 patient sera with the highest serovar Varillal titers, 67% of sera had IgG antibodies to rLipL32 or rLipL41, and 33% of sera had IgG antibodies to rLigA-Rep above cutoff value.

**DISCUSSION**

The diagnosis of leptospirosis relies mainly on serological methods. The current serological gold standard, MAT, is complex and time consuming. The performance of MAT requires knowledge of the prevalent Leptospira strains in a particular region and the maintenance of a large panel of leptospiral cultures. The agglutination of Leptospira is thought to be mediated by lipopolysaccharide (LPS)-specific total antibodies, including both IgM and IgG. The LPS varies among different serovars with low cross-reactivity; therefore, a large number of different serovars need to be cultured and maintained for agglutination tests, such as MAT. Leptospiral proteins with a high degree of sequence homology among various serovars have a potential advantage compared with serovarspecific LPS antigens. LipL32 and LigA each exhibits 99% amino acid sequence homology across a broad range of pathogenic Leptospira species, whereas a 95% homology has been shown for LipL41 and LigA. Recombinant proteins are much easier to prepare and standardize, which lead to better batch to batch

**Figure 3.** IgG ELISA results for 63 MAT-positive patient samples using recombinant proteins. Specific IgG against (A) rLipL32, (B) rLipL41, and (C) rLigA-Rep were tested. The x axis indicates the number of days after the onset of illness, and the y axis indicates the OD405. For three samples missing the days after onset of fever information, day 16 (medium day) was used to plot. The cutoff values were 0.199 for rLipL32, 0.330 for rLipL41, and 0.207 for rLigA-Rep (mean of 22 negative controls plus 2.3 SDs for 95% confidence level).
consistency compared with leptospiral LPS preparation using culture based methods.

In this study, we produced three recombinant immunogenic antigens, rLipL32, rLipL41, and rLigA-Rep, to explore the feasibility of using them for the detection of *Leptospira*-specific antibodies in ELISA. All three proteins showed similar overall sensitivity (62–65%) (Table 3) and high specificity individually (greater than 90%) (Table 3). Not every serum had detectable IgM or IgG against these recombinant antigens. Among 63 positive sera, 4, 4, and 11 only had IgM against rLipL32, rLipL41, and rLigA-Rep, respectively, and 10, 3, and 2 only had IgG against rLipL32, rLipL41, and rLigA-Rep, respectively. Of 50 IgG-positive samples, 17 of them were also IgM positive. There were 8, 2, and 1 patient sera that had specific antibodies against only rLipL32, rLipL41, and rLigA-Rep, respectively.

Guerreiro and others previously showed, using IgG immunoblotting with whole cell antigens, that rLipL32 was recognized by 57% and 84% of acute and convalescent phase sera, respectively, and that rLipL41 was recognized by 21% and 36% of acute and convalescent phase sera, respectively. Similar findings were also reported by Flannery and others using recombinant antigens LipL32 and LipL41 in IgG ELISA. The recombinant LipL32 was recognized by 56% and 94% of acute and convalescent phase sera, and rLipL41 was recognized by 24% and 44% of acute and convalescent phase sera. In both studies, the majority of the patient samples had the highest agglutinating titers to reference strains of the serovar Icterohaemorrhagiae. Table 4 showed that rLipL32 and LipL41 were recognized by 55% and 71% of days 0–7 and 21–44 sera, respectively, and that rLipL41 was recognized by 45% and 67% of days 0–7 and 21–44 sera, respectively. Our data indicated that, even with highly conserved proteins (such as LipL32 and LipL41), one antigen was unable to detect antibodies in all sera from different serovar infections. Our recombinant proteins have been derived from a strain of *L. interrogans*. Therefore, the test results were reasonably good, because the majority of this panel consists of infections with serovars that belong to *L. interrogans*. However, data from 15 Thai samples showed that these three recombinant antigens could also detect antibodies in samples with the highest agglutinating titers against other serovars, such as *Cynopteri* (*L. kirschneri*), *Panama* (*L. kirschneri*), and *Sejroe* (*L. borgpetersenii*) (Chen H-W and others, unpublished results). This result strongly suggests the broadness of the assay using the combination of conserved antigens.

All three antigens showed robust IgG and weak IgM interactions to the sera of infected patients. They could be the results of previous exposures to *Leptospira* infections, which is quite common in a highly endemic area. Several studies have documented IgG immunoblot reactivity to leptospiral proteins during acute-phase illness in the absence of specific IgM. The predominant humoral responses during acute-phase infection were thought to be IgM antibodies directed primarily against carbohydrate epitopes. These findings suggest that early host immune response to *Leptospira* infection is characterized by both IgM and IgG specific for different moieties, similar to processes observed in *Borreella* and *Treponema* infections. As shown in Table 4, more samples had detectable IgG than IgM in all three stages. There was no indication of early detection of IgM in the samples that we evaluated. The percentage of samples that had detectable IgG against recombinant antigens increased from early (68%; days 0–7) to late stage (95%; days 21–44). The purpose of the original study for febrile patients was to determine the seroprevalence of different diseases, and therefore, the outcomes from these leptospirosis patients were not followed.

The combination of results from rLipL32 and rLipL41 only gave a sensitivity of 84% and a specificity of 91% (Table 3). The combination of ELISA results from rLipL32, rLipL41, and rLigA-Rep showed an increase in the assay sensitivity to 90%, whereas the specificity dropped to 82% in the local control (Table 3). The low specificity is the cumulative non-specific interactions of rLipL32 and rLigA-Rep. No false positive was found among the local control against rLipL41 in ELISA. Both rLipL32 and rLigA-Rep had two false positives among the local control. To further improve the performance of the ELISA, we plan to include additional immunogens (such as LipL21 and OmpL1) to increase the assay’s sensitivity. The cross-reactive epitopes, which cause false positives, may be

### Table 3

Analysis of different combinations of ELISA data using rLipL32, rLipL41, and rLigA-Rep as the antigen (have detectable IgM or IgG)

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of sera</th>
<th>L32*</th>
<th>L41*</th>
<th>Lig*</th>
<th>L32 + L41*</th>
<th>L32 + Lig*</th>
<th>L41 + Lig*</th>
<th>L32 + L41 + Lig*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leptospirosis</td>
<td>63</td>
<td>41</td>
<td>39</td>
<td>40</td>
<td>53</td>
<td>52</td>
<td>45</td>
<td>57</td>
</tr>
<tr>
<td>Local control</td>
<td>22</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>

* L32, L41, and Lig represent rLipL32, rLipL41, and rLigA-Rep, respectively.
† Sera that have antibody levels above cutoff values against at least one antigen.
‡ Have detectable IgM or IgG.

### Table 4

Antibody responses against rLipL32, rLipL41, and rLigA-Rep according to days after onset of fever in leptospirosis patient sera (only 60 of 63 leptospirosis patient sera have the information for days after onset of fever)

<table>
<thead>
<tr>
<th>Range after onset of fever (days)</th>
<th>No. of sera</th>
<th>IgM</th>
<th>IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L32*</td>
<td>L41*</td>
<td>Lig*</td>
</tr>
<tr>
<td>0–7</td>
<td>22</td>
<td>1 (5)</td>
<td>2 (9)</td>
</tr>
<tr>
<td>8–20</td>
<td>17</td>
<td>2 (12)</td>
<td>4 (24)</td>
</tr>
<tr>
<td>21–44</td>
<td>41</td>
<td>4 (19)</td>
<td>2 (10)</td>
</tr>
</tbody>
</table>

* L32, L41, and Lig represent rLipL32, rLipL41, and rLigA-Rep, respectively.
† Sera that have antibody levels above cutoff values against at least one antigen.
‡ Have detectable IgM or IgG.
**Table 5**

Comparison of the IgG responses against rLipL32, rLipL41, and rLigA-Rep in 63 leptospirosis patient sera with primary infecting serovar by ELISA

<table>
<thead>
<tr>
<th>Serovar*</th>
<th>No. of sera</th>
<th>rLipL32</th>
<th>rLipL41</th>
<th>rLigA-Rep</th>
<th>Com†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bataviae</td>
<td>10</td>
<td>7 (70)</td>
<td>4 (40)</td>
<td>4 (40)</td>
<td>8 (80)</td>
</tr>
<tr>
<td>Bratislava</td>
<td>29</td>
<td>15 (52)</td>
<td>14 (48)</td>
<td>16 (55)</td>
<td>22 (76)</td>
</tr>
<tr>
<td>Icterohaemorrhagiae</td>
<td>12</td>
<td>9 (75)</td>
<td>7 (58)</td>
<td>9 (75)</td>
<td>10 (83)</td>
</tr>
<tr>
<td>Varillal</td>
<td>12</td>
<td>8 (67)</td>
<td>6 (87)</td>
<td>4 (33)</td>
<td>10 (83)</td>
</tr>
</tbody>
</table>

*If the sample reacts with more than one serovar by MAT, the serovar with the highest agglutinating titer is listed.
†Sera that have IgG against at least one specific antigen.

identified by pepscan and subsequently truncated from LipL32 or LipA to improve the assay’s specificity. This study is a pilot study to show the potential of using multiple recombinant antigens for the detection of *Leptospira*-specific antibodies. In addition, a larger number of samples with the information of the collection date after onset of fever and paired acute and convalescent phase samples will be needed for future study.

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