B-Cell-Specific Peptides of *Leptospira interrogans* LigA for Diagnosis of Patients with Acute Leptospirosis

Murugesan Kanagavel, a Santhanam Shanmughapriya, a Kumarasamy Anbarasu, b Kalimuthusamy Natarajaseenivasan a

Medical Microbiology Laboratory, Department of Microbiology, a and Department of Marine Biotechnology, b Bharathidasan University, Tiruchirappalli, India

Leptospirosis is a reemerging infectious disease that is underdiagnosed and under-recognized due to low-sensitivity and cumbersome serological tests. Rapid reliable alternative tests are needed for early diagnosis of the disease. Considering the importance of the pathogenesis-associated leptospiral LigA protein expressed in vivo, we have evaluated its application in the diagnosis of the acute form of leptospirosis. The C-terminal coding sequence of *ligA* (*ligA-C*) was cloned into pET15b and expressed in *Escherichia coli*. Furthermore, the B-cell-specific epitopes were predicted and were synthesized as peptides for evaluation along with recombinant LigA-C. Epitope 1 (VVIENTPGK), with a VaxiJen score of 1.3782, and epitope 2 (TALSVGGSK), with a score of 1.2767, were utilized. A total of 140 serum samples collected from leptospirosis cases during the acute stage of the disease and 138 serum samples collected from normal healthy controls were utilized for evaluation. The sensitivity, specificity, positive predictive value, and negative predictive value were calculated for the recombinant LigA-C-specific IgM enzyme-linked immunosorbent assay (ELISA) and were found to be 92.1%, 97.7%, 92.8%, and 97.5%, respectively. Epitopes 1 and 2 used in the study showed 5.1 to 5.8% increased sensitivity over recombinant LigA-C in single and combination assays for IgM antibody detection. These findings suggest that these peptides may be potential candidates for the early diagnosis of leptospirosis.

Leptospirosis is a worldwide zoonotic disease affecting humans in rural and urban settings and in industrialized and developing countries. It is also an important public health problem in many parts of India and causes epidemics, especially after natural disasters such as floods and heavy monsoons (1). It is caused by spirochetes of the genus *Leptospira*, a group of bacteria that are morphologically, physiologically, metabolically, antigenically, and genetically distinct from other microorganisms. Clinical manifestations of acute leptospirosis range from mild febrile illness to more severe icteric Weil’s disease, which is characterized by renal and liver failure. Globally, 300,000 to 500,000 humans are affected by leptospirosis annually, according to International Leptospirosis Society global data collection (2). Leptospirosis is underdiagnosed for many reasons, including difficulty in distinguishing clinical signs from those of other endemic diseases and a lack of appropriate diagnostic laboratory services (3). Misdiagnosis of leptospirosis leads to severe complications including kidney damage, liver failure, respiratory distress, and meningitis leading to death.

The diagnosis of leptospirosis by the microscopic agglutination test (MAT) and *Leptospira* culture can be performed only by reference laboratories and requires specially trained personnel to interpret the results. Moreover, the MAT requires paired serum samples to achieve sufficient sensitivity. The sensitivity of other rapid and less complicated serological techniques, such as the Lepto dipstick assay, the Lepto Dri Dot test, and immunofluorescence assays, is insignificant, especially during the early phase of the disease (4). Most of these assays employ antigens from nonpathogenic *Leptospira biflexa* strain Patoc I and detection by cross-reactivity with repeating disaccharides of lipopolysaccharide (5). Molecular methods, including conventional and real-time PCR assays, were used to overcome the aforementioned diagnostic difficulties. However, the molecular methods are expensive due to the need for specialized equipment and expensive reagents that are not available during outbreak situations in the field and for routine diagnosis.

Recombinant antigen-based serological tests are being developed for spirochetal infections such as Lyme disease and syphilis (6, 7). Recently, recombinant proteins of *Leptospira*, including LipL32, LigA, LigA-C, and Hsp60, were widely characterized for their diagnostic and immunogenic potential (3, 8–11). Several studies have emphasized surface-exposed or secreted leptospiral proteins to induce better humoral immune responses in humans (12, 13). Such surface-exposed immunoreactive proteins can thus play a major role in the diagnosis of leptospirosis.

After leptospiral infection, the host produces antibodies to a myriad of bacterial antigens expressed constitutively or expressed in vivo. Antibodies to the latter antigens are the diagnostic targets of antibody-based assays for early diagnosis, especially during the acute phase of the illness. The antigens that are expressed in vivo interact with the immune system and prime an immune response to eliminate leptospires. Antibody responses peak during the acute phase of infection. In contrast, antibodies to whole-cell lysates and constitutively expressed proteins continue to be present at high titers. Similar results have been observed with the p35 and p37 proteins of *Borreliaburgdorferi*, which are expressed in vivo (14). Among various *Leptospira* antigens expressed in vivo, the immunoglobulin-like proteins LigA and LigB are found only on the surface of freshly or recently isolated pathogenic *Leptospira*.
Leptospira cultures and microscopic agglutination test. A panel of 12 reference strains were used for the MAT, including the following serogroups: Australis (serovar Australis), Autumnalis (serovar Autumnalis, strain Akiyami A), Ballum (serovar Ballum, strain Mus 127), Bataviae (serovar Bataviae, strain Swart), Canicola (serovar Canicola, strain Hond Utrecht IV), Icterohaemorrhagiae (serovar Icterohaemorrhagiae, strain RGA), Grippotyphosa (serovar Grippotyphosa, strain Moskva V), Hebdomadis (serovar Hebdomadis, strain Hebdomadis), Javanica (serovar Poi, strain Poi), Pomona (serovar Pomona, strain Pomona), Sejroe (serovar Hardjo, strain hardjoprajitno), and Pyrogenes (serovar Pyrogenes, strain Salinem). In addition to the reference strains, 101 serovars were included to study the efficiency of the ELISAs (Table 1).

The purpose of the present study was to compare the performance of five different ELISAs for the serological diagnosis of leptospirosis. ELISAs based on recombinant LigA-C (rLigA-C), a whole-cell lysate of *Leptospira interrogans* serovar Australis, synthetic epitope 1, epitope 2, and a combination of epitopes 1 and 2 were evaluated. This study was performed with a panel of human serum samples collected from patients with clinical diagnoses of leptospirosis.

### MATERIALS AND METHODS

#### Leptospira cultures and microscopic agglutination test

A panel of 12 reference strains were used for the MAT, including the following serogroups: Australis (serovar Australis, strain Ballico), Autumnalis (serovar Autumnalis, strain Akiyami A), Ballum (serovar Ballum, strain Mus 127), Bataviae (serovar Bataviae, strain Swart), Canicola (serovar Canicola, strain Hond Utrecht IV), Icterohaemorrhagiae (serovar Icterohaemorrhagiae, strain RGA), Grippotyphosa (serovar Grippotyphosa, strain Moskva V), Hebdomadis (serovar Hebdomadis, strain Hebdomadis), Javanica (serovar Poi, strain Poi), Pomona (serovar Pomona, strain Pomona), Sejroe (serovar Hardjo, strain hardjoprajitno), and Pyrogenes (serovar Pyrogenes, strain Salinem). In addition to the reference strains, the clinical isolates and the nonpathogenic strain Patoc I of serovar Serumanga were maintained. Clinical isolates included serovar Canicola of serogroup Canicola, serovar Icterohaemorrhagiae of serogroup Icterohaemorrhagiae, and serovar Javanica of serogroup Javanica. Leptospiral reference strains and isolates were maintained in EMJH medium at the Medical Microbiology Laboratory, Bharathidasan University (Tiruchirappalli, India). The MAT was performed using 7-day-old cultures grown at 30°C in EMJH medium (17).

#### Patients, case definitions, and ethics

In total, 140 serum samples with MAT titers of ≥1:160 (see Table S1 in the supplemental material) were selected from a bank of 476 laboratory-confirmed samples (positive IgM ELISA results, isolation of leptospires from the blood, seroconversion, or 4-fold titer increases) collected during the early phase of illness (0 to 10 days after the onset of disease) through an active hospital-based surveillance program in Tiruchirappalli. A total of 138 seronegative healthy controls selected from a group of cases matched with respect to age (± 5 years) and sex and 775 patients with diseases other than leptospirosis were also included to study the efficiency of the ELISAs (Table 1).

Patients’ sera were analyzed from the following control groups: patients with typhoid (*n* = 107), patients with malaria (*n* = 96), patients with hepatitis (*n* = 110), patients with dengue (*n* = 86), and patients who were hospitalized with clinical suspicion of leptospirosis and subsequently were diagnosed as having another illness based on laboratory and radiological evidence (*n* = 376). Written informed consent was obtained from both case and control subjects before blood sampling. The study protocol was approved by the institutional ethics committee of Bharathidasan University.

### SDS-PAGE and immunoblotting

SDS-PAGE was performed on 10% polyacrylamide gels using a discontinuous buffer system, as described elsewhere (18). The affinity-purified proteins were mixed with 2X SDS-PAGE sample loading buffer (125 mM Tris–HCl, 4% SDS, 2% glycerol, 1% β-mercaptoethanol, 0.5% bromphenol blue) and boiled for 5 min before loading. Electrophoresis was carried out in a vertical electrophoretic mini-cell unit (Bio-Rad, Hercules, CA), in Tris-glycine running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS [pH 8.3]), for 2 h at 120 V. Proteins were transferred to nitrocellulose membranes (pore size, 0.2 μm; Schleicher and Schuell, Keene, NH) and blocked with 4% nonfat dry milk in Tris-buffered saline (20 mM Tris, 150 mM NaCl, 0.05% Tween 20 [pH 7.5]). Membranes were incubated with serum samples from patients or hyperimmune sera raised in rabbits, followed by incubation with secondary antibody (anti-human IgG or anti-rabbit IgG conjugated with horseradish peroxidase [HRP]; Sigma, St. Louis, MO), and bands were visualized using 4-chloro-α-naphthol (Sigma, St. Louis, MO).

#### Prediction of LigA-C-specific B-cell epitopes

Protein sequences of cloned *LigA* from the *L. interrogans* serovar Australis, strain Ballico, were retrieved from NCBI and subjected to BCPreps analysis (19). BCPreps identifies common B-cell epitopes. Epitopes with BCPreps scores of >0.8 and VaxiJen scores of >0.4 were predicted to be highly immunogenic epitopes.

### Peptide synthesis and purification

The peptides listed were synthesized by the solid-phase method using 9-fluorenylmethoxy carbonyl (Fmoc) chemistry on a solid support of 4-methyl-benzhydryl-

---

**TABLE 1** Case definitions and groupings of the patients included in the study

| Group | Description | No. of cases |
|-------|-------------|--------------|
| a     | Clinically suspected laboratory-confirmed leptospirosis<sup>a</sup> | 140 |
| b     | Clinically suspected laboratory-negative leptospirosis<sup>b</sup> | 439 |
| c     | Seronegative healthy controls | 138 |
| d     | Typhoid | 107 |
| e     | Malaria | 96 |
| f     | Hepatitis | 110 |
| g     | Dengue fever | 86 |

<sup>a</sup> Confirmed cases of leptospirosis.

<sup>b</sup> Clinically suspected but serologically negative.
amino acid composition (see Fig. S2B in the supplemental material). No reactivity was observed on immunoblots probed with MAT-negative patient sera.

**Prediction of B-cell epitopes.** In total, six peptides were predicted to be immunogenic, of which two peptides, with sequences of VVIENTPGK (epitope 1) and TALSVGSSK (epitope 2) and Vaxijen scores of 1.3782 and 1.2767, respectively, were selected for synthesis and ELISA validation. The epitopes were predicted to lie in the C-terminal region at positions 1084 to 1092 (epitope 1) and 1189 to 1197 (epitope 2). The conservation of the epitopes was determined by multiple sequence alignment. The LigA sequences of *L. interrogans* serovar Copenagheni strain Fiocruz L1-130 (GenBank accession number AAS69086), *L. interrogans* serovar Pomona (GenBank accession number ACH89909), *L. interrogans* serovar Icterohaemorrhagiae (GenBank accession number ACH89908), and *L. interrogans* serovar Austrails (GenBank accession number AFG28560), *L. interrogans* serovar Lai strain Lai (GenBank accession number ACK58260), *L. interrogans* serovar Kennewicki (GenBank accession number ACH98097), and *Leptospira kirschneri* serovar Grippotyphosa (GenBank accession number AAP04735) were retrieved from GenBank and analyzed with the BioEdit sequence alignment editor (version 7.1.3.0). The two epitopes were found to be highly conserved among different serovars of *L. interrogans* and between *L. interrogans* and *L. kirschneri* species (Fig. 1). Antibodies to these epitopes were readily detected in human sera in the format of epitope-blocking ELISA.

**Liga-C-, epitope 1-, and epitope 2-based IgM ELISAs.** The overall results of the recombinant LigA-C-, epitope 1-, and epitope 2-based ELISAs are shown in Table 2 and Fig. 2A, B, C, and D. The mean ± 2 SD absorbance values for seronegative healthy individuals were defined as the cutoff values to achieve diagnostic specificity of the ELISAs in comparison with the MAT. The cutoff values were determined to be 0.209 for whole-cell lysate, 0.205 for rLigA-C, 0.190 for epitope 1, 0.191 for epitope 2, and 0.194 for the combination of epitope 1 and epitope 2. The ELISAs demonstrated sensitivity and specificity values of 85.2% and 85.7% for whole-cell lysate, 92.1% and 97.7% for rLigA-C, 97.1% and 98.2% for epitope 1, 97.9% and 98.4% for epitope 2, and 97.9% and 99.1% for epitope 1 and 2, respectively, in serum samples from confirmed cases of leptospirosis.

**DISCUSSION**

Leptospirosis is recognized as a globally reemerging public health problem; in humans, this disease may be fatal due to the potential damage to multiple organs such as liver, lung, kidney, and brain...
The LigA sequences of *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 (GenBank accession number AAS69086), *L. interrogans* serovar Pomona (GenBank accession number ACH8990), *L. interrogans* serovar Icterohaemorrhagiae (GenBank accession number ACU87695), *L. interrogans* serovar Australis (GenBank accession number AFG28560), *L. interrogans* serovar Lai strain Lai (GenBank accession number ACK58260), *L. interrogans* serovar Kennewicki (GenBank accession number ACH98097), and *L. kirschneri* serovar Grippotyphosa (GenBank accession number AAP04735) were analyzed with the BioEdit sequence alignment editor. *s*, epitope conservation.

FIG 1 Conservation of the peptides among different serovars of *Leptospira*. The conservation of the epitopes was determined by multiple sequence alignment. The LigA sequences of *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 (GenBank accession number AAS69086), *L. interrogans* serovar Pomona (GenBank accession number ACH89909), *L. interrogans* serovar Icterohaemorrhagiae (GenBank accession number ACU87695), *L. interrogans* serovar Australis (GenBank accession number AFG28560), *L. interrogans* serovar Lai strain Lai (GenBank accession number ACK58260), *L. interrogans* serovar Kennewicki (GenBank accession number ACH98097), and *L. kirschneri* serovar Grippotyphosa (GenBank accession number AAP04735) were analyzed with the BioEdit sequence alignment editor. *s*, epitope conservation.

TABLE 2 Sensitivities, specificities, positive predictive values, and negative predictive values for IgM ELISAs using whole-cell lysate, LigA-C, epitope 1, epitope 2, and epitopes 1 and 2

| Antigen       | Sensitivity (%) | Specificity (%) | PPV* (%) | NPVa (%) |
|---------------|----------------|-----------------|----------|----------|
| Whole-cell lysate | 85.2          | 85.7            | 82.3     | 83.4     |
| LigA-C        | 92.1           | 97.7            | 92.8     | 97.5     |
| Epitope 1     | 97.1           | 98.2            | 94.4     | 99.1     |
| Epitope 2     | 97.9           | 98.4            | 95.1     | 99.3     |
| Epitopes 1 and 2 | 97.9        | 99.1            | 97.2     | 99.3     |

*a* PPV, positive predictive value.

*b* NPV, negative predictive value.

Recent studies have shown that leptospirosis, especially an early sensitive and reliable diagnostic test, would improve patients’ quality of life (26), as *L. interrogans* can rapidly disseminate to multiple organs and cause multiorgan system complications, including jaundice, meningitis, pulmonary hemorrhage, hepatic and renal dysfunction, and cardiovascular collapse. Therefore, it is important to identify novel candidate antigens to improve diagnostic methods to assist in early treatment.

Recently, the improved serological diagnosis of leptospirosis has been targeted with recombinant proteins. This may achieve high sensitivity and specificity because of the high concentrations of immunogenic antigens and specific antigenic moieties in the purified fractions (27). The identification and characterization of a new family of Big domain proteins (bacterial immunoglobulin-like proteins), referred to as Lig proteins, in pathogenic *Leptospira* have been reported (8, 9). Previously published articles demonstrated that LigA is unique to pathogenic *Leptospira* species and that *Leptospira*-infected hosts produced antibodies to LigA (8, 9).

Furthermore, these proteins have been proven to be potential vaccine candidates for immunoprotection in infected hamster models (28). However, the diagnostic potential of these proteins in human cases, especially during the acute stage of the illness, is unconvincing. Previous reports on N-terminal recombinant Lig-based Western blot analysis gave a specificity of 93%, owing to the cross-reactivity of sera from patients with dengue fever, hepatitis, Lyme disease, or positive VDRL test results (16). Similarly, Srimanote et al. (3) reported the diagnosis of human leptospirosis using recombinant C-terminal LigA and described achievable specificity values of 100% and 98% for IgG and IgM ELISAs, respectively. In this regard, recombinant LigA-C is considered a good antigen for detecting antibodies in the sera of patients with suspected leptospirosis during the acute phase of the illness.

In this study, five different ELISAs were carried out to study the sensitivity and specificity for samples collected during the acute stage of leptospirosis. The C-terminal portion of ligA (bp 2214 to 3771) was cloned into pET15b and expressed in *E. coli* BL21(DE3).

The purified recombinant proteins were assayed for the detection of IgM-specific antibodies, and the results were compared with those for the whole-cell lysate and the predicted B-cell-specific peptides alone and in combination. The IgM ELISAs specific for the whole-cell lysate and recombinant LigA-C revealed sensitivity values of 85.2% and 92.1%, respectively. Interestingly, the B-cell-specific peptide-based ELISA showed increased sensitivity of 98%, i.e., ~13% and ~6% higher than the values for the whole-cell lysate- and recombinant LigA-C-specific ELISAs, respectively, confirming the peptides as promising diagnostic candidates.

The whole-cell lysate-based ELISA offers reasonable sensitivity and the possibility of handling many samples at one time; the major drawback of this test system is the need for the maintenance of live leptospires for antigen preparation. Moreover, the antigenic preparations are generally crude in nature and from a single serovar, with lipopolysaccharide as the major antigenic component. Since lipopolysaccharide is serovar specific, the antigens may not detect antibodies produced against serovars other than that used for antigen preparation, thus limiting the widespread use of the assay (11). The full-length recombinant LigA-C can serve as an effective reagent for immunodiagnostic testing with multiple epitopes. However, the highly antigenic epitopes are buried in the complex structures of proteins and hence are unavailable for the induction of immunogenic responses, consequently yielding reduced immune responses and decreased sensitivity in ELISAs.

As a result, immunodiagnostic tests utilizing peptides offer several advantages over diagnostic tests that rely on more-complex biological materials. For instance, synthetic peptides represent chemically defined antigens and, because they are not derived from biological material, assay standardization and validation are often greatly simplified. Peptide reagents also offer flexibility in terms of antigen specificity, including species-specific diagnostic testing. Furthermore, by screening overlapping peptides within an immunogenic protein, the highly specific epitopes can be maintained while peptide epitopes that are cross-reactive or that demonstrate poor specificity are purged.

In conclusion, our results showed increased sensitivity and specificity for peptide-based ELISAs for the diagnosis of human leptospirosis during the early stage of the disease. Although diagnostic tests based on a single peptide may lack sensitivity in certain cases due to the dependence on a single antibody epitope, the use of elongated peptides or multiple peptides may reduce or elimi-
nate this potential problem in the near future. Thus, LigA-C peptide-based ELISAs should be considered MAT alternatives in primary and secondary health care centers, not only because of their simplicity and rapid performance but also because of their affordability in developing countries where leptospirosis has been established as an endemic disease.

ACKNOWLEDGMENTS
We acknowledge the Department of Biotechnology (DBT), Ministry of Science and Technology, Government of India, for the research and development grant (BT/PR6872/MED/14/892/2005) that it provided to carry out this study.

We are grateful to Noriko Fujii, Research Reactor Institute, Kyoto University (Osaka, Japan), for the synthesis of peptides.

REFERENCES
1. Ratnam S, Sundararaj T, Subramaniana S. 1983. Serological evidence of leptospirosis in human population following an outbreak of the disease in cattle. Trans. R. Soc. Trop. Med. Hyg. 77:94–98.
2. World Health Organization. 1999. Leptospirosis worldwide, 1999. Wkly. Epidemiol. Rec. 74:237–242.
3. Srimanote P, Wongdeethai N, Jieanampunkul P, Samonkiert S, Leepiyasakulchai C, Kalambaheti T, Prachayasittikul V. 2008. Recombinant ligA for leptospirosis diagnosis and ligA among the Leptospira spp. clinical isolates. J. Microbiol. Methods 72:73–81. http://dx.doi.org/10.1016/j.mimet.2007.10.012.
4. Cumberland P, Everard CO, Levet PN. 1999. Assessment of the efficacy of an IgM-ELISA and microscopic agglutination test (MAT) in the diagnosis of acute leptospirosis. Am. J. Trop. Med. Hyg. 61:731–734.
5. Toyokawa T, Ohnishi M, Koizumi N. 2011. Diagnosis of acute leptospirosis. Expert Rev. Anti Infect. Ther. 9:111–121. http://dx.doi.org/10.1586/eri.10.151.
6. Magnarelli LA, Ijdo JW, Padula SJ, Flavelli RA, Fikrig E. 2000. Serologic diagnosis of Lyme borreliosis by using enzyme linked immunosorbent assays with recombinant antigens. J. Clin. Microbiol. 38:1735–1739.
7. Noordhoek GT, Cockayne A, Schouls LM, Meloen RH, Stolz E, van Embden JD. 1990. A new attempt to distinguish serologically the subspecies of Treponema pallidum causing syphilis and yaws. J. Clin. Microbiol. 28:1600–1607.
8. Haake DA, Chao G, Zuerner RL, Barnett JK, Barnett D, Mazel M, Matsunaga J, Levet PN, Bolin CA. 2000. The leptospiral major outer membrane protein LjIL32 is a lipoprotein expressed during mammalian infection. Infect. Immun. 68:2276–2283. http://dx.doi.org/10.1128/IAI.68.4.2276-2283.2000.
9. Palaniappan RU, Chang YF, Jusuf SS, Artiushin S, Timoney JF, McDonough SP, Barr SC, Divers TJ, Simpson KW, McDonough PL, Mohammed HO. 2002. Cloning and molecular characterization of an immunogenic LigA protein of Leptospira interrogans. Infect. Immun. 70:5924–5930. http://dx.doi.org/10.1128/IAI.70.11.5924-5930.2002.
10. Matsunaga J, Barocchi MA, Croda J, Young TA, Sanchez Y, Siqueira I, Bolin CA, Reis MG, Riley LW, Haake DA, Ko AI. 2003. Pathogenic Leptospira species express surface-exposed proteins belonging to the bacterial immunoglobulin superfamily. Mol. Microbiol. 49:929–945. http://dx.doi.org/10.1046/j.1365-2958.2003.03619.x.
11. Natarajaseenivasan K, Shanmughapriya S, Velineni S, Artiushin SC, Timoney JF. 2011. Cloning, expression, and homology modeling of GroEL protein from Leptospira interrogans serovar autumnalis strain N2. Genomics Proteomics Bioinformatics 9:151–157. http://dx.doi.org/10.1016/S1672-0229(11)60018-1.
12. Natarajaseenivasan K, Vijayachari P, Sugunan AP, Sharma S, Sehgal

FIG 2 Evaluation of different ELISAs with sera from patients with different clinical manifestations. Groups with different clinical manifestations are indicated on the x axis and the optical density (OD) at 490 nm on the y axis. IgM responses to recombinant LigA-C (A), epitope 1 (B), epitope 2 (C), and the combination of epitopes 1 and 2 (D) are shown. Study groups were as described in Table 1. The horizontal line shows the cutoff value for each ELISA.
13. Guerreiro H, Croda J, Flannery B, Mazel M, Matsunaga J, Galvao Reis M. 2001. Leptospiral proteins recognized during the humoral immune response to leptospirosis in humans. Infect. Immun. 69:4958–4968. http://dx.doi.org/10.1128/IAI.69.8.4958-4968.2001.

14. Fikrig E, Barthold SW, Sun W, Feng W, Telford SR, Flavell RA. 1997. Borrelia burgdorferi p35 and p37 proteins, expressed in vivo, elicit protective immunity. Immunity 6:531–539. http://dx.doi.org/10.1016/S1074-7613(00)80341-6.

15. Matsunaga J, Sanchez Y, Xu X, Haake DA. 2005. Osmolarity, a key environmental signal controlling expression of leptospiral proteins LigA and LigB and the extracellular release of LigA. Infect. Immun. 73:70–78. http://dx.doi.org/10.1128/IAI.73.1.70-78.2005.

16. Croda J, Ramos JGR, Matsunaga J, Queiroz A, Homma A, Riley IW, Haake DA, Reis MG, Ko AI. 2007. Leptospira immunoglobulin-like proteins as a serodiagnostic marker for acute leptospirosis. J. Clin. Microbiol. 45:1528–1534. http://dx.doi.org/10.1128/JCM.02344-06.

17. Faine S, Adler B, Perolat P, Bolin CA. 1999. Leptospira and leptospirosis, 2nd ed. MediSci, Melbourne, Australia.

18. Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

19. El-Manzalawy Y, Dobbs D, Honavar V. 2008. Predicting linear B-cell epitopes using string kernels. J. Mol. Recognit. 21:243–255. http://dx.doi.org/10.1002/jmr.893.

20. Merrifield RB. 1963. Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. J. Am. Chem. Soc. 85:2149–2154.

21. Terpstra WJ, Lighthart GS, Schoone CJ. 1985. ELISA for the detection of specific IgM and IgG in human leptospirosis. J. Gen. Microbiol. 131:377–385.

22. Verma A, Artiushin S, Matsunaga J, Haake DA, Timoney JF. 2005. LruA and LruB, novel lipoproteins of pathogenic Leptospira interrogans associated with equine recurrent uveitis. Infect. Immun. 73:7259–7266. http://dx.doi.org/10.1128/IAI.73.11.7259-7266.2005.

23. Timoney JF, DeNegri R, Sheoran A, Forster N. 2010. Affects of N-terminal variation in the SeM protein of Streptococcus equi on antibody and fibrinogen binding. Vaccine 28:1522–1527. http://dx.doi.org/10.1016/j.vaccine.2009.11.064.

24. Harlow E, Lane D. 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

25. Dutta TK, Christopher M. 2005. Leptospirosis: an overview. J. Assoc. Physicians India 53:545–551.

26. Lin YP, McDonough SP, Sharma Y, Chang YF. 2010. The terminal immunoglobulin-like repeats of LigA and LigB of Leptospira enhance their binding to gelatin binding domain of fibronectin and host cells. PLoS One 5:e11301. http://dx.doi.org/10.1371/journal.pone.0011301.

27. Flannery B, Costa D, Carvalho FP, Guerreiro H, Matsunaga J, Da Silva ED, Ferreira AG, Riley IW, Reis MG, Haake DA, Ko AI. 2001. Evaluation of recombinant Leptospira antigen-based enzyme-linked immunosorbent assays for the serodiagnosis of leptospirosis. J. Clin. Microbiol. 39:3303–3310. http://dx.doi.org/10.1128/JCM.39.9.3303-3310.2001.

28. Silva EF, Medeiros MA, McBride AJ, Matsunaga J, Esteves GS, Ramos JG, Santos CS, Croda J, Homma A, Dellagostin OA, Haake DA, Reis MG, Ko AI. 2007. The terminal portion of leptospiral immunoglobulin-like protein LigA confers protective immunity against lethal infection in the hamster model of leptospirosis. Vaccine 25:6277–6286. http://dx.doi.org/10.1016/j.vaccine.2007.05.053.