Serodiagnosis of Equine Leptospirosis by Enzyme-Linked Immunosorbent Assay Using Four Recombinant Protein Markers

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Leptospirosis, caused by Leptospira spp., is one of the most common zoonotic diseases in the world. We tested four recombinant proteins of Leptospira interrogans, namely, rLipL21, rLoa22, rLipL32, and rLigACon4-8, to evaluate their potential for use as antigens for the diagnosis of equine leptospirosis. We employed equine sera (n = 130) that were microscopic agglutination test (MAT) negative and sera (n = 176) that were MAT positive for the 5 serovars that most commonly cause equine leptospirosis.

The sensitivity and specificity of ELISA compared to MAT were 82.39% and 86.15%, respectively, for LigACon4-8, 77.84% and 92.31%, respectively, for Loa22, 77.84% and 86.15%, respectively, for LipL32, and 84.66% and 83.85%, respectively, for LipL21. When one of the two antigens was test positive, the sensitivity and specificity of ELISA were 93.75% and 78.46%, respectively, for rLigACon-4-8 and LipL32, 93.18% and 76.15%, respectively, for rLigACon-4-8 and LipL21, 89.77% and 80.77%, respectively, for rLigACon-4-8 and Loa22, 91.48% and 78.46%, respectively, for LipL21 and LipL32, 93.75% and 76.92%, respectively, for LipL21 and LipL32, and 90.34% and 80.77%, respectively, for Loa22 and LipL32. In conclusion, we have developed an indirect ELISA utilizing rLigaCon4-8, rLoa22, rLipL32, and rLipL21 as diagnostic antigens for equine leptospirosis. The use of four antigens in the ELISA was found to be sensitive and specific, the assay was easy to perform, and the results concurred with the results of the standard Leptospira MAT.

MATERIALS AND METHODS

Bacterial strain. L. interrogans serovar Pomona (NVSL 1427-35-093002) was used for this study (12). Leptospira isolates were maintained on Eltinghausen, McCullough, Johnson, and Harris (EMJH) medium at 30°C. Growth of Leptospira was monitored using dark-field microscopy.

Sera. All equine sera were collected from 2010 to 2012 by the New York State Animal Health Diagnostic Center (AHDC), Cornell University, Ithaca, NY. These serum samples were either positive or negative by MAT for the most common serovars causing equine leptospirosis, including L. interrogans serovar Pomona, L. kirschneri serovar Grippotyphosa, L. interrogans serovar Icterohaemorrhagiae, and L. interrogans serovar Bratislava.

Cloning, expression, and purification of proteins. pLip32L was cloned into pGEX4T2 by using the primers ATAGCGGCCGCAGGTGC TTTCGGTGGTCTG (forward) and GCCACCTTTCGGTACCTTTTT AACC (reverse). The PCR products derived from the genes encoding LipL21 (amplified with primers CCGGTTTCTGTTCCAGTACTGA

Received 28 October 2013 Returned for modification 18 November 2013 Accepted 17 January 2014 Published ahead of print 22 January 2014

Editor: W. R. Waters

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doi:10.1128/CVI.00649-13

April 2014 Volume 21 Number 4
CACA [forward] and ATTCTCGAGTTATTGTTTGGAAACCTCTTGA
GCTTTTG [reverse]) and Loa22 (amplified with primers CGCGGATCC
GAAAAAAAAGAGGAATCC [forward] and ATTCTCGAGTTATTGG
gTGTTGCGGA [reverse]) were cloned into pET28 (Invitrogen), with a
6-histidine tag at the 5’ end of the inserted DNA. The oligonucleotide
primer pairs were designed for each gene, with the incorporation of XhoI
at the 5’ end and EcoRI at the 3’ end (restriction sites are underlined in
primer sequences). The PCR products and plasmid vector were double
digested with those two enzymes and then ligated. For rLigACon4–8 con-
struction, we used forward primer A (5’-GATCCACTCCAGCCTTA-
3’) and forward primer B (5’-CACTCCAGCCTTA-3’) (both
complementary to LigA4) plus reverse primer C (5’-AGCCTTAAGAATT
GGGGAGT-3’) and reverse primer D (5’-TAAGAATTGCGGGAGT-
3’) (both complementary to LigA8) to generate a sticky-ended PCR
product. Two pairs of primers (A-D and B-C) were used to run PCRs indi-
vidually, and the PCR products were phosphorylated by using T4 polynu-
cleotide kinase at 37°C for 2 h and then ligated into pET28 cut with BamHI
and HindIII. The obtained recombinant gene was transformed into
Escherichia coli DH5α as the host strain. The DNA insert of each clone was verified by
DNA sequencing, and the recombinant plasmid was then transformed
into E. coli BL21(DE3) (Stratagene, Santa Clara, CA) for expression. Pro-
tein expression and purification were performed as previously described
(11). The concentration of purified protein was then determined using the
Bradford method, and the protein was finally used for ELISA (11).

**Leptospira MAT.** The MAT was used as the reference method to de-
termine serum titers, using live *L. interrogans* as antigen, as previously
described (11, 13).

**ELISA.** Indirect ELISA was performed as previously described (11),
using purified LigACon4–8, LipL32, Loa22, and LipL21 proteins.

**Western blot analysis.** Western blot analysis was performed as previ-
ously described (11), using purified rLigACon4–8, rLipL32, rLoa22,
and rLipL21 antigens.

**Statistical analysis.** The performance of the ELISA was evaluated us-
ing the MAT as the reference method (gold standard) (11). First, we com-
pared the ELISA results for the individual recombinant proteins to the

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**FIG 1** Expression of recombinant proteins. Analysis of affinity chromatogra-
phy-purified recombinant fragments was performed by Coomassie brilliant
blue staining of SDS-PAGE gels. Lane M, protein marker; lane 1, LipL21; lane
2, Loa22; lane 3, LipL32; lane 4, LigACon4–8.

**FIG 2** Graphs of IgG ELISA reactivities of 306 equine sera. The x axis indicates the
MAT titers of the tested sera. The y axis indicates the ELISA readings (OD450).
various concentrations (25, 50, 100, and 200 ng/well) in 100
ative sera were employed as positive and negative reaction controls,
1:2,000, and 1:4,000 dilutions). The equine MAT-positive and -neg-
itive buffer were added to each well and incubated at 4°C overnight,
were considered MAT positive (11,14). As previously reported,
easy recovery and purification.

results of the MAT. The accuracy of ELISA relative to the MAT was mea-
tured in terms of sensitivity and specificity. Sensitivity is the probability of
the respective protein test results being positive given that the MAT results
were positive. Specificity is the probability of the respective protein test
results being negative given that the MAT results were negative. Second,
we compared sensitivities and specificities by using the ELISA results for
each one, two, three, or four recombinant proteins compared to those of
the MAT, using the same accuracy measures.

RESULTS

Cloning, expression, and purification of recombinant proteins. All
the recombinant proteins were expressed and purified as His-
tagged fusion or glutathione S-transferase (GST)-tagged proteins
as previously described (11). SDS-PAGE and Coomassie blue
staining of the purified recombinant proteins revealed protein
bands corresponding to the expected sizes of the proteins (Fig. 1).
All these proteins were expressed in soluble form, which allowed
easy recovery and purification.

MAT. Sera with titers of ≥100 against one or more serovars
were considered MAT positive (11, 14). As previously reported,
most seropositive cases were positive for multiple serovars (11).

Optimization of antigen concentration in ELISA. Proteins at
various concentrations (25, 50, 100, and 200 ng/well) in 100 μl
coating buffer were added to each well and incubated at 4°C overnight,
while the test serum concentration also varied (1:500, 1:1,000,
1:2,000, and 1:4,000 dilutions). The equine MAT-positive and –neg-
ative sera were employed as positive and negative reaction controls,
respectively. A serum MAT titer of 1:800 was selected as the optimum
dilution, based on its optical density at 630 nm (OD630) in the range
of 0 to 1.0. For rLipL21, rLipL32, and rLoa22, a protein concentration
of 100 ng/well was selected for performing the assay, while 50 ng/well
was selected for the LigACon4-8 protein.

Evaluation of ELISA in comparison with MAT and Western
blot analysis. One hundred thirty negative and 176 positive serum
samples were used in this experiment (306 total serum samples). All
four recombinant proteins reacted with MAT-positive equine serum
samples, and the results are shown in Fig. 2 and Table 1. The sensitiv-
ity and specificity of ELISA compared to the MAT were 82.39% and
86.2%, respectively, for rLigACon4-8, 77.8% and 92.3%, respectively,
for rLoa22, 77.8% and 86.2%, respectively, for rLipL32, and 84.7%
and 83.8%, respectively, for rLipL21 (Table 2). When two to four
proteins were used and were all positive, we considered the ELISA
result to be positive; the sensitivity and specificity of ELISA are shown
in Table 3. The sensitivity and specificity of ELISA if one of these two
to four proteins was positive and the ELISA result was considered to
be positive are also shown in Table 3. Western blots of all MAT-
positive and -negative samples are shown in Fig. 3. Among MAT-
negative serum samples, 21, 10, 18, and 18 were ELISA positive for
rLipL21, rLoa22, rLipL32, and rLigACon4-8, respectively (Table 3).
Among the MAT-positive serum samples, 27, 39, 39, and 31 were
ELISA negative, and 19, 25, 29, and 22 were Western blot analysis
negative, for rLipL21, rLoa22, rLipL32, and rLigACon4-8, respect-
ively (Table 4). Interestingly, five of these negative samples were positive
for at least one of these four recombinant proteins (Table 5). Four of
these negative serum samples had a MAT titer of 1:200, while the other
had a MAT titer of 1:400.

DISCUSSION

Leptospirosis is an important zoonotic disease in the United States
and throughout the world (15–17). Leptospirosis is also an impor-
tant disease of horses, causing abortions and uveitis (18–20). The
diagnosis of leptospirosis by MAT, bacterial culture, PCR, real-
time PCR, and/or histopathological examination has been re-
ported previously (21). Because of the serious drawbacks of
these assays, numerous attempts have been made to develop an ELISA
serodiagnostic test (22–30) or to develop a dual-path platform

| Protein | % Sensitivity | % Specificity |
|---------|--------------|--------------|
| rLipL21 | 84.66        | 83.85        |
| rLoa22  | 77.84        | 92.31        |
| rLipL32 | 77.84        | 86.15        |
| rLigACon4-8 | 82.39   | 86.15        |

One of two, three, or four proteins was
positive, and the result was considered
positive

| Protein | % Sensitivity | % Specificity |
|---------|--------------|--------------|
| rLipL21 and rLoa22 | 71.02 | 97.69 |
| rLipL21 and rLipL32 | 68.75 | 93.08 |
| rLipL21 and rLigACon4-8 | 73.86 | 93.85 |
| rLoa22 and rLipL32 | 65.34 | 97.69 |
| rLoa22 and rLigACon4-8 | 70.45 | 97.69 |
| rLipL32 and rLigACon4-8 | 66.48 | 93.85 |
| rLipL21, rLoa22, and rLipL32 | 60.23 | 99.23 |
| rLipL21, rLipL32, and rLigACon4-8 | 60.80 | 96.15 |
| rLoa22, rLipL32, and rLigACon4-8 | 60.23 | 99.23 |
| rLipL21, rLoa22, rLipL32, and rLigACon4-8 | 55.68 | 100.00 |

| Protein | % Sensitivity | % Specificity |
|---------|--------------|--------------|
| rLipL21 and Loa22 | 91.48 | 78.46 |
| rLipL21 and LipL32 | 93.75 | 76.92 |
| rLipL21 and LigACon4-8 | 93.18 | 76.15 |
| Loa22 and LipL32 | 90.34 | 80.77 |
| Loa22 and LigACon4-8 | 89.77 | 80.77 |
| LipL32 and LigACon4-8 | 93.75 | 78.46 |
| LipL21, Loa22, and LipL32 | 95.45 | 73.08 |
| LipL21, LipL32, and LigACon4-8 | 96.59 | 71.54 |
| LipL22, LipL32, and LigACon4-8 | 96.02 | 74.62 |
| LipL21, Loa22, LipL32, and LigACon4-8 | 97.16 | 67.69 |

TABLE 1 Results of MAT, ELISA, and Western blot analyses of serum samples used in this study

| Protein | No. of serum samples | MAT, ELISA, and Western blot analysis |
|---------|----------------------|--------------------------------------|
| LipL21  | 130                  | 109 negative, 93 positive, 176 positive, 149 negative, 116 positive |
| Loa22   | 130                  | 120 negative, 90 positive, 176 positive, 137 negative, 102 positive |
| LipL32  | 130                  | 112 negative, 100 positive, 176 positive, 137 negative, 115 positive |
| LigACon4-8 | 130             | 112 negative, 97 positive, 176 positive, 145 negative, 116 positive |

TABLE 2 Sensitivity and specificity of ELISA when a single protein was evaluated

| Protein | % Sensitivity | % Specificity |
|---------|--------------|--------------|
| rLipL21 | 84.66        | 83.85        |
| rLoa22  | 77.84        | 92.31        |
| rLipL32 | 77.84        | 86.15        |
| rLigACon4-8 | 82.39   | 86.15        |
We previously used the rLigA protein for diagnosis of equine and canine leptospirosis (11, 32, 33). We hypothesized that the use of multiple antigens in the ELISA would improve the sensitivity and specificity of this serologic test. In this study, we used 4 different recombinant antigens, rLigACon4-8, rLipL32, rLipL21, and rLoa22, for further single-antigen ELISA evaluation of equine serum samples.

We used equine serum samples collected from the Animal Health Diagnostic Center (AHDC) at Cornell University. The AHDC indicates that five serovars occur commonly in New York State, and these are used routinely in our MAT for equine leptospirosis. From 2010 to 2012, we collected 176 MAT-positive and 130 MAT-negative equine sera for further ELISA evaluation using four antigens. The MAT targets both IgM and IgG but is skewed toward IgG (1, 34); therefore, we used the rLigACon4-8, rLipL32, rLipL21, and rLoa22 proteins as the coated antigens to establish an ELISA for improved detection of specific IgG in sera from equine patients with positive MAT titers.

A 4-fold rise in titer or seroconversion has been used as a definitive criterion for the serologic diagnosis of active leptospirosis. This requires collecting serum samples from the same animal 3 or 4 weeks later, and this delay is not practical in the clinical setting. Alternatively, a single high titer in the MAT may be taken as evidence of active infection. Therefore, the WHO Leptospirosis Burden Epidemiology Reference Group and the U.S. Centers for Disease Control and Prevention (CDC) recently defined a MAT titer of 400 in a single serum specimen as evidence supporting laboratory confirmation (35,36). A defined positive titer is also needed for horses. However, to our knowledge, no such titer has been defined for the diagnosis of animal leptospirosis. Based on the results from this study and a previous study (11), a definition similar to that of the WHO and CDC may be applied to equine leptospirosis, i.e., a MAT titer of 400.

The use of recombinant proteins as ELISA antigens for the diagnosis of leptospirosis in humans and other mammals was reported previously (25–28,32, 37–39). We reported the use of the Lig protein in the diagnosis of equine leptospirosis (11, 33). Hartleben et al. reported that the sensitivity and specificity of the rLipL32 ELISA for swine leptospirosis were 100% and 85.1%, respectively (26). Joseph et al. reported that the sensitivity and specificity of the rLipL21 ELISA for bovine leptospirosis were 100% and 97%, respectively (27). It has been reported that the efficiency of rLipL32 and rLoa22 in the diagnosis of human leptospirosis is 75%, whereas that of rLip21 was reported as only 68% (23). Only a few published reports detail the diagnosis of equine leptospirosis. Further studies are needed to address the diagnosis of equine leptospirosis by ELISA.

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**TABLE 4** Comparison of MAT-negative, ELISA- and Western blot-positive results and MAT-positive, ELISA- and Western blot-negative results

| Protein   | No. of MAT-negative samples ELISA positive | No. of MAT-positive samples ELISA positive Western blot positive | ELISA negative Western blot negative |
|-----------|------------------------------------------|---------------------------------------------------------------|-------------------------------------|
| LipL21    | 21                                       | 17                                                           | 27                                  | 19                                   |
| Loa22     | 10                                       | 7                                                            | 39                                  | 25                                   |
| LipL32    | 18                                       | 12                                                           | 39                                  | 29                                   |
| LigACon4-8| 18                                       | 13                                                           | 31                                  | 22                                   |

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![FIG 3](A) Western blot results for sera that were MAT negative but ELISA positive. (B) Western blot results for sera that were MAT positive but ELISA negative. The numbers indicate the horse serum numbers.
TABLE 5 Results of ELISA and Western blot analyses of the 79 samples that were MAT positive but ELISA negative for at least one of the four antigens

| Serum no. | LipL21 | Loa22 | LipL32 | LigACon4-8 |
|-----------|--------|-------|--------|------------|
| 1         | −/+    | −/+   | −/+    | −/+         |
| 2         | −/+    | −/+   | −/+    | −/+         |
| 3         | +/+    | −/+   | +/+    | +/+         |
| 4         | −/+    | +/+   | +/+    | +/+         |
| 5         | −/+    | +/+   | +/+    | +/+         |
| 6         | −/+    | +/+   | +/+    | +/+         |
| 7         | −/+    | +/+   | −/+    | +/+         |
| 8         | −/+    | −/+   | +/+    | +/+         |
| 9         | −/+    | −/+   | +/+    | +/+         |
| 10        | −/+    | −/+   | −/+    | +/+         |
| 11        | −/+    | −/+   | −/+    | +/+         |
| 12        | −/+    | −/+   | −/+    | +/+         |
| 13        | −/+    | −/+   | −/+    | +/+         |
| 14        | −/+    | −/+   | −/+    | +/+         |
| 15        | +/+    | −/+   | −/+    | +/+         |
| 16        | +/+    | −/+   | +/+    | +/+         |
| 17        | +/+    | +/+   | +/+    | +/+         |
| 18        | +/+    | −/+   | +/+    | +/+         |
| 19        | +/+    | −/+   | +/+    | +/+         |
| 20        | +/+    | −/+   | +/+    | +/+         |

TABLE 5 (Continued)

| Serum no. | LipL21 | Loa22 | LipL32 | LigACon4-8 |
|-----------|--------|-------|--------|------------|
| 64        | +/+    | −/+   | −/+    | +/+         |
| 65        | +/+    | −/+   | −/+    | +/+         |
| 66        | +/+    | −/+   | −/+    | +/+         |
| 67        | +/+    | −/+   | −/+    | +/+         |
| 68        | +/+    | −/+   | +/+    | +/+         |
| 69        | +/+    | −/+   | +/+    | +/+         |
| 70        | +/+    | −/+   | +/+    | +/+         |

a −, negative; +, positive.

found that 21, 10, 18, and 18 MAT-negative serum samples tested positive by ELISA when rLipL21, rLoa22, rLipL32, and rLigACon4-8, respectively, were used as antigens. We further evaluated these ELISA-positive serum samples by Western blot analysis and found that 17 of 21, 7 of 10, 12 of 18, and 13 of 18 of the above-mentioned samples, respectively, were also Western blot positive. This suggests that these horses were infected previously but that the MAT antibody titers to *Leptospira* lipopolysaccharide antigens declined to levels below the detection threshold (<1:100).

We also found that 27, 39, 39, and 31 MAT-positive serum samples were negative by ELISA when rLipL21, rLoa22, rLipL32, and rLigACon4-8, respectively, were used as antigens. However, Western blot analysis indicated that only five of these ELISA-negative samples were negative for all four recombinant antigens. All others were positive for at least one of these antigens (Table 4). It is unknown why the results were negative for all four antigens. However, we speculate that horses infected with either different *Leptospira* serovars or strains have differential expression of these antigens in vivo. In conclusion, the ELISA developed in this research, utilizing rLipL21, rLoa22, rLipL32, and rLigACon4-8 as antigens, could increase the sensitivity and specificity of ELISA for detection of leptospirosis in horses. This ELISA may be able to replace or supplement the current equine MAT for the diagnosis of equine leptospirosis in the near future, after further validation with more defined equine serum samples.

ACKNOWLEDGMENTS

This work was supported in part by the Biotechnology Research and Development Corporation (BRDC) and the New York State Science and Technology Foundation and Center of Advanced Technology (CAT) to Y.-F.C. C.Y. was supported by a scholarship from the China Scholarship Council (grant 2009850564).

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