Development of an Enzyme-Linked Immunosorbent Assay Using a Recombinant LigA Fragment Comprising Repeat Domains 4 to 7.5 as an Antigen for Diagnosis of Equine Leptospirosis

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Leptospira immunoglobulin (Ig)-like (Lig) proteins are a novel family of surface-associated proteins in which the N-terminal 630 amino acids are conserved. In this study, we truncated the LigA conserved region into 7 fragments comprising the 1st to 3rd (LigACon1-3), 4th to 7.5th (LigACon4-7.5), 4th (LigACon4), 4.5th to 5.5th (LigACon4.5–5.5), 5.5th to 6.5th (LigACon5.5–6.5), 4th to 5th (LigACon4-5), and 6th to 7.5th (LigACon6-7.5) repeat domains. All 7 recombinant Lig proteins were screened using a slot-shaped dot blot assay for the diagnosis of equine leptospirosis. Our results showed that LigACon4-7.5 is the best candidate diagnostic antigen in a slot-shaped dot blot assay. LigACon4-7.5 was further evaluated as an indirect enzyme-linked immunosorbent assay (ELISA) antigen for the detection of Leptospira antibodies in equine sera. This assay was evaluated with equine sera (n = 60) that were microscopic agglutination test (MAT) negative and sera (n = 220) that were MAT positive to the 5 serovars that most commonly cause equine leptospirosis. The indirect ELISA results showed that at a single serum dilution of 1:250, the sensitivity and specificity of ELISA were 80.0% and 87.2%, respectively, compared to those of MAT. In conclusion, an indirect ELISA was developed utilizing a recombinant LigA fragment comprising the 4th to 7.5th repeat domain (LigACon4-7.5) as a diagnostic antigen for equine leptospirosis. This ELISA was found to be sensitive and specific, and it yielded results that concurred with those of the standard MAT.
fic potential in equine leptospirosis with an indirect ELISA using one of these recombinant proteins.

**MATERIALS AND METHODS**

**Bacterial strain.** The *L. interrogans* serovar Pomona (NVSL 1427-35-093002) was used for this study (28). Leptospira isolates were maintained on Ellinghausen-McCullough-Johnson-Harris (EMJH) medium at 30°C. Growth of the Leptospira isolates was monitored using dark-field microscopy.

**Sera.** All equine sera were collected from 2005 to 2009 by the New York State Animal Health Diagnostic Center (AHDC), Cornell University, Ithaca, NY. These serum samples were either positive or negative in MAT to the most common serovars causing equine leptospirosis, including *L. interrogans* serovar Pomona, *L. kirschneri* serovar Grippotyphosa, *L. interrogans* serovar Icterohaemorrhagiae, and *L. interrogans* serovar Hardjo, *L. interrogans* serovar Grippotyphosa, *L. interrogans* serovar Icterohaemorrhagiae/Copenhageni, and *L. interrogans* serovar Canicola. Briefly, serial 2-fold dilutions of the sera, starting with a dilution of 1:10, were mixed with an equal volume of viable *Leptospira* strains in a 96-well microtiter plate. After incubation at 30°C for 2 h, the samples were examined for agglutination by dark-field microscopy. Titers represented the highest serum dilution showing 50% agglutination of the *Leptospira* cells in the suspension.

**Slot-shaped dot blot assay.** A total of 600 ng of purified protein in 200 μl PBS was transferred onto nitrocellulose membranes (Schleicher & Schuell Biosciences, Inc.) by using a Bio-Dot slot format (SE) microfiltration apparatus (Bio-Rad) according to the manufacturer’s instructions. After washing with Tris-buffered saline (TBS), the membranes were removed and soaked in the blocking solution, Tris-buffered saline (20 mM Tris-HCl, 500 mM NaCl [pH 7.5]) containing 5% (wt/vol) nonfat powdered milk for 1 h at 37°C on a shaker to saturate the remaining protein-binding sites. The blocked membranes were then washed three times (15 min each) with Tris-buffered saline containing Tween (TTBS) and probed with MAT-positive equine serum at 4°C overnight. After washing, the membranes were transferred to the alkaline phosphatase-labeled goat anti-horse IgG (KPL, Inc., MD) solution and incubated for 1 h at 37°C. After this, the membranes were incubated in freshly prepared 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium (BCIP-NBT) color development solution (Invitrogen) for 10 to 30 min and then rinsed in deionized water to stop the reaction.

**Quantitative slot blot assay for LigA fragment interaction with equine anti-Leptospira-positive sera.** To determine the intensity of the interaction between the LigA fragments and equine anti-Leptospira antibodies, we used a quantitative slot assay as previously described (30). The membrane to be analyzed was scanned immediately following the blotting procedure. The scanned image of the membrane was analyzed by Phoretix 1D (TotalLab Ltd., England) image processing and analysis software, which can calculate peak height and area for each slot on the membrane. A line graph for each lane was plotted with pixel intensity units on the y axis and pixel location (position from left to right of the membrane in each lane) on the x axis. Thus, each peak in the graphs represented a slot containing detected recombinant protein interaction with antibodies.

**TABLE 1 Primers used to amplify and clone L. interrogans serovar-specific sequences**

| Recombinant LigA fragment | Primer sequence
|----------------------------|------------------|
| LigACon1-3 | CGGGATCCATGGTAAACCCTTAAATTCCGG (F) |
| LigACon7-5 | CGGTGCGATATCGTTACTCCAGCA (F) |
| LigACon6-7.5 | CGGGCCGCCACATTCGTATTAGA (F) |
| LigACon4-5.5-5.5 | CGGGGAAATCCTTCTACTATCCAATGCATGTATGTAATCTCCT (F) |
| LigACon4-5 | CGGGGAAATCCTTCTACTATCCAATGCATGTATGTAATCTCCT (F) |
| LigACon5.5-6.5 | CGGGGAAATCCTTCTACTATCCAATGCATGTATGTAATCTCCT (F) |
| LigACon4 | CGGGGAAATCCTTCTACTATCCAATGCATGTATGTAATCTCCT (F) |

* Underlined nucleotides indicate the restriction site added to facilitate cloning, F, forward; R, reverse.
ELISA. Purified proteins were diluted in coating buffer (0.05 M NaHCO₃-Na₂CO₃ buffer [pH 9.6]) at optimum concentration established by checkerboard titration. A total of 100 µl of the diluted antigen was used to coat 96-well microtiter plates (Corning, NY), incubated at 4°C overnight, and blocked with 1% bovine serum albumin in PBS. To determine the optimum dilution to test sera, 10 sera representing negative (MAT titer of <100) and positive (MAT titer of >800) serum samples were diluted 2-fold from 1:50 to 1:400 in 1% bovine serum albumin in PBS. Each dilution of serum was tested for Leptospira-specific antibodies. The dilution that provided a high optical density (OD) value while effectively differentiating between negative and positive sera was selected as the optimum dilution for ELISA. The optimum dilution of conjugates for the assay was also determined by checkerboard titration. The dilution that gave the highest OD was selected as the optimum dilution.

The test sera were diluted at optimum dilution in PBS containing 1% bovine serum albumin and 0.05% Tween 20 and then added to the wells for 1 h at 37°C. The IgG reactivity was detected with peroxidase-labeled anti-horse IgG (KPL, Inc., MD) and tetramethylbenzidine (TMB) 2-component microwell peroxidase substrate (KPL, Inc., MD). The plates were read at an optical density of 450 nm (OD₄₅₀) on a microtiter plate reader (BioTek, VT) after the addition of the same volume of TMB stop solution (KPL, Inc., MD).

Western blot analysis. Sera that were MAT negative and ELISA positive were further tested by Western blotting. Purified recombinant LigACon4-7.5 (rLigACon4-7.5) was transferred from the SDS-PAGE separation gel to a nitrocellulose membrane (Schleicher & Schuell Biosciences, Inc.) using a Mini Trans-Blot system (Bio-Rad, CA) according to the instruction manual. After washing with TBS, the membranes were removed and soaked in the blocking solution, Tris-buffered saline (20 mM Tris-HCl, 500 mM NaCl [pH 7.5]) containing 5% (wt/vol) non-fat powdered milk, for 1 h at 37°C on a shaker to saturate the remaining protein-binding sites. The blocked membranes were then washed 3 times for 15 min each with Tris-buffered saline containing Tween (TTBS). After washing, the membranes were subjected to assay using the equine serum to be tested as the primary antibody and 1:3,000-diluted, alkaline phosphatase-labeled goat anti-horse IgG (KPL, Inc., MD) as the secondary antibody. After this, the membranes were incubated in freshly prepared BCIP-NBT color development solution (Invitrogen) for 10 to 30 min and then rinsed in deionized water to stop the reaction. A serum sample that was both MAT and ELISA negative was used as the negative control and an experimental positive serum was used as a positive control.

Additional samples from experimentally infected horses. A total of 39 serum samples from 3 experimentally infected horses were included.

RESULTS

Cloning, expression, and purification of recombinant proteins. All the recombinant proteins were expressed as GST fusion proteins and the GST tags were excised by treatment with thrombin. SDS-PAGE and Coomassie blue staining of the purified recombinant proteins revealed the protein bands corresponding to the expected size of the proteins (Fig. 2). However, LigACon4-5 (lane 3) with an extra band is either a contaminant or its degraded product. All of these proteins were expressed in soluble form to retain their native conformation and allowed easy recovery and purification.

MAT. Serum with a titer equal to or higher than 1:100 against one or more serovars was considered MAT positive. As we reported before (31, 32), multiple titers against different serovars were detected in most seropositive cases.

Slot-shaped dot blot assay. All recombinant proteins tested reacted with the Leptospira-positive serum; however, the level of reactivity varied (Fig. 3). LigACon4-7.5 showed the highest intensity when analyzed by the image analysis software. Thus, recom-
binant protein LigACon4-7.5 was chosen as the candidate antigen for the ELISA.

Optimization of reagents for ELISA. A checkerboard titration technique was used to determine the optimum concentrations of reagents for ELISA. With the use of the equine serum with the high MAT titer, the optimum concentration of recombinant antigen yielding high specificity was obtained at 25 ng/well and the optimum dilutions of primary and secondary conjugated antibodies were found to be 1:250 and 1:500, respectively. The cutoff value of the ELISA was determined from 60 MAT-negative equine sera. The mean OD value was 232 with a standard deviation (SD) of 101. The cutoff value for estimation of positive and negative samples was 333 (mean +1 SD).

Detection of serum samples from experimentally infected horses. The earliest antibody response was detected at day 3 postchallenge with Leptospira. The OD reading of the ELISA increased as days postinfection increased (Fig. 4) and peaked at 14 days postinfection, which is consistent with the MAT results. However, the increasing of IgG as early as 3 days postinfection was unexpected. The early antibody response suggests that all three horses had been exposed to Leptospira before challenge, even though the MAT was negative before challenge.

Evaluation of ELISA in comparison with MAT. All together a total of 280 horse serum samples were studied with both ELISA and MAT. Sixty of the samples were negative and 220 positive by MAT; 76 were negative and 204 positive by ELISA. ELISA had a sensitivity of 87.2%, a specificity of 80.0%, and an accuracy of 85.7%, based on the assumption that the MAT test, considered the gold standard for Leptospira antibody detection, is 100% accurate (Fig. 5).

Western blot analysis. Of the sera that were negative in MAT but positive in ELISA, 10 recognized the LigACon4-7.5 fragment in the Western blot (WB) assay (Fig. 6). This indicated that some of these horses may have been infected before and that antibody levels declined below the detection threshold of MAT. However, these low antibody levels were detected by ELISA and confirmed by Western blotting when the LigACon4-7.5 antigen was used.

DISCUSSION
Although MAT is an imperfect reference test (33), it is still considered the gold standard for leptospirosis diagnosis in both humans and animals (2,32). Most state animal diagnostic laboratories in the United States use MAT for the serologic diagnosis of animal leptospirosis. Because of the serious drawbacks of the MAT assay, numerous attempts have been made to develop a serodiagnostic ELISA (10–18) or to develop a dual-path platform (DPP) assay (19). We previously used the LigACon protein for diagnosis of equine and canine leptospirosis (26, 27). The full length of LigA was poorly expressed in Escherichia coli, as previously described (27). The full length of LigA was poorly expressed in Escherichia coli, as previously described (27). Therefore, we were not able to use the full length of LigA in this study. The N-terminal 630 amino acids of LigA and LigB are conserved. However, the N-terminal conserved region is very large (62 kDa) (20). LigACon was expressed well in E. coli; however, it was in an insoluble form (inclusion body) (26, 34). Therefore, we hypothesized that one of several truncated LigA conserved regions would be expressed well and in a soluble form that could be useful as a source of antigens in a serologic-testing format for animal leptospirosis. We truncated the LigA conserved region into 7 fragments and performed a quantitative slot blot assay to assess the interaction of the LigA fragments with equine
anti-\textit{Leptospira}-positive sera. The rationale to use a quantitative slot blot assay was that the test proteins were not in denatured condition and thus retained their native conformation to a large extent. So, at least in principle, most of the antibodies from infected animals should interact with these nondenatured proteins (35). This is especially important for detection of antibodies that bind to higher-order protein structures. In this study, we found that LigACon4-7.5 gave the strongest response and thus it was chosen for further ELISA evaluation.

Results from the Animal Health Diagnostic Center indicate five serovars that occur commonly in New York State and are used routinely in our MAT for equine leptospirosis. We collected 220 MAT-positive and 60 MAT-negative equine sera from the 2005-2009 period, as well as equine serum samples from our previous challenge study (31) for further ELISA evaluation. Since the MAT targeted both IgM and IgG but skewed toward IgG (2, 32), we used a LigACon4-7.5 fragment as the coating antigen to establish an ELISA for improved detection of specific IgG in sera from equine patients with positive MAT titers. In this study, we found that 95% of the equine serum samples with MAT titers of 1:100 or 1:200 was positive by ELISA. This is not surprising, as a titer of 1:100 or 1:200 is considered low positive and may require other clinical data, follow-up serological testing, or a direct demonstration of the presence of \textit{Leptospira} in samples, such as a positive blood culture, to help establish the accuracy of the low-titer MAT results. This information was not available, so we cannot prove that these se-

![FIG 5](graph_of_elisa_samples.png) Graph of the ELISA samples showing the IgG ELISA reactivity of 220 equine sera. The x axis indicates the MAT titers of the tested sera. The y axis indicates the ELISA reading of OD\textsubscript{450}.

![FIG 6](western_blot_results.png) Western blot results of sera that were MAT negative but ELISA positive. Lanes 1 through 12, sera that are MAT negative and ELISA positive; lane 13, MAT positive and ELISA positive (positive control); lane 14, MAT negative and ELISA negative (negative control).
rum samples with low MAT titers are truly positive for leptospirosis.

MAT has some limitations for the diagnosis of leptospirosis. Currently, a 4-fold rise in titer or seroconversion is the most definitive criterion for diagnosis of active leptospirosis. Therefore, a second sample is required, which is often not practical in the clinical setting. In such circumstances, a single high titer in MAT may be taken as evidence of active infection. For human cases, laboratory case definitions have been developed for single (unpaired) MAT results. The WHO Leptospirosis Burden Epidemiology Reference Group recently defined a MAT titer of 1:400 in a single serum specimen as evidence supporting laboratory confirmation (http://www.who.int/zoonoses/diseases/lerg/en/). A similar definition has been made by the U.S. Centers for Disease Control and Prevention (CDC) (http://www.cdc.gov/NNDSS/script/casedef.aspx?CondYrID = 907&DatePub = 1/1/2013%2000:00 %20AM). However, to our knowledge, no such titer has been defined for animal leptospirosis diagnosis. A defined positive titer is also needed in horses. Based on the results from this study, a definition similar to those of the WHO and CDC may be applied to equine leptospirosis, i.e., a 1:400 MAT titer in a single serum specimen.

The sensitivity and specificity of this ELISA are 80.0% and 87.2%, respectively. These values are based on the assumption that the MAT is 100% accurate. If we had additional defined leptospira-positive serum samples for this study, the sensitivity and specificity of this ELISA could be higher. In our experimental challenge horses, we performed the MAT before challenge, and the MAT titers of these horses were all below 1:100. However, all three horses developed anti-LigACon4-7.5 IgG antibodies 3 days after challenge. It has been reported that 7 days after the onset of clinical symptoms in human patients, 62% had anti-LigB IgG antibodies as detected by WB analysis (13). Similar results with IgG antibody responses to other leptospiral recombinant proteins have also been reported (12, 36–38). This suggests that these horses were previously infected with Leptospira and developed a memory response upon re-exposure to Leptospira. However, another possible explanation for the robust IgG response to LigACon4-7.5 or other leptospiral antigens may be a rapid antibody class switch during the incubation period, as previously described (13, 32). This phenomenon has also been found in Lyme disease and syphilis patients (39–41).

Interestingly, 12 sera were negative by MAT but positive in our ELISA. We subjected these sera to WB analysis, and to our surprise, 10 out of 12 sera with a negative MAT titer were positive by WB analysis. This suggests that these horses were infected previously but the MAT antibody titers to Leptospira lipopolysaccharide antigens declined below the detection threshold (<1:100). Another possibility is that these horses were infected with other pathogens and developed cross-reacting antibody to LigACon4-7.5. Further study is needed to clarify this issue.

In conclusion, LigACon4-7.5 is a good candidate antigen to develop an ELISA to detect leptospirosis in horses. This ELISA may replace or supplement the current equine MAT for the diagnosis of equine leptospirosis in the near future after further validation with additional defined equine serum samples.

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