Competitive Enzyme-Linked Immunosorbent Assay for Detection of
*Leptospira interrogans* Serovar pomona Antibodies in Bovine Sera

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Received 9 May 2000/Returned for modification 14 August 2000/Accepted 13 October 2000

A competitive enzyme-linked immunosorbent assay (ELISA) using a specific monoclonal antibody (M898) was developed for detection of bovine serum antibodies to *Leptospira interrogans* serovar pomona. This assay was evaluated using field sera (n = 190) with serovar pomona microscopic agglutination test (MAT) titers of \( \geq 100 \) as the positive population (group A); field sera (n = 1,445) which were negative in the MAT (1:100 dilution) for serovar pomona (group B); and sera (from a specific-pathogen-free cattle herd [n = 210]) which were negative in the MAT (1:100 dilution) for serovars canicola, copenhageni, grippotyphosa, hardjo, pomona, and sejroe (group C). At the cutoff point recommended by receiver operating characteristic (ROC) curve analysis, of the combined ELISA results of serum groups A, B, and C, the sensitivity and specificity values were 93.7 and 96.3%, respectively. The value for the area under this ROC curve was 0.977, indicating a high level of accuracy for the ELISA. Similar results were obtained from the analysis of the combined results of serum groups A and B and from the analysis of the combined results of serum groups A and C.

In the Canadian cattle population, leptospirosis is predominantly caused by serovar hardjo (now generally accepted as *Leptospira borgpetersenii* serovar hardjo type hardjobovis) and serovar pomona (1, 6, 7, 8, 9, 12, 13, 14, 15). Other serovars such as grippotyphosa and icterohaemorrhagiae have also been detected but at relatively lower levels (6, 7, 13). Direct detection of these organisms by microscopic examination or culture is impractical due to the low success rate and the amount of time and labor required. Instead, leptospirosis is most often diagnosed serologically with the microscopic agglutination test (MAT) (2). The MAT however, despite its widespread usage and international recognition, is cumbersome with a number of limitations. These include the need to use hazardous live bacteria and the amount of time and labor required to test each serum sample against multiple serovars of this organism. In addition, the lack of standard operating procedures and source strains among laboratories and the subjective scoring of results may cause quality assurance difficulties. Due to the drawbacks of the MAT we are developing alternative diagnostic tests for the detection of *Leptospira* serovars which are of economic importance to Canada.

In a previous publication (20), we described two monoclonal antibodies (M897 and M898) that are suitable for incorporation into competitive enzyme-linked immunosorbent assays (ELISAs) for the specific detection of serum antibodies to serovar pomona. In this communication, we report the results of a validation study of a competitive ELISA that was developed with monoclonal antibody M898 for the detection of bovine serum pomona antibodies.

**MATERIALS AND METHODS**

*Bacterial culture and MAT.* The *Leptospira* organisms were cultured and the MAT was performed as previously described (20).

**Bovine sera.** Field serum samples submitted to Canadian Food Inspection Agency laboratories across Canada were collected and tested by the MAT. Of these sera, 190 with serovar pomona MAT titers of \( \geq 100 \) (group A) and 1,445 which were serovar pomona MAT negative at a 1:100 dilution (group B) were included in this study. Some of these sera also had MAT titers of \( \geq 100 \) for serovars other than pomona. Two hundred and ten sera (group C) from a specific-pathogen-free (SPF) herd of cattle were also tested. These sera were negative in the MAT at a 1:100 dilution for serovars canicola, copenhageni, grippotyphosa, hardjo, pomona, and sejroe. All sera were stored at \(-20^\circ\text{C}\) and thawed at room temperature before testing.

**ELISA.** The monoclonal antibody (M898) was produced as described (20). The antigen was prepared from serovar pomona cells as described (20) and then sonicated for 2 min with a 375-W cell disruptor (Heat Systems-Ultrasonics Inc., Farmingdale, N.Y.). The assay was performed as described (20) except for the following modifications. Batches of microtiter plates were coated with the antigen, incubated overnight at room temperature, and then frozen at \(-20^\circ\text{C}\). The plates were thawed at room temperature and washed before use. Four controls (each in quadruplicate wells) were included in every plate. In the first (uninhibited control), the bovine serum was replaced with phosphate-buffered saline-Tween (PBST). The second control consisted of a serovar pomona MAT-negative serum. Conditions of the assay were adjusted so that at 10 min of substrate-chromogen development, an optical density (OD) value of approximately 1.0 was obtained for the PBST and the negative serum controls. The third control was a medium-titer-positive serum which gave an optical density value of approximately 0.50 at 10 min, and the fourth control was a high-titer-positive serum which gave an optical density value of \(<0.10\) at 10 min. Both of the positive control sera were obtained from cows naturally infected with serovar pomona. In the control wells all other reagents were added in the exact amounts and sequence as described.

**Acceptance criteria.** Results of the entire plate were rejected unless the mean OD at 414 nm (OD\textsubscript{414}) values of the controls were within predetermined limits (established by performing the test at least 40 times) with a coefficient of variation of \( \leq 10\% \) for the medium-titer-positive serum, the negative serum, and the PBST controls. A coefficient of variation of \( \leq 50\% \) was acceptable for the high-titer-positive serum control, which gave OD\textsubscript{414} values of \(<0.10\). In addition, for plates with acceptable control OD\textsubscript{414} values, the results for test samples were rejected unless the coefficient of variation of the duplicate OD\textsubscript{414} values was \( \leq 10\% \), except for OD\textsubscript{414} values of \(<0.10\), when a coefficient of variation of \( \leq 50\% \) was accepted.

**Data expression and analysis.** The results of the assay were expressed as percent inhibition of the binding of the monoclonal antibody to the antigen (%I), which was calculated as described (11). Receiver operating characteristic (ROC) curve analysis (MedCalc Software, Mariakerke, Belgium) was performed on the ELISA results to determine the optimal cutoff point (at which the sum of the sensitivity and specificity values is highest) for distinguishing between positive and negative results. The area under the ROC curve (AUC), which can be used...
as a measure of the accuracy of the test, was also calculated. Separate analyses were performed on the ELISA results of (i) serum groups A and B; (ii) serum groups A and C; and (iii) serum groups A, B, and C.

RESULTS

In this competitive ELISA, the majority of the negative sera yielded %Is of $\leq 10$ and the majority of the positive sera yielded %Is of $\geq 20$. There was also an area of overlap involving relatively few numbers of both positive and negative sera (Fig. 1). This indicated that the cutoff point for differentiating between positive and negative samples would be %I between 10 and 20. ROC curve analysis was then used to optimize the cutoff point.

The ROC curve analysis of the combined ELISA results of serum groups A and B yielded an AUC of 0.975 and recommended a cutoff point of $\geq 13\%$ inhibition of the binding of the monoclonal antibody to the antigen. At this cutoff point, the sensitivity estimate was 93.7\% (± 3.5\% with 95\% confidence limits) and the specificity estimate was 96.3\% (± 0.97\% with 95\% confidence limits). The analysis of the combined results of serum groups A and C yielded an AUC of 0.987 and recommended a cutoff point of $\geq 12\%$ inhibition, with sensitivity and specificity estimates of 94.7\% (± 3.2\% with 95\% confidence limits) and 96.2\% (± 2.6\% with 95\% confidence limits), respectively. The analysis of the combined results of serum groups A, B, and C yielded an AUC of 0.977 and recommended a cutoff value of $\geq 13\%$, with a sensitivity estimate of 93.7\% (± 3.5\% with 95\% confidence limits) and a specificity estimate of 96.3\% (± 0.91\% with 95\% confidence limits).

The ELISA sensitivity and specificity estimates that were obtained from the three analyses and the respective AUC values are summarized in Table 1. The ROC curve obtained from the analysis of the combined ELISA results of serum groups A, B, and C is shown in Fig. 2.

DISCUSSION

Many countries stipulate that animals be tested for specific Leptospira serovars as part of their requirements for the international trade of livestock and their products. In addition, some countries also require that cattle which are resident in artificial insemination centers be similarly tested on a periodic basis. In Canada, cattle in artificial insemination centers are tested for a number of serovars, including canicola, copenhageni (represents icterohaemorrhagiae), grippotyphosa, hardjo, pomona, and sejroe. Due to the problems associated with the MAT, we have undertaken the development of sensitive and specific ELISAs to detect antibodies to each of the six serovars.

TABLE 1. Summary of the results of the ROC curve analyses performed on different combinations of the competitive ELISA data obtained from the three groups of sera tested

<table>
<thead>
<tr>
<th>Serum group combination*</th>
<th>Recommended cutoff (%I)</th>
<th>% Sensitivity (95% CL [%])$^+$</th>
<th>% Specificity (95% CL [%])$^+$</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>A + B</td>
<td>$\geq 13$</td>
<td>93.7 (± 3.5)</td>
<td>96.3 (± 0.97)</td>
<td>0.975</td>
</tr>
<tr>
<td>A + C</td>
<td>$\geq 12$</td>
<td>94.7 (± 3.2)</td>
<td>96.2 (± 2.6)</td>
<td>0.987</td>
</tr>
<tr>
<td>A + B + C</td>
<td>$\geq 13$</td>
<td>93.7 (± 3.5)</td>
<td>96.3 (± 0.91)</td>
<td>0.977</td>
</tr>
</tbody>
</table>

* Group A, serovar pomona MAT-positive field sera (n = 190); group B, serovar pomona MAT-negative field sera (n = 1,445); group C, sera from an SPF herd (n = 210).
$^+$ %I of the binding of the monoclonal antibody to the antigen.
$^+$ 95\% confidence limits (95\% CL) are given parenthetically.

FIG. 1. Frequency distribution of the competitive ELISA results of 1,655 serovar pomona MAT-negative (NEG) and 190 serovar pomona MAT-positive (POS) bovine sera. %I of the binding of the monoclonal antibody to the antigen is shown in 10% increments (x axis) against the number of observations (y axis).
listed above. The availability of such a package of ELISAs would then present a practical alternative to the MAT for the diagnosis of these important *Leptospira* serovars. As part of this endeavor, we have already reported an indirect ELISA (18) and a competitive ELISA (19) for detection of antibodies to serovar hardjo type hardjobovis and an indirect ELISA (17) for detection of antibodies to serovar pomona. In this communication, we report the development and evaluation of a monoclonal antibody-based competitive ELISA for detection of bovine antibody to serovar pomona. The monoclonal antibody (M898) that has been incorporated into this ELISA is very specific for serovar pomona. This monoclonal antibody does not cross-react with any of 13 other pathogenic serovars, including those known to occur in North America (1, 3, 6, 7, 8, 9, 12, 13, 14, 15, 16, 21, 22) and thus the most likely cause of cross-reaction in the local cattle population, nor does it cross-react with *L. biflexa* serovar patoc, an ubiquitous nonpathogen (20).

The ELISA data were subjected to ROC curve analysis. This type of analysis has been used to evaluate the ability of a test to discriminate between infected and healthy subjects (10, 23) and to compare the diagnostic performance of two or more tests (5). With ROC curve analysis, the sensitivity and specificity values are estimated for every possible cutoff point that is selected to distinguish between a positive and a negative result. The cutoff point at which the sum of the sensitivity and specificity estimates is highest is usually recommended. However, depending on the intended application of the test, a higher sensitivity or specificity than that recommended may be desired, and this can be achieved by an appropriate adjustment of the cutoff value. The ROC curve is obtained by plotting the true-positive rate (sensitivity) as a function of the false-positive rate (100 – specificity) that is associated with each cutoff point. The AUC can be used as a measure of the accuracy of the test. If the test cannot distinguish between infected and normal populations the AUC will be equal to 0.5 and the ROC curve will coincide with the diagonal. On the other hand, if the test is 100% sensitive and specific, then the AUC will be equal to 1 and the curve will reach the upper left corner.

The AUC values obtained from the three ROC curve analyses conducted on the ELISA data were all relatively high, indicating that this assay is very accurate. Each analysis also recommended approximately the same cutoff point, with similar sensitivity and specificity estimates. Two of the three analyses only differed by the source of the pomona-negative serum population included in each (sera from an SPF herd and negative field sera), and the third contained both of these negative serum populations. These results indicate that in regards to their reactivity in this ELISA, there was little difference between the SPF sera and the pomona-negative field sera that were used in this study. At either of the two recommended cutoff points, more than 96% of these pomona MAT-negative sera were also negative in the ELISA. The approximately 4% of these sera that were positive in the ELISA may have contained low levels of antibodies that were not detectable in the

![ROC curve](http://cvi.asm.org/)

**FIG. 2.** ROC curve obtained from analysis of the ELISA results of all of the bovine sera tested (groups A, B, and C). The true-positive rate (sensitivity [y axis]) is plotted against the false-positive rate (100 – specificity [x axis]) for each %I cutoff point applied. An optimal %I cutoff point of ≥13 is indicated.
MAT at a 1:100 dilution. It is also possible that some of these sera may have contained nonagglutinating anti-pomona antibodies which cannot be detected by a test such as the MAT, but which can be detected by an ELISA which measures the primary binding function of antibodies. At either of the two recommended cutoff points, this ELISA detected anti-pomona antibodies in approximately 94% of the sera that had pomona MAT titers of $\geq 100$. This indicated that the epitope recognized by monoclonal antibody M898 is highly immunodominant in cattle. The sera that were not detected by the ELISA could have been falsely positive in the MAT or perhaps contained antibodies to epitopes on the pomona antigen which are different from the one recognized by the M898 monoclonal antibody.

At this point it is not known whether this ELISA can distinguish between the antibodies produced in response to infection and those stimulated by vaccination. The MAT cannot make this distinction (4), and it would be useful to develop assays that can, so that vaccinated animals are not unnecessarily treated with antibiotics.

The repeatability of this ELISA is 100% when performed under the conditions described. The protocol for production of the antigen used in this ELISA is relatively simple and highly reproducible. The use of frozen antigen-coated plates allows for testing during every working day.

In conclusion, we report the development and validation of a highly sensitive and specific competitive ELISA for the detection of bovine antibodies to \textit{L. interrogans} serovar pomona. This test uses nonhazardous reagents, is repeatable, is scored objectively, is semiautomated, and can be subjected to stringent quality assurance protocols. This competitive ELISA has the potential to become a useful tool for the diagnosis of serovar pomona infection in cattle.

ACKNOWLEDGMENTS

We thank S. Duff for technical assistance with the MAT, the personnel from the Canadian Food Inspection Agency laboratories across Canada for collecting the field sera, and the Animal Diseases Research Institute (Lethbridge, Alberta, Canada) for donating the sera from the SPF herd. We also thank C. Elmgren for producing the monoclonal antibody.

REFERENCES