Rapid, Field-Adapted Indirect Enzyme-Linked Immunosorbent Assay for Detection of Antibodies in Bovine Whole Blood and Serum to Brucella abortus

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A simple, rapid, field-adapted indirect enzyme-linked immunoassay (FldELISA) for the detection of antibodies to Brucella abortus in whole blood and serum has been developed. This assay detects antibodies to B. abortus in approximately 15 min or less. Over a 3-month period, this assay has consistently identified immunized and nonimmunized animals, while the percent coefficient of variation for each immunized animal has been less than 20%. As with any indirect enzyme-linked immunoassay, quality control can be established and maintained. Using defined positive and negative sera, the sensitivity and specificity of the FldELISA was 100% and 94.2%, respectively. As a model, this test can be readily extended to other disease applications that use lipopolysaccharide or other stable antigens for the detection of antibodies, such as those to Salmonella spp., Escherichia coli, or Yersinia spp.

Since the development of the first agglutination test for brucellosis by Wright and Smith (21), veterinary laboratory workers have been developing tests to improve diagnostic performance and accuracy. Among the myriad tests developed were rapid agglutination tests for the detection of antibodies to Brucella abortus in cattle sera, such as the Rose Bengal Test (7), the Card Test (9), and the Buffered Antigen Plate Agglutination Test (BPAT) (1). These tests, using acidified antigens, were developed to improve accuracy, while at the same time they expedited turnaround time of results. The purpose of the acidified antigens was to reduce agglutination by immunoglobulin M, thus reducing nonspecific false-positive reactions. While rapid, the tests were largely laboratory based and subjective in the interpretation of results. With the exception of the BPAT, they did not significantly improve test accuracy (6).

The next evolution in rapid test technology for detection of brucellosis occurred with the development of immunochromatographic assays (2, 10, 20). These tests involve the attachment of B. abortus whole-cell antigen or lipopolysaccharide onto a compatible membrane (i.e., test strips) such as cellulose acetate or nitrocellulose, respectively. While faster than conventional enzyme-linked immunoassays, these tests were not field adapted.

In 2001, Nielsen et al. introduced a fluorescence polarization assay (FPA) for the diagnosis of bovine brucellosis (14) that was subsequently adapted for field use based on B. abortus O-polysaccharide conjugated with fluorescein isothiocyanate (12). The sensitivity and specificity relative to the BPAT and the competitive enzyme-linked immunoassay were 95.0% and 97.3%, respectively (15). Using this test, individual whole blood samples (EDTA treated) could be tested in less than 2 min chute side or in the field. The only limitation of this assay system was the initial size of the molecule required for labeling with fluorescein isothiocyanate, such as the B. abortus O-poly saccharide that was hydrolyzed to an average molecular mass of 20 to 30 kDa (12).

For enzyme-linked immunoassays, there is no limitation on the molecular size. We have modified an indirect enzyme-linked immunoassay (IELISA) with a previously reported diagnostic sensitivity and specificity of 95.9% and 100%, respectively (11). From the IELISA, a rapid field-adapted enzyme-linked immunoassay (FldELISA) for the detection of whole-blood and serum antibody to B. abortus using a battery-operated portable photometer for assessment of results was developed. This test relies on the attachment of a 50:50 mixture of B. abortus smooth lipopolysaccharide (SLPS) and rough lipopolysaccharide (RLPS) to polystyrene microstrips, followed by incubation with diluted blood and horseradish peroxidase-conjugated recombinant protein A/G, respectively.

MATERIALS AND METHODS

Experimental population group. Whole-blood samples were collected sequentially every 7 days postimmunization from five cows (C55, C85, C311, C367, and H11003) in Freund’s complete adjuvant and reimmunized periodically intramuscularly with B. abortus strain 1119-3 (1 × 1010) in physiological saline and reimmunized periodically intramuscularly with the same cell concentration in physiological saline.

Similar blood samples were collected from two additional nonimmunized cows as negative controls. C35 was also immunized subcutaneously with killed B. abortus strain 1119-3 (1 × 1010) in Freund’s complete adjuvant and reimmunized periodically intramuscularly with the same cell concentration in physiological saline.

Control sera. Two control sera were used. They consisted of a pooled strong positive anti-Brucella abortus serum and a negative serum. A minimum of three replicates per control per microstrip was used.

Validation sera. Negative sera from a random selection of Canadian cattle (n = 480) were obtained from a national survey conducted in 2002. Canada has been officially free of bovine brucellosis since 1984. Fifty-one Canadian bovine sera from animals from which B. abortus was isolated from tissues or secretions were used to determine sensitivity. These sera were obtained from a serum bank established prior to 1984.

Assay development and optimization. Concentrations and dilutions of reagents were determined through checkerboard titrations. The optimization of the assay, including incubation times, was determined as previously described (4).

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Photometers. Photometers used were Labsystems, Multiskan EX, and the battery-operated portable photometer (Biotek EL310) (Fig. 1).

Preparation of substrate chromogen. The initial stock supply was prepared using 0.3 g of 3,3'-5,5'-tetramethyl-benzidine (TMB) dissolved into 30 ml of dimethyl sulfoxide. To 120 ml of citrate buffer (pH 4.0), 6 ml of TMB and 0.6 ml of 3% hydrogen peroxide were added. The solution was thoroughly mixed and frozen at −70°C. After freezing, the substrate chromogen was lyophilized and subsequently reconstituted with pyrogen-reduced 18-MΩ or equivalent water to the original volume. The reconstituted substrate chromogen was then stored at room temperature.

Field-adapted indirect enzyme-linked immunoassay (FldELISA) for detection of antibody in whole blood and serum. Each well of polystyrene microstrips (NUNC 469922) was passively coated with 100 µl of 0.06 M carbonate buffer, pH 9.6, containing a 50:50 mixture of B. abortus SLPS at 1 µg/ml and RLPS at 5 µg/ml. The combined SLPS and RLPS antigen (17) in the microstrips was incubated overnight for more than 18 h at 25°C to 30°C. The next day the microstrips were emptied and allowed to air dry overnight, sealed for later use, and stored at room temperature. For the test, 5-µl volumes of serum controls and whole-blood or serum samples were added to 95 µl of 0.01 M phosphate-buffered saline, pH 7.2, containing 0.05% Tween 20 (PBST) into each well of the microstrips. The microstrips were shaken for 10 s and incubated for 5 min at ambient temperature. Following incubation, the microstrips were washed four times with PBST using an Eppendorf repeater adapted with an eight-channel dispensing head. After washing, 100 µl of an appropriate amount of horseradish peroxidase-conjugated recombinant protein A/G diluted in PBST was added to each well, shaken for 10 s, and incubated for 5 min. The microstrips were shaken for another 10 s prior to the measurement of the optical density (OD) at 620 or 650 nm in a photometer.

Data analysis. Test results were expressed as a percentage of the sample OD reading of the mean OD of the positive control included on each microstrip at the 5-min development time. Percent positivity (%P) was calculated with the following equation: %P = (OD of the test sample/mean OD of the positive control) × 100.

A one way analysis of variance (8) was performed on the raw optical density measurements of the six animals in the experimental population group to determine variation between days using reconstituted substrate chromogen.

Using receiver operating characteristics, the data from the validation sera were analyzed (8). This analysis determined the optimal cutoff value between the defined positive sera (n = 51) and the negative sera (n = 480) to achieve the optimal sensitivity and specificity estimates. In addition, the area under the curve was determined, indicating the accuracy of the field-adapted indirect enzyme-linked immunoassay.

The upper and lower control limits for the positive and negative controls (n = 78) were determined using three standard deviations as previously described (4).

Percent coefficient of variation (%CV) is a measure of dispersion about the mean and expresses the standard deviation as a percentage of the mean.

RESULTS

Sequential whole-blood samples were collected weekly for a 3-month period to determine if the field-adapted indirect enzyme-linked immunoassay could correctly identify positive and
negative animals (i.e., accuracy). The samples were tested, and the results were plotted as shown in Fig. 2. The mean %P for the immunized animals C55, C85, C311, C367, and C620 were 103.0%, 94.5%, 77.2%, 78.9%, and 89.7%, respectively. For the two negative control animals (C308 and C309), the mean %P was 9.6% and 7.8%, respectively. The %CV for the immunized animals were 8.0%, 6.1%, 15.3%, 18.8%, and 11.0%, respectively. The %CV for the two negative control animals were 65.3% and 51.4%, respectively.

Three replicates of each control serum, a strong positive and a negative, were included on each microstrip to determine serum control limits (Fig. 3). Each point represents a raw optical density measurement at 620 nm. The upper and the lower control limits were determined using three standard deviations from the mean. A downward trend in the positive control suggests the slow degradation of the reconstituted substrate chromogen. However, neither control has exceeded the lower control limit previously set.

Using the positive and negative control sera, reconstituted substrate chromogen stability was followed for 30 days as shown in Fig. 4. Each point represents the mean optical density of three measurements, and there were two measurements per

FIG. 2. Comparison of the field-adapted indirect enzyme-linked immunoassay. Percent positivity data (y axis) of the experimental group of 10 observations of different days postimmunization representing 3 months (x axis).

FIG. 3. Standard quality control chart with upper and lower control limits set at three standard deviations for the positive and negative control sera, respectively. The control limits and average means are depicted as dashed lines. The x axis shows the number of observations, while the y axis is in units of optical density at 620 nm.
day for each control. The substrate chromogen slowly declined, as evidenced by the trend line for the positive control. However, as shown in Tables 1 and 2 and Fig. 5, a one-way analysis of variance of the whole-blood samples of the experimental population group, which were also measured along with the control sera, indicated no significant difference (F = 1.0) between day measurements. The calculated F statistic (0.096) also was less than the tabulated F statistic (1.897), indicating that the population means were equal; thus, the null hypothesis was not rejected.

Based on the defined negative (n = 480) and positive (n = 51) validation sera, the sensitivities and specificities of the field-adapted indirect enzyme-linked immunosassay at various cutoffs were calculated by receiver operating characteristics and are shown in Table 3. With sensitivity and specificity of 100% and 94.2%, respectively, the optimal cutoff for percent positivity as shown by the software was 13.2%. The area under the curve was 0.994, indicating that in more than 99% of the cases the validation sera were correctly identified.

**DISCUSSION**

Using a tentative cutoff of 13.2% (17), the results from Fig. 2 show that the performance of the field-adapted indirect enzyme-linked immunoassay has been accurate (i.e., the ability of a test to correctly identify positive and negative animals) and repeatable (i.e., consistent results in repeated tests) over a 3-month period. In only three instances did the %P for the negative animals (C308 and C309) exceed 13.2%. Since the specificity of the validation sera was 94.2% (95% confidence interval, 91.7 to 96.1), these were not unexpected and were probably due to nonspecific interference (19). The %CV measures the dispersion around each mean, suggesting relatively little variation with the immunized animals, while the higher variation for negative sera was due to small differences in the low optical density values resulting in higher %CVs (5).

Figure 3 demonstrated the relative stability of both control sera until approximately day 17, when the positive control results began to decline. The decline was less noticeable with the negative control results due to the low optical density values. One reason for the decline was probably the slow deterioration of the reconstituted substrate chromogen. The data suggested that the optimal shelf life of the reconstituted substrate chromogen was between 10 and 17 days after reconstitution, confirming stability problems previously reported (3). However, in spite of the slow decline, the substrate chromogen was still usable for at least 30 days. The data are shown in Fig. 4, in which each point represents the mean optical density of three measurements with two independent measurements per day for each control. Again, a downward trend was evident but was less dramatic due to the mean OD values. Analysis of variance (Tables 1 and 2 and Fig. 5) of the data from the associated experimental group suggest that there was no significant difference (F = 1.0) between days up to day 30, further suggesting that the reconstituted substrate chromogen was usable for at least 30 days. This was collaborated by the F statistic.
TABLE 3. Comparison of sensitivities and specificities with 95% confidence intervals (95% CI) at various cutoffs for the FldELISA as well as the area under the curve, with standard errors and 95% CI*

<table>
<thead>
<tr>
<th>Cutoff</th>
<th>Sensitivity (95% CI)</th>
<th>Specificity (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥2.8</td>
<td>100.0 (93.0–100.0)</td>
<td>0.0 (0.0–0.8)</td>
</tr>
<tr>
<td>&gt;13.2b</td>
<td>100.0 (93.0–100.0)</td>
<td>94.2 (91.7–96.1)</td>
</tr>
<tr>
<td>&gt;14.9</td>
<td>98.0 (89.5–99.7)</td>
<td>94.2 (91.7–96.1)</td>
</tr>
<tr>
<td>≥16.6</td>
<td>98.0 (89.5–99.7)</td>
<td>94.4 (91.9–96.3)</td>
</tr>
<tr>
<td>&gt;16.9</td>
<td>96.1 (86.5–99.4)</td>
<td>94.4 (91.9–96.3)</td>
</tr>
<tr>
<td>&gt;20.1</td>
<td>96.1 (86.5–99.4)</td>
<td>95.4 (93.1–97.1)</td>
</tr>
<tr>
<td>&gt;20.4</td>
<td>94.1 (83.7–98.7)</td>
<td>95.6 (93.4–97.3)</td>
</tr>
<tr>
<td>&gt;20.6</td>
<td>92.2 (81.1–97.8)</td>
<td>95.6 (93.4–97.3)</td>
</tr>
<tr>
<td>&gt;24.7</td>
<td>92.2 (81.1–97.8)</td>
<td>97.5 (95.7–98.7)</td>
</tr>
<tr>
<td>≥28.9</td>
<td>88.2 (76.1–95.5)</td>
<td>97.5 (95.7–98.7)</td>
</tr>
<tr>
<td>&gt;29.4</td>
<td>88.2 (76.1–95.5)</td>
<td>97.9 (96.2–99.0)</td>
</tr>
<tr>
<td>&gt;33.0</td>
<td>86.3 (73.7–94.3)</td>
<td>97.9 (96.2–99.0)</td>
</tr>
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<td>&gt;40.4</td>
<td>86.3 (73.7–94.3)</td>
<td>99.2 (97.9–99.8)</td>
</tr>
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<td>&gt;42.3</td>
<td>82.4 (69.1–91.6)</td>
<td>99.2 (97.9–99.8)</td>
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<td>&gt;47.3</td>
<td>82.4 (69.1–91.6)</td>
<td>98.0 (96.0–100.0)</td>
</tr>
<tr>
<td>&gt;50.0</td>
<td>78.4 (64.7–88.7)</td>
<td>98.8 (96.8–100.0)</td>
</tr>
<tr>
<td>&gt;55.2</td>
<td>78.4 (64.7–88.7)</td>
<td>100.0 (99.2–100.0)</td>
</tr>
<tr>
<td>&gt;118.0</td>
<td>0.0 (0.0–7.0)</td>
<td>100.0 (99.2–100.0)</td>
</tr>
</tbody>
</table>

* Defined positive sera; 51; defined negative sera, 480; area under the receiver operating characteristics curve, 0.994; standard error, 0.008; 95% confidence interval, 0.983 to 0.999.

b The optimal cutoff as determined by receiver operating characteristics.

(Table 1), indicating no difference between the population means.

The use of combined antigens increased the ability of the field-adapted indirect enzyme-linked immunoassay (FldELISA) to detect antibodies to either smooth or rough lipopolysaccharide or both (17). Combined antigens are also more likely to detect antibodies in more animals exposed to SLPS or RLPS than enzyme-linked immunoassays using only SLPS or RLPS as an antigen, as reported by Nielsen et al. (17). The use of horseradish peroxidase-conjugated recombinant protein A/G with the binding characteristics of both protein A and G (18) as a universal detection agent would allow this assay to detect antibodies to all Brucella spp. in cattle, dogs, goats, sheep, bison, deer, and swine (16).

Compared to the field-adapted fluorescence polarization assay (12), the sum of the sensitivity and specificity of the FldELISA (194.2) marginally exceeded the sum of the field-adapted fluorescence polarization assay (192.3). The sum of the sensitivity and specificity of the IELISA (11) from which the FldELISA was modified was 195.9, a difference of 1.7. Compared to the field-adapted fluorescence polarization assay (FPA) can be used to test whole blood in a field setting. Like the FPA, this test can be extended to other disease applications using carbohydrate antigens for the detection of antibodies, such as Salmonella spp., Escherichia coli, or Yersinia spp. Quality control can be established and maintained to meet ISO 17025 accreditation standards as well.

ACKNOWLEDGMENT

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REFERENCES

14. Nielsen, K., D. Gall, P. Smith, W. Kelly, J. Yeo, K. Kenny, T. Heneghan, (WHO) website (http://www.who.int/diagnostics_laboratory/faq/simple_rapid_tests/en/) for rapid and simple tests for use in resource-poor settings. It was also quick and easy to perform, easily meeting the 10-min-to-2-h period shown on the website. Other criteria described on the WHO website were same-day results and the possibility to store samples at room temperature for extended periods of time, both of which the FldELISA meet. Additionally, the results from the FldELISA are easily interpretable.


