Evaluation of serological tests for detection of caprine antibody to *Brucella melitensis*

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**Abstract**

A total of 2213 goat sera were tested by the indirect enzyme immunoassay (IELISA), the competitive enzyme immunoassay (CELISA), the fluorescence polarization assay (FPA), complement fixation test (CFT) and the buffered antigen plate agglutination test (BPA T) for their antibody against *Brucella melitensis*. Of these, 528 blood samples collected in EDTA were tested in the field by the FPA. In addition, 249 goat milk samples were tested by IELISA (mELISA). The serum samples were classified based on their reactivity in the BPA T and CFT. A total of 716 sera were positive while 1497 gave negative reactions in both tests. Relative to the BPA T and CFT reactivity, the sensitivity and specificity of the assays were as follows: IELISA: 96.2 and 99.7%, CELISA: 93.6 and 99.4% and FPA: 88.7 and 98.9%, respectively. The bFPA gave sensitivity and specificity values relative to the BPA T and CFT of 92.3 and 61.6%, while the mELISA based on the serum BPA T and CFT of the paired animals gave a relative sensitivity value of 90.9% and a relative specificity value of 92.4%.

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1. **Introduction**

Control programs for brucellosis depend on presumptive diagnosis of infection by serological tests. The accuracy of the serological tests used, therefore, has considerable impact on the success of a program.
Thus, if the tests are prone to give false negative results, infected animals will be missed and false positive reactions lead to condemnation of animals. Both scenarios have an adverse effect on control campaigns. In addition, other factors are important, including the test cost, ease of performance, test precision, interference by antibody resulting from vaccination or cross-reacting antigens and turn around time for results.

A number of serological tests have been developed for diagnosis of brucellosis in cattle. Most of these tests have been used for diagnosis in other species without proper validation. The first serological test developed was a slow agglutination test for the diagnosis of human brucellosis (Wright and Smith, 1897). Since then, a number of modifications have been made to the agglutination test to increase the specificity and to decrease time to obtain test results (reviewed by MacMillan, 1990). The CFT was developed (Hill, 1963) but used mostly as a confirmatory test because of its complexity and expense. The prescribed tests for brucellosis in goats (and sheep) by the OIE are the buffered antigen plate agglutination tests and the CFT (OIE Manual of Standards, 2000).

Indirect enzyme immunoassays were developed as a replacement for the CFT and while the sensitivity of IELISA was an improvement, a relative lack of specificity resulted from interference by cross-reacting antibodies. These problems were largely overcome by the development of the CELISA (Nielsen et al., 1989; MacMillan et al., 1990) which excluded interfering antibody in most cases and it was rapid compared to the CFT. It was, however, not cost effective to perform a small number of tests, leading to the development of the FPA. The FPA has been adapted for detection of antibody in serum to both *Brucella abortus* and *B. suis* (Nielsen et al., 1996a, 1999). The premise for the FPA is that a small molecule in solution randomly rotates at a rate inversely proportional to its size. Using an attached fluorochrome and plane polarized light allows measurement of the rate of rotation through a given angle. Thus, a small molecule rotating at a high rate will revolve through the angle rapidly, resulting in a low polarization value. However, attachment of antibody to the small molecule causes a size increase resulting in a decrease in the rate of rotation and a higher polarization value. Based on this, *B. abortus* O-polysaccharide, labelled with fluorescein isothiocyanate, was used as an antigen to estimate antibody to *B. abortus* in blood, serum or milk. The FPA is a homogeneous assay which is rapid, relatively inexpensive and can be conducted under field conditions.

This study was undertaken to compare the FPA originally developed for detection of bovine antibody to *B. abortus* as a diagnostic tool for the presumptive diagnosis of *Brucella melitensis* infection in goats with those obtained using the IELISA and the CELISA and to evaluate the bFPA, using whole blood and the mELISA for detection of antibody in milk.

2. Material and methods

2.1. Test samples

Goat sera tested by the IELISA, CELISA and FPA were obtained from seven flocks in Mexico, from which, *B. melitensis* was isolated from at least one animal and a number of Canadian goats from approximately 20 flocks (considered negative for *B. melitensis* which has not been isolated in Canada). The sera were segregated into two groups: 716 sera that gave positive reactions in the BPA T and CFT, and 1497 sera which did not react in the BPA T/CFT. No Canadian sera reacted in both the BPA T and the CFT.

In the bFPA, a total of 528 blood samples were tested. In addition, 249 milk samples from goats of known BPA T/CFT status were tested by the mELISA.

2.2. Serological tests

The BPA T and Rose–Bengal agglutination test (RBT) were performed as prescribed by the OIE Manual of Standards (2000).

The CFT used serum diluted in 96 well plates, incubated at 5 °C as described by Samagh and Boulanger (1978). A reaction of 50% hemolysis at a dilution of 1:5 or higher was considered positive. A number of anti-complementary sera were excluded.

The IELISA and CELISA (Nielsen et al., 1996b; Biancifiori et al., 2000) were performed. For the IELISA, smooth lipopolysaccharide (SLPS) from *B. abortus* strain 1119.3 was used as the antigen, immobilized on polystyrene plates. Sera diluted 1:50 were tested. For milk, the dilution was 1:2. Reactivity was determined using a mouse monoclonal antibody specific for bovine IgG conjugated with horseradish
peroxidase. This monoclonal antibody has been shown to cross-react extensively with IgG of sheep and goats (Henning and Nielsen, 1992). For the CELISA, immobilized LPS was used as the antigen. Serum diluted 1:10 was added to each well at the same time as an equal volume of prediluted mouse monoclonal antibody specific for a common epitope of the O-polysaccharide (OPS) of the SLPS molecule. Reactivity of the mouse antibody was detected using a commercially available goat antibody to mouse IgG, conjugated with horseradish peroxidase. Hydrogen peroxide substrate and ABTS chromogen were developed for 10 min in both the IELISA and CELISA and optical density measurements were obtained without using a stopping reagent. For the IELISA and mELISA, data was calculated as percent positivity (% P) based on a strongly positive serum from each species included as a control in each plate. For the CELISA, data was calculated based on a buffer control (uninhibited) included in each plate and used to calculate the percent inhibition (% I). All enzyme immunoassay (ELISA) plates contained the following controls: a strongly positive, a weakly positive, a negative serum and a buffer control (no serum).

The FPA was detailed by Nielsen et al. (1996a). Briefly, B. abortus S1119.3 O-polysaccharide hydrolyzed to an average molecular weight of 22 kDa was conjugated with fluorescein isothiocyanate and used as an antigen. Goat blood collected in EDTA or sera were diluted 1:25 in 0.01 M Tris buffer, pH 7.2 with 0.15 M NaCl, 15 mM EDTA and 0.05% Igepal CA630 (previously NP40) for testing. Background activity was measured in each serum or blood sample using a fluorescence polarization analyzer (Diachemix Corp., Whitefish Bay, Wisconsin, USA) and after incubation for 15 s or a minimum of 2 min for serum with antigen, a second reading was obtained. The results were recorded in millipolarization (mP) units.

2.3. Data analysis

Receiver operator characteristic (ROC) analysis was used to determine optimum cut-off values of each test using MedCalc software (Schoonjans et al., 1995). The optimum cut-off value is that which gives the highest sum of the sensitivity and specificity values. Relative sensitivity and specificity values were calculated for the IELISA, CELISA and FPA using the goat sera classified as positive or negative by the BPA T/CFT both of which are OIE prescribed tests. The IELISA data was expressed as a percent of a strongly positive serum or milk (% P) included with each test. The CELISA data was expressed as percent inhibition (% I) relative to an uninhibited (buffer) control included with each test. The FPA results were expressed in millipolarization units (mP). Area under the ROC curve (AUC) was determined. The AUC is an indication of test accuracy. For a simple determination of the most effective assay, the sensitivity and specificity values were added and their sum was considered an index of performance.

Negative and positive predictive values were calculated for the IELISA, CELISA, FPA, mELISA and bFPA at 0.01, 0.10, 1.0, 10 and 20% disease prevalences.

3. Results

Optimum cut-off values for the IELISA, CELISA, FPA, bFPA and mELISA were 51% P, 25% I, 88 mP, 103 mP and 51% P, respectively, as determined by ROC analysis. Confirmation of the infection by bacteriological culture was not available for the goat sera used in this study. The BPAT and CFT were used as selection criterion and the sensitivity and specificity values presented in Table 1 for the tests are relative to the BPAT/CFT (OIE prescribed tests). The IELISA performed better than the CELISA and the FPA. Vaccination with B. melitensis Rev1 frequently results in sera being BPAT, CFT and IELISA positive and CELISA and FPA negative (Biancifiori et al., 2000), most likely resulting in

<table>
<thead>
<tr>
<th>Test</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PI</th>
<th>AUC</th>
</tr>
</thead>
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<tr>
<td>IELISA</td>
<td>96.2</td>
<td>99.7</td>
<td>195.9</td>
<td>0.990</td>
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<tr>
<td>CELISA</td>
<td>95.6</td>
<td>99.4</td>
<td>193.0</td>
<td>0.987</td>
</tr>
<tr>
<td>FPA</td>
<td>88.7</td>
<td>96.9</td>
<td>187.6</td>
<td>0.981</td>
</tr>
<tr>
<td>bFPA</td>
<td>92.3</td>
<td>61.6</td>
<td>153.9</td>
<td>0.842</td>
</tr>
<tr>
<td>mELISA</td>
<td>90.9</td>
<td>92.4</td>
<td>183.3</td>
<td>0.954</td>
</tr>
</tbody>
</table>

Some goats (n = 532) were tested by the bFPA and some matched milk samples (n = 249) were tested by mELISA. All values are relative to results of BPAT/CFT data. A performance index (PI), the sum of the percent sensitivity and specificity is indicated for each test.
the low sensitivity values observed for the CELISA and FPA in this study.

The bFPA, testing 528 blood samples immediately after collection, resulted in a sensitivity value of 92.3% and a specificity value of 61.6% relative to BPA T/CFT test performed subsequently in the laboratory. One reason for the low specificity value was difficulties in hemolyzing the red blood cells which appear somewhat more resistant to lysis compared to bovine erythrocytes, leading to elevated mP values.

The mELISA performed quite well considering the quality of the milk samples, many of which resembled butter more than milk. The results of 90.9% sensitivity and 92.4% specificity were determined using the BPA T/CFT obtained with the matching serum samples.

Based on the sensitivity and specificity shown in Table 1, the negative and positive predictive values were calculated for disease prevalences of 0.01, 0.10, 1.0, 10 and 20% as shown in Fig. 1. Positive predictive value is the probability that the disease is present when the test is positive. Conversely, the probability that the disease is not present when the test is negative is known as the negative predictive value. In Fig. 1, as the disease prevalence increases, the positive predictive value for the IELISA, CELISA and FPA serum approaches 100%. The negative predictive value is the similar for all tests (approximately, 100%).

4. Discussion

The sensitivity and specificity values estimated in this communication are relative to the BPA T/CFT, the two serological tests prescribed by the OIE Manual of Standards (2000) for the serological diagnosis of caprine brucellosis.

The most suitable cut-off value for each test may be established in a number of different ways (Gall and Nielsen, 2001), however, ROC analysis has a number of advantages in that the sensitivity and specificity estimates for a number of different cut-off values are calculated. The cut-off value resulting in the maximum sum of the percent sensitivity and percent specificity (the performance index) is generally considered the

![Fig. 1. Comparison of negative and positive predictive values for the IELISA, CELISA, FPA, mELISA and the bFPA.](image-url)
optimum value for that assay, however, other cut-off values may easily be selected if other sensitivity or specificity values were desired. ROC analysis also provides a calculation of the area under the curve (AUC), an indication of the expected accuracy of the test. Thus, an AUC of 0.96 indicates that 96% of the time, the assay will correctly identify an animal as positive if the test value is above the cut-off value.

For a new test for the presumptive diagnosis of brucellosis to be acceptable, it must perform as well or better than the prescribed test(s) or possess an advantageous attribute. Direct comparison of performance relative to an old test is not reasonable as a new test which outperforms prescribed tests will always be assumed incorrect if there is disagreement. The data reported for the diagnosis of B. melitensis in goats, based on a relative comparison with prescribed tests indicates that the IELISA, outperforms the CELISA and FPA slightly, the performance indices being 195.9, 193.0 and 187.6, respectively (Table 1). However, the differences in performance are mostly due to lower sensitivity values for the CELISA and the serum FPA, a phenomenon likely related to the classification of sera based on their reactivity in the BPA T/CFT. This is not a good criterion to use in animal populations which may have been vaccinated, possibly more than once with B. melitensis Rev1. Since the IELISA also tends to detect vaccinal antibody in cattle and sheep (Nielsen et al., 1989; Biancifiori et al., 2000), and therefore, most likely in goats as well, it is reasonable that it should agree more with the BPA T/CFT which also detect some vaccinal antibody, giving reactions which are eliminated by the higher specificity of the CELISA and serum FPA. Thus, based on the data, it is difficult to assess the usefulness of the IELISA, CELISA and FPA for serology for caprine brucellosis, except to say they are comparable and possibly superior to the BPA and CFT. The ELISA techniques are relatively complicated and expensive laboratory assays and are not cost effective for field use. The FPA is a very simple and inexpensive test compared to the CFT and ELISAs and it can be adapted for use outside the laboratory setting. If the prevalence of the disease is high as shown in Fig. 1 then the IELISA, CELISA and FPA would be useful in the initial eradication of the disease, especially the FPA for its diagnostic speed (2 min per samples versus 1.5 h for the IELISA or CELISA) even though the sensitivity was slightly lower than the IELISA or CELISA.

The bFPA did not perform as well as expected in terms of its relative specificity (61.6%) although it is provided reasonable sensitivity (92.3%) as can be seen in Table 1. A likely explanation for the low specificity may be that goat erythrocytes appear more difficult to lyse than those of other species. It was found that freshly collected EDTA anticoagulated blood did in the majority of cases partly lyse, leaving residual intact erythrocytes to interfere with fluorescence polarization readings, both of the blank and of the final reading taken 15 s later. Further work is required on this aspect of the assay, hopefully resulting in an increased specificity value.

The mELISA for milk antibody to B. melitensis performed quite well considering the condition of many of the 249 milk samples. The sensitivity value was 90.9% with a specificity of 92.4% (Table 1). Although the milk samples were freshly obtained, many of the animals suffered from various degrees of mastitis, resulting in milk of considerable viscosity that could not be tested in a milk ring test. The mELISA may prove to be a useful screening tool, at least for flock exposure to B. melitensis as it is obtained inexpensively in a non-invasive fashion, a considerable economic advantage.

For detection of antibody to B. melitensis in goats, the FPA appears to have some advantages given that it eliminates some reactivity due to vaccinal antibody in cattle (Nielsen et al., 1996a) and presumably in other species and is technically simple, rapid and economical compared to the ELISAs.

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References


