Application of the Fluorescence Polarization Assay for Detection of Caprine Antibodies to Brucella melitensis in Areas of High Prevalence and Widespread Vaccination

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The screening Rose Bengal test (RBT), the buffered plate agglutination test (BPAT), and the confirmatory complement fixation test (CFT) are currently approved by the World Organization for Animal Health (OIE) for diagnosis of goat brucellosis. However, RBT (at 3% or 8% cell concentration) is known to be affected by viral antibodies. In the present study, Mexican and Canadian OIE tests were compared with the fluorescence polarization assay (FPA), alone or in combination, using indirect and competitive enzyme-linked immunosorbent assays as classification variables for goat sera obtained from an area of high prevalence and widespread vaccination. The relative sensitivities and specificities were, respectively, 99.7% and 32.5% for RBT3, 92.8% and 68.8% for RBT8, 98.4% and 84.8% for Canadian CFT, 83.7% and 65.5% for Mexican CFT, and 78.1% and 89.3% for FPA. The use of FPA as the confirmatory test in combination with other tests significantly increased the final specificities of the screening tests alone; BPAT, RBT3, and RBT8 plus FPA resulted in final specificities of 90%, 91.2%, and 91.3%, respectively, whereas for the combinations RBT3 plus Mexican CFT, RB8 plus Mexican CFT, and BPAT plus Canadian CFT, specificities were 65.5%, 63.2%, and 91.7%, respectively. We suggest that FPA may be routinely applied as an adaptable screening test for diagnosis of goat brucellosis and as a confirmatory test for screening test series. Some advantages of FPA are that its cutoff can be adjusted to improve its sensitivity or specificity, it is a low-cost and easy-to-perform test of choice when specificity is relevant or when an alternative confirmatory test is not available, and it is not affected by vaccination, thus reducing the number of misdiagnosed and killed goats.

The World Organization for Animal Health (OIE) (15)-approved tests for diagnosis of brucellosis in cattle are the buffered Brucella antigen (BBA) tests (Rose Bengal test [RBT] and buffered plate agglutination test [BPAT]), the complement fixation test (CFT), the indirect (IELISA) and competitive (CELISA) enzyme-linked immunosorbent assays (ELISAs), and the fluorescence polarization assay (FPA). The BBA tests, the CFT, and the IELISA are affected by antibodies resulting from immunization with Brucella abortus S19, whereas CELISA and the FPA are not significantly affected (2, 9, 10, 15). For goats, the OIE-accepted tests (15) are RBT and the CFT, although they have not been validated using statistical analysis for sheep and goats compared with similar studies of cattle (13); in addition, they have low specificities (probabilities of correctly identifying as positive those animals that are truly positives [18]) of RBT fulfills requirements for surveillance of free areas at the flock level and that RBT and CFT should be used in a test series procedure to obtain accurate individual sensitivities in test-and-slaughter programs (7, 15).

RBT, or the card test (CT), is easy to perform and inexpensive and can be developed in the field or the laboratory; in contrast, CFT is cumbersome and costly, which results in a very slow, expensive, and difficult diagnostic procedure that is not easy to develop in countries like Mexico (16), where the disease is endemic and most diagnosis is performed by RBT alone, causing the unnecessary killing of goats and increasing the cost-effectiveness of the eradication. RBT, BPAT, and CFT antigens are prepared from B. abortus S1119-3 (1, 17). RBT antigen cells are stained with Rose Bengal and adjusted to a concentration of 8% cells (wt/vol) (RBT8) in a buffered diluent; BPAT uses crystal violet and brilliant green dyes and is adjusted to 11% cells (wt/vol) in a buffered diluent, and CFT uses unstained cells at a concentration of 4.5%. If CFT is not available or cannot be used simultaneously with RBT in eradication programs (15), RBT antigen can be modified to 3% cells (wt/vol) (RBT3) to increase its sensitivity (3, 5, 15), as shown by Díaz et al. (4), who found that RBT3 had 19% more sensitivity than RBT8 in a study using infected, nonvaccinated
goats; however, the low concentration of cells in the antigen decreases its specificity.

The smooth lipopolysaccharide (LPS) is the most relevant reacting antigen in conventional serological tests for brucellosis (1), and antibodies from *B. melitensis* Rev.1 vaccination usually interfere with the diagnosis (3, 5, 15). To avoid this, CELISA is used to inhibit binding of vaccinal but not field strain antibodies in some cases (12). Biancifiori et al. (2) found that CELISA (using CT and CFT as screening tests) resulted in a 99.4% sensitivity and 98.9% specificity in nonvaccinated ovine/caprine samples, whereas in vaccinated samples, CELISA had values of 89.0% sensitivity and 90.3% specificity. In another study using Mexican goats with unknown vaccination statuses, Nielsen et al. (14) showed that the sensitivity and specificity of indirect ELISA (IELISA) were 96.2% and 99.7%, whereas CELISA had 93.6% and 99.4%, respectively, relative to BPAT and CFT. Despite these promising results, ELISAs need to be properly standardized in order to be approved for goats by the OIE (15).

Several studies on the detection of goat antibodies to brucellosis with FPA, relative to BPAT and CFT results, have been published. Nielsen and Gall (11) reported that FPA had 94.9% and 99.4% sensitivity and specificity, respectively. Similarly, using Mexican goat serum samples, Nielsen et al. (13, 14) found 88.7 to 92.7% and 98.9 to 99.8% sensitivity and specificity, respectively, whereas a study on the evaluation of Mexican goat serum samples from an area of high prevalence and widespread vaccination (16) observed 83.5% sensitivity, 82.2% specificity, and 88.2% accuracy for FPA, relative to RBT3 and CFT results. FPA also possessed higher specificity (84.1%) than RBT3 (65.7%) and showed 95.8% specificity when using 702 negative samples from nonvaccinated animals (16); it was suggested that if CFT is not used as a confirmatory test, RBT3 is not appropriate for goats in areas of high prevalence and widespread vaccination and that the use of FPA may help to decrease the number of misdiagnoses caused by false positives (16).

The present study was undertaken to compare the performances of OIE-approved tests (15) RBT3, RBT8, BPAT, CFT, and FPA in detecting *Brucella* antibodies in goat serum samples from an area of high prevalence and widespread vaccination, using a combination of IELISA and CELISA (IC-ELISA) to classify goat sera from an area of high prevalence and widespread vaccination and to evaluate test combination series, using easy-to-perform tests as screening tests and a secondary confirmatory test to screen positive samples.

**MATERIALS AND METHODS**

Two approaches were taken to compare the performances of OIE-tests versus that of FPA in the diagnosis of brucellosis. In the first approach, we evaluated the efficacies of tests to confirm samples previously classified as positive and negative for each combination of classification criteria. For this, results for Mexican (RBT3, RBT8, and CFTm) and Canadian (BPAT CFTc and FPA) tests were compared using goat serum samples classified as negative or positive by IC-ELISA. In the second approach, using the test results obtained in step one, we simulated various test series combinations. In these, an easy-to-perform and high-sensitivity screening test was followed by a high-specificity confirmatory test in order to improve the final result and determine the most useful procedures that might be routinely applied in field and laboratory testing, for an area with high prevalence and widespread vaccination. According to the results for the first approach, the easy-to-perform Mexican tests (RBT3 and RBT8), Canadian BPAT, and FPA were selected as screening tests, and the positive serum samples were confirmed with high-specificity tests.

**Serum samples.** For this study, 534 goat serum samples were obtained from the Laboratorio de Inmunología y Virología (LIV) serum bank, Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León, México. These samples were obtained as a part of the eradication program in Nuevo León State, Mexico, where vaccination with Rev.1 may reach 80% of the goat population and the prevalence of *Brucella* infection is estimated to be between 2% and 4% (J. G. Muraira, personal communication). Previous RBT3 and CFT sample results were also obtained from LIV; however, since sera from goats with proven *B. melitensis* infections were not available, and the vaccination statuses of the animals were unknown, they were classified as positives or negatives using a test series with ELISA as the screening test and CELISA as the confirmatory test for positives, as described above.

Serum aliquots from some of the samples were identified and sent frozen overnight to the OIE Regional Reference Laboratory and Brucellosis Centre of Expertise of the Ottawa Laboratories, Fallswfield (OLF), Nepean, Ontario, Canada, for BPAT, CELISA, and IELISA testing. The remaining samples were sent for RBTs testing to the Laboratorio Regional Central de Monterrey (LRCM), an accredited Mexican brucellosis laboratory which also performed the CT and CFTm tests.

**Serological tests.** We compared the Mexican and Canadian tests; the Mexican (m) serological tests included RBT3, RBT8, and CFTm, whereas the Canadian (c) tests were BPAT, CFTc, IELISA, and CELISA. The tests RBT3, RBT8, and CFT were performed at OLF using antigens and procedures according to the NOM (19), which are the same as those described by the OIE (15). BPAT and CFTc were performed at OLF, using antigens and procedures previously described by the OIE (15).

FPA was performed at LIV as previously described (10), using *B. abortus* fluorescein isothiocyanate-labeled O-polsaccharide as the tracer (OLF). In brief, 25 μl of each goat serum sample was diluted in 1 ml of 0.1 M Tris buffer. After the mixing, a blank reading was obtained using a microplate photometer (FP) analyzer (Sentry FP; Diachemix LLC., Grayslake, IL); 10 microliters of the tracer was then added to the diluted sample, and after 2 min of incubation, a final reading was obtained. Poole goat control samples (strongly positive, weakly positive, and negative sera) were prepared by adjusting their readings to the respective standard FPA OLF bovine serum readings and were used daily as controls for FPA.

IELISA and CELISA were performed at OLF according to Nielsen et al. (10). For IELISA, smooth LPS (SLPS) extracted from *B. abortus* strain 1191-3, immobilized on a polystyrene matrix, was used as the antigen. Serum samples diluted 1:50 were added after the 96-well plates were washed with phosphate-buffered saline—Tween 80. Reactivity was then determined using a mouse monoclonal antibody specific for bovine immunoglobulin G (IgG) and conjugated to horseradish peroxidase. This monoclonal antibody has been shown to strongly cross-react with IgG of sheep and goats (8). For CELISA, SLPS was used as the antigen, a positive serum sample (50 μl) was diluted 1:10 and added to each well, followed by addition of an equal volume of pre-diluted mouse monoclonal antibody specific for a common O-polsaccharide epitope of the SLPS molecule. Reactivity to the mouse antibody was detected using a commercially available goat antibody to mouse IgG conjugated with horseradish peroxidase. Hydrogen peroxide (H₂O₂) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) enhancer (ABTS, Sigma-Aldrich, St. Louis, MO) were used as a substrate. Reaction samples for CELISA and IELISA were then incubated for 10 min, and optical densities were determined by spectrophotometry at 414 nm. Tests at OLF and LRCM were performed blindly, and their respective results were included in a database. It was not possible to perform all tests on all serum samples due to depletion.

**Data expression for individual serological tests and test combinations.** (i) **IELISA.** Selected IC-ELISA samples were considered negatives if IELISA results were negative or if IELISA results were positive and CELISA results were negative; samples were considered positives if IELISA and CELISA results were positive. (ii) **Individual (screening) tests.** For RBT3, RBT8, and BPAT, positive or negative results were determined by the presence or absence, respectively, of visible agglutination (15, 19). For CFTc (14), positive results were determined by 50% hemolysis of the indicator cells at 1:5, whereas for CFTm (19), 1:4 serum dilutions were used as the cutoff. The FPA cutoff value was determined by receiving operator characteristic (ROC) analysis (10). For IELISA, data were calculated as percent positivity based on a strongly positive serum included as a control in each plate, whereas for CELISA, percent inhibition was calculated using a buffer control (uninhibited) included in each plate. All ELISA plates contained the following controls: a strong-positive, a weak-positive, and a negative serum sample and a buffer control without serum.
TABLE 1. Numbers of positives and negatives for various tests, using goat serum samples previously selected with I/C-ELISA

| No. of samples | No. of samples identified | Test | No. of samples identified |
|---------------|---------------------------|------|---------------------------|
|               | identified by I/C-ELISA as: | Test | identified as:             |
| 508           | Positive                   | 370  | Negative                  | 138 RBT3 | 364  | 116 CFTm | 84  |
| 518           | Positive                   | 371  | Negative                  | 147 BPAT | 365  | 119 CFTc | 84  |
| 345           | Positive                   | 242  | Negative                  | 103 FPA | 189  | 92 RBT8 | 64  |
| 316           | Positive                   | 223  | Negative                  | 93 RBT3 | 207  | 64 CFTm | 55  |
| 385           | Positive                   | 301  | Negative                  | 84 CFTc | 292  | 55 BPAT | 49  |
| 532           | Positive                   | 381  | Negative                  | 151 RBT8| 380  | 49 BPAT | 20  |

(iii) Test combinations. For test combinations, BPAT, RBT3, and RBT8 were selected as screening tests, and CFTm, CFTc, FPA, and BPAT were used as confirmatory tests for the positive samples in screening tests relative to I/C-ELISA. Negative results were indicated by negative screening tests or positive screening tests plus negative confirmatory tests, whereas positive results were represented by positive confirmatory tests.

Data analysis. In the first approach, ROC analysis, with the aid of the MedCalc program (Frank Schoonjans, V.8.1.0.0), was used to evaluate the efficacies of tests to discriminate results previously classified as positive and negative for each combination of classification criteria. ROC analysis plots the true positive rate relative to the false positive rate at different cutoff points, which are selected for screening or confirmatory purposes (18). In addition, it provides information about the cutoff value, the sensitivity, the specificity, and the area under the ROC curve (AUC), which is an indicator of the accuracy of the test (10). The performance index (PI) was calculated by adding the sensitivity and specificity values for each test and test combination (7). In the second approach, to obtain the final specificity, the MedCalc program was also used to perform ROC analysis of the confirmatory tests (CFTc, FPA, CFTm, and BPAT) on the samples identified as positive in the screening tests (BPAT, RBT3, and RBT8). The optimum cutoff value was calculated by the MedCalc program; this value differed according to whether the tests were used alone or in combination, to increase the sensitivity or specificity as necessary (see Tables 2 and 4).

RESULTS

Serological test performance. The AUCs, sensitivities, specificities, and PI s for different tests and test combinations were compared using goat serum samples, previously classified using I/C-ELISA. The numbers of goat serum samples selected as positive and negative with the I/C-ELISA combination were shown in Table 1, whereas Table 2 shows the overall performances of the different evaluated tests. We observed that the test accuracies (AUC x 100) were 91.5% for CFTc, 89.7% for BPAT, 86.8% for FPA, 80.8% for RBT8, 80.5% for CFTm, and 66.1% for RBT3, whereas the PI s, from high to low, were those for CFT, BPAT, FPA, RBT8, CFTm, and RBT3 (Table 2).

Comparison of test series performances. The numbers of goat serum samples selected as positive and negative with the I/C-ELISA combination, in an approach used to select the confirmatory tests that improve the specificities of screening tests, are shown in Table 3, and the final specificities and PI s obtained by the confirmatory test are shown in Table 4. The PI s, from high to low, were those for RBT3 plus FPA (>99 mP), RBT3 plus FC-4 (the official NOM combination), BPAT plus CFTc, BPAT plus FPA (>98 mP), and the rest of the combinations (Table 4).

DISCUSSION

We compared the performances of OIE-approved diagnosis tests versus those of FPA and test combinations, using goat serum samples from an area of high prevalence and widespread vaccination. Sera were obtained for the brucellosis campaign in northeast Mexico, and some of these were used in a recent study on brucellosis diagnosis (16). A test series procedure, consisting of a combination of IELISA as the screening test and CELISA as the confirmatory test for positive samples (I/C-ELISA), was chosen to classify the samples, since the infection status for proven B. melitensis isolation was not available, and the vaccination status for the B. melitensis Rev.1 strain was unknown; the performance indicators for the evaluated tests were relative to the I/C-ELISA results. IELISA has been reported as more sensitive but less specific than CELISA, which has been shown not to be significantly influenced by antibodies resulting from cross-reactions and vaccination (12). Diagnostic procedures for test series are used to increase the diagnosis specificity; in these, the sensitivity is obtained by a screening test, and the specificity is increased with a confirmatory test.

In the present study, CFTc, BPAT, and FPA were observed to have better accuracies (91.5%, 89.7%, and 86.8%, respectively) and PI s (183.2, 179.4, and 167.4, respectively) than RBT8, CFTm, and RBT3 (accuracies of 80.8%, 80.5%, and 66.1%, respectively; PI s of 149.2, 132.2, respectively). We also observed a significant difference between

TABLE 2. Performances of individual serological tests using goat serum samples, relative to I/C-ELISA results

| Test     | Cutoff | AUC  | Sensitivity (%) | Specificity (%) | PI    |
|----------|--------|------|-----------------|-----------------|-------|
| CFTc     | 1:5    | 0.915| 98.4            | 84.8            | 183.2 |
| BPAT     | 1      | 0.897| 98.4            | 81.0            | 179.4 |
| FPA      | >88 mP | 0.868| 57.7            | 97.1            | 154.8 |
| FPA      | >98 mP | 0.868| 78.1            | 89.3            | 167.4 |
| FPA      | >88 mP | 0.868| 87.6            | 57.3            | 144.9 |
| RBT8     | 1      | 0.808| 92.8            | 68.8            | 161.6 |
| CFTm     | 1:4    | 0.805| 83.7            | 65.5            | 149.2 |
| RBT3     | 0      | 0.661| 99.7            | 32.5            | 132.2 |

a See data expression and interpretation in Materials and Methods.

b Fluorescence polarization assay. The cutoff of >88 mP was selected by the software as the mP value that maximizes the sensitivity and specificity.

c Results for fluorescence polarization assays with cutoffs of >100 and >88 mP are presented for different cutoff selections.

TABLE 3. Numbers of positives and negatives for various test series, using I/C-ELISA-negative samples

| No. of I/C-ELISA negatives | Test 1 | No. of test 1 positives | Test 2 | No. of test 2 negatives |
|-----------------------------|--------|-------------------------|--------|------------------------|
| 137 BPAT                    | 27 CFTc | 16                      |
| 139 BPAT                    | 20 FPA  | 18                      |
| 103 RBT3                    | 68 FPA  | 62                      |
| 84 RBT3                     | 84 CFTm | 73                      |
| 121 RBT3                    | 72 BPAT | 72                      |
| 151 RBT3                    | 102 CFTm| 55                      |
| 93 RBT3                     | 65 RBT8 | 41                      |
| 151 RBT8                    | 101 CFTm| 87                      |
| 70 RBT8                     | 23 FPA  | 2                       |
| 91 RBT8                     | 29 BPAT | 20                      |

* Test 1-positive samples were retested with test 2 to obtain numbers of false-positive samples.
Canadian and Mexican CFTs; the sensitivities and specificities for CFTc were 98.4% and 84.9%, and those for CFTm were 83.7% and 65.5%, respectively (Table 2). Previous reports on CFT (3, 6, 11) support our findings; they obtained sensitivities of 88.5%, 85.9%, and 95.9%, respectively. In contrast, Díaz et al. (4) found that CFT possessed 100% specificity for serum samples from noninfected animals. Furthermore, the sensitivity and specificity of FPA (>98 mP) relative to those of I/C-ELISA were found to be 78.1% and 89.3% (PI = 167.4), respectively, which were lower than those relative to BPAT-CFTm results reported in a previous study (88.7% and 98.9%, respectively) (14) but similar to those relative to RBT3 and CFTm reported elsewhere (82.1% and 82.2%) (16). These conflicting results may be explained by the fact that RBT and CFT results can be affected by antibodies induced by the Rev.1 vaccine (5, 7, 15); in this regard, Ramírez-Pfeiffer et al. (16) found a 35.9% false-positive rate with RBT3 compared with CFT results in areas of high prevalence and widespread vaccination of goat brucellosis.

Our present results showed that RBT3 had the lowest performance index score among the evaluated tests. In addition, the observed test differences may be due to the selection of serum samples (14); this is because, unlike RBT and CFT, new tests differentiate between antibodies from infected and vaccinated animals and may give negative results for serum samples obtained from vaccinated or revaccinated goats, thus making conventional test results favorable. In addition, the differences between results for Mexican and Canadian tests agreed with a study by Gall and Nielsen (7), who analyzed 37 different sets of bovine test results, published between 1969 and 2003, and observed that the average PIs for RBT and CFT were 167.6 and 172.5, respectively, with a higher coefficient of variation (14.8% and 14.1%, respectively) than the rest of the tests included in the study. On the other hand, BPAT and FPA yielded the highest PIs and the lowest PI variabilities (193.1 [3.3%] and 196.4 [4.4%], respectively).

Our results for single-test performance also showed that FPA with >98 mP yielded 89.3% specificity, and by modification of its cutoff value to >108 mP, the specificity was increased to 97.1% (Table 2). This cutoff adjustment of sensitivities and specificities for accommodation of different epidemiological situations makes FPA a convenient test for use in the diagnosis of goat brucellosis when sensitivity is not critical or when other confirmatory tests are not available, particularly in low-income areas with widespread vaccination, like those commonly found in Mexico.

Confirmatory tests, including CFT or ELISAs, are difficult to perform in most Mexican laboratories because of the vaccination programs. Our results agreed with Ramírez-Pfeiffer et al. (16) in that RBT3, despite its high sensitivity (99.7%), had the lowest specificity (65.5%) and that it is necessary that all positives be confirmed with other tests to diminish the unnecessary killing of false-positive goats. Furthermore, our results with screening tests may suggest the use of RBT8, which has lower sensitivity but higher specificity than RBT3; this is supported by the fact that RBT8, with 82.8% sensitivity, yields 68.8% specificity, which is still higher than the 65.5% specificity obtained by the combination RBT3 plus CFTm>1:4; although this may allow the presence of a small number of undetected infected goats in the flock, it will be more affordable than RBT3 when a confirmatory test is not available.

With test combinations, we observed that the use of FPA as a confirmatory test for the screening tests RBT8, RBT3, and BPAT increased the specificities from 68.8%, 32.5%, and 81% to 91.3%, 91.2%, and 90%, respectively, which were higher values than those provided by any other test combination (Table 4). For instance, the specificity of RBT3 plus CFTm>1:4, a combination of the present NOM goat tests in Mexico (19), was converted from 32.5% to 65.5%, which represented the lowest value of all the combinations and was closer to the value of 61.6% previously described for BPAT by Nielsen et al. (14) for nonvaccinated goats. However, when CFTm>1:8 was used as a confirmatory test for RBT3-positive samples, the specific-
ity increased from 32.5% to 90.5%; this combination could be easily adopted by the NOM to reduce the number of false positives. Similarly, when CFTm > 1:8 was used as a confirmatory test for RBT8, the specificity increased from 68.8% to 94.7%, despite the relatively low sensitivity (92.8%) of RBT8. It is interesting to note that FPA (> 98 mP) used as a single test has 89.3% specificity, which increased to 91.2% (> 99 mP) or 97.1% (> 108 mP) when combined with RBT3 (Table 4).

The results from the present study agreed with those reported by Nielsen et al. (13), who suggested that FPA can be applied for serological diagnosis of brucellosis in small ruminants, and if the I/C-ELISA series is better than the present conventional testing (RBT3 plus CFT), it is likely that the combination of a BBA test with a confirmation of positives by FPA may be routinely applied as an adaptable screening test for diagnosis of goat brucellosis and as a confirmatory test for screening test series, particularly in laboratories with limited resources. Moreover, our results may favor the use of FPA alone in areas of high prevalence, widespread vaccination, and low resources instead of the current regime, because one of the advantages of FPA is the possibility of adjusting the cutoff values, which may increase or decrease the sensitivity or specificity for use in different epidemiological situations. For example, a cutoff of > 88 mP provides 87.6% sensitivity instead of 78.1%, which is provided with a cutoff of > 98 mP (Table 2), whereas FPA with > 108 mP provides 97.1% specificity instead of 89.3%, which is provided with > 98 mP. The cost of the FP reader, however, can be compensated for by reducing the killing of goats by misdiagnosis; in addition, the reader may be used in the diagnosis of brucellosis in cattle and swine (15), and other tracers to various diseases are being developed, making FPA a versatile technology of choice.

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