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Use of the *Brucella melitensis* Native Hapten To Diagnose Brucellosis in Goats by a Rapid, Simple, and Specific Fluorescence Polarization Assay▽

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The performance of the fluorescence polarization assay (FPA) using the recently described *Brucella melitensis* native hapten and the *Brucella abortus O*-polysaccharide tracer was evaluated and compared with those of The World Organization for Animal Health tests related to indirect and competitive enzyme-linked immunosorbent assays as classification variables for goat sera obtained from a high-prevalence area where vaccination was performed; test series were also evaluated to increase the final specificity of the tests. Our results showed that the respective relative sensitivity and specificity were 99.7% and 32.5% for the rose Bengal test with a 3% cell concentration (RBT3), 92.8% and 68.8% for the rose Bengal test with 8% cell concentration (RBT8), 98.4% and 84.9% for the Canadian complement fixation test (CFT), 83.7% and 65.5% for the Mexican CFT, 98.4% and 81.0% for the buffered plate agglutination test (BPAT), and 78.1% and 89.3% for the fluorescence polarization assay (FPA). The use of the FPA as the secondary test significantly increased the final specificities of test combinations; the screening tests BPAT, RBT3, and RBT8 plus FPA resulted in 90%, 91.2%, and 91.3% final specificities, respectively, whereas for the combinations RBT3 plus Mexican CFT, RBT8 plus Mexican CFT, and BPAT plus Canadian CFT, the specificities were 65.5%, 63.2%, and 91.7%, respectively. The results suggested that the FPA may be routinely applied as an adaptable screening test for diagnosis of goat brucellosis, since its cutoff can be adjusted to improve its sensitivity or specificity, it is a rapid and simple test, it can be the 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 goats wrongly slaughtered due to misdiagnosis.

Brucellosis is the most prevalent bacterial disease transmitted from animals to humans worldwide, with 500,000 annual human cases (18). In particular, *Brucella melitensis* mainly infects goats, causing a disease that affects the reproductive tract. This disease, in turn, causes great economic losses because of induced abortions; *B. melitensis* is also considered the most pathogenic species for humans (9). The World Organization for Animal Health (OIE) currently approved tests for diagnosis of brucellosis in goats (and sheep) are the rose Bengal test (RBT), the buffered plate agglutination test (BPAT), and the complement fixation test (CFT) (24). They have been statistically validated only for cattle, not for sheep and goats (16), and can be affected by vaccinal antibodies (1, 4, 7, 8, 16, 24). To increase the sensitivity of the RBT in screening procedures, the test is modified at a 3% cell concentration (RBT3) instead of the 8% (RBT8) that is used for cattle (1, 7, 24); however, in vaccination areas with high prevalence of disease, such as Mexico, this procedure possesses a sensitivity of 99.7%, and the specificity is reduced to as low as 32.5% (20). Nevertheless, when the CFT is used as a confirmatory test for RBT3-positive samples, the specificity increases to 65.5% relative to the test series indirect enzyme-linked immunosorbent assay (IELISA) and competitive ELISA (CELISA) (19, 20). Gel precipitation tests using polysaccharide haptens were the first tests described for differentiating infected from vaccinated animals (5); however, according to others, their sensitivity was insufficient for large-scale diagnosis (14) and they are not recommended for diagnosis by the OIE.

Despite the reduction of false positives, a large number of goats may be culled in test-and-slaughter programs. This makes it critical to evaluate and develop new test procedures with high sensitivity and specificity to significantly improve the diagnosis of brucellosis. Studies have shown that IELISA, CELISA, and the fluorescence polarization assay (FPA) might

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be useful. Despite its high sensitivity, IELISA has a relative lack of specificity, and interference from cross-reacting antibodies may arise (15). This weakness was largely overcome by the development of CELISA (10, 17) and the FPA (12), since ELISAs and the FPA are not significantly affected by antibodies resulting from immunization (3, 12, 13, 17, 24) and are faster than the CFT (16). Previous evaluations of the FPA (11, 15, 16, 19, 20) using the *O*-polysaccharide (OPS) conjugated with fluorescein isothiocyanate (FITC) as a tracer (OPS tracer) in goats and sheep, have shown that the diagnostic performance results might be affected by different selection criteria for positive reference standards and by the quality of sera used; however, there appears to be agreement that the OPS tracer might be a useful tool for the diagnosis of brucellosis in small ruminants (9, 15, 16, 19, 20).

We have recently (20) developed a novel FITC-conjugated tracer for use in the FPA, based on the *B. melitensis* native hapten (NH) described by Diaz et al. (5) for diagnosis of bovine brucellosis using radial immunodiffusion, which in an initial evaluation with positive and negative Mexican field goat serum samples, selected by test series approved by the OIE (card test [with 3% suspension] and CFT), performed better than the OPS tracer. The present study was undertaken to evaluate the novel NH tracer for detecting *Brucella* antibodies in goat serum relative to bacteriology-, PCR-, and ELISA-positive samples and to validate its performance on vaccinated goats and in test series that can be easily adopted for use by any laboratory or in the field.

(This work was part of the Ph.D. thesis of Carlos Ramírez-Pfeiffer.)

MATERIALS AND METHODS

For the FPA evaluation using *B. melitensis* NH tracer, the following studies were performed.

(i) In the first study, FPA/NH tracer performance in detecting *Brucella* antibodies in infected goats was determined; (ii) in a second study, the NH tracer potential to detect antibodies in samples obtained from vaccinated goats was evaluated; and (iii) in a third study, the NH tracer potential to improve the final specificity of tests, when used as a confirmatory test in combination with screening tests (RBT3, RBT8, or BPAT) for positive samples, was evaluated.

Goat serum samples. Goat serum samples, together with their RBTs (RBT8 and RBT3, with 8% and 3% cell concentrations, respectively), CFT, FPA with OPS tracer, ELISAs, and PCR results, were obtained from the serum bank of the Laboratorio de Inmunología y Virología (LIV) of the Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León (N. L.), México. Most of them had been used in previous studies (19, 20) and had been kept frozen at −70°C. These samples had been obtained as part of the eradication campaign in the northeast part of Mexico and had been tested by RBT3, RBT8, and CFT at the Laboratorio Regional Central de Monterrey (LRCM) N. L. (Comité de Fomento y Protección Pecuaria de Nuevo León, Guadalupe, N. L., México) according to the Mexican official norm (22), which applies procedures and antigens described elsewhere (1, 24). Additional results for ELISAs, BPAT, and Canadian CFT (CFTc) were performed at the Reference Laboratory and Brucellosis Centre of Expertise of the Ottawa Laboratories (Fallowfield, Nepean, Ontario, Canada) (OLF), as previously described (12). The results of PCR performed according to the method of Bailey et al. (2) were obtained at the Laboratorio de Biotecnología of the Instituto Nacional de Investigaciones Forestales y Agrícolas y Pecuarias located in the LRCM. Isolation and identification of *Brucella* spp. was performed at LIV using goat milk samples obtained from nonvaccinated goats according to previously described procedures (1).

For this study, a total of 381 serum samples were classified as positive in the following cases: (i) sera obtained from an area where nonvaccinated goats were raised for at least 5 years and with the presence of flock abortions and human cases of Malta fever in which *Brucella* spp. were isolated from goats' milk (8 samples) and (ii) samples from bacteriologically positive flocks that were positive by PCR (40 samples) or positive by both IELISA and CELISA (333 samples). No signs of disease were observed in the goats when they were sampled, nor were tests for other diseases performed on the samples. "Negative" samples were obtained from Canada (96 samples) (a brucellosis-free country where vaccination is not done) or were considered negative if they were obtained from nonvaccinated flocks from northeast Mexico without a history of abortion, with two consecutive negative tests by RBT3, and with negative IELISA and CELISA results (125 samples) and where no human Malta fever cases were observed; in addition, "vaccinated" samples were obtained from vaccinated animals after at least two consecutive negative tests with RBT3 and also vaccination with *B. melitensis* strain Rev. 1.

NH tracer preparation and FPA procedure. NH was obtained from *B. melitensis* strain Rev. 1 in a previous work (21) at the Centro Nacional de Investigaciones Disciplinarias en Microbiología of the Instituto Nacional de Investigaciones Forestales Agrícolas y Pecuarias, following the extraction method described by Díaz et al. (6) for use in FPA. In brief, to obtain the crude NH-containing pelleted fraction (6), killed cultured cells were pelleted, suspended in distilled water, centrifuged, precipitated twice with ethanol, and lyophilized; then, NH was FITC conjugated at LIV as previously described (12). The amounts of tracer and serum samples for testing were standardized to yield polarization fluorescence reading values similar to those obtained from FPA using the OIE OPS tracer and FPA bovine standard serum controls (produced at OLF and kindly supplied by Klaus Nielsen).

Once the NH tracer was standardized, FPA was performed at LIV following the procedure previously described by Nielsen et al. (12). In brief, for the NH tracer, 10 μl of each goat serum sample was diluted in 1.0 ml of 0.1 M Tris buffer in a test tube or in 700 μl of this buffer in each microplate well, instead of the 25 μl used for the OPS tracer. After the ingredients were mixed, a blank reading was obtained; 10 μl of the tracer was then added to the diluted sample, and after 2 min of incubation, a final reading was obtained.

Strong-positive, weak-positive, and negative goat FPA serum controls were prepared by pooling goat serum samples and adjusting their readings to the respective OIE brucellosis FPA bovine standard serum readings (kindly supplied by Klaus Nielsen), and they were used daily as controls for FPA tests. It was not possible to perform all tests on all serum samples due to sample depletion.

FPA readers. Two fluorescence polarization readers were used: the Fluorescence Polarization Analyzer (Sentry FP, model PS-1; Jolley Consulting and Research Inc. Diachemix, Grayslake, IL), in which test tubes were used for testing, and the Multimode Reader DTX 880 (Beckman-Coulter, Inc., Fullerton, CA), in which 96- or 254-well black plates could be used. Data are expressed in milli-polarization units (mP).

Data analysis. To evaluate the diagnostic performance of the FPA or its ability to discriminate diseased from healthy cases, based on its accuracy, sensitivity, and specificity values, receiver operating characteristic (ROC) analysis (MedCalc software, V.9.2.1.0, from Frank Schoonjans) was used to determine the statistical differences in pairwise nonparametric comparisons of areas under the ROC curves (AUCs) (25). We used a *P*-value cutoff of ≤0.05 for significance. A ROC curve analysis is a graph that plots the true rate in the function of the false-positive rate at different cutoff points that are a consequence of their individual test sample results. The ROC graph is a plot of all of the sensitivity/specificity pairs resulting from continuously varying the decision threshold over the entire range of results observed. In each case, the ROC plot depicts the overlap between the two distributions by plotting the sensitivity versus 1 minus the specificity for the complete range of decision thresholds, and it determines the optimal cutoff value based on achieving the highest possible sensitivity and specificity without a suspect range (25). This is especially useful for free-ranging populations in which resampling of suspect animals is impossible. RBT3 was not included in this comparison, as it had been proved previously that its performance is poorer than that of RBT8 (20).

In addition, the sensitivity and specificity values of tests were added to obtain the performance index (PI), which is a value that is useful for comparing test performances (13); when the sensitivity and specificity of one test are 100%, the resulting PI value is 200.

RESULTS

Test performance using the NH tracer. Table 1 shows a comparison of individual test performances related to the status of goat serum samples. It was observed that the AUC of the FPA/NH tracer at >101 mP was 0.944, followed by CFTc

TABLE 1. Performances of individual tests related to the status criteria for sample selection

Test	Cutoff	No. of positive samples	No. of negative samples	AUC	Sensitivity (%)	Specificity (%)	Performance index
NH	>101 mP	335	221	0.944	85.7	91.9	177.8
CFTc	>0	359	138	0.916	98.6	84.8	183.4
BPAT	>0	360	147	0.896	98.3	81.0	179.3
OPS	>98 mP	281	103	0.874	79.4	89.3	168.8
RBT8[a]	>0	264	95	0.747	81.1	68.4	149.5
CFTm[b]	>8	332	84	0.730	55.4	86.9	142.3
CFTm[c]	>4	332	84	0.730	74.7	65.5	140.2
RBT3[d]	>0	381	153	0.662	99.7	32.7	132.4

[a] RBT8 is used as the official screening test in Mexico for cattle (22).
[b] CFTm (>8) is used as the official cattle confirmatory test in Mexico (22).
[c] CFTm (>4) is used as the official goat and sheep confirmatory test in Mexico (22).
[d] RBT3 is used as the official cattle-screening test in Mexico (22).

(0.916), BPAT (0.896), OPS (>98 mP; 0.874), RBT8 (0.747), Mexican CFT (CFTm) (for >4 and >8 cutoffs; 0.730), and RBT3 (0.662). In addition, despite the NH tracer's relatively low sensitivity (85.7%), its specificity was higher (91.9%) than those of other tests, whereas the OPS tracer yielded 79.4% sensitivity and 89.3% specificity, and in regard to the plate agglutination tests, the BPAT had 98.3% sensitivity and 81.0% specificity, RBT8 had 81.1% sensitivity and 68.4% specificity, and RBT3 had the highest sensitivity (99.7%) but the lowest specificity (32.7%). The pairwise comparison of test AUCs showed that there was no significant difference ($P > 0.05$) between NH, BPAT, and OPS, but they were significantly different ($P < 0.05$) from RBT8 and CFT; in addition, there was no significant difference ($P > 0.05$) between RBT8 and CFTm.

Performance of NH tracer on vaccinated goats. In the second study, goat serum samples obtained from flocks with no evidence of infection and with 1 to 4 months of Rev. 1 vaccination were considered "vaccinated." Although it was not possible to test all vaccinated samples with all tests, NH tracer (≤81 mP) had the fewest (72.9%) positive reactions when 107 vaccinated-goat serum samples were tested, compared with the other evaluated tests (OPS tracer, 98.2% positive reactions; RBT3 and RBT8, 98.2% positive reactions; 281, 283, and 282 samples, respectively).

Performance of NH tracer in test series. In the third study, goat serum samples that tested positive with RBT3, RBT8, and BPAT were considered "positive on screening tests" and were tested with FPA with NH and OPS tracers, CFTm, and CFTc as confirmatory tests for test series evaluation. Table 2 shows that when RBT3 was used as the screening test (with an initial sensitivity of 99.7% and a specificity of 32.7%) and the positive samples were confirmed with secondary tests, the final specificities of the diagnostic procedures increased to 94.5% (NH), 91.2% (OPS), 86.9% (CFTm), 81.1% (CFTc), 65.5% (CFTm), and 62.1% (RTB8), whereas when RBT8 was used as the screening test (with an initial sensitivity of 81.1% and a specificity of 68.4%) and the positive results were confirmed with NH, CFTs, or OPS, the final specificities of the diagnostic procedures increased to 96.3% (NH), 94.7% (CFTm), 91.3% (OPS), and 85.2% (CFTc). Finally, when BPAT was used as the screening test (with an initial sensitivity of 98.3% and a specificity of 81%) and the positive samples were confirmed with NH or OPS, the final specificities of the diagnostic procedures increased to 95.6% (NH) and 90.0% (OPS). As can be seen in Table 2, in all cases, the PI was in accord with the increase in the final specificity. It is notable that the NH tracer PI (177.8) (Table 1), as a single test, was higher than those of all of the test series (Table 2), except when RBT3 was used as

TABLE 2. Comparison of test series performance used to improve the final specificity, using a screening test and a secondary test for positives testing

Screening test	Performance indicator value for screening test			No. of samples		Confirmatory test	Performance indicator value for test series		
	Cutoff	Sensitivity	Initial specificity	Negative	Positive		Cutoff	Final specificity	PI
RBT3	>0	99.7	32.7	297	91	NH tracer	>108 mP	94.5	194.2
				245	68	OPS tracer	>99 mP	91.2	190.9
				297	84	CFTm	>1:8	86.9	186.6
				358	95	CFTc	>0	81.1	180.7
				297	84	CFTm	>1:4	65.5	165.2
RBT8	>0	81.1	68.4	205	27	NH tracer	>108 mP	96.3	164.7
				179	19	CFTm	>1:8	94.7	163.1
				181	23	OPS tracer	>97 mP	91.3	159.7
				200	20	CFTc	>0	85.2	153.6
BPAT	>0	98.3	81	278	23	NH tracer	>108 mP	95.6	193.9
				231	20	OPS tracer	>98 mP	90	188.3

the screening test and FPA was used with NH (194.2) or OPS tracer (190.9).

DISCUSSION

Recently, an FPA novel tracer based on FITC-conjugated *B. melitensis* NH (NH tracer) for detection of antibodies against *Brucella* was evaluated with positive and negative Mexican field goat serum samples selected by test series approved by the OIE (card test, 3%, and complement fixation test). The NH tracer produced values of 97.2% accuracy, 95.7% sensitivity, 99% specificity, and 194.7 PI, which were higher than those obtained with the OPS tracer (93.8%, 91.3%, 99%, and 190.3, respectively) (21). However, as it is known that the OIE tests are affected by Rev. 1 vaccinations, the present work aimed to (i) evaluate NH tracer in detecting *Brucella* antibodies using bacteriology, PCR, and ELISAs to select "positive" samples; (ii) evaluate NH tracer performance with Rev. 1-vaccinated goats; and (iii) evaluate its performance as a secondary test in test series that can be easily adopted for use in laboratories with little infrastructure or in the field.

The results of the first study with infected goats showed that NH tracer's accuracy (94.4%) and PI (177.8) were higher than those of other tests, including the OPS tracer (accuracy and PI, 89.6% and 168.8, respectively). Such results have some similarity to the results of conventional tests; in a previous evaluation of the tracers using RBT3 and CFTm to select samples, we also obtained better accuracy and PI for the NH tracer (87.5% and 163.8) than for the OPS tracer (83.5% and 156.9). Nielsen et al. (13), using OPS (16), found the respective values to be 96.1% and 187.6% relative to BPAT and CFT, whereas Minas et al. (11), also using the OPS tracer, found an accuracy of 98.7% and a PI of 191.8 in nonvaccinated goats. The differences in test performance between studies may be associated with the tests/antigens selected and the serum quality; other individual factors, such as age, stage of pregnancy, health status, and stage of infection; the type of husbandry, management, and animal breeds (11); and, in addition, as in this case, the kind of tracer used in FPA.

Although in the evaluation of tests with vaccinated-goat serum samples, the antibody response was followed up to 4 months after vaccination, it was expected that both the NH and OPS tracers would yield similar and lower percentages of positives than RBT, but according to our results, the NH tracer yielded 72.9% positives whereas, surprisingly, the OPS tracer yielded 94.3% (and the RBT3 and RBT8 yielded 98.2%). It is difficult to explain the differences between the tracers, but they may be related to the postvaccination period evaluated, making it necessary to extend it for future studies.

The Rev.1 *B. melitensis* NH tracer's higher performance, compared with the OPS S1119-3 *B. abortus* tracer observed in the present work, might be due to the production of higher-affinity antibodies directed against NH in homologous *B. melitensis* infections in goats than those cross-reacting with *B. abortus* OPS; however, to date, it is not clear why the OPS tracer showed a high percentage of reactions with vaccinated-goat serum samples in a homogeneous FPA format. However, whatever the explanation for this, there is an advantage for the NH tracer in an FPA format, because according to our results, it is more accurate in areas in which vaccination is being done and it may reduce the killing of goats in areas where test-and-slaughter programs are being run.

In the third study, we evaluated serial testing, considering that this procedure may optimize the specificity and the predictive value of a positive test result, making it an important part of the disease eradication campaigns in which positive animals are culled from flocks (i.e., for goat brucellosis) (23). Unlike parallel testing, in which animals positive in any test are considered to be affected, thus emphasizing sensitivity, serial testing analysis is conducted sequentially, and only those samples that are positive in an initial screening are tested again with the confirmatory test and only samples that are positive in all tests are considered "affected" (23). For this, we used BPAT, RBT3, and RBT8 as screening tests because they showed higher sensitivity than other tests, they are easy to perform, and they are used worldwide, whereas FPA (with NH and OPS tracers) and CFTm were selected as confirmatory tests because of their relatively high specificity, which ensures a higher final specificity than with other tests. Our results showed that all test series in which NH was used as a confirmatory test yielded higher final specificity than others, including the OPS tracer series. The difference observed between NH and OPS performances as confirmatory tests may be due to the fact that NH alone had 91.9% specificity versus 89.3% for the OPS (Table 1), and it may be a result of the higher affinity of goat antibodies for *B. melitensis* NH tracer directed to *B. melitensis* field strain infection than that of antibodies reacting to *B. abortus* OPS tracer.

In addition, it is noteworthy that the use of RBT3 plus NH instead of RBT3 plus CFTm (>1:4), which is the only procedure accepted in Mexico for goat testing (22), might significantly reduce the number of false positives, from 67.3% or 34.5% to 5.5%, if only RBT3 or RBT3 plus CFT (>1:4) are used; this may prevent goats from being culled according to the current Mexican regulations (22).

In summary, our results demonstrated that the FPA with the *B. melitensis* NH tracer yielded better diagnostic performance results for detection of antibodies against *Brucella* in goat serum samples than other tests, including FPA using *B. abortus* OPS tracer. The performance values of the FPA found in the present study support its use as a secondary test for RBT3 and make the NH a tracer of choice to be used in test series for goat brucellosis in areas with high vaccination rates and high prevalence; nevertheless, more studies are needed to evaluate the FPA in long-term vaccination studies. In addition, our results agreed with those of previous studies by others in which FPA was observed to perform better than the currently approved tests for diagnosis of *B. melitensis* infection in small ruminants (11, 14, 15, 18, 19) and showed that it is a viable solution for low-income countries where the goat industry is developing, because it is a simple, rapid, and low-cost test that does not require highly trained technicians like other confirmatory tests. In addition, it can be performed in or outside the laboratory, and it is considered that the reduction in the cost of replacement of wrongly killed goats justifies the initial investment in the test.

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