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ABSTRACT: A number of serological tests were compared for the detection of antibodies to *Brucella abortus* in bison (*Bison bison*). The performance of the fluorescence polarization assay (FPA) in both the preliminary evaluation and a subsequent blind validation indicated that this test was the most suitable for serological diagnosis of brucellosis in bison. The sensitivity and specificity in the preliminary evaluation were 92.1% and 99.4%, respectively. The sensitivity and specificity in a subsequent blind study were 96.3% and 97.6%, respectively. In a double blind study conducted on bison vaccinated with *B. abortus* strain 19, the data suggests that the FPA can differentiate bison infected with *B. abortus* from bison vaccinated with *B. abortus* strain 19. Both the indirect immunoassay (IELISA) and the competitive immunoassay (CELISA) performed nearly as well as the FPA. The buffered antigen plate agglutination test (BPAT) and the complement fixation test (CFT) did not perform as well as the FPA, CELISA or the IELISA in both studies. The FPA is a homogeneous assay eliminating the washing steps and reducing incubation to minutes rather than hours saving on time, equipment, materials, reagents and cost. These attributes, together, with its excellent sensitivity and specificity make the FPA an attractive test for the detection of serum antibodies to *Brucella abortus* in bison.

Key words: Bison, *Brucella abortus,* competitive enzyme immunoassay, fluorescence polarization assay, validation.

INTRODUCTION

The first published paper on the serological diagnosis of brucellosis using an agglutination test was by Wright and Smith (1897). Since then, the diagnosis of brucellosis has been accomplished using a variety of tests that vary in their ability to detect antibodies to *Brucella abortus* and as a consequence their diagnostic sensitivity and specificity. All these tests and their subsequent modifications were initially developed for use in cattle. Traditionally, agglutination tests which were sensitive but not highly specific were used as screening tests and positive results were confirmed using more accurate but labor intensive tests such as the complement fixation test. Primary binding assays have greater accuracy and some can differentiate animals vaccinated with *B. abortus* strain 19 from infection induced by field strains (MacMillan et al., 1990; Nielsen et al., 1989, 1995, 1996b; Rylatt et al., 1985). These assays can be tailored for diagnosis of brucellosis in non-cattle species that may impact on the cattle industry. As early as 1917, brucellosis in bison (*Bison bison*) was a concern (Mohler, 1917) and has remained so, particularly in cattle ranches or farms adjacent to game ranches (Bulmer, 1989) or National Parks such as Yellowstone (USA) (DeYoung, 1973; Cohn,

1991). It is important to accurately diagnose brucellosis in bison (Tessaro, 1986, 1989), since Canadian cattle are considered free of bovine brucellosis and infected bison might pose a significant threat to agriculture and export markets.

Primary binding assays for brucellosis were developed to improve the diagnostic sensitivity and specificity of serological tests for cattle and can be adapted for use in bison. The indirect enzyme immunoassay (IELISA) is highly sensitive and can be modified for bison using a monoclonal antibody produced against the bovine immunoglobulin light chain that cross reacts with the bison immunoglobulin light chain (Henning and Nielsen, 1992). However, the IELISA cannot differentiate vaccinal antibody or antibody from cross reacting microorganisms from antibody resulting from field infection. The competitive enzyme immunoassay (CELISA) which can differentiate as described by Nielsen et al. (1995, 1996a) is also capable of detecting antibodies to *B. abortus* in other species.

The fluorescence polarization assay (FPA) (Nielsen et al., 1996b) like the CELISA has the capability to differentiate field infection from vaccine and cross reactions, requires minimal manipulations, uses less equipment and can be completed in less than five minutes (Nielsen et al., 1996b). Because of this and its performance characteristics, it is a near ideal test for the detection of antibody to *B. abortus* in bison.

MATERIALS AND METHODS

Serological tests

The buffered antigen plate agglutination test (BPAT) was performed as described in the Office International des Epizooties (1996). Manual of standards for diagnostic tests and vaccines.

The complement fixation test (CFT) was done as described by Samagh and Boulanger (1978). The IELISA was performed as described by Nielsen et al. (1996a). The IELISA used smooth lipopolysaccharide (sLPS) from *B. abortus* as the antigen adsorbed onto a polystyrene microplate and followed stepwise by the application of the diluted serum samples; murine monoclonal antibody anti-bovine light chain (M4-1) conjugated with horseradish peroxidase (Henning and Nielsen, 1992) and substrate/chromogen. Divalent cation chelating agents (EDTA/EGTA) were added to the serum diluent to minimize nonspecific interactions (Nielsen et al., 1994) and the microplate was washed between each step with 0.01 M, pH 7.2 phosphate buffer saline containing 0.15 M NaCl and 0.05% Tween-20 (PBS/T).

The CELISA was performed as described by Nielsen et al. (1996a). As in the IELISA, the CELISA used sLPS antigen adsorbed onto a polystyrene microplate. After incubation and washing, diluted serum samples were added immediately followed by the addition of a murine monoclonal antibody (M84) specific for a *B. abortus* O-polysaccharide epitope. The serum samples were diluted in PBS/T containing EDTA/EGTA (Nielsen et al., 1994). The serum samples and the M84 were mixed for three minutes and incubated for thirty minutes. After incubation and washing, commercial goat antimouse IgG horseradish peroxidase conjugated antibody (heavy and light specific) was added which was followed by the addition of substrate/chromogen after incubation and washing.

The FPA was performed as described by Nielsen et al. (1996b). The assay uses *B. abortus* O-polysaccharide conjugated with fluorescein isothiocyanate (FITC). The assay involves the addition of a serum at 1/100 in 2 ml of 0.1 M sodium phosphate, pH 7.4, containing 0.15 M NaCl, 0.1% sodium azide and 0.05% lithium dodecyl sulfate (PBSAL). The sample was tested in a fluorescence polarization analyzer (FPM-1, Jolley Consulting and Research Inc., Round Lake, Illinois, USA) to obtain a baseline measurement. Next a predetermined amount of the conjugated antigen in 0.01 M sodium phosphate, pH 7.4 containing 0.15 M NaCl and 0.1% sodium azide is added, mixed and incubated for approximately two minutes to allow for interaction between the antigen and antibody that may be present. After incubation, the sample was measured in the fluorescence polarization analyzer. In the presence of antibody, a high millipolarization (mP) result was obtained, while in the absence of an antibrucella antibody, a low mP result was apparent.

Serum samples

Bison sera for the preliminary evaluation were obtained from areas where no clinical or epidemiological evidence of brucellosis was apparent for the specificity studies. Of these, approximately, 1,000 samples initially submitted

for diagnostic screening of brucellosis were tested by the BPAT, CELISA and IELISA. These samples were later supplemented with another 1,807 diagnostic samples from the same source for the comparison of the CFT and FPA (2,807 samples). Vaccination of bison with *B. abortus* strain 19 or RB51 is not currently practiced in Canada.

Positive samples for the preliminary study (*n* = 38) were collected from bison from which field strains of *Brucella abortus* had been isolated from various tissues after slaughter. These samples were collected from bison in Wood Buffalo National Park (*n* = 5), the United States (*n* = 15) and Ontario (*n* = 18).

In a subsequent blind study bison sera from 223 animals of known culture status were evaluated using the cutoffs previously determined. Nine sera were not tested due to insufficient volume and of the 214 remaining sera, 54 were from animals in which *B. abortus* had been isolated.

Bison sera from three animals inoculated with *B. abortus* strain 19 were evaluated with the CELISA and FPA tests in a double blind study. They were subsequently challenged with *B. abortus* strain 2308.

Data handling and analysis

A combination of statistical analysis and graphical presentation were used to determine the initial cutoff and the diagnostic sensitivity and specificity of the various assays for bison. The data was first analyzed using receiver operating characteristics (ROC) analysis (Schoojans et al., 1995). This analysis determined the cutoff to achieve the optimum sensitivity and specificity, as well as determining sensitivity and specificity values for other cutoffs. For visual confirmation, frequency distribution of the data was used.

The ELISA software used for the CELISA and IELISA was developed at the Animal Diseases Research Institute (ADRI) and is available upon request from (W. Kelly, Canadian Food Inspection Agency, Animal Diseases Research Institute, Nepean, Ontario, Canada).

RESULTS

The preliminary data presented in Table 1 compares the actual sensitivity based on culture results and the specificity based on no previous clinical or epidemiological evidence of brucellosis in the bison. The sensitivity of the BPAT (92.1%) did not exceed the other assays except for the CFT (89.5% with the anti-complementary (AC)

TABLE 1. Comparison of sensitivity and specificity estimates for serological tests for brucellosis in bison.

Test	% sensitivity (*n*)	% specificity (*n*)	Performance index[g]
BPAT[a]	92.1 (38)	91.7 (1,000)	183.8
CFT[b]	89.5 (38)	95.5 (2,807)	185.0
CFT[c]	97.4 (38)	65.1 (2,807)	162.5
CELISA[d]	92.1 (38)	98.4 (1,000)	190.5
IELISA[e]	100 (38)	96.2 (1,044)	196.2
FPA[f]	92.1 (38)	99.4 (2,807)	191.5

[a] Buffered antigen plate agglutination test, either positive or negative.
[b] Complement fixation test cutoff ≥ 1/5. AC refers to anti-complementary samples. AC reactions treated as negative.
[c] Anticomplementary reactions treated as positive.
[d] Competitive enzyme immunoassay using a cutoff of 29 percent inhibition.
[e] Indirect enzyme immunoassay using a cutoff of 39 percent positivity.
[f] Fluorescence polarization assay using a cutoff of 85 millipolarization units.
[g] Performance index is percent sensitivity plus percent specificity.

results treated as negative). The specificity of the BPAT (91.7%) did not exceed the other assays except for the CFT (65.1% with the AC results as positive). The sensitivity/specificity of the complement fixation test did not exceed the other tests depending on how the AC results were treated.

The specificity of the FPA was 99.4%, exceeding both the CELISA (98.4%) and IELISA (96.2%). Although, the sensitivity of the IELISA was 100%, it is unable to distinguish animals that have been vaccinated with strain 19 or infected with cross-reacting microorganisms from animals infected with *B. abortus,* probably accounting for its lower specificity (96.2%).

Both the CELISA and FPA are multi species assays with the capability to distinguish *Brucella* infected animals from *B. abortus* strain 19 vaccinated animals and from animals infected with cross-reacting microorganisms. Although these two assays both had sensitivity values of 92.1%, the specificity of the FPA was higher at 99.6% thus giving it a slight advantage over
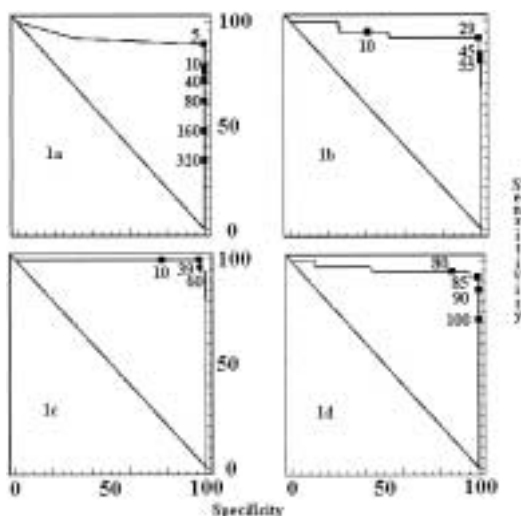
FIGURE 1a–d. The receiver operating characteristic curve is presented for each assay. The cutoff value for each assay is in the upper right-hand corner of each curve. Figure 1a is the complement fixation test with a optimal cutoff value greater than or equal to a 1/5 dilution. Figure 1b is the competitive enzyme immunoassay with a optimal cutoff value of 29% inhibition. Figure 1c is the indirect enzyme immunoassay with an optimal cutoff value of 39% positivity. Figure 1d is the fluorescence polarization assay with an optimal cutoff value of 85 millipolarization units.

the CELISA resulting in 10 fewer animals per 1,000 incorrectly diagnosed.

The cutoffs for each assay except for the BPAT were initially determined using ROC analysis and are presented in Figure 1a to 1d. This analysis determines sensitivity and specificity values at various cutoff values and determines the optimal sensitivity and specificity for each assay and provides the ability to compare assays using different measurement units. For instance, the CFT uses dilutions, the CELISA percent inhibition, the IELISA percent positivity and the FPA millipolarization units. The ROC analysis of the CFT data as presented in Figure 1a included anti-complementary results which were treated as negative results. This resulted in an optimal sensitivity of 89.5% and a specificity of 95.5% at a cutoff greater than or equal to 1/5 dilution. One of the difficul-

ties with the CFT is the interpretation of anticomplementary data. As indicated in Table 1 this can affect the determination of the sensitivity and specificity of the assay. The other assays do not have this problem.

In Figure 1b, the cutoff of the CELISA was 29% inhibition giving an optimal sensitivity of 92.1% and a specificity of 98.4%, while in Figure 1c the cutoff of the IELISA was 39% positivity for an optimal sensitivity of 100% and specificity of 96.2%. Figure 1d, the ROC curve for the FPA, determined a cutoff of 85 mP for an optimal sensitivity of 92.1% and specificity of 99.4%.

Receiver operating characteristics analysis can also determine the area under the curve (AUC). As an example, an AUC of 0.95 indicates that a randomly selected individual animal from a positive population will have a test value greater than a randomly selected individual animal from a negative population 95% of the time. The lowest AUC was for the CFT (0.923) while the highest AUC was for the IELISA (0.998). However, since the IELISA is unable to distinguish cattle infected with *B. abortus* from those vaccinated with strain 19 or infected with cross reacting microorganisms, the FPA with an AUC of 0.960 is a more accurate test followed by the CELISA with an AUC of 0.945.

The frequency distribution of the data for each assay is presented in Figure 2a to 2d. From these figures it would be difficult to determine the cutoff for each assay as some overlap between the negative and positive populations are apparent. This is quite obvious in Figure 2b for the CELISA and Figure 2d for the FPA data. Therefore, ROC analysis was used to determine the cutoff values for optimal sensitivity and specificity. These values were then visually confirmed using frequency distributions. The advantage of using frequency distributions in combination with ROC analysis is the ability to visualize the number of false positives and false negatives after the cutoff has been determined. For instance,
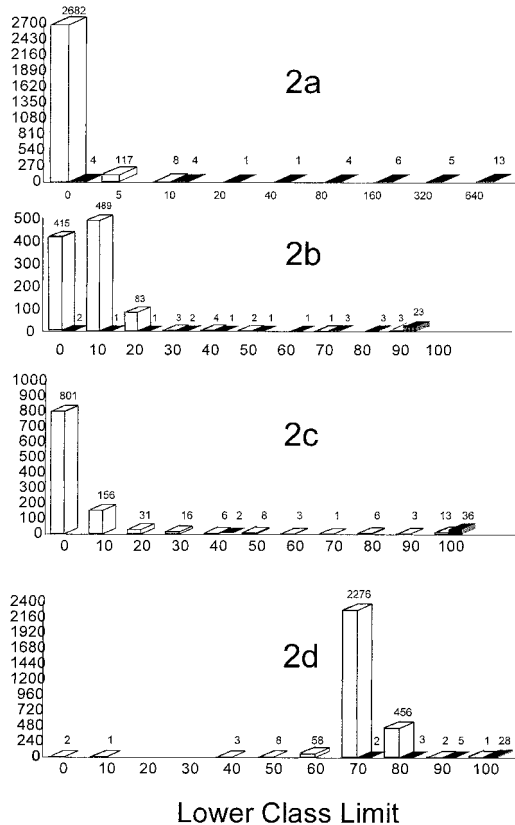
Number of Observations

FIGURE 2a–d. The frequency distribution for each assay is presented. The overlap of data varies for each distribution. Open bars are negative and closed bars are positive. The data above each bar, indicate the number of observations for that class interval. Figure 2a is the complement fixation test. Figure 2b is the competitive enzyme immunoassay. Figure 2c is the indirect enzyme immunoassay. Figure 2d is the fluorescence polarization assay.

a cutoff of 85 mP for the FPA assay in Figure 2d indicates that at least three samples were false positive results. Similarly, a cutoff of 39% for the IELISA in Figure 2c indicated that it had more false positive results than the FPA. This was true for the other assays as well.

In a blind study (Table 2) conducted on 214 samples of which 54 samples were from animals from which *B. abortus* had been isolated the CFT (ACs included), CELISA, FPA and IELISA had an actual sensitivity of 96.3%, respectively. The

BPAT had a sensitivity of 81.5%. The BPAT, IELISA and the FPA had the highest specificity of 97.6%. The CFT and the CELISA had a specificity of 91.7% and 94.1%, respectively.

Data presented in Table 3 compare the FPA and the CELISA using sera from bison inoculated with *B. abortus* strain 19 and subsequently challenged with *B. abortus* strain 2308. With one exception (4 wk PI) both the FPA and the CELISA were able to differentiate post-inoculation sera from post-challenged sera. *Brucella abortus* was isolated in two of the three bison after challenge.

The performance index presented in Tables 1 and 3 is the sum of the sensitivity value plus the specificity value. Both the IELISA and the FPA have the highest index in both studies. The BPAT has the lowest index in both studies.

DISCUSSION

The data presented in Tables 1, 2, and 3 indicated that the most suitable test overall for diagnosis of brucellosis in bison was the FPA followed by the IELISA, CELISA, CFT and BPAT as indicated by the sensitivity values, specificity values, AUC and the performance index. The BPAT and CFT did not perform as well as the primary binding assays, due in part to their reliance on secondary properties of antibodies such as the ability to agglutinate or to fix complement.

These assays were less specific than the primary binding assays and were developed for the detection of serum antibody to *Brucella abortus* in cattle. They were never subjected to the same scrutiny for validation as present day primary binding assays. As well, the data reported in the literature (Thorne et al., 1978; Davis et al., 1990; Nicoletti, 1992) suggests that no single test could be relied upon to definitively diagnose brucellosis in bison.

The IELISA used a murine monoclonal produced against bovine immunoglobulin light chain which cross-reacts with bison immunoglobulin light chain (Henning and

TABLE 2. Comparison of sensitivity and specificity estimates for serological tests for the detection of bison serum antibody to *Brucella abortus* in a blind study.

Test	Sensitivity (%) (n = 54)	Specificity (%) (n = 160)	Performance index[g]
Buffered Antigen Plate Agglutination Test[a]	81.1	97.6	178.7
Complement Fixation Test[b]	94.3	91.7	186.0
Complement Fixation Test[c]	96.3	91.7	188.0
Competitive enzyme immunoassay[d]	96.3	94.1	190.4
Indirect enzyme immunoassay[e]	96.3	97.6	193.9
Fluorescence polarization assay[f]	96.3	97.6	193.9

[a] Buffered Antigen Plate Agglutination Test, either positive or negative.
[b] Complement Fixation Test cutoff ≥ 1/5. AC refers to anticomplementary samples. AC reaction treated as negative.
[c] Anticomplementary reaction treated as positive.
[d] Competitive enzyme immunoassay using a cutoff of 29 percent inhibition.
[e] Indirect enzyme immunoassay using a cutoff of 39 percent positivity.
[f] Fluorescence polarization assay using a cutoff of 85 millipolarization units.
[g] Performance index is percent sensitivity plus percent specificity.

Nielsen, 1992) resulting in a sensitivity value of 100%. The FPA and the CELISA can distinguish over 95% of the cattle vaccinated with strain 19 (Nielsen, 1995, 1996b) or exposed to cross-reacting microorganisms from cattle infected with *B. abortus.* The BPAT, CFT and the IELISA cannot distinguish cattle vaccinated with *B. abortus* strain 19 or exposed to cross-reacting microorganisms from cattle infected with *B. abortus.* Strain 19 vaccination of bison has been attempted in the past in both Wood Buffalo National Park (Choquette, 1961) and in the United States in private herds (Flagg, 1983; Davis et al., 1991) and strain 19 vaccinated bison are regularly imported into Canada from the United States. The data presented in Table 3 suggests that both the FPA and CELISA can differentiate bison infected with *B. abortus* from bison vaccinated with *B. abortus* strain 19.

For acceptance of a new test for the presumptive diagnosis of brucellosis, it would be required to perform as well as or better than the in-use tests or possess an advantageous attribute. The data in Table 2 validates the preliminary data presented in Table 1 suggesting that the FPA is the most suitable test for the diagnosis of brucellosis in bison reducing the need to use a series of unvalidated conventional serological tests.

TABLE 3. Comparison of the fluorescence polarization assay and competitive enzyme immunoassay with bison inoculated with *B. abortus* strain 19 and challenged with *B. abortus* strain 2308 in a double blind study.

Animal	Time	Culture	FPA[c]	CELISA[d]
Bison 7	52 wk PI[a]		66.5	21
Bison 7	4 wk PC[b]		110.4	75
Bison 7	Necropsy (12 wk PC)		99.4	71
Bison 8	52 wk PI		73.6	28
Bison 8	4 wk PC	+	118.2	62
Bison 8	Necropsy (12 wk PC)	+	180.3	99
Bison 9	4 wk PI		88.5	32
Bison 9	52 wk PC		66.6	7
Bison 9	Necropsy (12 wk PC)	+	90.2	48

[a] PI = weeks after inoculation with 2.3×10^{10} CFU of *B. abortus* strain 19.
[b] PC = weeks after intra conjunctival challenge with 1×10^7 CFU of *B. abortus* strain 2308 at 180 days gestation.
[c] Fluorescence polarization assay using a cutoff of 85 millipolarization units.
[d] Competitive enzyme immunoassay using a cutoff of 29 percent inhibition

The BPAT and the FPA are comparable in cost and less expensive than the CELISA or the IELISA. The most expensive test to perform is the CFT. The sensitivity and specificity of the CFT are not comparable to those of the FPA or CELISA.

The data demonstrates that brucellosis tests such as the BPAT and CFT which were developed for cattle may not be the

most suitable tests for bison or other non-traditional livestock. The bison industry in North America continues to expand (80,000 to 100,000 bison; Davis et al., 1990), placing bison in close contact with traditional livestock species. Some herds are located close to known sources of infection such as Wood Buffalo National Park in Canada and Yellowstone National Park (USA). When testing bison, it is important that the most accurate test be used to minimize the risk of missing infected animals.

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