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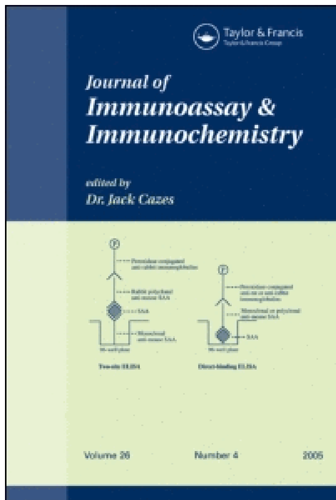

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FLUORESCENCE POLARIZATION ASSAY FOR THE DIAGNOSIS OF BRUCELLOSIS: A REVIEW

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FLUORESCENCE POLARIZATION ASSAY FOR THE DIAGNOSIS OF BRUCELLOSIS: A REVIEW

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SUMMARY

Fluorescence polarization assay (FPA) is based on the rotational differences between a small soluble antigen molecule in solution (labelled with a fluorochrome) and the antigen molecule complexed with its antibody. A small molecule will rotate randomly at a rapid rate, resulting in rapid depolarization of light, while a larger complex molecule will rotate slower and depolarize light at a reduced rate. The rate change in depolarization can be measured. The FPA is a homogeneous assay which does not require removal of unreacted reagents and can, therefore, be performed very quickly and, given portable equipment, in the laboratory and in the field. The latter obviates the need for shipping samples and eliminates waiting for results, as well as reducing test costs.

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The FPA technology has been developed and validated for the serological diagnosis of brucellosis in cattle, swine, sheep, goats, bison, and cervids. Sufficient cross reactivity of the common epitopes of *Brucella abortus*, *B. melitensis* and *B. suis* O-polysaccharide (OPS) allowed for the use of a single antigen for all species of smooth *Brucella* and animals. The OPS prepared from *B. abortus* S1119.3 was conjugated with fluorscein isothiocyanate (FITC). The FPA was initially developed for testing serum; however, the technology has been extended to testing whole blood and milk from individual animals or bulk tank samples pooled from 2000 or fewer animals. The accuracy of the FPA equalled or exceeded those obtained using other serological tests such as the buffered antigen plate agglutination test (BPAT), the milk ring test (MRT), the complement fixation test (CFT), the indirect enzyme immunoassay (IELISA), and the competitive enzyme immunoassay (CELISA).

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INTRODUCTION

Brucellosis is a world wide zoonotic disease affecting domestic animals and man. The first description was provided in 1888 by Bruce,(1) directing the Mediterranean Fever Commission set up to investigate morbidity and mortality among British military personnel stationed on Malta. As it turned out, the soldiers consumed untreated local goat milk and milk products from animals infected with *B. melitensis*. This avenue of transmission is still the major cause of human brucellosis. In 1897, Bang(2) determined that *B. abortus* was a cause of abortion in cattle and the same year, Wright and Smith(3) described the first serological test for brucellosis. Since then, a number of serological tests, including numerous agglutination tests, agar gel immunoprecipitation tests, and complement fixation tests, have been developed and many modified to enhance performance. These have been reviewed by MacMillan.(4)

In 1930, Buck(5) introduced a vaccine, *B. abortus* S19, against cattle brucellosis. This vaccine was instrumental in controlling and eliminating bovine brucellosis in many areas of the world; however, in many instances, it also confounded serological diagnosis of the infection because the serological tests, except for an agar gel immunodiffusion test developed by Diaz et al.,(6) could not distinguish antibody resulting from vaccination from that due to infection.

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In 1976, Carlsson and coworkers(7) introduced a primary binding assay for detection of antibody to *B. abortus* in man. This assay was an indirect enzyme immunoassay (IELISA) which was very sensitive, but lacked specificity when applied to bovine brucellosis, as it detected residual antibody to *B. abortus* S19 and also to cross reacting microorganisms. It was, however, shown to be a useful diagnostic test with both serum and milk where vaccination was not practiced and with unvaccinated species.

The cross reactivity problems of the IELISA were partly solved with the development of competitive enzyme immunoassays (CELISA) which, based on the use of higher affinity monoclonal antibody, were able to eliminate low affinity cross reacting antibody and, in some cases, also residual antibody to *B. abortus* S19. Primary binding assays have been reviewed.(8)

While ELISAs have been developed for use outside the laboratory, these tests, such as pregnancy tests, are too expensive for mass diagnosis and, as a consequence, serological diagnosis has been performed on samples collected on the farm and shipped to the laboratory. After testing, the results would be reported to the field personnel who, in turn, would report to the owner of the animals. This lengthy procedure is not only expensive, but it also could result in spread of disease by aborting animals during the waiting period. As a result, it would be very useful for the control and eradication of brucellosis to have a test procedure which could be done rapidly *in situ* with the same accuracy as the ELISAs and at a reasonable cost. This led to the development of the FPA, a homogeneous assay which can be done in minutes using serum, whole blood, or milk from individual animals or from bulk milk tanks. The FPA is as accurate as the CELISA and more accurate than other serological tests. It is applicable to a number of species of animals infected with a number of species of *Brucella*.

The mechanism of the FPA was reviewed recently.(9) Briefly, molecules in solution rotate randomly. The rate of rotation is dependent on the molecular size, tertiary structure, and a number of other factors such as temperature and viscosity. In general, a small molecule will rotate more rapidly than a larger molecule and will, therefore, depolarize plane polarized light more rapidly. Therefore, a small antigen, less than 50 kilodaltons, conjugated with a fluorochrome will rotate at a reduced rate when combined with antibody and, thereby, depolarize light at a decreased rate. The shift in depolarization rate can be measured by assessing the time it takes for molecules to rotate through an angle of approximately 68.5°.

The FPA for the serological diagnosis of brucellosis uses OPS prepared from *B. abortus* S1119.3, hydrolyzed to an average molecular

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weight of 22 kd and conjugated with FITC. The assay is performed by diluting serum, blood, or milk in an appropriate buffer, obtaining a baseline reading for the sample, adding the OPS-FITC and allowing an appropriate incubation period of two minutes or less. A final reading is obtained, from which the background activity is subtracted. The result is expressed in millipolarization units (mP) and a low reading indicates that the sample was negative. The FPA has been developed and validated for serological diagnosis of cattle, sheep, goats, bison, and cervids infected with smooth species of *Brucella*.

EXPERIMENTAL

Samples

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Serum

Serum samples should be free from particulate matter such as bacteria. Hemolysed sera do not pose a problem. Serum dilutions are tested as follows: cattle, bison, cervids 1:100; goats 1:40; sheep, swine 1:25.

Whole Blood Samples

Blood collected in EDTA anticoagulant may be tested immediately, after storage or freezing. Blood is tested at a dilution of 1:25.

Milk

Milk may be tested directly, after storage, after freezing, and in a deteriorated state. Milk is diluted 1:25 for testing.

Bulk Milk Samples

Two mL. of milk from bulk tanks from up to 2000 cattle were tested undiluted.

Samples were collected from *Brucella sp.* infected or serologically positive (as determined by the serological tests prescribed for diagnosis by the country of collection) and from non-exposed animals collected in areas with no epidemiological, clinical, or serological evidence of

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brucellosis. The following numbers of samples were tested for validation purposes:

Species	Positive	Negative
Cattle		
Serum	1084[1]	23755
Whole blood (fre)[2]	100	424
Whole blood (sto)[3]	236	969
Milk (ind)[4]	1086	2974
Milk (blk)[5]	12[6]	361
Swine	401[1]	14037
Bison		
Serum	91[1]	1160+
Whole blood[2]	38	36
Goats	1093	1817
Sheep	71[1]	1286
Cervids	626[1]	2631

[1]Indicates samples from animals from which *Brucella sp*. was isolated. Other positive samples were collected from animals with positive serological reactions.
[2]Refers to whole blood samples collected in the field and tested immediately.
[3]Refers to whole blood samples collected and shipped to the laboratory for testing (stored).
[4]Refers to milk samples collected from individual cows.
[5]Bulk milk tank samples.
[6]Positive bulk tank milk samples were artificially created by mixing milk from a confirmed positive animal with negative bulk milk.
+Varied between 1160 and 2967 samples among tests due to depletion of some samples.

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Diluent

The diluent buffer for all assays except the bulk milk assay was 0.01 M tris, pH 7.2, and contained 10 mM EDTA, 0.15 M NaCl and 0.05% Igepal CA 630. This buffer is stable and causes lysis of cells in whole blood and some dispersion of fat globules when used with milk samples.

Antigen

The OPS from *B. abortus* S1119.3 is prepared by hydrolyzing the OPS from the bacteria with 2% acetic acid at 121°C for 15 minutes. After cooling, 4% trichloroacetic acid is added and the precipitate removed by centrifugation at 10.000 × g for 15 minutes. After neutralization, the supernatant solution was dialized against distilled water for 24 hours and

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then against 0.01 M phosphate buffer, pH 6.8, containing 0.1 M NaCl. The dialized material was then passed through a column packed with immobilized polymyxin B to remove lipid A. The column effluent was dialized against water and freeze dried. An amount of 3 mg of OPS was hydrolyzed with 100 μL of 0.1 N NaOH at 37°C for 60 minutes and conjugated with 33 mg FITC (100 mg per mL in dimethyl sulfoxide) at 37°C for 60 minutes. The conjugated material was then applied to a 1 × 5 cm column packed with DEAE Sephadex A 25 equilibrated with 0.01 M phosphate buffer, pH 7.4. The effluent with this buffer was discarded and the material eluted with 0.1 M phosphate buffer, pH 7.4 was kept and used as the antigen. The amount to be used per test was determined with the FP Sentry analyzer as the volume that gave a total intensity reading of 250,000, usually 10 μL.

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FP Assay

Serum, Whole Blood, and Individual Milk Samples

A background reading for indigenous fluorescence was obtained with the appropriately diluted and thoroughly mixed sample in a 10 × 75 mm borosilicate glass tube using a Sentry FP analyzer (Diachemix Corp., Grayslake, IL, USA). Antigen-FITC was added, the reagents mixed and, for whole blood, a second reading was taken after 15 seconds of incubation while all serum and milk assays were incubated for 2 minutes. The second reading in the analyzer automatically subtracted the background reading and the result was presented in millipolarization units [mP](11,12).

Bulk Milk Tank Samples

2 mL of milk were acidified with 10 μL 1.0 gm/mL citric acid, mixed rapidly with a Vortex mixer for 3 minutes to congeal the fat, and centrifuged at 10,000 rpm for 6 minutes. One mL of the whey was added to a 10 × 75 mm borosilicate glass tube containing 0.5 mL lyophilized 1.0 M sodium dithionate dissolved in 0.04 M tris, pH 10.2, containing 10 mM EDTA. After mixing, a background reading was obtained with the Sentry FP analyzer, antigen-FITC was added and mixed and, after a minimum of two minutes incubation, a final reading was obtained with the analyzer.(13)

In all cases, manipulations were done at ambient temperature and all chemicals were obtained from Sigma Chemical Co., St. Louis, Mo, USA.

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Other serological tests were performed on all samples for comparison, including the buffered antigen plate agglutination test [BPAT];(14) the complement fixation test [CFT];(15) the indirect enzyme immunoassay with serum and milk [sIELISA, mIELISA];(16) the competitive enzyme immunoassay [CELISA];(16) and the milk ring test where applicable [MRT].(4)

Data Expression for the Serological Tests Used to Diagnose Brucellosis

BPAT	Positive or negative, depending on the presence of visible agglutination.
CFT	50% Hemolysis of the indicator system at a 1:5 dilution or greater.
IELISA	Positivity % relative to a positive reference serum.
CELISA	Inhibition % of the competing monoclonal antibody, relative to a buffer control (no competition).
FPA	Millipolarization (mP) units equal to or above the cutoff value determined by receiver operator characteristic analysis.

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Data Evaluation

Cutoff values between positive and negative reactions were established by analyzing the data using MedCalc software.(17) Initially, a dot plot was prepared followed by receiver operator characteristic analysis (ROC). The ROC analysis provided optimum sensitivity and specificity values (the sum of the sensitivity and specificity values were at a maximum). Sensitivity and specificity values were added to provide a performance index (PI).

RESULTS AND DISCUSSION

The FPA was initially developed for detection of bovine antibody to *B. abortus*.(11,18–20) The dot plot for cattle sera tested by the FPA is presented in Figure 1. From the plot, it is clear that a cutoff value of 89.9 mP give maximum discrimination between positive and negative samples. The software does not provide sensitivity and specificity values accurately and, as a result, ROC analysis was performed (Figure 2). From the ROC analysis, a cutoff value of 90 mP gave a sensitivity value of 99.3% and a specificity value of 99.98%. All other data from the FPA and other

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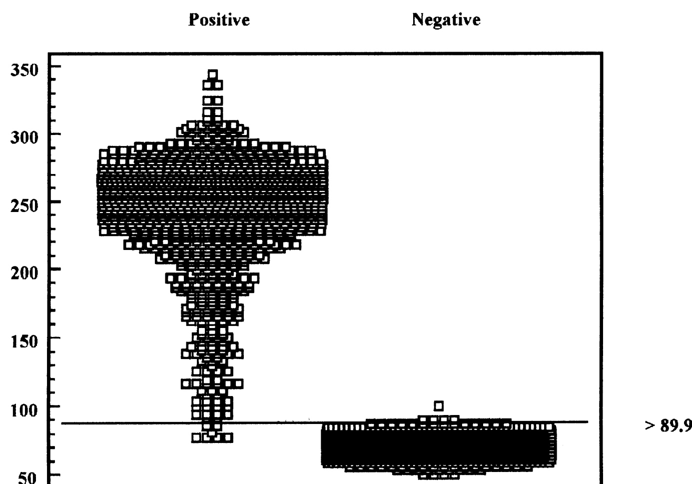

Figure 1. MedCalc dot plot of sera from 1084 confirmed cases of bovine brucellosis and 23755 negative cattle sera (of Canadian origin). The cutoff value was determined to be 89.9 mP. The Y-axis is in millipolarization (mP) unit.

serological tests was manipulated in a similar fashion and, as a result, the dot plots and the ROC curves have not been included. The results are summarized in Table 1a.

Based on performance index values (PI), the FPA performed as well as the CELISA and IELISA and better than the CFT and BPAT with bovine sera (Table 1a). The negative samples used for this study were of Canadian origin and therefore from an unvaccinated population. It was shown previously that the IELISA was considerably less specific that the FPA and CELISA when testing vaccinated animals (11). Sera sometimes activate complement in the absence of antigen and a diagnostic decision cannot be made for such anti-complementary sera. This leads to rebleeding and delays in obtaining results. Where anticomplementary reactions occurred with the test samples, data was analyzed twice, considering those sera as positive or negative.

When testing whole blood, the cutoff value depended on the length of time elapsed after the sample was obtained. If tested immediately, a cutoff value of 105 mP was determined as optimum and it was necessary to obtain the final reading on the sample after 15 seconds (rather than 2 minutes) of incubation to avoid increasing autofluorescence in the sample.(21) From Table 1b, the 15 second reading appeared to cause a loss in sensitivity and specificity relative to the FPA performed on matching serum samples. Since

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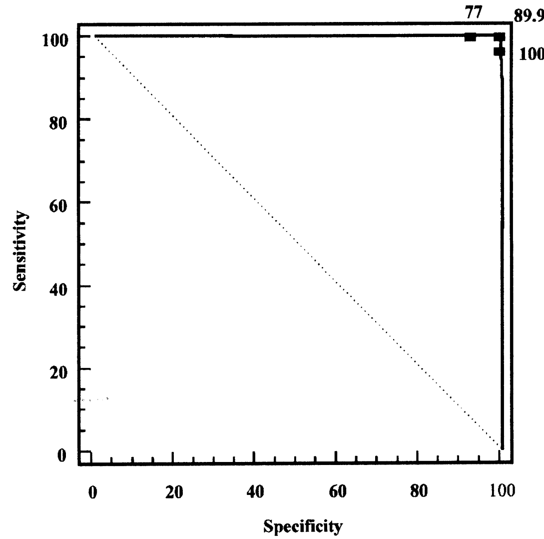

Figure 2. Receiver operator characteristic (ROC) analysis of the same sera as in Figure 1 using MedCalc software. The cutoff remained 89.9, and the sensitivity was determined to be 99.30% and the specificity was 99.96%. Cutoff values of 77 or 100 mP would increase sensitivity or specificity, respectively. The diagonal line, the 'chance' line indicates the area below which the test would provide a 50% chance of being correct. Thus the area under the curve is representative of the accuracy of the test. In this case the area is 0.999, indicating a test accuracy of 99.9%.

Table 1a. Summary of Serological Tests Using Serum from Cattle. Results Were Obtained Using 1084 Serum Samples from Cattle from Which *Brucella sp*. Was Isolated and 23755 Serum Samples from Canadian Cattle (Free from Brucellosis Since 1985)

Serum	FPA	CELISA	IELISA	CFT		BPAT
Cutoff	90	30	46	1:5		+/−
% Sensitivity	99.3	100	100	97.1[1]	87.9[2]	98.6
% Specificity	100	99.8	99.8	93.1	99.8	97.9
PI	199.3	199.8	199.8	190.2	187.7	196.5

Data obtained if anticomplementary reactions were considered as [1]positive and [2]negative.

the data was compiled using only 100 positive and 424 negative samples, it is possible that testing more samples will increase the PI values. It is also worthwhile noting that early data was probably less accurate, due to problems with precipitates in phosphate buffer dissolved in non-distilled

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Table 1b. Summary of FPA Using Whole Anticoagulated Blood (FPAfwb) Tested Immediately After Collection Compared to Results Obtained with the FPA and CFT Using Matched Serum Samples. Samples that Gave a Positive Reaction (n = 100) in the BPAT and CELISA Were Considered as Positive and the % Sensitivity Is Relative to Those Tests. Samples that Gave Negative Reactions (n = 424) in the BPAT and CELISA Were Considered Negative for Determination of the Relative Specificity Value

	FPA (fwb)	CELISA	IELISA	CFT	BPAT	FPA (Serum)
Cutoff	105	NA	NA	1:5	NA	90
% Sensitivity	95.0			100		100
% Specificity	97.3			66.7[1] 98.1[2]		100
PI	192.3			166.7 198.1		200

Data obtained if anticomplementary reactions were considered as [1]positive and [2]negative.

Table 1c. Anticoagulated Whole Blood Which Was not Tested Immediately Was Tested by the FPA (swb) and by the CFT and Serum FPA Using Matched Serum Samples. Sample Were Considered as Positive (n = 236) if Positive Reactions Were Obtained Using the BPAT and CELISA and Negative (n = 969) if Both Tests Gave Negative Results

	FPA (fwb)	CELISA	IELISA	CFT	BPAT	FPA (Serum)
Cutoff	95	NA	NA	1:5	NA	90
% Sensitivity	98.6			97.5		98.6
% Specificity	98.9			66.5		100
PI	197.5			164.0		198.6

Table 1d. Milk Samples from Individual Animals Were Tested by the mFPA, mIELISA, and MRT. Positive Samples (n = 1086) Were Selected Based on the *Brucella* Ring Test Reaction in the Country of Origin. Negative Samples Were of Canadian Origin (n = 2974)

	FPA (m)	CELISA	mIELISA	CFT	BPAT	MRT
Cutoff	95	NA	20	NA	NA	+/−
% Sensitivity	100		100			99.1
% Specificity	99.3		98.7			99.7
PI	199.3		198.7			198.8

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Table 1e. Positive (n = 12) Bulk Tank Milk Samples Were Artificially Created by Adding Milk from a Bacteriologically Confirmed Case of Brucellosis to Negative Bulk Milk. Samples from Canadian Herds Were Used for Negative Data (n = 361) to Generate a Cutoff Value. Without a Sensitivity Estimate, the PI Value Could Not Be Determined

	FPA (m)
Cutoff	140
% Sensitivity	Insufficient data
% Specificity	100
PI	

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water and solubility problems with the detergent, lithium dodecyl sulfate, at lower temperatures. These problems have been resolved by using a tris based buffer and Igepal CA630 (formerly NP40) detergent. The data was compiled using the BPAT and CELISA as reference tests and, therefore, no data was included for those tests. The IELISA was not done due to its lack of specificity where *B. abortus* S19 vaccination was practiced.

For whole blood samples tested more than 15 minutes after bleeding, a cutoff of 95 mP was selected as optimum (Table 1c). The PI for these samples was 5.2% higher than for the field tested samples (Table 1b) and approached the PI obtained with matched serum samples tested in the FPA, the sensitivity of the two formats being identical and the serum FPA being marginally more specific. The PI of the whole blood FPA exceeded the PI for the CFT performed on matching serum samples. Again, further testing may improve the PI for the whole blood FPA. No data was reported for the BPAT and CELISA which were used as reference tests.

An FPA was developed for testing individual milk samples(12) and for bulk tank milk samples(13) for antibody to *B. abortus*. The individual milk samples tested were selected from herds confirmed infected by bacteriology and based on a positive MRT performed locally. The samples were then shipped and retested in the MRT as well as the mIELISA and the FPA. The FPA performed as well as the IELISA and the MRT (there is a small discrepancy between the original MRT by which the positive samples were selected, possibly due to sample deterioration during shipping). Use of the MRT for sample selection caused sensitivity to be inflated. The reported sensitivity values for the MRT is about 90%.(22, 23) These data are presented in Table 1d. The MRT sensitivity data should be considered control data indicating that sample quality and technical aspects of the test were comparable between the initial sample collection and final testing.

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The FPA for testing bulk tank was recently developed and has only been used with artificially created positive samples by mixing milk whey from a confirmed case of brucellosis with negative milk. Therefore, statistical analysis of the sensitivity is not possible (Table 1e). It was possible to repeatedly detect a positive sample, among 2000 negative samples using this technology.(13) For both milk assays, the negative milk samples were of Canadian origin.

Serological tests for the diagnosis of porcine brucellosis perform poorly compared to the same tests used for bovine serology. Specifically, the sensitivity values are lower, with the FPA and IELISA performing best (and the CFT if anticomplementary sera were considered as negative). It would appear that some pigs do not produce detectable levels of antibody in spite of being infected with *B. suis*, as shown by isolation of the causative organism.(24,25) Similarly, the assay specificities are generally low, leading to low PI estimates (Table 2). The question arises if serological tests can be used to diagnose individual pigs infected with *B. suis* or if serological tests are only useful for herd diagnosis. It is of interest to note that a commonly used screening test, the BPAT, failed to detect antibody in 22.9% of the samples. The highest PI was established for the FPA, making it the choice test for diagnosis.

Serum samples were available from 91 bison from which *Brucella sp.* had been bacteriologically isolated.(26) All the serological tests failed to detect antibody in 2 of the sera and the FPA and CELISA failed to detect antibody in a further 3 sera with the CFT and BPAT failing with 4 and 16 sera, respectively. The commonly used screening test, the BPAT, performing worst (Table 3a). The specificity values were similar, although the BPAT had more false positive reactions than the other tests. The most accurate performance overall was with the FPA, marginally higher than the IELISA; however, in a situation where sensitivity was essential, the IELISA would be the test of choice.

Table 2. Samples Obtained from 401 Swine from Which *B. suis* Was Isolated and 14037 Canadian Swine Sera Were Tested by the FPA, CELISA, IELISA, CFT, and BPAT

	FPA	CELISA	IELISA	CFT		BPAT
Cutoff	84	28	10	1:5		+/−
% Sensitivity	93.5	90.8	94.1	93.3[1]	58.1[2]	77.1
% Specificity	97.2	96.6	87.9	99.9	95.5	95.9
PI	190.7	187.4	182.0	193.2	153.6	173.0

Data obtained if anticomplementary reactions were considered as [1]positive and [2]negative.

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The FPA was also shown to be useful with whole blood from bison (Table 3b). Blood samples collected and tested in the field were divided into positive and negative groups based on the BPAT and CELISA. All negative samples were also negative in the FPA and of the positive samples, the FPA failed to detect antibody in 1 sample.

Goat serum samples selected on the basis of their reaction in the rose bengal and rivanol agglutination tests showed excellent agreement with the BPAT and the CFT (Table 4). The relative sensitivity values of the FPA and CELISA were 4 to 5% lower, most likely due to animal selection using isotype specific agglutination tests and/or residual vaccinal antibody not being detected by these assays. The relative specificity values for all assays were very similar. These data highlight one of the problems frequently encountered when validating new tests by comparing the data to those obtained with other, accepted tests, usually of lower performance. Thus, it is often thought that the new test is wrong in favour of the older test. In spite of the data, it would appear advisable to use tests that distinguish vaccinal antibody in areas where vaccination is practiced. In addition, the selection criteria used for the goat sera favour the agglutination tests, making them appear more accurate than in reality.

Table 3a. Serum Samples Obtained from 91 Culture Positive Bison Were Tested. The Number of Negative Bison Sera Varied as Follows Due to Depletion of Samples: FPA (n = 2967); CELISA (n = 1160); IELISA (n = 1204); CFT (n = 2967); and BPAT (n = 1160)

	FPA	CELISA	IELISA	CFT	BPAT
Cutoff	85	28	33	1:5	+/−
% Sensitivity	94.5	94.5	97.8	92.3	85.7
% Specificity	99.5	97.4	95.4	99.4	91.7
PI	194.0	191.9	193.2	191.7	177.4

Table 3b. Relative Sensitivity and Specificity of Anticoagulated Whole Blood Samples from 38 Bison with Positive BPAT and CELISA Tests and 36 Bison Samples that Gave Negative Results in the BPAT and CELISA Using Matched Serum Samples

Cutoff	100
% Sensitivity	97.4
% Specificity	100
PI	197.4

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Table 4. Goat Serum Samples, (n = 1093), Giving Positive Results in the Rose Bengal and Rivanol Agglutination Tests and 1817 Goats that Were Negative Were Tested by the FPA, CELISA, CFT, and BPAT. Sensitivity and Specificity Values Are Relative to the BPAT and CFT

	FPA	CELISA	IELISA	CFT		BPAT
Cutoff	88	27	NA	1:5		+/−
% Sensitivity	94.9	95.4		95.9[1]	99.7[2]	99.9
% Specificity	99.4	99.0		100	100	100
PI	194.3	194.4		195.9	199.7	199.9

Data obtained if anticomplementary reactions were considered as [1]positive and [2]negative.

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Table 5. Sheep Serum Samples from 71 Sheep from Which *B. melitensis* Was Isolated and 1286 Canadian Sheep Were Tested by the FPA, CELISA, IELISA, CFT, and BPAT

	FPA	CELISA	IELISA	CFT	BPAT
Cutoff	78	26	9	1:5	+/−
% Sensitivity	91.5	76.1	93.0	83.1	77.5
% Specificity	98.6	99.7	97.6	99.5	99.8
PI	190.1	175.8	190.6	182.6	177.3

Samples of sheep serum from 71 confirmed cases of infection with *B. melitensis* demonstrated that the most sensitive assays were the IELISA, FPA, CFT, BPAT, and CELISA, in that order. The specificity values for all assays were very similar, resulting in the IELISA and the FPA being the most accurate tests (Table 5). Surprisingly, the CELISA detected the lowest number of sera from infected animals, approximately the same number as the BPAT, resulting in nearly one quarter of infected animals not being detected. This was a repeatable finding which may possibly result from serum obtained from animals in very early stages of infection with antibody of insufficient affinity to compete with the monoclonal antibody.

The relative sensitivity and specificity for detection of antibody to *Brucella sp*. in cervids was nearly identical for the FPA, CELISA, IELISA and BPAT with the CFT giving somewhat lower PI values (Table 6). The BPAT was used as the selection criterion for some of the serum samples and that explains its high PI value. The results provided are a composite of data from elk, reindeer, caribou, and red deer samples. The values for the individual species were reported elsewhere.(27)

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Table 6. Relative Sensitivity and Specificity Obtained with Serum Samples from Cervids (Positive Samples, Were Based on the BPAT or Isolation of *Brucella sp*. from 320 Reindeer, 102 Caribou, 102 Elk, and 102 Red Deer. Negative Samples Were from 658 Reindeer, 308 Caribou, 351 Elk, and 1314 Red Deer). Sera Were Tested by the FPA, CELISA, IELISA, CFT, and BPAT. The Data Was Combined for All Species (Individual Data May be Found in Gall et al, 2001)

	FPA	CELISA	IELISA	CFT	BPAT
Cutoff	87	27	11	1:5	+/−
% Sensitivity	99.0	99.0	100	100	99.0
% Specificity	99.3	99.8	98.7	90.6[1] 54.4[2]	100
PI	198.3	198.8	198.7	190.6 154.4	199.0

Data obtained if anticomplementary reactions were considered as [1]positive and [2]negative.

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The ROC analysis of the results for the different species and serum/milk/whole blood samples indicate that a unified cutoff value between positive and negative results for the various species tested cannot be established for the FPA, CELISA, and IELISA. It is possible that ROC analysis is not the ideal method of establishing the cutoff value, as it does not take into account day to day variations in the test environment. Therefore, a cutoff value determined, for instance, by testing a number of negative samples and adding a constant to the mean daily or for each sample lot may be more desirable.(28) Such a protocol, however, would lead to standardization problems as the supply of control sera would undoubtedly vary. An international standardization scheme would be fairly easy to accomplish using standard sera available only from national reference laboratories which, in turn, would prepare national standard sera that could be supplied to diagnostic laboratories or, alternately used as reference controls for locally established control samples. For these purposes, bovine monoclonal antibodies specific for the *Brucella* OPS would be of value; however, such reagents are not available currently.

Table 7 summarizes the data. For all species tested, except for goats, some cervids and the CELISA used with sheep sera, the primary binding assays, the FPA, CELISA, and IELISA, ranked equivalent of higher in performance index than the conventional tests, the BPAT and the CFT. This may be partly explicable by the selection criteria used for goat and cervid sera thought to be from exposed animals as agglutination tests were used for the initial screening and, in some cases, for confirmation. In the case of goats, the positive population was at least partly vaccinated, accounting for the lower sensitivity values compared to the other tests. The reason for the low value of the CELISA when used with sheep sera

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Table 7. Summary of Serological Test Performance with Sera from Various Species. The Values Are the Performance Indices (PI) from the Above Tables. The PI Value Is the Maximum Sum of the % Sensitivity and % Specificity Determined by ROC Analysis

	Bovine	Swine	Bison	Goat	Sheep	Cervids
BPAT	196.5	173.0	177.4	199.9*	177.3	199.0*
CFT						
AC−	190.2	193.2	191.7	195.5*	182.6	190.6
AC+	187.7	153.6		199.7*		154.4
IELISA	199.8	190.6	193.2		190.6	198.7
CELISA	199.8	187.4	191.9	194.4	175.3	198.8
FPA	199.3	190.7	194.0	194.3	190.1	198.3

*Denotes test partly used to establish exposure of the animals. These values are therefore inflated.

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is not understood. The IELISA, while a very sensitive test for serum antibody, does not have the capability of distinguishing vaccinal antibody in most cases. In addition, both it and the CELISA are laboratory tests, necessitating shipment of collected samples to a central testing facility at considerable cost and time. The FPA, on the other hand, is a homogeneous assay which may be performed in the field with accuracy approaching that of the laboratory based assays. The use of the FPA has been extended to serum, whole blood (may be frozen and thawed before testing), and milk, both from individual animals and from bulk tank samples from as many as 2000 cattle. Thus, the FPA can be used for detection of antibody to the smooth *Brucella sp.* in most domesticated animals very rapidly, at a relatively low cost and without the need for shipping samples. This will allow much greater progress in the control of brucellosis.

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