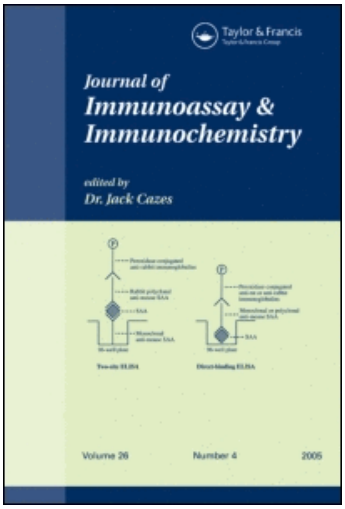

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FLUORESCENCE POLARIZATION ASSAY: APPLICATION TO THE DIAGNOSIS OF BOVINE BRUCELLOSIS IN ARGENTINA

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ABSTRACT

A homogeneous fluorescence polarization assay (FPIA) for detection of bovine antibody to *Brucella abortus* was validated in Argentina. Sera were defined based on their reactivity in the buffered antigen plate agglutination test (BPAT) and the competitive enzyme immunoassay (CELISA). Sera negative in these tests were collected from farms without evidence of brucellosis (n=733). Sera positive in the two tests were collected from cattle on farms from which *B. abortus* was isolated from at least one animal (n=1039). Sera from cattle vaccinated 26, 89, 240 and 272 days previously with *B. abortus* strain 19 were collected and tested. A cut-off value of 87 mP was determined for the FPIA, resulting in relative sensitivity and specificity values of 98.1 and 99.6%. The specificity for *B. abortus* strain 19 vaccinated cattle was 64.9% (26 days post vaccination, DPV), 92.1% (89 DPV), 98.6% (242 DPV) and 97.1% (272 DPV). These values were compared to those obtained with the BPAT, the CELISA, the indirect ELISA, the complement fixation test and the 2-mercaptoethanol agglutination test. Sera from 18 cattle which were vaccinated and revaccinated with *B. abortus* strain 19 were also tested by the same assays and the FPIA was found to be 100% specific. The use of the FPIA as a diagnostic test for brucellosis is discussed.

(KEY WORDS: Homogeneous assay, fluorescence polarization assay, *Brucella abortus*, serology).

INTRODUCTION

The fluorescence polarization assay (FPIA), described by Jolley (1), has been used for detection of drugs (2-4) and macromolecules (5-7). An FPIA for detection of bovine antibody to *Brucella*

abortus was developed in Canada using Canadian sera to represent the negative population (bovine brucellosis was eradicated more than a decade ago and vaccination with *B. abortus* strain 19 is very rare). Positive sera were obtained from individual cattle from which *B. abortus* was isolated. Data also indicated the FPIA was capable of distinguishing antibody resulting from field infection with *B. abortus* from vaccinal antibody (8). This was the first demonstration of the usefulness of this homogeneous assay for the presumptive diagnosis of an infectious disease.

The FPIA is very simple, involving the blank reading of a diluted serum in a fluorescence polarization analyzer, addition of antigen, labelled with a fluorochrome and then after two minutes of incubation, a final reading in the analyzer. Thus the assay takes only a few minutes to perform and is adaptable to use in the field as well as in the laboratory. Because the FPIA is more accurate than the buffered antigen plate agglutination test (BPAT), based on data obtained in Canada (8) and it can be performed in the same time or less, the former test is a valuable addition to campaigns against brucellosis. Thus the elimination of a large percentage of animals with residual vaccinal antibody and animals exposed to cross-reacting microorganisms results in fewer sera requiring confirmatory testing. In addition, animals that are difficult to handle may be presumptively diagnosed while held in a chute, eliminating the requirement for capturing them twice. This is particularly useful when testing wildlife for brucellosis.

Since bovine brucellosis was eradicated in Canada in 1984, it was felt that to validate the results of the FPIA, it should be applied to a trial in an area in which bovine brucellosis occurred and where extensive vaccination with *B. abortus* strain 19 was practiced. Bovine brucellosis is prevalent in some areas of Argentina and an extensive control program is currently being implemented, including mandatory calfhood vaccination. In a previous report (9), preliminary FPIA data was presented on a small population of *B. abortus* exposed and non-exposed cattle in Argentina. In that study, the specificity of the FPIA was 99.1% and the sensitivity was 95.5%, relative to the rose-bengal agglutination and the complement fixation tests.

In this communication, application of the FPIA to the presumptive diagnosis of bovine brucellosis in Argentina is reported on a larger population of cattle including 1882 sera obtained from sequential bleedings of *B. abortus* strain 19 vaccinated calves and 18 sera from calves vaccinated twice.

MATERIALS AND METHODS

Serum Samples

Serum samples were obtained from Argentinian herds with no serological, epidemiological or clinical evidence of brucellosis. Sera negative in both the buffered antigen plate agglutination test (BPAT) and the competitive enzyme immunoassay (CELISA) were used (n=733). In addition, one serum which was BPAT negative and CELISA positive and two sera that were BPAT positive and CELISA negative were also tested.

Serum samples (n=1039) from cattle on premises from which *B. abortus* was isolated from at least one animal were used. All serum samples were positive in the BPAT and the CELISA. The positive population included cattle with residual vaccinal antibody. Additional sera from 46 cattle positive in the BPAT and negative in the CELISA obtained from premises with proven infection were also tested.

Sera from calves vaccinated with 3-5 x 10^9 live *B. abortus* strain 19 26 days previously (DPV, n=605), 89 DPV (n=610), 240 DPV (n=283) and 272 DPV (n=384) were tested in the FPIA, BPAT, CELISA, indirect enzyme immunoassay (IELISA), complement fixation test (CFT) and the 2-mercaptoethanol agglutination test (2-ME). The sera obtained at the various times were not necessarily always from the same animals.

Sera were also obtained from calves that had been vaccinated twice (n=18) about 90 days after the second vaccination. These sera were also assayed for antibody to *B. abortus* by the above tests.

Test Procedures

The buffered antigen plate agglutination test (BPAT) was performed as described by Angus and Barton (10) and modified to the Office International des Epizooties procedure (11). The CFT was done as described in the Public Health Monograph (12). The 2-mercaptoethanol agglutination test (2-ME) used the methodology of Alton et al (13).

The procedures for the IELISA and CELISA were described by Nielsen et al (14). Briefly, both assays used *B. abortus* strain 1119.3 smooth lipopolysaccharide as the antigen. Antigen was passively attached to polystyrene 96 well plates. In the IELISA, serum diluted 1:50 in 0.01M phosphate buffer, pH 7.2 and containing 0.15M NaCl, 0.05% Tween 20 (PBST) and 15 mM each of EDTA and EGTA (PBST/EDTA/EGTA) was added, followed by monoclonal antibody specific for a bovine IgG_1 heavy chain epitope and conjugated with horseradish peroxidase and hydrogen peroxide/2,2'-azonobis(3-ethylbenz-thiazoline sulfonic acid) [substrate/chromogen]. Four wash cycles were included between each step using PBST. In the CELISA, serum diluted 1:10 in PBST/EDTA/EGTA was added at the same time as a monoclonal antibody specific for a common determinant of B. abortus O-polysaccharide, appropriately diluted, followed by a commercial goat anti-mouse IgG (H and L chain specific) antibody conjugated with horseradsh peroxidase and substrate/chromogen. Four eash cycles using PBST were included between each step.

For the IELISA, data was expressed as percent positivity relative to a strong positive serum control included on each 96 well plate. For the CELISA, data was expressed as percent inhibition using a buffer control included on each plate as an unhibited control value.

The FPIA was done as outlined by Nielsen et al (8). Briefly, 20 ul of serum was added to 2 ml of 0.1M phosphate buffer, pH 7.0, containing 0.01% NaN_3, 0.15M NaCl and 0.5% lithium dodecyl sulfate. The diluted serum was incubated at ambient temperature for 2 minutes and a blank reading in a fluorescence polarization analyzer was taken. Antigen, *B. abortus* O-polysaccharide conjugated with fluorescein isothisocyanate, appropriately diluted, was added, mixed and incubated for two

minutes after which a final reading was done in the fluorescence polarization analyzer. A low reading indicated no reaction between antigen and antibody and the serum was considered negative. Data are expressed in millipolarization units (mP).

Statistical Analysis

The data were classified into serologically positive and negative populations using the optimal cut-off value for the FPIA as determined by receiver operator characteristic analysis (ROC) using MedCalc software (15). The cut-off values for the other assay were described elsewhere (16) and are indicated in Table 2.

Based on the number of positive or negative results obtained with sera positive or negative in the BPAT and the CELISA, relative sensitivity and specificity values were calculated for the FPIA. For specificity values for sera from vaccinated animals, the same cut-off values were used.

RESULTS

The optimal cut-off value between positive and negative data for the FPIA was determined to be 87 mP by ROC analysis (Figure 1). Using this cut-off the sensitivity and specificity of the FPIA relative to sera positive or negative in the BPAT and the CELISA were 98.1% and 99.6%, respectively. All sera that were either BPAT positive and CELISA negative or vice versa (n=49) were negative in the FPIA.

When testing sera from the *B. abortus* strain 19 vaccinated calves, specificity values for the BPAT, 2-ME, CFT, IELISA, CELISA and the FPIA were determined for each bleeding date. These data are presented in Table 1. Thus less than one month after vaccination, both the CELISA and the FPIA detected antibody in about one third of the vaccinated cattle, while the BPAT, CFT, 2-ME and IELISA were positive in nearly all the sera. Three months after vaccination, less than 10% of the sera were positive in the CELISA and FPIA. In contrast, the BPAT and the IELISA detected antibody

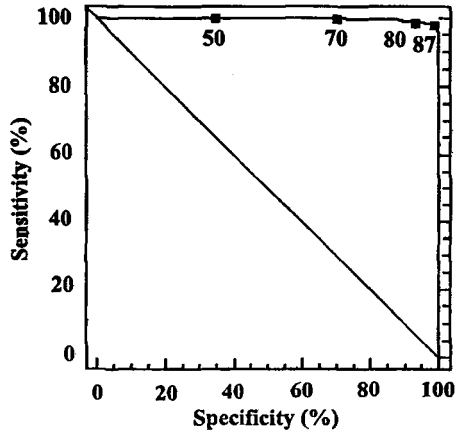

FIGURE 1: Receiver operator characteristic analysis of fluorescence polarization assay results obtained with sera from brucellosis free and brucellosis infected herds. Relative sensitivity and specificity are based on sera being negative or positive in the buffered antigen plate agglutination test and the competitive enzyme immunoassay. The area under the curve is 0.998. An area of 1.00 is perfect while an area of 0.50 is indicative of the result being obtained by chance.

in about 75% of the sera and the 2ME and CFT gave positive reactions in about a third of the animals.

Data obtained with sera from 18 cattle, vaccinated with *B. abortus* strain 19 and then revaccinated and tested using the above assays about 90 days later are presented in Table 2. This population was selected based on reactivity in the BPAT and the CFT tests (all but one serum also gave 2-ME positive reactions).

DISCUSSION

In many countries, serological diagnosis is based on reactivity in a rapid inexpensive screening test and confirming positive reactions with a more specific test(s) which is usually more difficult, time consuming and expensive to perform. In Argentina, most initial testing is done with the BPAT or rose bengal agglutination test and positive reactions are confirmed with the 2-ME agglutination

TABLE 1

Serological results, expressed as % negative animals in the test sample, of calves vaccinated with a full dose of *B. abortus* strain 19 at various times post vaccination.

DPV	BPAT	2-ME	CFT	IELISA	CELISA	FPIA
26	0.8	3.2	7.0	3.2	72.8	64.9
89	24.2	61.3	75.1	27.7	92.1	92.1
240	75.0	98.9	99.3	94.0	99.6	98.6
272	82.8	98.6	98.1	70.7	95.5	97.1

DPV - days post vaccination
BPAT - buffered antigen plate agglutination test
2-ME - 2-mercaptoethanol agglutination test
CFT - complement fixation test
IELISA - indirect enzyme immunoassay
CELISA - competitive enzyme immunoassay
FPIA - fluorescence polarization assay

test or the CFT. In Argentina, calfhood vaccination with *B. abortus* strain 19 is mandatory. The agglutination tests, the CFT and the IELISA are unable to distinguish vaccinal antibody from that induced due to field infection. The CELISA is capable of this differentiation in most cases. Therefore, serum samples used in this study were selected based on positive serological reactions in the BPAT and the CELISA. It is realized that this is not a perfect method, however, in the absence of data on the isolation of *B. abortus* from individual animals, it is the most suitable. If other test combinations were used, for instance BPAT and 2-ME, reactions due to vaccinal antibody would not be eliminated and the primary binding assays would appear to be less sensitive.

In a previous study (9), a small number of sera tested gave some preliminary indications that the FPIA would be a useful test in a control/surveillance program in several countries, including the US, Argentina, Mexico and Chile. Thus using a cut-off of 94 mP, 709 sera selected based on positive screening and confirmatory tests were 95.5% positive in the FPIA while 215 sera deemed negative in the screening test were 99.1% negative in the FPIA. The current study extended this data and by using more realistic selection criteria, increased the FPIA sensitivity and specificity.

TABLE 2

Serological results obtained with sera from animals vaccinated twice with *B. abortus* strain 19 and bled approximately 90 days post vaccination.

Animal	BPAT	2-ME	CFT	IELISA	CELISA	FPIA
1	+	N	10	12	6	84
2	+	100	20	52	6	78
3	+	25	10	43	7	84
4	+	25	10	29	1	77
5	+	25	10	47	18	82
6	+	50	20	28	18	75
7	+	25	10	43	20	67
8	+	25	10	59	61	70
9	+	25	10	27	-11	67
10	+	50	20	50	21	49
11	+	25	10	24	46	67
12	+	200	40	59	71	67
13	+	50	20	17	35	66
14	+	50	20	19	24	82
15	+	25	10	19	52	75
16	+	200	40	13	10	36
17	+	25	10	120	54	84
18	+	50	10	28	32	58

Animal - identification
BPAT - buffered antigen plate agglutination test - result: + or -.
2-ME - 2-mercaptoethanol agglutination test - result: reciprocal titer 25 or over is +.
CFT - complement fixation test - result: reciprocal titer of 10 or over is +.
IELISA - indirect enzyme immunoassay - result: 40%P or over is +.
CELISA - competitive enzyme immunoassay - result: 40%I or over is +.
FPIA - fluorescence polarization assay - result: 87mP or over is +.

The sensitivity value obtained for the FPIA using 1039 serum samples from *B. abortus* infected herds was 98.1% relative to positive results in the BPAT and the CELISA. This value is lower than expected, probably due to the lower specificity of the BPAT (48%) and the CELISA (97.2%) when testing cattle with residual vaccinal antibody (17 and Table 1). This would result in some sera giving positive results in the BPAT and CELISA but negative results in the FPIA, appearing as false negative data. Alternately, the FPIA may have given a few false positive reactions.

The specificity of the FPIA relative to the BPAT and the CELISA was 99.6%. Thus 4 of 733 sera gave FPIA results above the cut-off value of 87 mP. A number of explanations are possible. Most

likely is that the serum samples contained bacterial growth which when suspended in the diluent buffer caused deflection of light when reading the blank value resulting in too small a correction for serum autofluorescence thereby giving an inflated final reading. The underlying mechanism for this phenomenon is not fully understood as light scatter would be expected to be similar in the presence or absence of the soluble antigen. However, the effect has been noted on nearly all samples contaminated with insoluble materials.

Additional sera from the positive or negative populations that were positive in the BPAT but negative in the CELISA or vice versa were all negative in the FPIA.

In testing serum samples from *B. abortus* strain 19 vaccinated calves, it is clear that the CELISA and the FPIA were capable of eliminating 65 to 70% of positive reactions at 26 days post vaccination. In contrast, the specificity of the BPAT, CFT, 2-ME and IELISA were 0.8%, 7.0%, 3.2% and 3.2%, respectively. Similarly, at 89 days post vaccination, over 92% of the positive reactions were eliminated by the CELISA and the FPIA while the other assay gave specificity values ranging from 24.2% to 75.1%. At 240 and 272 days post vaccination, the 2-ME, the CFT, the CELISA and the FPIA were over 95% specific, while the BPAT gave 75% and 83% specificity values for those times. The IELISA was 94% specific on day 240 and for inexplicable reasons, the specificity dropped to 70.7% by day 272 post vaccination. It should be recalled that the sera tested at the different times were not all from the same animals, possibly accounting for any discrepancies. These data are presented in Table 1.

Cattle vaccinated with a full dose of *B. abortus* strain 19 and then revaccinated at a later date often cause conventional serological tests to be positive for an extended period of time. In Table 2, data from 18 serum samples from such animals, taken about 90 days after the second vaccination and tested by the serological tests described above, are summarized. The FPIA did not detect antibody in any of the sera while the CELISA detected low reactions in 4 sera. The IELISA gave positive results with 8 sera and the 2-ME detected antibody in 17 of the sera. The BPAT and the CFT

gave positive results with all 18 sera. This would obviously cause diagnostic problems as the BPAT, followed by the CFT or the 2ME test are commonly used for serological diagnosis.

The mechanism by which the CELISA and the FPIA discriminate vaccinal antibody from antibody due to infection is not known. For the CELISA it has been hypothesized that it is a combination of low antibody affinity, specificity to limited or occluded epitopes and/or serum antibody levels are responsible for the lack of reactivity after the initial antibody peak. However, with the FPIA, the mechanism is most likely different and may result from low antibody affinity and production of less reactive isotypes. The increase in specificity with time after vaccination is probably a result of declining total antibody levels. If a serum sample is positive in the FPIA two to three months after vaccination with *B. abortus* strain 19, there is a small chance of it being residual antibody due to the vaccine, however, there is a more than 90% chance that it is due to infection and such an animal should be retested.

From the data presented, it is clear that the FPIA performs well in areas where brucellosis occurs and where vaccination with *B. abortus* strain 19 is widely used. Because of the ease of performance, the less likelihood of error, the rapidity and the cost-effectiveness of the FPIA it will be a valuable asset in a campaign to control and/or eradicate brucellosis. While the fluorescence polarization analyzer used in this study was a laboratory instrument, a smaller, less expensive and portable model is being manufactured. This would make on farm, sales barn and abattoir testing possible, resulting in decreased cost of shipping and reduced turn around time for results. Finally, it will be a near ideal test for use with wildlife such as bison and deer, as the animals would only be required to be captured once for testing.

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