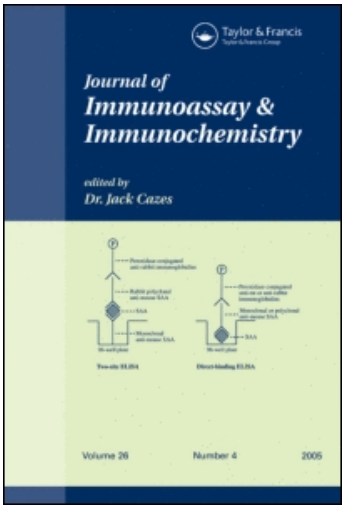

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VALIDATION OF THE FLUORESCENCE POLARIZATION ASSAY FOR DETECTION OF MILK ANTIBODY TO *BRUCELLA ABORTUS*

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ABSTRACT

A fluorescence polarization assay (FPA) for detection of antibody to *Brucella abortus* in individual milk samples was developed and validated. Samples from 190 cattle from which *B. abortus* was isolated; milk samples from cattle in herds infected with *B. abortus* (n = 1086) and positive in the milk ring test (MRT), as well as milk samples from Canadian cattle (with no evidence of brucellosis, n = 2974) were tested by the indirect enzyme immunoassay (IELISA) and the FPA. The sensitivity (based on samples from culture positive cattle) and specificity (based on Canadian milk samples) of the IELISA and the FPA were 100%. The relative sensitivity value obtained with milk from cattle of infected herds and the specificity values of the IELISA were 98.5 and 99.9%, respectively. The relative sensitivity and specificity of the FPA with the same samples were 82.2 and 99.4% using a cut-off value of 90 millipolarization units (mP). The low relative sensitivity value of the FPA was shown, by competitive enzyme immunoassay (CELISA), to be due to vaccinal antibody (assumed as vaccinal antibody against *B. abortus* S19 is excluded by the FPA and CELISA but not by the MRT and the IELISA), present in some of the milk samples. The FPA is a homogeneous assay which, unlike the MRT and the IELISA, may be used for testing in the field.

INTRODUCTION

The milk ring test (MRT) for the diagnosis of brucellosis has been widely used for screening for antibody to *Brucella abortus*. The MRT works on the principle that fat globules with antibody molecules will rise to the surface of the milk and form a cream layer. If the milk contains antibody to *B. abortus*, the antibody will attach to antigen (stained for visualization) added to the milk and form a visible layer at the top of the milk. The degree of colour is indicative of the amount of antigen in the cream and, hence, the amount of antibody. The MRT is a good screening test which may be used on individual samples or, by increasing the volume of test sample, can be used on bulk tank samples. The MRT may give false reactions with milk collected shortly after parturition, when milk obtained in the late stages of the lactation cycle or when milk from mastitic cows is tested.(1) In addition, the MRT is not suitable for testing milk samples stored for more than 48 hours.

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To overcome these problems, a number of indirect enzyme immunoassays (IELISA) have been developed. The initial assays used crude preparations of antigen and polyclonal anti-immunoglobulin reagents conjugated with enzyme.(2–5) The performance of these assays did not greatly exceed that of the MRT; however, the newer generation of enzyme immunoassays, using more refined antigen and monoclonal anti-immunoglobulin conjugate, have enhanced accuracy. Thus, the sensitivity and specificity of an IELISA, using purified smooth lipopolysaccharide antigen and mouse monoclonal antibody specific for a heavy chain epitope of bovine IgG_1, and incorporating divalent cations to reduce non specific binding, was 100% and 99.9%, respectively, when testing milk samples from a *B. abortus* non-vaccinated population.(6)

Animals vaccinated with *B. abortus* strain 19 sometimes have residual antibody to the immunodominant epitopes of lipopolysaccharide (LPS) antigen. Some of these epitopes are shared between the vaccine strain and pathogenic strains. Some of the epitopes are also shared among the species of *Brucella* which have smooth LPS in the outer cell wall. This sharing of epitopes and, hence, the antibody response causes diagnostic problems as some serological tests such as the agglutination tests, the complement fixation test, and the indirect ELISA are unable to distinguish such antibodies. Other tests, such as the CELISA and the FPA, have been found to be able to distinguish vaccinal antibody from that induced by pathogenic strains in over 90% of the sera tested. Development of newer vaccines, such as *B. abortus* RB51, which has rough LPS in the cell wall, has decreased this problem.

Milk is a desirable sample for antibody testing because it is easy to obtain by non-invasive means and tests may be adapted to screening a number of animals with a single test. A test which is technically simple, compared to the IELISA, and which has the potential for being performed on the farm rather than in the laboratory, would be ideal if it had the same performance characteristics as the enzyme immunoassay.

The fluorescence polarization assay (FPA) has been developed to test milk for antibody to *B. abortus* in individual samples. The assay works on the principle that molecules in solution randomly rotate at a rate inversely proportional to their size. Thus, a small molecule labelled with a fluorochrome will depolarize plane polarized light at a more rapid rate than a larger molecule. The rate of rotation, when a small molecule is complexed with a large molecule (such as an antibody molecule), can be measured. This test is a homogeneous assay, requiring no removal of unbound reactants. It is a primary binding assay, relying only on the ability of the antibody to react with its antigen. It is simple and relatively inexpensive and it may be performed almost anywhere.

EXPERIMENTAL

Positive Milk Samples

Milk samples (n = 190) from individual cattle from which *B. abortus* was cultured and which were positive in the MRT (2+ or higher) were tested by IELISA and FPA.

Milk samples (n = 1086) were obtained from individual cattle in herds infected with *B. abortus* in Argentina, Chile, and Mexico. The bacterium was isolated from at least one animal per herd. All samples were positive in the MRT, reactivity ranging from 1+ to 4+. Extensive vaccination with *B. abortus* strain 19 is practised in each country. All milk samples were tested by the IELISA and the FPA. A number of milk samples (n = 146) that gave low results in the FPA were centrifuged at 15,000×g for 20 minutes to remove the lipid and the whey tested by the CELISA.

Negative Milk Samples

Milk samples (n = 2974) were obtained from individual Canadian cattle. All samples were tested by the IELISA. The MRT was not performed. Canada has been free from brucellosis in domestic animals for more than 15 years.

Serological Tests

The IELISA was performed with *B. abortus* smooth lipopolysaccharide (SLPS) as the antigen and used milk diluted 1:2 with 0.01 M phosphate buffer, pH 6.3, and containing 0.15 M NaCl, 15 mM EDTA, and 15 mM EGTA (PBSEDTA). A monoclonal antibody specific for a heavy chain epitope of bovine IgG_1 and conjugated with peroxidase(7) was used as the detection reagent. Hydrogen peroxide and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) were used to develop colour. A value of 20% positivity, relative to a standardized positive serum sample, was considered positive for milk and 46% positivity was considered positive for serum samples.(6)

The CELISA was described previously for use with bovine sera.(8) Milk whey was tested at a 1:20 dilution. Values of 30% inhibition, relative to a non-inhibited control, were considered as positive.

The FPA was performed as previously described for serum,(9) except the milk samples were diluted 1:25 for testing. Briefly, 40 uL of whole milk were diluted into 1.0 mL of 0.01 M phosphate buffer, pH 7.2, with 0.15 M

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NaCl, 0.1% sodium azide, and 0.05% lithium dodecyl sulfate (PBSAL). A baseline evaluation of fluorescence polarization was obtained using an FP Sentry (Diachemix Corp., Wisconsin, USA) fluorescence polarization analyzer. This analyzer is portable, with power and software to perform the FPA supplied by a laptop computer, and is free-standing depending only on the laptop battery capacity. It is adaptable to a 12 volt outlet and to 110–220 volt power. Data are automatically recorded and files may be transmitted electronically. Antigen (10 μL) consisting of alkali hydrolyzed *B. abortus* o-polysaccharide conjugated with fluorescein isothiocyanate(10) was added, mixed well, and a final fluorescence polarization reading was obtained after two minutes of incubation. Results were expressed in millipolarization units (mP).

Data Analysis

Data analysis was performed using frequency distributions of the serological results. This allowed for the establishment of an initial cut-off value between positive and negative samples and the determination of the assay sensitivity and specificity for that cut-off. Receiver operator characteristic (ROC) analysis(11) was done to provide sensitivity and specificity values for other cut-off points, and a value for the area under the curve was calculated. The area under the curve is indicative of the accuracy of the test; the closer to 1.00, the more accurate it is. An optimized cut-off point was determined from the ROC analysis (the maximum sum of the sensitivity and specificity values for that cut-off point).

RESULTS

Milk samples from culture positive cattle (n = 190) were all positive in the FPA and IELISA.

The frequency distributions for the positive and negative milk samples was plotted and an initial cut-off of 85 mP was established for the FPA. The cut-off for the IELISA had previously been determined to be 20% or higher, relative to a positive control included with each test. Receiver operator characteristic (ROC) analysis of the FPA data fine tuned the optimum cut-off value at 90 mP, using the 190 positive and 1944 (a randomly selected subset of the 2974 negative samples tested), giving 100% sensitivity and 99.1% specificity values. The 1086 samples from infected premises were tested and gave a relative FPA sensitivity value of 82.2%; the 2974 negative samples gave an FPA specificity value of 99.4%. The ROC curve for these

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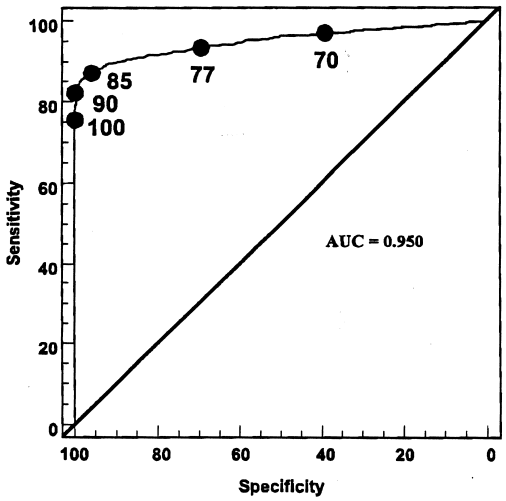

Figure 1. Receiver operator characteristic (ROC) analysis of data obtained with a fluorescence polarization assay. The x-axis represents assay specificity (%) and the y-axis sensitivity (%) at various cut off values. The diagonal line is the 'chance' line. If the ROC curve is at or below this line the result obtained may be due to chance only. The area under the curve (AUC = 0.950) is indicative of the assay accuracy (an AUC of 1.00 is perfect). Thus the accuracy is 95%. The optimum cut off value, 85 mP, is the maximum sum of the sensitivity and specificity values.

data is presented in Figure 1. The area under the curve, an indication of the accuracy of the test, indicates that 95+/−0.6% of the time there is a 95% chance of the test giving the correct result.

Given the relatively low sensitivity value of the FPA, it was realized that this may result from milk samples derived from cattle with residual antibody to *B. abortus* strain 19. Therefore, whey was prepared by centrifugation to remove lipids which interfere with the CELISA from 146 milk samples that gave lower FPA values. The whey samples were tested by the FPA and the CELISA. The cutoff for the CELISA was 30% inhibition, relative to an uninhibited control included with each test. These data are presented in Table 1.

DISCUSSION

Presumptive diagnosis of brucellosis, using the MRT, is a prescribed diagnostic test by the OIE; however, according to MacMillan,(1) the MRT

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Table 1. Results Obtained with 146 Whey Samples Prepared from Milk Samples that Gave Lower FPA Results. All Whey Samples Were Tested by the IELISA, CELISA, and FPA. MRT Results Are Those Originally Obtained with the Milk Samples

Number	MRT	IELISA	CELISA	FPA
6	+	−	−	−
3	+	−	−	+
3	+	+	−	+
3	+	+	+	−
115	+	+	−	−
13	+	+	+	+
3	+[1]	−	−	−

[1]These samples gave a 1+ reaction in the MRT.

is not accurate when testing milk from animals with mastitis or animals at either end of the milking cycle. In addition, it has been suggested that the actual sensitivity of the test may be about 90%.(12)

Based on results of this study and previously published data, the most accurate test using milk is the IELISA, with sensitivity and specificity values approaching 100%. The IELISA, however, cannot distinguish antibody resulting from vaccination with *B. abortus* strain 19 from antibody resulting from exposure to pathogenic strains. In addition, this test is suitable for the laboratory and it is technically too difficult in its current format to use in the field (alternative procedures, such as the dipstick would be useable in the field but would also be more costly). Therefore, a simple, inexpensive and accurate test would be useful. The FPA has been tested on a number of positive (based on milk ring and IELISA tests) and negative milk samples. The initial result, giving sensitivity and specificity values of 82.2% and 99.4% at a cut-off of 90 mP (Figure 1), was not encouraging. It should, however, be recalled that the positive milk samples were not derived from cattle which were individually proven to be infected with *B. abortus*, but rather from populations from which *B. abortus* had been isolated from a limited number of animals.

It should also be recalled that all positive milk samples came from cattle which had been vaccinated with *B. abortus* strain 19. Since the FPA, in most cases, does not detect antibody resulting from vaccination with *B. abortus* strain 19,(9) it is possible that the positive milk samples that gave negative results in the FPA contained residual vaccinal antibody, giving false positive reactions in the MRT and IELISA. To test this hypothesis, 146 positive milk samples that gave lower FPA results were centrifuged to

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remove the lipids which interfere with the CELISA. These samples were then tested by the CELISA, which does not detect vaccinal antibody in over 97% of sera tested. From Table 1, it is clear that there is good agreement between the CELISA and the FPA, indicating the likelihood that most of the positive milk samples that gave lower FPA results contained residual vaccinal antibody. Therefore, the actual sensitivity of the FPA is probably considerably higher than indicated by the ROC analysis. Thus, if the whey samples (Table 1) that were negative in CELISA and negative in FPA ($n = 124/133$ with 13 samples giving positive results with both the FPA and CELISA for a total of 146), they could originate from non-infected vaccinated cattle and the actual sensitivity of the FPA would then be 99.4% ($n = 1086 - 124 = 962$ of which 6 were FPA negative; Table 1), approximating the value obtained with milk from culture positive cattle.

The FPA, based on the validation data presented, is recommended as a serological test for milk. The assay is standardizable on a large scale, is easy to perform, is adaptable to field use, and is relatively inexpensive. Adoption of this test would enhance the ability to accurately diagnose brucellosis and contribute to control and eradication of this disease.

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