

## Fluorescence Polarization Assay for Detection of *Brucella abortus* Antibodies in Bulk Tank Bovine Milk Samples

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**A simple, rapid, inexpensive fluorescence polarization assay for the detection of antibodies to *Brucella abortus* in bulk tank milk samples at the farm level or at dairies with a sensitivity and specificity of 100 and 95.9%, respectively, is described. The assay detects antibodies to *B. abortus* in 15 min by testing undiluted whey produced by chemical and physical manipulation of milk from bulk tanks. This sampling is noninvasive and therefore costs less and is less stressful than blood-based tests. The assay is specific and can detect antibodies at levels below that of the indirect enzyme immunoassay for milk and the fluorescence polarization assay for individual milk samples. Use of this test would make programs for surveillance of dairy animals and eradication of *B. abortus* more cost-effective.**

The presence of *Brucella abortus* bacterial infection in milk was reported by Schroeder (E. C. Schroeder, E. S. R. 27:281, 1912) in 1912, and the presence of agglutinins in the milk of infected animals was reported by Cooledge (L. H. Cooledge, J. Agr. Res. 5:871, 1916). A laboratory test for the diagnosis of bovine brucellosis using milk samples was not attempted until 1937, when the milk ring test (MRT) was developed by Fleischhauer (G. Fleischhauer, Berl. Tierarzt. Wochenschr. 53:527-528, 1937). At the time, this test was considered highly sensitive (G. Fleischhauer and G. Hermann, Berl. Tierärztl. Wochenschr. 54:333, 1938) due to its ability to detect antibodies in milk from one infected animal mixed with milk from 5 to 10 cows negative for this pathogen. However, there were many shortcomings, including false-positive reactions associated with the MRT, as listed in Table 1. Nicoletti (10) showed that the MRT correctly identified 88.5% of animals in which *B. abortus* was isolated and 77.4% of animals in which *B. abortus* was not isolated. Similar sensitivity and specificity (89 and 86%, respectively) based on culture status were obtained by Hunter and Allen (8).

Using undefined antigens and polyclonal anti species immunoglobulin enzyme conjugates for detection (2, 7, 19), indirect enzyme immunoassays (indirect ELISA) attempted to eliminate some difficulties inherent in the MRT and to improve on detection of antibodies to *B. abortus*. The subjectivity inherent in interpreting the MRT was removed and sensitivity of the ELISAs was adequate to detect a single infected cow in bulk milk samples from herds of 100 or more cattle (2, 6, 13). However, problems associated with nonspecific reactions (12) and detection of residual antibodies due to vaccination (11, 15, 20) remained using these ELISAs.

An improved indirect ELISA using purified smooth lipopolysaccharide, a monoclonal antibody specific for the epitope of bovine immunoglobulin G1 and divalent cation chelating

agents (EDTA and EGTA) to reduce nonspecific reactions (13) was developed. The sensitivity (based on samples from herds infected with *B. abortus*) and specificity (based on samples from brucellosis-free herds) obtained by this assay were 96.5 and 99.9%, respectively. Evaluation of this assay under Argentinian field conditions resulted in relative sensitivity (based on matched serum samples that were positive on the complement fixation test) and specificity (based on samples from herds free of brucellosis for 5 years) values of 99.6 and 99.1%, respectively (22). Although the assay had improved specificity, tolerated poor quality samples, and allowed batch processing, it was relatively expensive, time-consuming, and labor-intensive and could only be done in a laboratory in its present format.

Recently, a homogeneous fluorescence polarization assay (mFPA) for detection of milk antibodies to *B. abortus* with a sensitivity (based on samples from culture positive cattle) and specificity (based on cattle with no evidence of brucellosis) of 100 and 99.1%, respectively, was developed (15). This assay has the capability to discriminate cattle vaccinated with *B. abortus* strain 19 from cattle infected with *B. abortus* (15). However, the mFPA involves collecting milk samples from individual animals and diluting samples to 1:25, decreasing the assay sensitivity.

The purpose of this study was to develop an FPA for detection of antibodies to *B. abortus* in bulk tank milk samples (bmFPA) with improved detection capability, improved diagnostic sensitivity and specificity, and simplified collection and dilution techniques.

### MATERIALS AND METHODS

**Preparation of reagents.** All chemicals were from Sigma-Aldrich Chemical Company, St. Louis, Mo.

A trizma base reagent grade was used in the preparation of 0.04 M TRIS buffer with 0.01 M EDTA. Both were dissolved in pyrogen-reduced 18-M $\Omega$  water. The pH was adjusted to 10.2 with 0.06 M NaOH.

A 1.0 M sodium dithionite (sodium hydrosulfite) solution was prepared in 0.04 M Tris-0.01 M EDTA buffer and allowed to equilibrate overnight before the preparation of a 0.25 M solution diluted in 0.04 M Tris-0.01 M EDTA buffer. Aliquots of the 0.25 M solution (0.5 ml) were dispensed into borosilicate glass

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TABLE 1. Problems associated with the milk ring test

Description	Reference
Decolorization induced by bacterial growth after long incubation periods could affect the strength of the reaction and subsequent interpretation. ....	3
The MRT was subject to nonspecific reactions caused by testing colostrum or milk from cows with mastitis. ....	4
In addition to the subjective nature of the test it was also recognized that results were dependent on the ability of laboratory personnel to read the test correctly. ....	5
It was known that antibodies against cross reacting organisms would produce reactions in the MRT resulting in false positives. ....	8
MRT depends on the presence of cream, making the test difficult to perform. The cream content varies with individual animals and would be diluted with pooled or bulk tank samples. ....	16
Partial freezing of negative milk could result in weak nonspecific reactions. ....	16
Animals vaccinated as calves produced weak reactions upon maturity. ....	23

tubes (10 by 75 mm), frozen at -70°C, and then lyophilized for approximately 24 h. Due to the light and moisture sensitivity of the lyophilized reagent, storage in a desiccant container at room temperature away from light sources was required.

**Negative milk samples.** Repeated sampling of Canadian milk bulk tanks were obtained for a total of 219 bulk tank samples from 13 bovine herds. Canada has been officially free of brucellosis in cattle since 1985.

**Positive milk samples.** A total of 39 positive milk samples (from which *B. abortus* was isolated from at least one animal in the herd) consisted of 23 Canadian bank samples and eight bulk tank samples from Baja California, Mexico. Eight artificially constructed bulk tank samples were also included, consisting of individual milk samples (positive and negative) tested on the FPA for individual milk samples (*n* = 193) from different positive herds in Mexico (from which *B. abortus* was isolated from at least one animal in the herd) and combined to simulate different herd sizes ranging from *n* = 16 to *n* = 37.

**Milk controls.** Commercially available 2% skim milk was used with whey obtained from milk of an animal naturally infected with *B. abortus*, to construct an artificial, positive milk sample (1/100 dilution). The artificial, positive milk sample and the unused 2% skim milk were freeze-dried and used as positive and negative controls (*n* = 43), respectively. As well, repeat titrations (*n* = 10) of the same artificially constructed positive sample were performed to determine detectability in different herd sizes.

**Milk treatment and bmFPA.** Before testing, 2-ml milk controls and test samples were treated with 10 µl of 1-g/ml of citric acid, resulting in the precipitation of casein after approximately 10 repeated inversions. Subsequently, the milk samples were mixed using a vortex for 3 min at maximum speed to congeal the fat in the milk sample. The resultant skim milk sample (1.8 ml) was dispensed into 2-ml microcentrifuge tubes and centrifuged at ≥5,600 × *g* using a portable microcentrifuge for approximately 6 min per sample. After centrifugation, 1 ml of the whey was removed with a pipette from the supernatant and dispensed into a borosilicate glass tube containing lyophilized sodium dithionite. After through mixing (5 s) on a vortex to ensure a homogeneous mixture of the whey sample, a blank measurement was obtained using a portable fluorescence polarization analyzer (Sentry FP; Diachemix Corporation, Grayslake, Ill.). A predetermined amount (10 µl) of *B. abortus* *O*-polysaccharide conjugated with fluorescein isothiocyanate (14) was added and thoroughly mixed for 5 s. After incubation at room temperature for at least 2 min, a second measurement was determined with the blank subtracted. Data from this assay were expressed as millipolarization (mP) units. (In the presence of antibodies, a high mP result was obtained, while the absence of an antibrucella antibody resulted in a low mP reading).

**mELISA.** The indirect enzyme immunoassay for milk (mELISA) was done as described by Nielsen et al. (13). Briefly, the mELISA used *B. abortus* smooth lipopolysaccharide as the antigen immobilized on 96-well polystyrene plates followed by sequential addition of test samples (diluted 1:2), controls, diluted murine monoclonal antibody specific for bovine immunoglobulin G1 (conjugated with horseradish peroxidase), and substrate or chromogen, with appropriate wash procedures and incubation periods. Optical densities were assessed after 10 min at 414 nm with results expressed as percent positivity (%P) using the

following equation: %P = optical density of test sample × 100/optical density of a strong positive control from a bovine from which *B. abortus* was isolated.

**mFPA.** The mFPA was done as described by Nielsen et al. (15). Briefly, the milk was diluted 1:25 in 0.01 M Tris buffer containing 0.15 M NaCl, 10 mM EDTA, and 0.05% Igepal CA630 and was mixed. After mixing, a blank reading was obtained using the portable fluorescence polarization analyzer, and this was followed by the addition of appropriately diluted *O*-polysaccharide conjugated with fluorescein isothiocyanate; the solution was mixed and equilibrated for 2 min. After equilibration, a reading was obtained. Results were expressed in mP units. In the absence of anti-*Brucella* antibodies (negative milk) the result was low mP values; high mP values resulted from the presence of *Brucella* specific antibodies (positive milk).

**Data analysis.** Using statistical software (17), upper control limits (UCL) and lower control limits (LCL) (±3 standard deviations [SD]) were obtained for the positive and negative controls. With the same software, using previously defined negative (*n* = 219) and positive (*n* = 39) milk samples, the sensitivity, specificity, and an associated cutoff were determined using receiver operating characteristics.

**RESULTS**

UCL and LCL of the positive and negative controls ±3 SD from the mean (18) for the bmFPA were determined by testing each control over a 6-month period for a total of 43 observations. For the positive control the UCL and LCL were 339.42 and 223.72 mP, respectively (Fig. 1). Similarly, the UCL and LCL for the negative control were 137.55 and 79.71 mP, respectively (Fig. 1). The running average for positive and negative controls were 281.57 and 108.62 mP, respectively (dashed lines in Fig. 1). No control data exceeded the UCL and LCL and rarely exceeded ±2 SD as shown in Fig. 1. The percent coefficient of variation (%CV) of the positive control was 6.85%, while the %CV of the negative control was 8.87%.

Using the calculated negative cutoff of 140 mP, a detection limit of one infected animal in approximately 2,000 negative animals ±2 SD was estimated, as presented in Fig. 2, where dilutions on the *x* axis represent postulated herd sizes. The %CVs at a 1:1600 dilution and a 1:3,200 dilution were 9.8 and 14.4%, respectively.

The sensitivity, specificity, and cutoff of the bmFPA were determined using receiver operating characteristics analysis based on a *B. abortus*-negative population of 219 samples and a *B. abortus*-positive population of 39 samples. The sensitivity and specificity with 95% confidence limits (CL) of the bmFPA (negative cutoff = 140 mP) were 100% (95% CL, 88.8 to 100 mP) and 95.9% (95% CL, 92.1 to 98.0 mP), respectively, as presented in Table 2. Similarly, the sensitivity and specificity of the mFPA were 76.9 and 100%, respectively, while the sensitivity and specificity of the mELISA were 94.9 and 91.8%, respectively.

Presented in Table 3 are the positive and negative predictive values of the bmFPA (cutoff, 140 mP) for disease prevalences of 0.01, 0.1, 1, 10, 15, and 20% and a sensitivity and specificity of 100 and 95.9%, respectively. As the disease prevalence increases, the positive predictive value (PPV) increases while the negative predictive value (NPV) remains unchanged. A PPV is the probability of the disease being present when the test is positive and an NPV is the probability the disease is not present when the test is negative.

**DISCUSSION**

The development of a simple, rapid, inexpensive FP assay with excellent accuracy suitable for testing bulk tank milk sam-

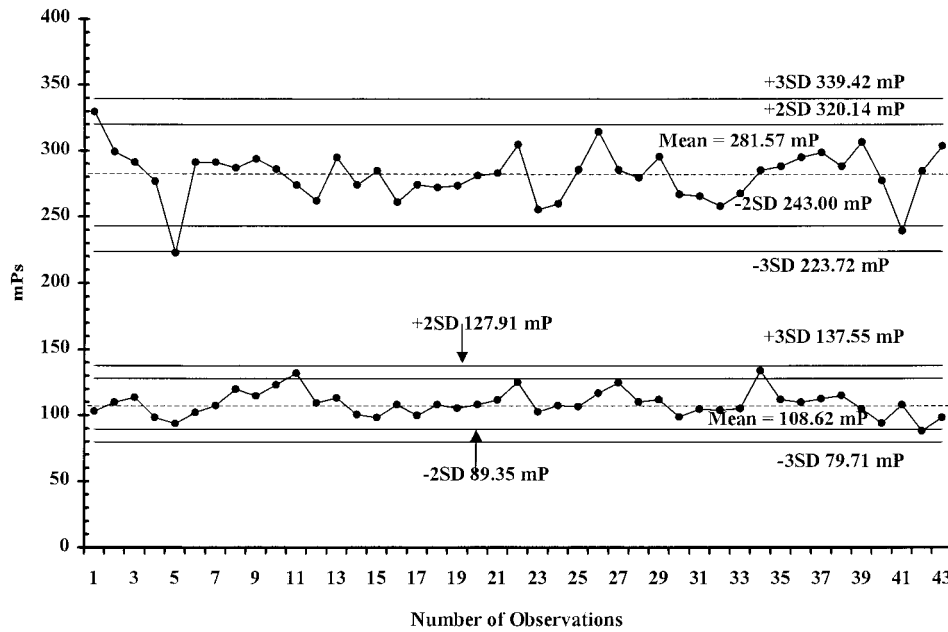


FIG. 1. Standard quality control charts with UCL and LCL set at 3 SD for the positive and negative controls. The running average is depicted as a dashed line. The x axis shows the number of observations, while the y axis is in mP units.

ples at the farm level or dairy level would be a desirable addition to diagnostic tests for controlling bovine brucellosis.

The bmFPA is a simple, cost-effective, rapid (less than 15 min), inexpensive assay developed for testing bulk tank milk samples. The noninvasive sampling procedure would result in further reductions in the costs of blood collection material and labor and stress-related symptoms in cattle, such as reduced milk production. It would be particularly useful in individual

dairy herds with high prevalence of brucellosis, where samples reacting on the bmFPA would most likely have antibody to *B. abortus* infection. At a prevalence of 20%, the positive predictive value was 85.9% (Table 3). This indicates that more than 8 out of every 10 samples testing positive by the bmFPA could have antibody to *B. abortus* infection. Because of the high NPVs (Table 3), samples testing negative by the bmFPA would mostly likely not be *B. abortus* infected.

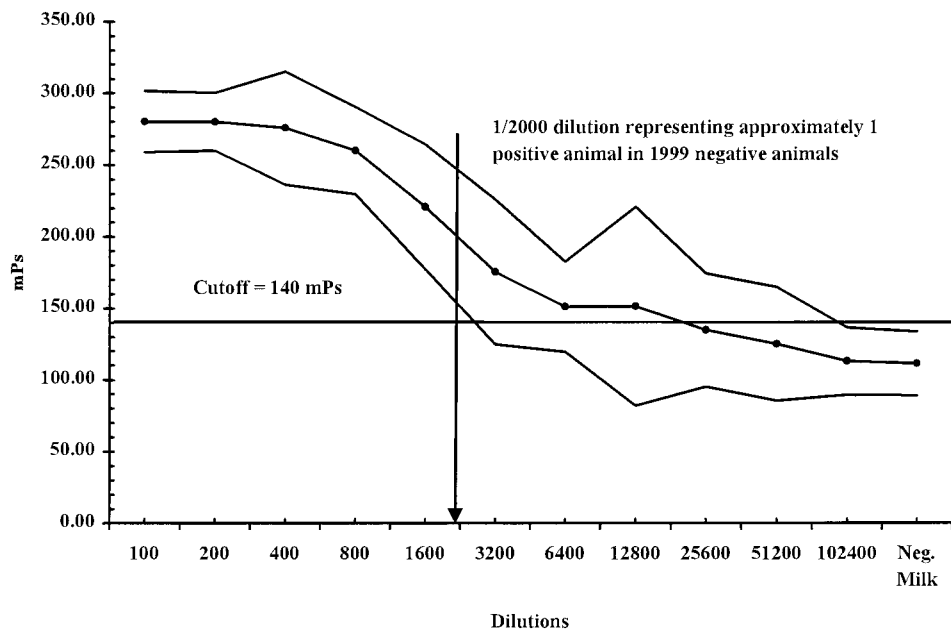


FIG. 2. Mean ( $n = 10$  repeat titrations) titration curve  $\pm 2$  SD of a *B. abortus*-positive milk sample artificially constructed to simulate one infected animal in a bulk milk tank for herds of various sizes. Values plotted along the x axis are the dilutions representing the hypothetical herd sizes, while those plotted along the y axis are in mP units.

TABLE 2. Sensitivities and specificities of various tests

Test (cutoff)	% Sensitivity (95% CL)	% Specificity (95% CL)
bmFPA (140 mP)	100 (88.8–99.8)	95.9 (92.1–98.0)
mFPA (87 mP)	76.9 (60.2–88.3)	100 (97.8–99.9)
mELISA (20% P)	94.9 (81.4–99.1)	91.8 (87.1–94.9)

Incorporation of sodium dithionite in the undiluted whey reduced the background fluorescence reading levels, possibly caused by riboflavin (21) allowing the assay to detect antibodies in samples that were negative for *B. abortus* by the mFPA and the mELISA. Riboflavin (vitamin B<sub>2</sub>) also imparts a greenish color (optical properties as reported by the Dairy Science and Technology of the University of Guelph [<http://www.foodsci.uoguelph.ca/dairyedu/chem.html>]) to the whey, again probably due to riboflavin fluorescence. Without sodium dithionite, the capacity of the photo multiplier tube of the FPA reader was easily overwhelmed (see Sentry FP operator manual [Diachemix Corporation] and FPM-1 fluorescence polarization analyzer operator manual, revision 2.0 [Jolley Consulting and Research, Inc., Grayslake, Ill.]), resulting in negative polarization values, negative intensity values, and erroneous millipolarization results. Addition of sodium dithionite allowed antibody in the whey to be measured undiluted, compared with the mFPA which was performed at a 1:25 dilutions.

The bmFPA demonstrated the best sensitivity (Table 2) compared with the mFPA and the mELISA. Since the milk samples were not diluted, the sensitivity of the bmFPA (100%) exceeded the sensitivities of the mFPA (76.9%) and the mELISA (94.9%). Low antibody levels produced from cross-reacting organisms (8) such as *Escherichia coli* 0:116, *Salmonella enterica* serovar Urbana 0:30, and *Pseudomonas maltophilia* strain 555 would not be diluted out in the bmFPA, which may explain the lower specificity (95.9%) of the bmFPA compared with the mFPA (100%), in which the milk was diluted 1:25. These and other organisms are capable of evoking low levels of antibodies reacting with *Brucella* antigens (8). Autofluorescence of milk components other than riboflavin could cause false-positive reactions, resulting in the lower specificity. Interference from lipids in the fresh milk samples could trap the enzyme conjugate used in the mELISA, resulting in a lower specificity (91.8%) of this test.

The quality control data presented in Fig. 1 demonstrate remarkably low day-to-day and test-to-test variation for the bmFPA, and the bmFPA compares favorably with the ELISA formats, where coefficients of variation of <20% among negatives were considered good. The bmFPA displayed good qual-

ity control over a 6-month period involving different batches of reagents, buffer, and controls. The control data never exceeded the UCL and LCL. Control samples were simple and inexpensive to prepare in-house, which would simplify their production locally and for international standardization.

The detection limit of this assay was determined by using 10 repeat titrations of an artificially constructed positive sample as presented in Fig. 2. Using the cutoff of 140 mP, the bmFPA in theory could detect one animal with *B. abortus* antibodies milk in more than 2,000 animals negative for *B. abortus*, which exceeded the mELISA (one infected animal in 100 animals) and the MRT (one infected animal in 5 or 10 animals) using similar methodology (2, 6, 13; Fleischhauer and Hermann, Berl. Tierärztl. Wochenschr. 54:333, 1938). The average dairy herd size in Canada is approximately 48 animals; therefore, a test that can detect one *B. abortus*-positive animal in 2,000 *B. abortus*-negative animals would be ideal for noninvasive screening for the detection of antibodies to *B. abortus* in milk. Analogously, a bulk tank with a capacity of 1,150 liters is sufficient to hold milk from 40 dairy cows producing on average 29 liters of milk per day, again making this test ideal for noninvasive screening. Because milk samples are not diluted in the bmFPA, animals at early stages of infection may be detected sooner with this test than with the mFPA that requires samples to be diluted 1:25 possibly diluting samples with low antibody levels to extinction.

Twenty-three milk samples banked from animals from which *B. abortus* had been isolated were used in this study. It was apparent from the data in this study that antibody activity in the stored milk samples could still be detected after 20 years of storage (samples were collected in 1981 and 1982). In all these samples the casein and total milk solids had precipitated. Treatment of these samples was the same as that of the freshly collected samples. An equal number of banked samples were not useable due to an irreversible aggregation phenomenon known as age gelation caused by aggregation of the micelles into long chains forming a three-dimensional network (<http://www.foodsci.uoguelph.ca/dairyedu/chem.html>).

Further studies are required to evaluate and validate the bmFPA for field use; however, this study showed that the bmFPA was a simple, rapid, and inexpensive test that compared favorably with traditionally used tests (mELISA) for detection of milk antibodies to *B. abortus*. The assay would be useful in surveillance and eradication programs, reducing costs significantly. Evaluation of this method for the detection antibodies to *B. melitensis* in milk from small ruminants should be considered, as the MRT works poorly with milk from sheep and goats (1).

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TABLE 3. Predictive values of bmFPA<sup>a</sup>

Prevalence	PPV	NPV
0.01	0.2	100
0.10	2.4	100
1.00	19.8	100
10.0	73	100
15.0	81.1	100
20.0	85.9	100

<sup>a</sup> Comparison of PPVs and NPVs of the bmFPA (negative cutoff = 140 mP) with a test sensitivity and specificity of 100 and 95.9%, respectively.

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