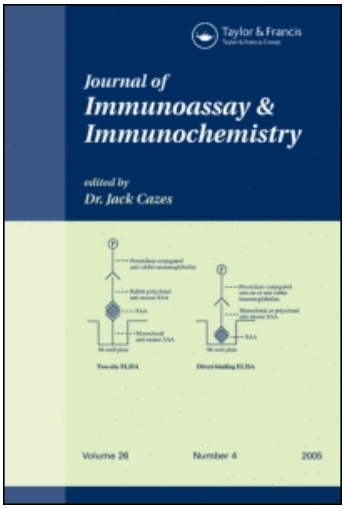

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

FIELD TRIAL OF THE BRUCELLOSIS FLUORESCENCE POLARIZATION ASSAY

K. Nielsen[a]; D. Gall[a]; R. Bermudez[b]; T. Renteria[b]; F. Moreno[b]; A. Corral[b]; O. Monroy[b]; F. Monge[b]; P. Smith[a]; J. Widdison[a]; M. Mardrueno[b]; N. Calderon[b]; R. Guerrero[b]; R. Tinoco[b]; J. Osuna[b]; W. Kelly[a]
[a] Animal Diseases Research Institute, Nepean, Canada [b] Instituto de Investigaciones en Ciencia Veterinarias, Unidad de Laboratorios de Diagnostico, Universidad Autonoma de Baja California, Mexicali, Mexico

Online publication date: 08 July 2002

To cite this Article Nielsen, K. , Gall, D. , Bermudez, R. , Renteria, T. , Moreno, F. , Corral, A. , Monroy, O. , Monge, F. , Smith, P. , Widdison, J. , Mardrueno, M. , Calderon, N. , Guerrero, R. , Tinoco, R. , Osuna, J. and Kelly, W.(2002) 'FIELD TRIAL OF THE BRUCELLOSIS FLUORESCENCE POLARIZATION ASSAY', Journal of Immunoassay and Immunochemistry, 23: 3, 307 — 316
To link to this Article: DOI: 10.1081/IAS-120013030
URL: http://dx.doi.org/10.1081/IAS-120013030


MARCEL DEKKER, INC. • 270 MADISON AVENUE • NEW YORK, NY 10016

JOURNAL OF IMMUNOASSAY & IMMUNOCHEMISTRY
Vol. 23, No. 3, pp. 307–316, 2002

FIELD TRIAL OF THE BRUCELLOSIS FLUORESCENCE POLARIZATION ASSAY

K. Nielsen,[1,*] D. Gall,[1] R. Bermudez,[2] T. Renteria,[2] F. Moreno,[2] A. Corral,[2] O. Monroy,[2] F. Monge,[2] P. Smith,[1] J. Widdison,[1] M. Mardrueno,[2] N. Calderon,[2] R. Guerrero,[2] R. Tinoco,[2] J. Osuna,[2] and W. Kelly[1]

[1]Animal Diseases Research Institute, 3851 Fallowfield Rd., Nepean, Ontario, Canada K2H 8P9
[2]Universidad Autonoma de Baja California, Instituto de Investigaciones en Ciencia Veterinarias, Unidad de Laboratorios de Diagnostico, Mexicali, Mexico

ABSTRACT

Fluorescence polarization assay (FPA) is a homogeneous technique which was applied to the serological diagnosis of bovine brucellosis. Because of its simplicity and because it may be performed very rapidly, it was an ideal test to adapt to field use. The FPA was used to test cattle on six dairy farms in Baja California, Mexico. Anticoagulated blood, serum, and milk were collected from each animal. The anticoagulated blood was tested immediately on the farm while serum and milk were tested subsequently in the laboratory. Cattle on one

*Corresponding author. E-mail: nielsenk@inspection.gc.ca

DOI: 10.1081/IAS-120013030 1532-1819 (Print); 1532-4230 (Online)

MARCEL DEKKER, INC. • 270 MADISON AVENUE • NEW YORK, NY 10016

farm ($n = 140$) were thought not to be infected with *Brucella abortus* and the other farms were thought to have high prevalence of the infection. The whole blood FPA (FPA(bld)) did not detect antibody in any of the cattle on the first premise. This finding was confirmed using a number of other serological tests, including the buffered antigen plate agglutination test, the complement fixation test, the indirect and competitive enzyme immunoassays, and the FPA using serum and milk. Cattle on the other premises ($n = 1122$) were tested in a similar fashion. The sensitivity of the FPA(bld), relative to the serum FPA (considered the definitive test), was 99.1% and the relative specificity of the FPA(bld) was 99.6%. These results compared favourably with those obtained using the other serological tests.

Key Words: Fluorescence polarization assay; Field test; Comparative serology; Brucellosis

INTRODUCTION

The fluorescence polarization assay (FPA), for detection of antibody to *Brucella sp*., was validated as a serological test for bovine brucellosis using serum,[1–3] and for a number of other species.[4–6] The premise of the FPA is that a small molecule rotates rapidly in a random fashion when in solution. If the molecule is labelled with a fluorochrome, the rate of rotation may be measured using polarized light and assessing depolarization. If the size of the molecule is altered, enlarged by attachment of an antibody, its rate of rotation is slower and the polarized light is depolarized more slowly. The change in rate of rotation is almost instantaneous and may be measured without removing unreacted reagents. This makes the FPA a very simple and rapid test to perform and, therefore, is an ideal candidate for field use, allowing considerable savings in shipping charges, time, and overhead costs.

Because the fluorescence polarization analyzer is computer controlled, data may be stored and communicated electronically for tracking and record purposes. Preliminary investigations suggested that the FPA was adaptable to testing anticoagulated blood chuteside[7] and using milk.[8] To validate the use of the FPA with whole blood and milk, further testing was required at the farm level and more milk data was needed. This communication reports data obtained by testing whole blood on six dairy farms, five with and one without brucellosis, and comparing the

data to that obtained in the laboratory using matched serum and milk samples.

EXPERIMENTAL

Samples

Six herds were selected. One ($n = 140$) was without serological and epidemiological evidence of brucellosis for several years. The other herds ($n = 1122$) were selected based on a high prevalence of serologically positive animals in previous herd tests. Clotted blood, blood anticoagulated with EDTA, and milk were collected from each cow. The anticoagulated blood was tested immediately after collection. Clotted blood was centrifuged and the serum was removed and frozen at -20°C until tested. Milk samples were stored at 4°C until tested.

Serological Tests

Brucella abortus S1119.3 was used as a source of antigen for all tests.

The buffered antigen plate agglutination test was performed according to the OIE Manual.[9] Briefly, 80 μL of serum was mixed with 30 μL of antigen for 8 min and agglutination was assessed visually.

The CFT of Samagh and Boulanger[10] was used. Serum dilutions, starting at 1 : 5 were incubated with antigen and three CH_{50} units of guinea pig serum overnight at 4°C. Indicator system, sheep erythrocytes sensitized with rabbit antibody, was added, incubated at 37°C, and the degree of hemolysis was graded.

The indirect serum enzyme linked immunosorbent assay (IEL(srm)), the milk IELISA (IEL(mLk)), and the competitive ELISA (CELISA) utilized smooth lipopolysaccharide (LPS) antigen passively attached to a polystyrene matrix. For the IEL(srm), serum diluted 1 : 50 and, for the IEL(mLk), milk diluted 1 : 2 was added for 30 min followed by monoclonal antibody specific for a heavy chain epitope of bovine IgG_1 (M23), conjugated with horseradish peroxidase (HRPO) for an additional 30 min. Substrate (1.0 mM H_2O_2) and chromogen (4.0 mM ABTS) were added for 10 min with constant shaking after which colour development was assessed in a spectrophotometer at 414 nm. A washing procedure took place between each step. Results were expressed as a percent of a strong positive control added to each polystyrene plate along with a weak positive control, a negative and a buffer control. The IEL(mLk) also utilized a positive and negative

milk control. For the CELISA, serum diluted 1 : 10 was added to an appropriately diluted monoclonal antibody specific for a common epitope of the *O*-polysaccharide portion of LPS for 30 min. The detection reagent was a goat anti-mouse IgG-HRPO (Jackson ImmunoResearch, West Grove, PA, USA) appropriately diluted and added for 30 min followed by the substrate/chromogen as for the IEL(srm). A washing procedure was performed between each step. Data was expressed as a percentage of an uninhibited control. All ELISA procedures are detailed in Ref. [11].

The fluorescence polarization assay (FPA) was described in Ref. [6]. Briefly, serum was diluted 1 : 100, blood and milk 1 : 50 in 0.01 M *tris*, pH 7.0, containing 0.15 M NaCl, 10 mM EDTA and 0.05% Igepal CA630 and mixed. After an initial measurement to assess non-specific fluorescence in an FPM Sentry Analyzer (Diachemix Corp., WI, USA), a small molecular weight *O*-polysaccharide fragment (approximately 22 kd average) conjugated with fluorescein isothiocyanate (Sigma Fine Chem Corp., MO, USA) was added and mixed. The serum and milk samples were incubated for 2 min and a final polarization measurement was made. The whole blood was incubated with antigen for 15 s before assessed for polarization. The analyzer subtracted background fluorescence and provided a net result in millipolarization units (mP). In all cases, controls consisting of a strong positive and a weak positive serum as well as sera from a *B. abortus* S19 vaccinated and a negative animal were included. For the serum and milk FPAs, the cutoff between positive and negative results was 90 mP while for the whole blood FPA, the cutoff was set at 95 mP.[7]

Data

All samples giving a positive result were retested as is common diagnostic procedure. The data was compiled and the % agreement with the serum FPA was calculated using receiver operator characteristic analysis.[12] All sensitivity and specificity estimates are relative to the serum FPA. Kappa analysis was performed comparing results of the various tests.

RESULTS

The results for the herd ($n = 140$) without any evidence of brucellosis are compiled in Table 1. Using the serum FPA(srm) as the definitive test, the specificity of the other assays used were calculated. For each test the cutoff is indicated.

Table 1. Specificity Values (%) for the Serological Tests Relative to the Serum FPA(srm)

Test	Cutoff	Specificity (%)
FPA(bld)	95 mP	100
CELISA	31% I	97.9
IEL(srm)	62% P	100
BPAT	+/−	100
CFT AC−	1 : 5	100
CFT AC+	1 : 5	97.8
FPA(mLk)	87 mP	99.3
IEL(mLk)	32%P	100

The CELISA gave three false positive reactions, all of which were negative in all other tests. The sera that gave false positive results were different in each test. In the CFT, 3 sera were anticomplementary (AC) and a diagnosis could not be made. Therefore 2 values were calculated in which the AC sera were considered positive or negative.

The results for the herds with *B. abortus* infection and the negative herd ($n = 1262$) were also based on the serum FPA(srm). In these herds, 228 animals gave positive results in the FPA(srm). Relative sensitivity values were calculated based on the reactivity of these 228 sera in other tests. Similarly, the specificity data was calculated relative to the 1034 sera which were negative in the FPA(srm). These data are presented in Table 2. Of the 1034 negative sera, 36 (3.5%) fixed complement in the absence of antigen (AC) and some of the positive sera gave some AC reactivity but it did not exceed the level of the specific complement fixation and therefore did not interfere with the diagnosis.

The reaction observed in tests of animals 57J and 941O in Table 3 (positive values are highlighted) could be due to a mixup of the labels on the blood/serum/milk tubes prior to testing. This was confirmed by testing the blood in CELISA (CEL bld) and IEL(bld) in which it gave positive reactions, agreeing with the FPA(bld) while serum and milk data with the exception of the BPAT (BPA srm) and the IEL(srm) both of which could be positive due to residual antibody to *B. abortus* S19. Serum 393O is interesting in that it may represent an animal in the very early stages of infection with *B. abortus*, giving positive results only in the (FPA srm) and IEL(srm) and in the milk FPA(mLk) and IEL(mLk).

Kappa statistics comparing the FPA(bld) to the other serological tests were determined and are presented in Table 4.

The remainder of the discrepancies were sporadic.

Table 2. Sensitivity and Specificity Values (%) Relative to the FPA(srm) and Using the Same Cutoff Values as Table 1

Test	Sensitivity (%)	Specificity (%)	P.I.	Prob.
FPA(bld)	99.1	99.6	198.7	99.8
CELISA	98.7	98.4	197.1	99.7
IEL(srm)	99.1	96.4	195.5	99.4
BPAT	96.5	98.3	194.8	97.4
CFT AC−	98.2	98.8	197.0	98.9
CFT AC+	98.2	95.3	193.5	98.9
FPA(mLk)	89.5	98.2	187.7	96.4
IEL(mLk)	96.5	96.3	192.8	98.2

Since the 36 AC sera were not FPA(srm) positive, they were not considered in the calculation of the CFT sensitivity value. A summary of the sera that did not agree with the FPA(srm) is presented in Table 3. The PI column refers to the performance index, the sum of the percent sensitivity and specificity values. The prob. column indicates the probability of the test being correct 95% of the time, based on the area under the curve of the receiver operator characteristic (ROC) analysis (not shown).

DISCUSSION

There are considerable advantages to chuteside testing of animals for evidence of infectious disease, obtaining results before the animal is released. It not only saves shipping costs and delay in obtaining results but it decreases loss of production by reducing stress with a single restraint procedure. This is particularly useful when testing wild animals such as bison. It would be even more advantageous if milk (where available) could be tested as it is very cheap to obtain by non-invasive methods. In this study we have tested cattle on six dairy farms for brucellosis using the FPA at the farm. Of the six farms, one was selected because there had been no evidence of brucellosis in the cattle for a period of time. The other five farms were thought to have a relatively high prevalence of brucellosis. All animals were bled (using EDTA as an anti-coagulant and clotted blood) and milk samples were obtained. The EDTA-blood samples were tested immediately. The serum and milk samples were tested subsequently in the laboratory. For this study, the FPA test using serum was used as the standard for comparison to the FPA(bld) using EDTA-blood, CELISA, IELISA, BPAT and CFT using serum and FPA and IELISA using milk. From Table 1, it is clear that all the tests performed well, the CELISA giving three false positive reactions and the FPA (mLk)

Table 3. Summary of Sera Not in Agreement with the FPA(srm)

#	FPA srm	CEL srm	IEL srm	BPA srm	CFT Srm	FPA bld	CEL Bld	IEL bld	FPA MLk	IEL mLk
57J	76.2	27	25	N	N	***265.7***	***99***	***65***	84.9	15
393O	***92.8***	24	***106***	N	N	90.4	24	30	***87.0***	***58***
941O	78.5	0	***118***	***P***	N	***158.7***	***50***	***59***	82.2	70

Table 4. Kappa Statistics Comparing the Results of the Various Tests

Test 1	Compared to	Test 2	Kappa Value
FPA(bld)		CFT (AC−)	0.963
FPA(bld)		CELISA	0.943
FPA(bld)		BPAT	0.937
FPA(bld)		FPA(mLk)	0.879
FPA(bld)		IELISA(srm)	0.879
FPA(bld)		IELISA(mLk)	0.878
FPA(bld)		CFT (AC+)	0.874

with one false positive reaction, while the CFT gave 3 AC reactions. Thus the specificity of all the serological tests were initially established using a herd with no evidence of brucellosis.

The data for the six herds was combined for analysis. This data is presented in Table 2. Receiver operator characteristic analysis (ROC) was performed on the data from each assay and the sensitivity and specificity values were used to determine a performance index (P.I.) as well as the area under the ROC curve (Prob.) which is a measurement of the test accuracy (the 95% probability that the result is correct) are also presented in Table 2. The FPA(bld) and the CELISA performed best, resulting in 99.8 and 99.7% probabilities (at the 95% confidence level) that the results were correct. All the other tests were more than 96% accurate. From the data it was clear that the sensitivity and specificity values obtained with the FPA(bld) made it a valuable asset for screening at the farm level while the FPA(mLk) was somewhat less sensitive (9.6%) and slightly less specific (1.4%). These results may improve when more data is accumulated and when milk samples can be tested fresh at the chute (it was decided not to test the milk in situ due to the risk of live *Brucella sp*. being present in some samples). A technique for inactivating organisms in the milk without influencing the results is under investigation. Thus it remains to be determined if milk may be used to screen for brucellosis antibodies.

Table 3 lists the results obtained with 3 samples that gave inconsistent results in the various tests. The negative serum and milk results and positive blood results obtained with sample 57J is most likely a result of mislabelled vials. The explanation(s) for samples 393O and 941O remain undetermined, however, it is interesting to note that if sample 393O is from an animal in the early stages of infection, the FPA(srm) and IEL(srm) were the most sensitive assays for antibody detection but antibody was also found in milk in this particular animal. All other discrepancies were sporadic, with only one assay giving an uncorrelated result. Overall, there was agreement between the

various tests and discrepancies could easily be overcome by retesting animals that gave positive FPA(bld) results before release.

The data presented for the FPA(bld) is an improvement on earlier data which gave a sensitivity value of 95.3% and a specificity value of 97.3%[7,8] and a considerable increase in sensitivity from previous data (a gain of 3.8%) with a slightly smaller increase in specificity (2.3%). This may be a result of operators becoming more familiar with the test protocol, better equipment and better and more samples for analysis.

Based on observations presented, the FPA(bld) performed as well as the CELISA and showed an almost 100% agreement with the FPA(srm) which was previously shown[6] to presumptively diagnose brucellosis as or more accurately than the BPAT, CFT, IEL(srm) and CELISA, assays sanctioned by the OIE.[9] However, kappa statistics showed an overall excellent agreement between all the tests used (Table 4). It was interesting to note that the best kappa agreement of the FPA (bld) assay was with the CFT when AC reactions were considered negative, closely followed by the CELISA and the BPAT.

Because it can be performed in 15–30 s, repeat testing of animals giving a positive reaction is realistic. The assay itself is robust, simple to perform, and relatively inexpensive. Therefore, it is suitable for use as a screening test at the farm level and it should be considered as a replacement for other screening tests such as the rose bengal and the buffered antigen plate agglutination tests (BPAT), both of which are prone to give false negative reactions with highly positive sera.[9]

REFERENCES

1. Nielsen, K.; Gall, D.; Jolley, M.; Leishman, G.; Balsevicius, S.; Smith, P.; Nicoletti, P.; Thomas, F. A Homogeneous Fluorescence Polarization Assay for Detection of Antibody to *Brucella abortus*. J. Immunol. Meth. **1996**, *195*, 161–168.
2. Dajer, A.; Luna-Martinez, E.; Zapata, D.; Villegas, S.; Gutierrez, E.; Pena, G.; Gurria, F.; Nielsen, K.; Gall, D. Evaluation of a Fluorescence Polarization Assay for the Diagnosis of Bovine Brucellosis in Mexico. Prev. Vet. Med. **1999**, *40*, 67–73.
3. Samartino, L.; Gregoret, R.; Gall, D.; Nielsen, K. Fluorescence Polarization Assay: Application to the Diagnosis of Bovine Brucellosis in Argentina. J. Immunoassay **1999**, *20*, 115–126.
4. Gall, D.; Nielsen, K.; Forbes, L.; Davis, D.; Elzer, P.; Olsen, S.; Balsevicius, S.; Kelly, L.; Smith, P.; Tan, S.; Joly, D. Validation

of the Fluorescence Polarization Assay and Comparison to Other Serological Tests for theDetection of Serum Antibodies to *Brucella abortus* in Bison. J. Wildlife Dis. **2000**, *36*, 469–476.

5. Gall, D.; Nielsen, K.; Forbes, L.; Cook, W.; Leclair, D.; Balsevicius, S.; Kelly, L.; Smith, P.; Mallory, M. Validation of the Fluorescence Polarization Assay and Other Serological Tests for the Detection of Brucellosis in Cervids. J. Wildlife Dis. **2001**, *37*, 110–118.
6. Nielsen, K.; Gall, D. Fluorescence Polarization Assay for the Diagnosis of Brucellosis: A Review. J. Immunoassay Immunochem. **2001**, *22*, 183–201.
7. Nielsen, K.; Gall, D.; Smith, P.; Kelly, W.; Yeo, J.; Kenny, K.; Heneghan, T.; McNamara, S.; Maher, P.; O'Connor, J.; Walsh, B.; Carroll, J.; Rojas, X.; Rojas, F.; Perez, B.; Wulff, O.; Buffonu, L.; Salustio, E.; Gregoret, R.; Samartino, L.; Dajer, A.; Luna-Martinez, E. Fluorescence Polarization Assay for the Diagnosis of Bovine Brucellosis: Adaptation to Field Use. Vet. Microbiol. **2001**, *80*, 163–170.
8. Nielsen, K.; Smith, P.; Gall, D.; Perez, B.; Samartino, L.; Dajer, A.; Rojas, X.; Kelly, W. Validation of the Fluorescence Polarization Assay for Detection of Milk Antibody to *Brucella abortus*. J. Immunoassay Immunochem. **2001**, *22*, 203–211.
9. MacMillan, A.P.; Stack, J.; *Bovine Brucellosis*. *OIE Manual of Standards for Diagnostic Tests and Vaccines*; OIE: Paris, 2000; 328.
10. Samagh, B.; Boulanger, P. Adaptation of the Technicon Autoanalyzer II for an Automated Complement Fixation Test for Detection of Antibody to *Brucella abortus* in Bovine Serums. Proc. 21st Ann. Meet. Am. Assoc. Vet. Lab. Diagnost. 1978; 347.
11. Nielsen, K.; Gall, D.; Kelly, W.; Vigliocco, A.; Henning, D.; Garcia, M. *Immunoassay Development: Application to Enzyme Immunoassay for the Diagnosis of Brucellosis*; Agriculture and Agri-Food monograph A62-45/1996E.
12. Schoonjans, F.; Zalata, A.; Depuydt, C.; Comhaire, F. MedCalc: A New Computer Program for Medical Statistics. Computer Meth. Progr. Biomed. **1995**, *48*, 257–267.

Received December 5, 2001
Accepted January 9, 2002
Manuscript 3058