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Fluorescence Polarization Assay for the Diagnosis of Anti-Brucella abortus Antibodies in Cattle Serum: Adaptation for its Use in Microplates and Comparison with Conventional Agglutination Tests

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Abstract

Bovine brucellosis, which is endemic in Argentina, is controlled by vaccination and slaughter of infected cattle. Conventional agglutination tests and primary binding assays like ELISA and fluorescence polarization assay (FPA) are used for the identification of infected cattle. The FPA has many advantages over the agglutination tests, however most accredited laboratories still use the conventional agglutination tests. FPA has been extensively evaluated in its original format of 10mm x 75mm glass tubes, while there are little reports on its performance in the 96-well microplate format. The aim of the present study was to set the conditions for the use of a commercially available antigen (the O-polysaccharide from *B. abortus* 1119-3 conjugated with fluorescein isothiocyanate) for the FPA assay in a 96-well microplate format, and to compare its diagnostic performance with the conventional agglutination tests currently used in Argentina. Serum samples were obtained from 149 cows and 20 bulls belonging to free and infected herds from different regions of Argentina. Two dilutions of serum and antigen were assayed and the fluorescence polarization was detected with a Beckman DTX 880 multimode reader. Results: The FPA conducted using antigen and serum at 1:100 dilution was as efficient as the test using antigen at 1:20 and serum at 1:10. Eight percent sera from an endemic region gave false positive reaction in the agglutination tests compared to FPA, while 8% of samples giving suspicious result in agglutination test resulted negative in FPA. Due to its high testing capacity, economy of antigen, lack of false positive reactions, and avoidance of re-testing of cattle with suspicious result in agglutination tests, the FPA conducted in microplates is a suitable test to be implemented in the control and eradication of brucellosis in Argentina.

Keywords: Brucellosis; Fluorescence polarization; Agglutination; Microplate; Serology

Abbreviations: FPA: Fluorescence Polarization Assay; BPAT: Buffered Plate Antigen Test; SAT: Serum Agglutination Test; β-ME: β Mercaptoethanol; O-PS: O-Polysaccharide

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Introduction

Bovine brucellosis is one of the most common bacterial zoonosis in the world and is responsible for important economic losses in cattle. The infection in cattle is mainly due to *Brucella abortus* and is characterized by the death and expulsion of the foetus between the fifth and eighth months of gestation. Diagnosis of brucellosis is complicated due to its variable incubation time and the absence of clinical signs other than abortion. Serological techniques are the mainstay of diagnosis and mass testing programmes [1,2].

Brucellosis is endemic in Argentina. A national survey in 2004 showed that 12.4% of the beef farms were seropositive to *Brucella*, and that the apparent prevalence was 2.15% [3]. The National Program of Control and Eradication of Bovine Brucellosis in this country involves the vaccination of female calves between 3 and 8 months of age with *B. abortus* S19 and slaughter of infected cattle. In Argentina, the identification of infected cattle is carried out by serological tests: buffered plate antigen test (BPAT) and indirect ELISA are used as screening tests, while serum agglutination test with β-mercaptoethanol (SAT/β-ME), fluorescence polarization assay (FPA), competitive ELISA and complement fixation tests are used as complementary. At present, complement fixation test is considered the confirmatory test. All tests detect antibodies to the O-chain of smooth lipopolysaccharide induced by vaccination or by *Brucella* field strains [4,5]. Occasionally, false positive reactions were found in healthy adult bovine induced by cross reaction antibodies stimulated by the administration of vaccines composed of Gram negative bacteria, but the cross reaction between these bacteria and *B. abortus* is dependent on the test used. Thus, extensive cross reaction was observed with the indirect ELISA with much less reactivity in the FPA and the competitive ELISA [6].

The FPA is a primary binding homogeneous immunoassay used for the detection of anti-*B. abortus* O-PS (O-polysaccharide) antibodies. The method is based on the principle that the rate of rotation of a small labeled antigen molecule in solution is altered if antibody is bound to it, and this change in rotation can be measured. Nielsen et al. [7] developed a FPA using the O-PS from *B. abortus* conjugated with fluorescein isothiocyanate as a tracer for detection of antibody to *B. abortus* in bovine serum. The main advantages of the FPA are its rapidness, simplicity, specificity, sensitivity, objectivity of results, and the ability to early distinguish vaccinated animals from those infected with *Brucella* spp. [8,9]. Diagnostic performance of the FPA was determined in well characterized samples from Canada, resulting in 99% sensitivity and 99.96% specificity. Due to these properties, the World Organization for Animal Health [2] has recommended the FPA assay as a compulsory test for the international trade of cattle [2]. The test has been extensively used with sera from different species of animals and in areas of various prevalences of brucellosis [10-12]. It was originally developed to be conducted in 10mm x75mm glass tubes in a single cell analyzer. The availability of microplate readers favors the automation of the test, but there are no recommendations from the international or national authorities of animal health regarding the procedure of the test in microplates. Furthermore, there are little reports on the performance and experimental conditions for the FPA test in microplate readers [13-15].

The aim of the present study was to adapt a commercially available kit for the FPA assay to the 96-well microplate format, and to compare its diagnostic performance with the conventional agglutination tests currently used in Argentina. Different dilutions of both serum and commercial antigen were assayed, and the polarization of the fluorescence was obtained with a multimode microplate reader. Once the optimal conditions for the FPA assay were established, its diagnostic performance was compared to that of the conventional agglutination tests in sera from an endemic area of Argentina.

Material and Methods

Samples

Serum samples were obtained from 20 bulls and 36 cows belonging to free herds where vaccination of female calves between 3 and 8 month of age is the routine. The status of these free herds was certified by the National Authority of Animal Health (SENASA), hence the totality of susceptible cattle in these herds have proven to be serologically negative in 3 consecutive samples through a period of at least 1 year. One hundred and thirteen serum samples were obtained from herds located in an endemic area (province of Santiago del Estero, Argentina). These herds had a history of abortion storms with culture evidence of *Brucella abortus*. Samples were stored at -20°C until used.

Serum samples from free herds and samples from endemic herds that tested positive in the agglutination tests (BPAT and SAT/β-ME) were used in preliminary experiments to set the optimal conditions (volume of total reaction, integration time, dilution of reagents) of the FPA in microplates. The comparative performance of agglutination tests versus FPA was evaluated using the samples obtained from the endemic area.

Fluorescence polarization assay

The antigen, the diluent of sera and the negative and positive control sera were provided in the FPA kit produced by Laboratorio Biológico Tandil (Tandil, Buenos Aires, Argentina). The antigen was composed by the O-polysaccharide from *B. abortus* 1119-3 conjugated with fluorescein isothiocyanate. All the components of the kit were produced according to the recommendations published by the OIE, on the basis of the protocol of Dr Klaus Nielsen.

The test was carried out in 96-well black polypropylene microplates (Greiner Bio One, Frickenhausen, Germany). In order to set the optimal conditions to perform the FPA assay in microplates, we first tested some experimental conditions and settings of the equipment including the volume of total reaction and the integration time of the polarimeter. Briefly, serum samples were clarified by centrifugation (2 min at 10000 rpm). Appropriate dilution of sera was prepared directly in the wells of the microplate and a blank reading was performed. When antigen was used at a 1:100 final dilution, a 1:10 pre-dilution was prepared as to avoid pippeting low volumes of antigen; when final dilution of antigen was 1:20, it was used directly from the undiluted stock. The test mixture was incubated with orbital shaking for 2 min, and after two additional min of incubation the polarization of the fluorescence was measured. A Beckman multimode reader (Model DTX 880, Beckman Coulter, Brea, California) with Multimode Detection Software version 2.1.0.17 was used. DTX parameters used for FPA measurements were 485nm excitation, 535nm emission and 0.65 gain. Results of total polarization obtained with blanks (diluted serum) and the total reaction mixture (diluted serum plus antigen)

were exported to an Excel® spreadsheet (Microsoft Corp, Redmond, Washington) in which the following formula was used to calculate the mP value:

$$mP = \frac{(V_t - V_b) - G(H_t - H_b)}{(V_t - V_b) + G(H_t - H_b)}$$

Where, V_t = vertical intensity of the test after the tracer was added; V_b = vertical intensity of the test before the tracer was added; H_t = horizontal intensity of the test after the tracer was added; H_b = horizontal intensity of the test before the tracer was added; G = G factor =0.65 [13]. The criteria for interpretation of results were according to that established by the national authorities of animal health, SENASA (Servicio Nacional de Sanidad Animal y Calidad Agroalimentaria): positive if mP was ≥105 mP, negative if the value was <94 mP and indeterminate when the mP value was between 94 and 105.

Agglutination tests

Conventional agglutination tests (BPAT and SAT/β-ME) were carried out and interpreted according to the procedures recommended by the SENASA [16]. Antigens were obtained from Laboratorio Biológico Tandil (Tandil, Argentina). BPAT was used as screening test, and positive samples were further tested in SAT with and without β-ME in parallel. Table 1 shows the criteria for the interpretation of results of these tests in cows older than 18 months that were vaccinated as calves with *B abortus* S19. Antigen and control sera for these tests were provided by Laboratorio Biológico de Tandil.

Statistical analyses

The comparisons of test conditions (dilution factor of serum and antigen) for the FPA was analyzed by McNemar´s t test for paired data, using the GraphPad online calculator, 2002-2005 by GraphPad Software Inc. available at http://www.graphpad.com/quickcalcs/index.cfm.

Results

We did not find statistical significant differences in the results when performing the reaction in 100 or 200μl of final volume, nor in the setting of the integration time between 0.05 and 1.25 sec (data not shown). We then set the final volume in 200μl and the integration time in 0.25 sec, and compared different proportions of both serum and antigen. Preliminary experiments with a limited number of samples showed no significant differences in the mP units obtained when antigen and sera were combined in different proportions (data not shown). To decide which proportion of serum and antigen performed better, we then compared, using a panel of 144 samples, the conditions recommended by the SENASA and OIE for the test

BPA	SAT	β-ME	Interpretation
Negative			Negative
Positive	≤ 50 IU	Negative	Negative
Positive	≤ 25 IU	≤ 25 IU	Negative
Positive	25 to 50 IU	I25 IU	Negative
Positive	I100 IU to I200 IU	Negative	Suspicious
Positive	≥ I50 IU	≥ I50 IU	Positive
Positive	200 IU	Negative	Positive

IU: International Units

Table 1: Criteria for interpretation of results from conventional agglutination tests in female cattle vaccinated between 3 and 8 month of age in Argentina.

		Condition A	
		Positive	Negative
Condition B	Positive	20	0
	Negative	3	115

Condition A: serum and antigen were both used at a 1:100 dilution

Condition B: serum was used at 1:10 and antigen at 1:20

Table 2: Comparative performance of the fluorescence polarization assay for the detection of antibodies against the O-polysaccharide of *B. abortus* using different dilutions of the sera and antigen.

Number of samples	Conventional agglutination tests[a]	Fluorescent polarization assay[b]
27	Positive	Positive
1	Positive	Suspicious
9	Positive	Negative
9	Suspicious	Negative
67	Negative	Negative

[a]BPAT and SAT/β-ME were carried out and interpreted according to the procedures recommended by the SENASA

[b]FPA was carried out in microplates as described in Materials and Methods

Table 3: Comparative performance of fluorescence polarization assay with conventional agglutination tests in serum samples from an endemic region of Argentina.

performed in 10mm x 75mm borosilicate glass tubes (serum and antigen at a 1:100 dilution) with that used by Minas et al. [14] in sheep and goat sera and McGiven et al [15] in bovine sera (serum at a 1:10 dilution and antigen at 1:20).

Six from the 144 serum samples tested in both conditions gave indeterminate result in any of the conditions tested and were excluded from the comparison. Table 2 shows the results obtained from the comparison of samples tested using the two different proportions of reactants. Ninety seven percent (135/138) gave concordant result in the two variants of the assay, while 3 samples resulted positive only when tested at 1:100 dilution. These differences were not statistically significant (Mc Nemar´s test, p=0.2482). The 3 samples that resulted positive at 1:100 dilution were also positive in the conventional agglutination tests, applying the criteria for interpretation of the results currently used in Argentina (title ≥ 200 in SAT or ≥ 50 incomplete in β−ME, Table 1).

We then set the dilution at 1:100 for both serum and antigen for the comparison of the performance of the FPA assay with the conventional agglutination tests. Results from this comparison are summarized in Table 3. Concordance between results from the agglutination tests and FPA was observed in 94 from 113 samples (83.2%). Seventeen from the 18 samples giving discordant results (positive or suspicious by the agglutination tests and negative in the FPA) were also tested by FPA using sera at a 1:10 dilution and antigen at 1:20 and resulted negative (the remainder sample could not be tested in this latter condition because of insufficient volume). Ten from 113 samples (8.8%) resulted positive in the conventional agglutination tests while negative or suspicious in FPA. Eigth percent of samples (9/113) giving suspicious result in the agglutination tests, which should be re-tested in order to give a definitive result, were defined as negative in the FPA.

Discussion

Although the FPA test has been recommended by the SENASA, most accredited laboratories still use the conventional agglutination tests for screening and confirmation of seroreactivity to *B. abortus*, mainly because of the lack of the necessary equipment. Nowadays, the acquisition of multimode microplate readers including the fluorescence polarization mode of reading is being considered, as this equipment can be employed for many applications. Most fluorescence polarization analyzers have fixed reading parameters, while they can be modified in the microplate analyzers, like the Beckman Coulter DTX-880 we have used in the present study.

We compared two different proportions of the two reagents, serum and antigen, because we have not found official recommendations (OIE or SENASA in Argentina) for the procedure of the test in microplates. As to that we have found in the literature, there is only one study in which the FPA was conducted in microplates with cattle sera [15]. In two other studies the FPA was carried out in microplates using sheep and goat sera [14,17]. In one of these studies the authors used the 1:100 dilution of both reagents while in the others serum was used at 1:10 and antigen at 1:20. Our results show that the proportions currently used for the procedure in glass tubes (dilution 1:100 for both the serum and antigen) performed as well as the variant with antigen at 1:20 and serum at 1:10. The main advantage of using the former option is that there is an economy of 80% in antigen.

While the conventional agglutination tests have widely contributed to the control of the disease, they have some disadvantages like subjectivity, they are time-consuming, and may give false positive results due to cross reaction with other Gram negative bacteria and residual antibodies generated by vaccination [7-9].

The FPA has first been validated using samples from Canada where brucellosis has been eradicated and vaccination with *B.abortus* S19 is rare. The cut off has been adapted for the testing of samples from Argentina, where cattle are vaccinated between 3 and 8 month of age [9]. Samartino et al. [14] evaluated the performance of the FPA in samples from argentinian cattle in which the serological status was defined according to its reaction in BPAT and competitive ELISA, finding that the FPA aided in the definition of samples that had discordant results between BPAT and ELISA.

The fact that FPA was negative in all samples giving suspicious result in the conventional agglutination tests is beneficial as it avoids the need to re-test these samples that, in our study, accounted for an 8% of the total. An additional 8% of samples which were positive in the agglutination tests and resulted negative in the FPA are considered presuntive false positive reactors. False positive reaction in agglutination test are well documented [18] and mainly due to cross reaction with antibodies to other Gram negative bacteria like *Yersinia enterocolítica*, *Pasteurella multocida* and *Escherichia coli* [19,20]. In a study with serum samples from Argentina, Samartino et al. [21] showed that specificity of the agglutination tests with the use of β-mercaptoethanol was above 98% in cattle after nine month of vaccination with *B. abortus* S19. Then, as the β-ME test was used in the sequence of tests carried out in the present study, it seems likely that the false positive reactions were mainly due to cross reaction with other Gram negative bacteria.

FPA performed in microplates increases testing capacity, is an accurate diagnostic test that has many advantages compared with other methods used for serological diagnosis of bovine brucellosis. Moreover, our results show that it is also possible to use less antigen than that used in the scarce previous reports.

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