Прилози, Одд. биол. мед. науки, МАНУ, XXXI, 1, с. 133–143 (2010)
Contributions, Sec. Biol. Med. Sci., MASA, XXXI, 1, p. 133–143 (2010)
ISSN 0351–3254
УДК: 636.39.09:[616.98:579.841.93.083.3(82)

EVALUATION OF THE FLUORESCENCE POLARIZATION ASSAY (FPA) FOR DIAGNOSIS OF *BRUCELLA MELITENSIS* INFECTION OF GOATS IN ARGENTINA

Nicola AM., Elena S., Alonso B., Esteves Madero J.

Reference Laboratory OIE for Brucellosis
National Service of Animal Health (DILAB - SENASA)
Martínez, Buenos Aires, ARGENTINA

A b s t r a c t: *Aim:* Determine the optimal cut-off and the use of Fluorescence Polarization Assay (FPA) to improve the detection of brucellosis in individual goats in Argentina.

Methods: Sera from 96 goats from a flock with abortion due to *B. melitensis* biovar 1 were used to assess the efficacy of the FPA to detect brucellosis in goats. FPA results were compared with those of the Buffered Antigen Plate Agglutination test (BPAT) confirmed by Seroagglutination in tube (SAT), the competitive enzyme-linked immunosorbent assay (c-ELISA) and the indirect enzyme-linked immunosorbent assay (i-ELISA).

Sera from 554 goats free from brucellosis were tested with the BPAT, SAT, c-ELISA and i-ELISA to determine its Specificity. Vaccination had not been performed in the flocks evaluated.

Results: The most appropriate cut-off was selected for the FPA by using MedCalc software. It was fixed at 87 mP giving a sensitivity and specificity of 98.1% (CI 89.9–99.7) and 92.8% (CI 90.4–94.7). The relative sensitivity compared with i-ELISA and c-ELISA was 97% and 92.9% respectively. The relative specificity compared with i-ELISA and c-ELISA was 97.5% and 98% respectively. The kappa measures of agreement between tests was higher than 0.75

Conclusion: The high correlation between FPA results and other serological methods with sera goats is indicative of the excellent performance of FPA technique in diagnosis of caprine brucellosis and we endorse it as a recommended method.

Key words: *Brucella melitensis;* serological diagnosis; fluorescence polarization assay; Argentina.

Introduction

Brucellosis is still one of the most important and serious bacterial diseases in Argentina, and the causative bacteria (*Brucella abortus* in cattle, *Brucella suis* in swine and *Brucella melitensis* in sheep and goats) are transmitted to humans through contact with infected livestock or by consumption of contaminated dairy products that cause serious public health risks with severe economic consequences.

The most reliable and the only unequivocal method for diagnosing animal brucellosis is isolation of *Brucella* spp. Although the currently in use serological tests effectively detect brucellosis on a flock basis, they are insufficient to detect infection in an individual animal due to missing identification of some true infected animals as judged by *Brucella* isolation. This is a major problem in monitoring areas of low prevalence of brucellosis and where trade in goats may introduce *Brucella* in areas free from brucellosis.

While the efficacy of FPA to detect *Brucella abortus* in cattle has been extensively evaluated in recent years [1, 2], relative little information is available on the efficacy of this method in detecting *Brucella melitensis* in goats [3]. The objectives of the present study were to compare the FPA results with those of the BPAT (confirmed by SAT), i-ELISA, and the c-ELISA and to establish whether the use of the FPA is a meaningful addition to the diagnosis of caprine brucellosis.

Fluorescence polarization immunoassay (FPA) makes use of molecular rotational properties, considered a homogenous test due to measuring antibody binding to antigen directly, eliminating the need for separation procedures. The principle of the method relies on a fluorescent dye attached to a small antigen (or antibody fragment) that is excited by plane-polarized light at the appropriate wavelength. The rate of rotation of the antigen molecule is reduced when its molecular size is increased by its binding to antibody (or antigen). This change in rate can be measured as milipolarization (mP) units [4, 5].

The FPA shows great potential as a diagnostic test due to its ease of use and potentially wide application.

Material and methods

Positive serum samples

Ninety-six goats from a flock from which *B. melitensis* biovar 1 was isolated from aborted foetuses and milk samples were chosen for this study. The animals came from areas in northwest Argentina in which vaccination had not been performed. The sera samples from each individual was examined using BPAT (confirmed by SAT), c-ELISA, FPA and i-ELISA.

The sera positive for BPAT and SAT with a titre equal or higher than 1 : 50, were considered as a true positive for this study.

Negative Serum samples

Brucella-free goats. Five hundred fifty-four goats were from three flocks free of brucellosis in which vaccination had not been performed and with no clinical or epidemiological evidence of brucellosis and with negative results for BPAT and SAT were selected as true negative.

Bacteriological procedures. Abortion material and milk samples were processed according to standard procedures for the bacteriological diagnosis [6].

Serological Tests

The BPAT [7] confirmed by SAT [8], c-ELISA, i-ELISA and FPA are the conventionally used tests [9–11] for diagnosis of bovine brucellosis and they are applicable for diagnosis in goats. All tests (except SAT) were conducted according to the OIE (Manual for Diagnostic Tests and Vaccines for Terrestrial Animals [12], which gives details of all diagnostic methods. The SAT was performed as previously described [8]. Briefly, the SAT antigen is a 4.5% (wet weight/Volume) *Brucella* suspension in physiological saline plus 0.5% phenol (PS) with a pH ranging from 6.4 to 7.0 that is diluted 1 : 100 in PS before use. The test was done in tubes (13 by 100 mm) arranged in rows of four. Decreasing quantities of 80, 40, 20, and 10 ml of serum were placed into the tubes, and 2 ml of appropriately diluted antigen was added to obtain dilutions of 1 : 25, 1 : 50, 1 : 100, and 1 : 200. The tubes were shaken and placed in an incubator at 37°C for 48 h. The results were read by observing the tubes against a black background with a light source behind. A titre of up to 1 : 50 was considered positive. All of the antigens used were prepared in our laboratory from a concentrated cell suspension of smooth *Brucella abortus* 1119–3 by using antigens supplied by the National Veterinary Services Laboratories, U.S. Department of Agriculture, as a reference.

The FPA was performed as previously described [1, 12], the sera samples were diluted 1 : 25 for testing in a 10 mm–75 mm glass tube. Briefly 40 µl of serum were diluted into 1.0 ml of Tris buffer (0.01 M Tris (1.21 g), containing 0.15 M sodium chloride (8.5 g), 0.05% Igepal CA630 (Sigma) (500 µl) (formerly NP40), 10 mM EDTA (3.73 g) per litre of distilled water, pH 7.2). A baseline evaluation of the serum sample fluorescence polarization level was obtained using FP Sentry 1000 (Diachemix LLC, Milwaukee, Wisconsin, USA), and subsequently 10 µl of antigen (O-polysaccharide of *Brucella abortus* strain 1119–3 labelled with fluorescein isothiocyanate (FITC) was added to each tube and mixed well. After two minutes of incubation, the tubes were read again, and the results were expressed in milipolarization units (mP).

Data Analysis

Data was analysed using MedCalc software [13], and the cut-off that resulted in the maximum sum of the sensitivity and specificity (with 95% confidence intervals) values was calculated for the FPA.

The data were sorted into positive and negative populations based on the BPAT and SAT (titre equal or higher than 1 : 50) as a confirmatory test (in series).

The sensitivity and specificity of the FPA, relative to the SAT, c-ELISA and i-ELISA (plus 95% confidence limits), and the kappa measures of agreement between tests were calculated. The data were also plotted in a receiver-operating characteristics (ROC) curve to determine a suitable cut-off value.

Results

The serological results from infected flocks with each of the tests are summarized in Table 1. Four samples were negative to FPA but positive in the other tests. One serum was negative to c-ELISA and positive for the other tests. Two samples were negative to i-ELISA and positive for the others. One of these samples was from an animal with culture positive.

Table 1 – Табела 1

Results of four serological tests run on sera from Brucella melitensis infected flock
Резултати од четири серолошки тестови направени на серуми од инфицирани стада со B. melitensis

FPA (n = 96)		BPAT (n = 96)		SAT (n = 96)			i-ELISA (n = 75)		c-ELISA (n = 86)	
Neg	Pos	Neg	Pos	Neg	1 : 25	≥ 1 : 50	Neg	Pos	Neg	Pos
10	86	0	96	12	11	73	8	67	2	84

Cut-off values were predetermined for the brucellosis serological tests to be ≥ 1 : 50 for the SAT, 47 (% *P*) for the i-ELISA, 20 (% *I*) for the c-ELISA.

Analisys of data for FPA gave a mP threshold of 87 mP. (Fig. 1) resulting in the highest sensitivity and specificity combination values of 98.1% (CI 89.9– 99.7) and 92.8% (CI 90.4–94.7) respectively.

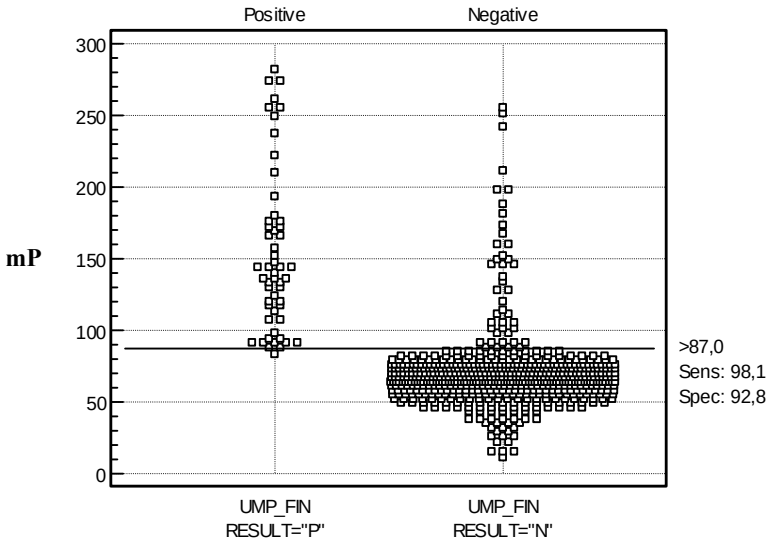

Figure 1 – Interactive dot diagram
Слика 1 – Интерактивен точкест дијаграм

Milipolarization unit distribution of 73 positive and 554 negative samples from *Brucella melitensis* infected and uninfected flocks, respectively; using a FPA 87 mP cut-off point.

The relative sensitivity compared with i-ELISA and c-ELISA was 97% and 92.9 respectively. The relative specificity compared with i-ELISA and c-ELISA was 97.5 and 98% respectively. The relative specificity to the BPAT was 98.9% (data not shown). The area under the ROC curve (Fig. 2) was 0.971 with a CI of 0.955 to 0.982.

Finally, kappa was 0.7842 between the BPA plus SAT and the FPA results; the kappa for the i-ELISA and FPA was 0.8744 and 0.8861 for the c-ELISA and FPA (Table 2).

Fourteen isolates of *Brucella melitensis* biovar 1 were obtained from infected flocks. All tests detected all culture-positive cases, except one sample by i-ELISA.

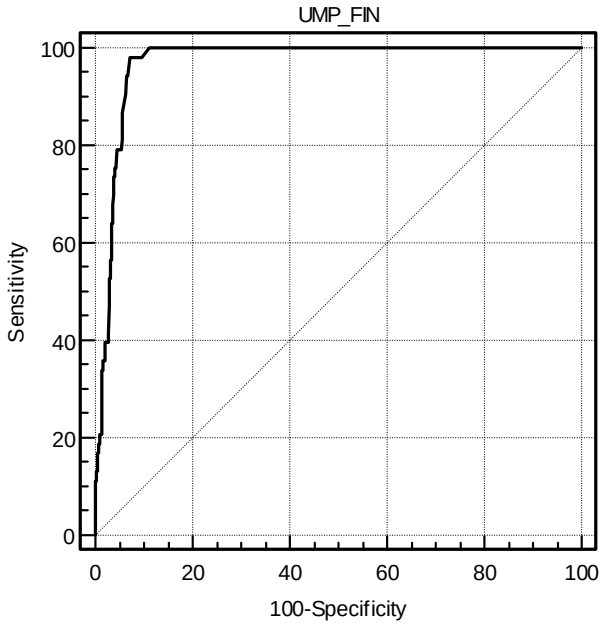

Figure 2 – Receiver operator characteristic (ROC) curve for the FPA
Слика 2 – Крива на Receiver operator characteristic (ROC) за FPA

Receiver operator characteristic (ROC) curve for the fluorescence-polarization assay for caprine brucellosis. The optimum cut-off value was calculated to be 87 mP. The area under the curve is 0.971 (1.00 would indicate a perfect test)

Table 2 – Табела 2

Test agreements for the fluorescence-polarization test (FPA), Seroagglutination in tube (SAT), c-ELISA and i-ELISA
Совпаѓање на тестовите fluorescence-polarization test (FPA), Seroagglutination in tube (SAT), c-ELISA и i-ELISA

Test	Kappa	95% CI
FPA agreement with BPA + SAT	0.7842	0.7115–0.8569
FPA agreement with i-ELISA	0.8744	0.8141–0.9347
FPA agreement with c-ELISA	0.8861	0.8329–0.9393

Discussion

The objectives of the present study were to compare the FPA results with those of the BPAT, SAT, i-ELISA and c-ELISA, and to establish whether the use of the FPA is a meaningful addition to the diagnosis of caprine brucellosis. Since diagnostic errors occur the relative sensitivity and specificity of a test are used to assess its efficacy to detect infection [2]. The only indisputable diagnostic test for brucellosis is isolation of the causative organism from fluids or tissues of suspected hosts. However, bacteriological isolation cannot always be relied on to prove the presence or absence of infection in individual animals and has some major drawbacks in that it is very time-consuming, expensive, operator-hazardous and not amenable to mass testing. Diagnosis is therefore made by serological testing [3, 5].

The diagnosis of brucellosis in Argentina is based mainly on the BPAT as a screening test and the SAT as a confirmatory test (in series). However, this combination of tests has a lower sensitivity than the CFT, which could result in infected animals being left in the herd. The complement-fixation test (CFT) has been established also as a confirmatory test in Argentina but this practice has a number of problems such as technical difficulties with the CFT procedure, long distances between the farms and the laboratories where the test is done, sera being hemolyzed and decomposed in some cases, and a number of anticomplementary sera. This makes the CFT in some areas an impractical solution for Argentinean conditions.

Among the serological tests, the agglutination tests were first used and in time they were improved. They are very sensitive but their specificity is relatively low [14]. In the classical serological tests, agglutination and complement fixation tests, the patterns are dependent on visual examination of antibody increments of 100% and therefore highly subjective [15].

The CFT is more accurate, but requires many steps and is a complicated procedure. Primary-binding assays, such as the indirect enzyme immunoassay (i-ELISA and c-ELISA), were developed to improve the sensitivity and specificity of serological testing.

The FPA is relatively inexpensive, requiring only a simple buffer, labelled antigen, a reusable glass tube and the equipment required to measure fluorescence polarization, which costs approximately the same as a photometer for enzyme immunoassay. Similar to other primary binding assays and unlike the conventional tests, data are obtained electronically, eliminating subjectivity and providing rapid analysis, a permanent record and easy data dispersal.

These findings, although preliminary, clearly endorse the use of the assay for the diagnosis of caprine brucellosis and indicate that the FPA cut-off value for goats in Argentina would be 87 mP, this value was similar (89 mP) to

that found by other studies in Mexico [16]. The sensitivity and specificity found for the FPA was higher than in previous studies for the diagnosis of brucellosis in goats [16, 17]. Even so, many aspects require additional research. False positive reactions for FPA may be attributed by the poor quality of some contaminated samples (if, however, false positive results often occur the test is not reliable). In addition, the performance of the test has been relied on using serum samples from unvaccinated goats. It is most likely, that in a vaccinated population, the increased sensitivity would have found positive reactors due to the vaccine. This drawback is in general applicable to i-ELISA and less to c-ELISA.

The agreement between FPA and the other tests was calculated by kappa index, the values obtained indicate a substantial agreement and almost perfect, following criteria based on the interpretation of Landis and Koch (1977), where (0.00–0.20) is slight, (0.21–0.40) is fair, (0.41–0.60) is moderate, (0.61–0.80) is substantial and (0.81–1.00) is almost perfect [18].

The FPA technique offers the advantage of simplicity of preparation and is less time-consuming. Implementation takes about 5 min to complete and has been demonstrated to be an accurate test for the detection of antibodies to *B. melitensis* in this study. Because of the ease of the procedure, it could be adapted for veterinary laboratories with facilities and equipment of low complexity. Regional laboratories could utilize the same technology, reducing errors of interpretation.

The FPA, based on the evaluation data presented, is recommended as a serological test for caprine brucellosis, capable of being a supplemental test in monitoring *B. melitensis* in Argentina. The application of the FPA is feasible due to its reproducibility and the ease of standardization on a large scale, adaptable to field use, relatively inexpensive and the objectivity of the assessment of positivity and the possibility of adjusting the cut-off point according to different epidemiological situations (improving sensitivity or specificity) are advantages not provided by the BPAT. Considering Argentinian field conditions the FPA for goat brucellosis would be an ideal confirmatory test to the official screening test such BPAT.

Acknowledgements

We would like to take this opportunity to thank Dr. Klaus Nielsen and the Canadian Food Inspection Agency for provision of reagents and equipment (FP Sentry 1000).

REFERENCES

1. Nielsen K., Lin M., Gall D., Jolley M. (2000): Fluorescence polarization immunoassay: detection of antibody to *Brucella abortus*. *Methods;* 22: 71–6.

2. Nielsen K., Smith P., Gall D., Perez B., Samartino L., Nicoletti P. *et al*. (2001): Validation of the fluorescence polarization assay for detection of milk antibody to *Brucella abortus*. *J Immunoassay Immunochem;* 22(3): 203–11.

3. Garin-Bastuji B., Blasco JM., Marin C., Albert D. (2006): The diagnosis of brucellosis in sheep and goats. *Small Rumin. Res.;* 62: 63–70.

4. Lucero Nidia E., Escobar Gabriela I., Ayala Sandra M., Silva Paulo P., Nielsen K. (2003): Fluorescence polarization assay for diagnosis of human brucellosis. *Journal of Medical Microbiology;* 52: 883–7.

5. Nielsen K., Gall D., Smith P., Vigliocco A., Perez B., Samartino L. *et al*. (1999): Validation of the fluorescence polarization assay as a serological test for the presumptive diagnosis of porcine brucellosis. *Vet. Micriobiol.;* 68: 245–53.

6. Alton GG., Jones LM., Angus RD., Verger JM. (1988): Serological methods In: *Techniques for the Brucellosis Laboratory*. Institut National de la Recherche Agronomique (INRA) Paris: 17–60.

7. Angus RD., Barton CE. (1984): The production and evaluation of a buffered plate antigen for use in a presumptive test for brucellosis. *Dev Biol Stand;* 56: 349–56.

8. Servicio Nacional de Sanidad y Calidad Agroalimentaria (SENASA). (1998): Manual de Diagnóstico serológico de Brucelosis Bovina: 17–23.

9. Gall D., Colling A., Marino O., Moreno E., Nielsen K., Perez B. *et al*. (1998): Enzyme immunoassays for serological diagnosis of bovine brucellosis: A trial in Latin America. *Clin. Diagn. Lab. Immunol.;* 5: 654–61.

10. McGiven JA., Tucker JD., Perrett LL., Stack JA., Brew SD., MacMillan AP. (2003): Validation of FPA and cELISA for the detection of antibodies to *Brucella abortus* in cattle sera and comparison to SAT, CFT, and iELISA. *J. Immunol. Methods*; 278; 171–8.

11. Stack JA., Perrett LL., Brew SD., MacMillan A P. (1999): Competitive ELISA for bovine brucellosis for testing poor quality samples. *Vet. Rec*.; 145:735–6.

12. World Organisation for Animal Health (OIE). (2009): Manual of Diagnostic Tests and Vaccine for Terrestrial Animals – Chapter 2.4.3 Bovine Brucellosis and Chapter 2.7.2 Caprine and ovine brucellosis (excluding Brucella ovis); OIE, Paris.

13. Schoonjans F., Zalata A., Depuydt CE., Comhaire FH. (1995): MedCalc: a new computer program for medical statistics. *Comput. Meth. Programs Biomed.;* 48(3): 257–62.

14. Dajer A., Luna-Martínez E., Zapata D., Villegas S., Gutierrez E., Peña G. *et al.* (1999): Evaluation of a fluorescence-polarization assay for the diagnosis of bovine brucellosis in México. *Preventive Veterinary Medicine;* 40: 67–73.

15. Nielsen K., Smith P., Yu WL., Elmgren C., Nicoletti P., Perez B. *et al*. (2007): Second generation competitive enzyme immunoassay for detection of bovine antibody to *Brucella abortus*. *Vet Microbiol;* 124: 173–7.

16. Ramirez-Pfeiffer C., Nielsen K., Marin-Ricalde F., Rodríguez-Padilla C., Gomez- Flores R. (2006): Comparison of fluorescence polarization assay with card and complement fixation tests for the diagnosis of goat brucellosis in a high-prevalence area. *Vet Immunol Immunopathol;* 110(1–2): 121–7.

17. Nielsen K., Gall D., Smith P., Balsevicius S., Garrido F., Ferrer MD. *et al.* (2004): Comparison of serological tests for the detection of ovine and caprine antibody to *Brucella melitensis*. *Rev Sci Tech;* 23(3): 979–87.

18. Landis JR., Koch GG. (1977): The measurement of observer agreement for categorical data. *Biometrics;* 33: 159–74.

Резиме

ЕВАЛУАЦИЈА НА ФЛУОРЕСЦЕНТНАТА ПОЛАРИЗАЦИЈА (FPA) ЗА ДИЈАГНОЗА НА ИНФЕКЦИЈА СО *BRUCELLA MELITENSIS* КАЈ КОЗИТЕ ВО АРГЕНТИНА

Никола АМ., Елена С., Алонсо Б., Естевес Мадеро Ј.

OIE референтна лабораторија за бруцелоза
Национален сервис за анимално здравство (DILAB - SENASA)
Мартинез, Буенос Аирес, АРГЕНТИНА

Цел: Да се одреди оптималниот момент во употребата на флуоресцентна поларизација (ФПА) за подобрување на откривањето на бруцелозата кај индивидуалните кози во Аргентина.

Методи: Серуми од 96 кози од стадо со абортуси причинети од *B. Melitensis* соj 1 беа употребени за да се одреди ефикасноста на FPA за откривање на бруцелоза кај козите. Резултатите од FPA беа споредени со резултатите добиени од тестот на аглутинација на плочка со пуфериран антиген (Buffered Antigen Plate Agglutination test – BPAT), потврдено со сероаглутинација во епрувета (SAT), компетитивен ензимски имун тест (competitive enzyme-linked immunosorbent assay – c-ELISA) и индиректен ензимски имун тест (indirect enzyme-linked immunosorbent assay – i-ELISA).

Серуми од 554 кози кои не беа заразени со бруцелоза, се тестираа со BPAT, SAT, c-ELISA и i-ELISA за да се одреди нивната специфичност. Испитуваното стадо не беше вакцинирано.

Резултати: Најадекватниот момент за употреба на FPA беше одреден со употреба на MedCalc софтверот. Тој беше одреден на 87 mP со сензитивност и специфичност од 98,1% (CI 89,9–99,7) и 92,8% (CI 90,4–94,7). Релативната сензитивност споредена со i-ELISA и c-ELISA беше 97% и 92.9% соодветно. Релатив-

ната специфичност споредна со i-ELISA и c-ELISA беше 97,5% и 98% соодветно. *Kappa* мерките за потврда меѓу тестовите беше повисока од 0,75.

Заклучок: Високата корелација меѓу резултатите од FPA и останатите серолошки методи со серуми од кози е индикативен за одлични перформанси на FPA техниката во дијагноза на бруцелозата кај козите и ја прифаќаме како препорачан метод.

Клучни зборови: *Brucella melitensis,* серолошка дијагноза, флуоресцентна поларизација, Аргентина.

Corresponding Author:

Nicola Ana María
Reference Laboratory OIE for Brucellosis
National Service of Animal Health
(DILAB – SENASA) Av. Fleming 1653
CP 1640, Martínez, Buenos Aires,
ARGENTINA
Tel/Fax: +54 11 48361992

E-mail: anicola@senasa.gov.ar