Journal of Medical Microbiology (2003), **52**, 883–887 DOI 10.1099/jmm.0.05217-0

Fluorescence polarization assay for diagnosis of human brucellosis

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Fluorescence polarization immunoassay (FPA) uses molecular rotational properties to measure antibody binding to antigen directly. The potential use of this method was assessed in comparison to a competitive enzyme immunoassay (CELISA) and conventional serological tests for the diagnosis of brucellosis on a total of 587 human sera. Based on 340 sera from asymptomatic blood donors with no evidence of brucellosis, the specificity of the FPA was 97·9 % using a cut-off value of 72 mP. Sera from *Brucella*-infected patients (11 *Brucella melitensis*, 32 *Brucella abortus*, 32 *Brucella suis* and one *Brucella* sp.) yielded a sensitivity estimate of 96·1 %. In tests on 84 sera from suspected brucellosis patients, the FPA detected 80 cases. Of 87 sera from patients with probable infection, 15 were detected by both CELISA and FPA, three by CELISA only and four by FPA only. The discrepancies in both groups involved sera with low, declining titres. The FPA uses a sample of 40 µl serum, takes about 5 min to complete and has been demonstrated to be accurate for the detection of antibodies to *B. abortus*, *B. melitensis* and *B. suis* and for identifying patients suffering relapses. Because of the ease of the procedure, it could be readily adopted for use in clinical laboratories and blood banks.

Received 10 February 2003
Accepted 7 July 2003

INTRODUCTION

Human brucellosis is diagnosed on the basis of clinical findings and laboratory studies that include bacteriological and serological tests. The conventional serological tests are insufficiently sensitive and/or specific to be used individually and, as a result, a panel of tests is frequently used, often leading to difficulty in interpreting the results. To overcome some of these problems, newer serological tests based on primary binding assays have been developed, mainly to improve test sensitivity and specificity. A competitive enzyme immunoassay (CELISA) for the detection of serum antibody to *Brucella* has been shown to be a suitable test for human brucellosis (Lucero *et al.*, 1999). The CELISA uses a mAb specific for a common and repeating epitope on the polysaccharide portion of the smooth lipopolysaccharide molecule of *Brucella* (S-LPS) to compete with antibody in the sample. This results in an assay with higher specificity than other assays because it frequently eliminates cross-reactions with other antigens.

Abbreviations: CELISA, competitive ELISA; FPA, fluorescence polarization assay.

Fluorescence polarization immunoassay (FPA) makes use of molecular rotational properties, 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. The method has been applied to the detection of bovine antibody to *Brucella abortus*, resulting in a sensitive and specific test (Nielsen *et al.*, 2000). The assay, which can be completed in a few minutes, requires a one-step serum dilution, assessment of background fluorescence, addition of labelled antigen and, finally, measurement of antigen–antibody interaction. In this study, we compare results obtained with the FPA with the CELISA and conventional tests for the diagnosis of human brucellosis.

METHODS

Serum samples. Serum samples were divided into the following groups: 340 sera from blood donors (18–62 years old) with no clinical or

epidemiological evidence of brucellosis and negative plate agglutination test (PAT), Rose Bengal test (RB), buffered antigen plate agglutination test (BPA), standard tube agglutination test (TAT), cold complement fixation test (CF) and CELISA; 76 sera from patients from whom *Brucella* sp. was isolated; 84 sera from suspected brucellosis patients with clinical symptoms compatible with brucellosis and positive PAT, RB, BPA, TAT, CF and CELISA at any titre and 87 sera from patients probably infected (slaughterhouse workers and blood donors) but free of clinical symptoms compatible with brucellosis yet positive for one or more of the serological tests at low titres.

Serological tests. PAT, RB, BPA, TAT and CF were performed as described previously (Centro Panamericano de Zoonosis, 1981; Angus & Barton, 1984; Alton *et al.*, 1988; Lucero & Bolpe, 1998). The antigens used in the above tests were prepared at the Administración Nacional de Laboratorios e Institutos de Salud (ANLIS) 'Dr C. G. Malbrán', using antigens from the National Veterinary Services Laboratories, US Department of Agriculture, for reference purposes.

CELISA involved adsorption of *Brucella* S-LPS antigen to polystyrene plates and was performed as described previously (Nielsen *et al.*, 1995, 1996; Lucero *et al.*, 1999). Briefly, plates with antigen added were incubated overnight at 4 °C followed by the addition of mouse mAb specific for a common epitope of the S-LPS molecule and diluted test sera. After the incubation period, diluted goat anti-mouse IgG antibody conjugated with horseradish peroxidase was added, followed by the addition of substrate and chromogen.

If the test was positive, the serum competed with the mAb for the epitope sites and inhibited binding of the mAb to the S-LPS antigen and subsequent colour development. Results for control and test sera were expressed as percentage inhibition (PI) of mAb activity against the antigen. For interpretation, sera with PI values of 28 % or more were considered positive. The S-LPS (*B. abortus* S 1119-3), mAb (M84) and control sera (strongly positive and weakly positive bovine anti-*Brucella* serum and negative bovine serum) were standardized and supplied by the Brucellosis Center of Expertise and OIE Reference Laboratory, Animal Diseases Research Institute (ADRI), Canada. In addition to bovine serum controls, positive and negative reference human sera were included in each CELISA plate. The conjugate, pre-adsorbed with bovine, equine and human serum protein, was from Jackson Laboratories.

FPA. FITC was conjugated to O-polysaccharide (OPS) extracted from S-LPS of *B. abortus* S 1119-3 and standardized at ADRI (Nielsen *et al.*, 2000). For testing, control and test sera were diluted 1 : 50 in 2 ml 0·01 M sodium phosphate, pH 7·4, containing 0·15 M NaCl, 0·1 % sodium azide and 0·05 % lithium dodecylsulfate (PBSAL), in a 12 × 75 ml glass tube. A serum blank measurement was obtained with a Fluorescence Polarization Analyser (FPM-1; Jolley Consulting and Research), with the lamp feedback off and the heater set at 37 °C. Twenty microlitres FITC-conjugated OPS was added and, after gentle mixing and incubation at room temperature for at least 2 min, a second measurement was made with the FPM-1, which automatically subtracted the blank reading. Positive and negative reference human sera were included with each lot of 30 samples tested. Data, expressed as millipolarization units (mP), were indicative of the amount of antibody present in the serum sample.

Bacteriological examination. *Brucella* organisms were isolated from three blood cultures incubated in 10 % CO_2 using a liquid medium (1·5 g NZ amine, 0·5 g Primatone, 0·2 g yeast extract, 0·1 g glucose, 0·5 g NaCl, 0·01 g sodium bisulphite, 2·5 g sodium citrate, 100 ml distilled water). A bottle holding 25 ml liquid medium was inoculated with 5 ml blood (Garcia-Carrillo & Lucero, 1993). Cultures were kept for 45 days before being considered negative. A suspected *Brucella* culture was subcultured on solid medium for identification. The strains were typed as recommended by the former ICSB Subcommittee on the Taxonomy of the Genus *Brucella* (Corbel & Brinley-Morgan, 1984) at ANLIS 'Dr C. G. Malbrán'.

Data analysis. An initial cut-off value was calculated from the FPA data obtained from the negative sera. Diagnostic sensitivity and specificity were calculated by plotting the data for negative and positive samples using a frequency histogram. In addition, the data were studied using receiver operator characteristic (ROC) analysis, which calculates the most suitable cut-off value, estimated by comparing the range of sensitivity and specificity values (Metz, 1978; Schoonjans *et al.*, 1995).

RESULTS AND DISCUSSION

The 340 negative sera yielded a mean FPA value of 60·95 mP with a standard deviation of 5·28 mP. A frequency distribution showed a characteristic skewing of the results for the serum samples (data not shown). The cut-off values were confirmed by ROC analysis (Fig. 1) using 76 sera of *Brucella*-infected patients as positive sera (defined on the basis of positive clinical history, cultural results and reactions with TAT, CF and CELISA). Thus, if a cut-off value of 72 mP was selected (mean + 2SD), the sensitivity and specificity values were respectively determined to be 96·1 and 97·9 % (seven false-positives and three false-negative results were evident; Table 1).

Seventy-six isolates of *Brucella* were obtained from patients. Of these, typing results yielded 11 isolates of *Brucella melitensis* [biovar 1 or 1a (atypical strains; data not shown)], 32 *B. abortus* (biovar 1, except for three isolates of biovar 2 and one of S19), 32 *Brucella suis* (biovar 1 or 1a; Corbel *et al.*, 1984) and one isolate that was not typed. In general, the results of serological tests used were consistent, but some discrepancies were found (Table 2). The FPA detected 73 culture-positive cases (ranging from 73 to 255 mP), CELISA 74, CF 73 and TAT 73 (but three sera had titres < 1 : 100).

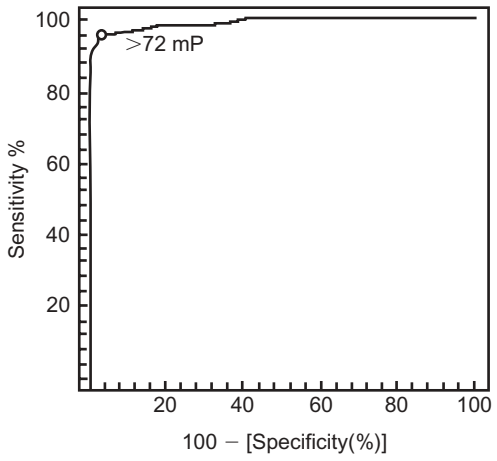

Fig. 1. ROC analysis of sensitivity (%) plotted against 100−specificity (%) for various cut-off values of the FPA for detection of antibody to *Brucella* sp. A value of 72 mP gave the maximum sum of sensitivity and specificity and was considered to be the optimum cut-off value.

Table 1. Sensitivity and specificity determinations for FPA data

FPA results are compared with culture results for the positive population and absence of clinical/epidemiological/serological evidence of brucellosis for the negative population. A cut-off value of 72 mP was used for the FPA. Sensitivity = (73/76) × 100 = 96·1 %. Specificity = (333/340) × 100 = 97·9 %.

FPA	Culture	
	Positive	**Negative**
Positive	73	7
Negative	3	333
Total	76	340

The serological results of three cases not detected by FPA at admission but detected during post-therapy monitoring are shown in Table 3. Relapse was observed in five cases with positive results of blood culture following therapy.

There is a linear relationship between antibody levels and mP output of the FPA to a plateau indicating maximum reactivity, somewhere around 250 mP, that can be shown by using dilutions of positive sera.

Using a cut-off value of 72 mP, FPA detected 80 of 84 sera from suspected brucellosis patients.

Among the 87 sera from probably infected patients without clinical symptoms, the screening test results were PAT (66, 1 : 25; 20, 1 : 50; 1, 1 : 100), RB (12 positive), BPA (76 positive) and the confirmative tests TAT (68, 1 : 25; 17, 1 : 50; 1, 1 : 100), CF (19 positive; 1 anticomplementary), CELISA (18 positive) and FPA (19 positive). PAT detected 87 cases and TAT 86; the discrepancy could be due to the occurrence of a non-specific reaction, cross-reacting antigens or false-positive results.

Cross-reactions with antibodies against other micro-organisms do occur in CELISA and FPA, but at a much reduced rate compared with conventional serological tests. No evidence of cross-reactions was found for sera from cattle exposed to *Yersinia enterocolitica* O9 and *Escherichia coli* O : 157 tested with CELISA and FPA (K. Nielsen, unpublished data).

A large number of serological tests has been used for the diagnosis of human brucellosis, demonstrating the lack of an ideal diagnostic technique and exhibiting limitations with patients in the early phases of the disease, persons exposed

Table 3. Serological data from three cases of brucellosis receiving treatment, not detected by FPA at admission

Time refers to months after initial isolation of *Brucella* sp. (species/biovar in parentheses). N, Negative; +/−, weakly positive; NS, serum not available. See Table 2 for explanation of values shown.

Time (months)	TAT	CF	CELISA	FPA
Case 10 (*B. melitensis* 1a)				
0	400	5	44	63
3	25	N	19	70
4	25+/−	N	14	70
6	N	N	11	68
12	N	N	10	62
Case 40 (*B. abortus* 1)				
0	800	160	70	66
3	800	320	51	98
5	400	160	50	90
7	200	80	47	97
12	100	40	36	65
Case 42 (*B. abortus* 1)				
0	800	80	73	NS
2	400	80	67	68
6	200	40	56	124
9	100	10	57	80
10	50	5	42	86
12	100	10	44	78
13	50	5	43	76

Table 2. Serological results for sera from *Brucella* sp. culture-positive patients where disagreement between tests was observed

Results considered negative are in bold. Data shown are: TAT, reciprocal of the highest dilution to cause agglutination; CF, reciprocal dilution causing inhibition of haemolysis (N, negative); CELISA, PI (cut-off is 28 PI); FPA, mP (cut-off is 72 mP). Case 32 was a relapse.

Case	TAT	CF	CELISA	FPA	Species/biovar
4	200	320	**10**	78	*B. melitensis* 1
10	400	5	44	**63**	*B. melitensis* 1a
28	**50**	10	57	83	*B. abortus* 2
32	**50**	40	90	219	*B. abortus* 1
40	800	160	70	**66**	*B. abortus* 1
52	200	**N**	90	218	*B. suis* 1a
59	**50**	**N**	43	78	*B. suis* 1

professionally and patients who relapse (Ariza *et al.*, 1995; Araj, 1999; Osoba *et al.*, 2001). A marked characteristic of brucellosis is its tendency to produce relapses once treatment is concluded (Solera *et al.*, 1998). The clinical symptoms are often misleading and the disease may present hepatosplenic, osteoarticular, gastrointestinal, neurological and/or cardiovascular complications.

The predominant isotype associated with brucellosis in man appears to be IgG, and is detectable very early in the immune response. Screening tests using acidified antigen preparations, such as BPA and RB, and confirmatory tests such as CF and CELISA have demonstrated an analytical bias in favour of the detection of this isotype. The CELISA has shown 99·7 % specificity and 98·3 % sensitivity and is relatively easy to perform (Lucero *et al.*, 1999).

Using a total of 587 sera in the present study, we investigated a single-step FPA assay as a rapid test for the diagnosis of human brucellosis and as a method to monitor the course of treatment. Tests of 340 sera from healthy people yielded an FPA specificity of 97·9 % when a cut-off value of 72 mP was selected. Seven sera gave false-positive reactions, ranging from 74 to 78 mP. Adjusting the cut-off value upwards, specificity may be increased but some sensitivity is lost.

With 76 sera from *Brucella*-infected patients, the FPA gave a positive result for 73 sera with a sensitivity value of 96·1 % using the 72 mP cut-off value. The three false-negative sera gave FPA results ranging from 63 to 68 mP. Case 10 was followed up over a 12-month period; however, the FPA titre did not become positive at any time and, after 3 months, all tests were negative. Cases 40 and 42 were FPA-positive 3 months after admission and titres declined slowly over a 12-month period (Table 3). Forty-four patients from this group were monitored for 2–34 months after initial diagnosis by clinical and laboratory studies including serological tests. In nearly all cases, agreement between CELISA and FPA was good (data not shown). In the same group of patients, FPA and CELISA detected the five cases of relapse (one *B. melitensis*, one *B. abortus*, three *B. suis*).

Since a negative blood culture does not exclude the presence of the infection (Yagupsky, 1999) and, at present, there are no definitive criteria to establish that brucellosis has been cured, this study included patients with negative blood culture but clinical symptoms, positive conventional serological tests and CELISA, those most likely infected or who had been exposed to the organism. Of 84 sera monitored from these suspected brucellosis patients, 80 were detected by FPA; the remaining four were patients with declining serological titres.

Of the 87 sera from patients probably infected with *Brucella* sp., 15 were detected by both FPA and CELISA while three were positive only by CELISA and four only by FPA. Among the latter four patients, one was a meat-packing-plant worker, one a rural worker, one had previous brucellosis history and one was a blood donor.

The discrepancies involve low-titre sera, particularly where titres were declining. In some areas where brucellosis is endemic, there is a high prevalence of antibodies in the healthy population, making individual clinical histories important for correct interpretation of serological test results. By adjusting the cut-off value, CELISA and FPA allow decisions to be made regarding the particular situation depending on requirements of specificity and sensitivity.

An interesting question is whether only immunoglobulins and not other serum components bind to the labelled substrate, but CELISA and FPA are based on the same antigen (OPS). We have evidence that the reaction in CELISA is based on antibody activity and not on other serum proteins, and the high correlation between positive FPA values and cultural evidence of infection as well as other serological evidence indicates a specific antibody response.

The FPA method can be run with a serum sample of 40 µl, takes about 5 min to complete and has been demonstrated to be an accurate test for the detection of antibodies to *B. abortus*, *B. melitensis* and *B. suis*. Because of the ease of the procedure, it could be adapted for clinical laboratories and blood banks. The test 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.

ACKNOWLEDGEMENTS

We are grateful to Dra Ana M. Vigliocco and Romina Ramondino at the Comisión Nacional de Energía Atómica, Buenos Aires, Argentina, for their help.

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