ELSEVIER

Journal of Immunological Methods 278 (2003) 171–178

JIM
Journal of
Immunological Methods

www.elsevier.com/locate/jim

Validation of FPA and cELISA for the detection of antibodies to *Brucella abortus* in cattle sera and comparison to SAT, CFT, and iELISA

J.A. McGiven*, J.D. Tucker, L.L. Perrett, J.A. Stack, S.D. Brew, A.P. MacMillan

Department of Bacterial Diseases, FAO/WHO Collaborating Centre for Reference and Research on Brucellosis, Veterinary Laboratories Agency, Woodham Lane, New Haw, Weybridge, Surrey, KT15 3NB, UK

Received 21 August 2002; received in revised form 27 January 2003; accepted 8 April 2003

Abstract

The fluorescence polarisation assay (FPA) is a recently described test for the serological diagnosis of *Brucella* infection. It has many methodological advantages over older, more established tests and can be performed in a fraction of the time. To validate the FPA, serum samples from 146 confirmed (by culture) *Brucella*-infected cattle were tested in conjunction with serum samples from 1947 noninfected cattle. The competitive ELISA (cELISA) was validated using these positive reference samples and 1440 negative samples, while data for the indirect ELISA (iELISA) was generated from 6957 negative samples plus the positive sera. Published diagnostic specificity (DSp) data for the complement fixation test (CFT) and serum agglutination test (SAT) was used in conjunction with the test results on the positive sera to obtain diagnostic specificity plus diagnostic sensitivity (DSn). After selection of a cutoff for the FPA and cELISA, the diagnostic specificity and sensitivity total for each test were compared. The results, with 95% confidence intervals, were: FPA (195.7 ± 2.79), iELISA (195.0 ± 2.70), cELISA (194.9 ± 3.48), CFT (191.7 ± 4.45), and SAT (180.4 ± 6.33). The data presented supports the use of the FPA in diagnosis of brucellosis and questions the use of the SAT and CFT for either screening or confirmatory testing.

Keywords: *Brucella*; FPA; ELISA; CFT; SAT; Validation

1. Introduction

The genus *Brucella* includes six species, *Brucella abortus*, *B. melitensis*, *B. suis* (in which smooth strains are virulent), *B. canis*, *B. ovis*, (naturally occurring virulent rough forms), and *B. neotomae*. *Brucella* is the causative agent of brucellosis which in man causes undulant fever (Dalrymple-Champneys, 1960). *Brucella* infection in cattle is characterised by an inflammatory response affecting cells of the reticuloendothelial system and of the placenta during pregnancy. This

Abbreviations: FPA, fluorescence polarisation assay; DSp, diagnostic specificity; DSn, diagnostic sensitivity; CFT, complement fixation test; SAT, serum agglutination test; RBPAT, Rose Bengal plate agglutination test; PCR, polymerase chain reaction; OIE, Office International Des Epizooties; mP, millipolarisation units; RFU, relative fluorescent unit; iELISA, indirect ELISA; cELISA, competitive ELISA; TW-ROC, two-way receiver operating characteristic; EU, European Union.

* Corresponding author. Tel.: +44-1932-357227.
E-mail address: j.mcgiven@vla.defra.gsi.gov.uk (J.A. McGiven).

0022-1759/03/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S0022-1759(03)00201-1

often results in the death and expulsion of the foetus between the fifth and eighth month of gestation. During abortion, large numbers of brucellae are released which may, in turn, cause the infection of other animals in the herd (Manthei and Carter, 1950).

Diagnosis of brucellosis in cattle is complicated by variable incubation time and the absence of clinical signs other than abortion. Microbiological isolation of the causative organism confirms the diagnosis (Morgan, 1977), and this result is taken as the 'Gold Standard' against which other tests are compared. However, it is time consuming and requires expensive laboratory facilities due to the contagious and zoonotic nature of the organism. Polymerase chain reaction (PCR), based on *Brucella*-specific primers, on blood samples is a potentially powerful tool (Fekete et al., 1990), but there may be problems of sensitivity as bacteraemia may not be constant.

Serological techniques are the mainstay of diagnosis and mass testing programmes. The most successful of the serological diagnostic tests for *B. abortus*, *B. melitensis*, and *B. suis* are based on the detection of antibodies to the LPS antigen of smooth *Brucella* strains. The immunodominant epitope of the LPS is the O-chain which is a homopolymer of 1,2-linked *N*-acylated 4-amino-4, 6-dideoxy-α-D-mannopyranosyl residues (Caroff et al., 1984). Traditional and well-documented techniques for serological diagnosis include the Rose Bengal plate agglutination test (RBPAT), serum agglutination test (SAT), complement fixation test (CFT), and, more recently, the indirect ELISA (iELISA) and competitive ELISA (cELISA) being put into more regular use. The fluorescence polarisation assay (FPA) (Nielsen et al., 1996) has a shorter history of use and has yet to become established within the routine testing procedures of most National Brucellosis Reference Laboratories.

Fluorescence polarisation measures the excitation by plane polarised light of a fluorescent molecule (Perrin, 1926). Measurement of returned photons in the planes parallel and perpendicular to the excitation plane allows for the assessment of the rotation of the fluorophore. Other factors being constant, then the rate of rotation of this molecule is inversely proportional to its size (Nasir and Jolley, 1999). Thus, the rotation of a fluorescent molecule (fluorophore) conjugated to, in this case, *Brucella* O-chain, will slow if bound by anti-*Brucella* LPS antibodies. The FPA is rapid and requires no solid phase bound reagent or removal of excess reagents. It is host species-independent and can also be conducted on whole blood (Nielsen et al., 2001a) and milk (Nielsen et al., 2001b). High throughput serological testing for brucellosis by FPA has been enabled by the development of 96-well plate readers. The use of this equipment for FPA has not previously been validated.

The FPA shows great potential as a diagnostic test due to its ease of use and potentially wide application. However, its performance must withstand close scrutiny against the more conventional tests, and the results of such examinations are described below. The bovine populations tested in these validation studies were derived from Northern Europe, including Germany, The Netherlands, Eire, and Britain. The diagnostic sensitivity (DSn) and diagnostic specificity (DSp) data can be extrapolated to a target population of such animals.

2. Materials and methods

2.1. FPA method

The FPA was conducted using a 96-well microtitre plate format. Test buffer was prepared by the addition of 0.836 g of sodium monophosphate monohydrate ($NaH_2PO_4 \cdot H_2O$), 1.49 g of sodium triphosphate dodecahydrate ($Na_3PO_4 \cdot 12H_2O$), 9 g of sodium chloride (NaCl), and 0.5 g of lauryl sulphate ($C_{12}H_{25}O_4Sli$), per litre of distilled water, with a final pH of 7.5. The first step of the test required the addition of 180 μl of buffer to 20 μl of test serum within each test well. Control samples were added to each plate, a minimum of 4 wells for the negative control [equilibrated to the Office International Des Epizooties (OIE) ELISA International Standard anti-*Brucella* Serum: negative] and also 4 high positive control wells. Buffer and test sample were mixed during addition by repeated pipette action, and the test plates were incubated for 2 min at room temperature (18–24 °C) on a rotary shaker (125 rpm).

After initial incubation, the test plate was read on a Tecan Polarion Fluorescence Polarisation microplate reader (reader set to gain of 115, number of flashes per well at 20, and filters of 485 nm for excitation and 535 nm for emission) to obtain a background reading, in

relative fluorescent units (RFUs), for each sample. Subsequently, 10 μl of antigen (Brucella O-polysaccharide conjugated isothiocyanate fluorophore: supplied by Diachemix, Whitefish Bay, WI, USA) was added to each well and mixed by pipette action followed by a further 2-min incubation as above. The plate was read again as before to obtain the raw parallel and perpendicular data for each sample. This data was converted to millipolarisation units (mP) by the formula: $mP=((Iv-Ih)/(Iv+Ih))\times 1000$, where Iv = the intensity of parallel light and Ih = intensity of perpendicular light (Perrin, 1926).

2.2. SAT, CFT, and ELISA methods

SAT, CFT, and iELISA were standardised and conducted according to the OIE Manual of Standards for Diagnostic Tests and Vaccines (Corbel and MacMillan, 1996). Additionally, the iELISA conforms to the standards set out in the annex of the current European Directive 64/432 in that it detects a 1/16 dilution (further diluted by 1/200 as per test protocol) of the OIE ELISA International Standard anti-*Brucella* serum: strong positive, while finding the negative serum negative. All iELISA reagents are produced at the VLA, Weybridge, UK. The cELISA is conducted using a monoclonal antibody specific to the O-chain polysaccharide portion of *Brucella* LPS (Stack et al., 1999) as recommended by the OIE Manual.

2.3. Serum samples

For the validation of the FPA, 1947 blood samples from *Brucella* negative animals (by virtue of their presence in Britain: officially brucellosis-free since 1985) collected from August to October 2001 were tested. Due to the difficulty of acquisition, only 146 serum samples from *Brucella* culture positive (*B. abortus*) animals were tested. These samples came from Germany, France, Eire, and from Britain pre-1985. All serum samples from animals confirmed positive by culture (herein described as 'positive reference serum') were tested by CFT, SAT, iELISA, cELISA, and FPA. A further 1440 negative samples (from Britain in 2002) were tested by cELISA for purposes of validation. A total of 6957 negative sera from Britain, collected during August to October 2001, was used to assess the performance of the iELISA.

2.4. Test validation

The most appropriate cutoff was selected for the FPA and cELISA by using a two-way receiver operating characteristic (TW-ROC) analysis (Greiner et al., 1995) that plots the DSn (true positives/true positives + false negatives) and DSp (true negatives/true negatives + false positives) as a function of cutoff. This is often the test value which produces the highest combined DSn and DSp, as has been chosen here.

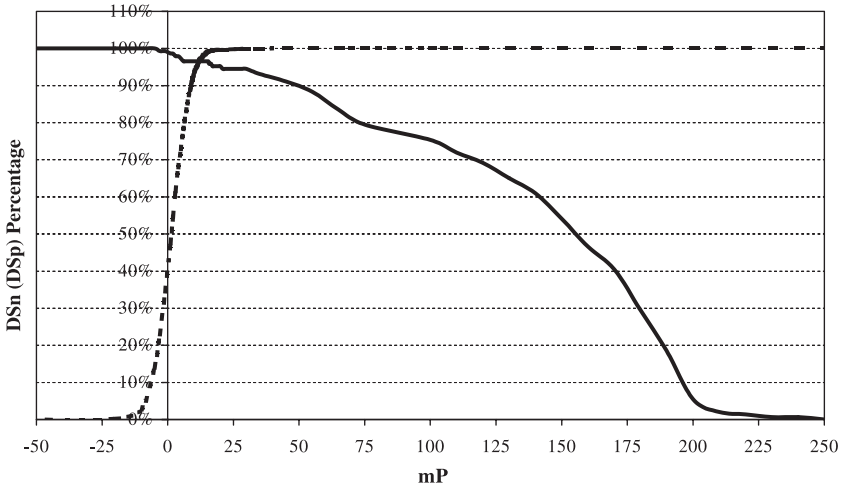

Fig. 1. TG-ROC analysis of FPA results from 1947 *Brucella* uninfected cattle serum samples and 146 serum samples from *Brucella* culture positive animals. Solid line shows DSn; broken line shows DSp.

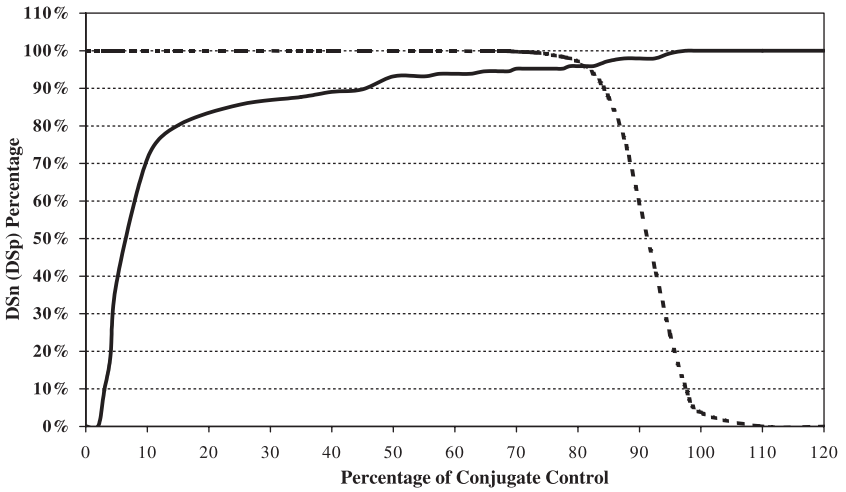

Fig. 2. TG-ROC analysis of cELISA results from 1440 *Brucella* uninfected cattle serum samples and 146 serum samples from *Brucella* culture positive animals. Solid line shows DSn; broken line shows DSp.

The intersection of the DSn and DSp line is the test value where the two parameters are equal; however, this is rarely the same value as the point where the combined values are highest. Confidence intervals for DSn and DSp can be determined (Jacobson, 1998) to show the range within which the parameters are likely to be when the test is used in the target population. Further statistical analysis was conducted on the results for the independent samples (negative reference sera) using two sample *t*-tests, and on the paired samples (positive reference sera) using a two-way binomial test, to look for differences in DSp and DSn, respectively.

As a field trial, 259 samples from Irish herds infected with *B. abortus* were tested by FPA, iELISA, and cELISA to compare the relative performance of each of the tests. Analysis of theses samples was performed by determining the percentage of results that were in agreement between tests and testing those in disagreement by McNemars test for paired data (with Yates' correction) to investigate significant differences.

3. Results

To assess the data from the FPA method, it was necessary to obtain results that were as precise as possible. To ensure high-quality results, test plates were accepted and rejected on the basis of the absolute values of the control serum used and the degree of variation between multiple wells of the same control serum.

A cutoff for the FPA was evaluated by subtracting the average plate negative control result from each test sample result and by analysing this data from known noninfected cattle sera and positive reference sera using TW-ROC analysis (Fig. 1). The cutoff which provided the highest combined DSn and DSp values was chosen. This was a value of 15.5 mP above the negative control.

The DSp data for the cELISA was also evaluated by TG-ROC analysis (Fig. 2). The cutoff that maximised

Table 1
Diagnostic specificity and sensitivity values for each method

Parameter	CFT	SAT	iELISA	cELISA	FPA
Test cutoff	20 IUs	30 IUs	10%	70%	15.5mP
DSp (%)	99.9 (± 0.20)	98.9 (± 0.65)	97.8 (± 0.34)	99.7 (± 0.28)	99.1 (± 0.44)
Total no. of samples	995[a]	995[a]	6957	1440	1947
DSn (%)	91.8 (± 4.46)	81.5 (± 6.30)	97.2 (± 2.65)	95.2 (± 3.47)	96.6 (± 2.95)
Total no. of samples	146	146	146	146	146
DSp + DSn	191.7 (± 4.45)	180.4 (± 6.33)	195.0 (± 2.70)	194.9 (± 3.48)	195.7 (± 2.79)

Values in parentheses indicate 95% confidence interval.
[a] Data from Emmerzaal et al. (2002).

Table 2
Comparison of differences between test diagnostic sensitivities listing *P* values

Test	SAT	CFT	cELISA	FPA	iELISA
SAT		0.0007*	0.0000*	0.0000*	0.0000*
CFT			0.0625	0.0156*	0.0078*
cELISA				0.5000	0.2500
FPA					1.0000
iELISA					

Results from each test for 146 positive reference sera were compared. The distribution of the samples differentially diagnosed when two tests were compared was analysed using a binomial distribution. The null hypothesis states that a sample differentially diagnosed by two tests is equally likely to be either positive for test A and negative for test B, or negative for test A and positive for test B ($P = 0.5$). The *P* values in the table show the probability (to 4 decimal places) of the observed test results occurring through chance if the null hypothesis is correct.

* Results are significantly different ($P < 0.05$).

the combined DSp and DSn was at 70% of the conjugate control (positive sera give lower values). Table 1 shows the number of samples tested by each method, the cutoff for each method, and the DSn and DSp for each—including the 95% confidence intervals. DSp data for the SAT and CFT are from a recent validation trial of an iELISA to be used in the Netherlands (Emmerzaal et al., 2002) where the CFT and SAT method conformed to the European Union (EU) (directive 64/432) and OIE requirements as is also the case in Britain. The DSp values for the iELISA method are calculated from British surveillance data where the British herd is screened by this method before any positive samples are subsequently tested by SAT and CFT for confirmation. This table clearly shows that the performance of the SAT is comparatively substandard, while the CFT is also inferior compared with the ELISAs and the FPA. The results for the iELISA, cELISA, and FPA showed the FPA to have the highest combined DSp and DSn. The confidence limits in Table 1 for the DSp + DSn revealed some overlap for all tests but the SAT. Testing for significant differences between these results is restricted by the combination of data from independent (negative reference sera) and dependent (positive reference sera) samples. Testing for significant differences ($P < 0.05$) between the DSp for each test, used with the cutoff as described in Table 1, revealed the following magnitude of DSp: (CFT = cELISA)>(FPA = SAT)>iELISA. Significant differences between the tests, based on the results from the positive reference sera alone and using a binomial distribution ($P = 0.5$), were found as described in Table 2. These results show that the iELISA and FPA are significantly more sensitive than the CFT and SAT, but not the cELISA. The SAT was significantly less sensitive than all other tests.

The Pearson product-moment correlation coefficient (Fig. 3) of the continuous FPA and iELISA results from the positive reference sera shows a highly significant ($P < 0.01$, $n = 146$) positive correlation ($r = 0.683$). Of the 146 positive serum samples, 140 tested positive for both tests, 3 were negative for both, with 2 iELISA positive but FPA negative and only 1 iELISA negative but FPA positive.

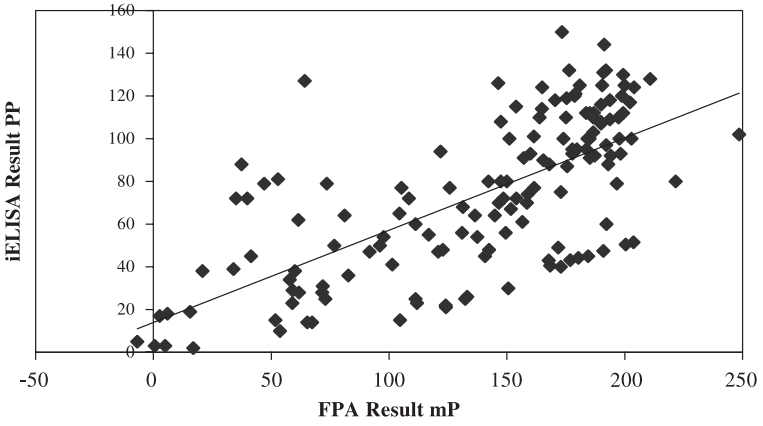

Fig. 3. FPA test results against iELISA results (expressed as percentage of positive control) for the 146 serum samples from culture positive animals.

Table 3
Test results from *B. abortus*-infected herds from Eire

	FPA	iELISA	cELISA
Number positive	166	168	158
% Pos.	64.09%	64.86%	61.00%
Number negative	93	91	101
% Neg.	35.91%	35.14%	39.00%
Test comparison	FPA vs. iELISA	iELISA vs. cELISA	cELISA vs. FPA
Result agreement	90.7%	93.1%	91.5%
Significance*	$P>0.1$	$P<0.05$	$P>0.1$

Significance: $P<0.05$ = tests are significantly different at the 95% confidence level.

*McNemars paired test With Yates' correction.

The samples from infected herds in Eire were tested by iELISA, cELISA, and FPA. The results (Table 3) show that the iELISA method finds the highest number of samples positive, with the cELISA finding the least number of samples positive. When the tests were compared to each other, the results for each sample were in agreement over 90% of occasions. Although the cELISA and the iELISA showed the highest degree of agreement, the samples in disagreement were heavily weighted towards iELISA positive and cELISA negative results. This split was less profound in the other two test comparisons. McNemar's test with paired samples (with Yates' correction) reflects this and revealed that there was no significant difference in the proportion of positive samples between the iELISA and FPA, or the FPA and cELISA methods ($P>0.05$). However, there was a significant difference between the iELISA and cELISA results ($P<0.05$).

4. Discussion

Diagnostic procedures for brucellosis should be specific, sensitive, and detect all stages of the infection. Currently, no such test exists. Yet, serological testing and notification in cases of abortion with subsequent serological and cultural examination, with an identification system in accordance with EU regulations, are the main requirements to be fulfilled in order to retain official brucellosis-free status as a Member State.

The current British brucellosis surveillance strategy uses the described iELISA method to screen nondairy herds (dairy herds are monitored by the bulk milk iELISA which is a minor adaptation of the serum iELISA used). ELISAs are easy to perform, to automate, produce objective results, rapidly lend themselves to Quality Assurance programmes, and can cut the cost of staff training due to the universality of the ELISA method for different applications. Samples that are positive by iELISA are subjected to further testing by both CFT and SAT for confirmation.

The SAT is recognised as inferior to other tests (Corbel and MacMillan, 1996). The CFT is recognised as a highly sensitive and specific test when correctly performed, yet its comparative underperformance in this study questions these properties. The CFT also has many practical drawbacks, not least of all which is its relative technical complexity. The test is also subject to anticomplementary reactions and will not work on haemolysed samples. Furthermore, prozone reactions caused by competition between C′ activating and non-C′ activating IgG isotypes (McGuire et al., 1979), and possibly IgM (Nielsen and Duncan, 1988), require serum to be tested at multiple dilutions. Yet, it must be recognised that these problems did not prevent the successful eradication of brucellosis (as defined by EU directive 64/432) from many countries using these tests (e.g. Britain and The Netherlands).

The cutoff of the FPA was determined using TG-ROC. The cutoff value of 15.5 mP was selected to maximise total DSp and DSp. The cELISA, although a more established method, was also evaluated by TG-ROC to obtain a cutoff of 70% of the conjugate control. This maximised the total DSp and DSn. Data was not available to perform the same analysis on the SAT, CFT, and iELISA. As these methods have strictly defined cutoff criteria, this method of evaluation was not necessary. Although cutoffs can be chosen to fall at the test value that provides the highest combined DSn and DSp, in practice, other factors also need to be considered when selecting a cutoff. The relative costs of false negative and false positive samples should be evaluated. If it will be more costly to get false negatives, then the test cutoff can reflect this by being moved in favour of increasing sensitivity. This might be the case in serious conditions where the false positives can be screened using a second, possibly more expensive test, but the consequences of missing a true positive will be severe.

Table 1 shows the DSp and DSn results for each test. For DSp + DSn, the results are in the following order of magnitude: FPA>iELISA>cELISA>CFT>

SAT. The conjecture that these relative performances can be extrapolated to the target population is difficult to prove statistically where the confidence limits for the result overlap those for other tests. This is because the DSn + DSp results are from both dependent samples (positive reference sera) and independent samples (negative reference sera). At the test cutoffs shown in Table 1, statistical analysis relating to the DSp alone shows the following significant differences: (CFT = cELISA)>(FPA = SAT)>iELISA. The results from the 146 positive reference samples for each of the tests were analysed using a two-tailed binomial test. The results from two tests were compared, and only the samples which were diagnosed differently in each test were considered. The null hypothesis was that these differentially diagnosed samples had an equal chance ($P = 0.5$) of being positive for test A and negative for test B, or negative for test A and positive for test B. A significant rejection of this hypothesis, that $P \neq 0$, concludes that one test is more sensitive at detecting positives than the other. These results (Table 2) show that the iELISA and FPA are significantly more sensitive than the CFT and SAT, but not the cELISA. The SAT is significantly less sensitive than all other tests.

The sampling methods for validation trials are of the utmost importance. Reliable confidence intervals for the test parameters DSn and DSp can only be achieved when the samples are selected at random from a selection of the population which has a proportionate representation (to the potential target population) of all parameters that may affect test outcome, for example, age of animal, sex, diet, breed, location, accommodation, etc. Serum samples from animals confirmed as *Brucella* positive by cultural identification are in short supply due to the nature of the disease and its distribution. Therefore, it is very difficult to select for such factors and obtain sufficient samples. However, a relatively large number of positive animals were sampled in this study. In previous validation studies of the FPA, despite the number of samples being indicated, it has not always been clear how many animals were sampled to obtain the positive reference sera set. As such, the confidence intervals are included to serve as only a guide to the uncertainty of the results, but in this instance have added validity over previous investigations.

Of equal, if not more, importance than the absolute results is the relation of the tests to one another in terms of their diagnostic performance. It should also be noted that the 'Gold Standard' of positive culture data is only a gold standard specificity. Far less is known about the sensitivity of culture. As a result, even the most stratified culture positive serum samples may be biased towards overstating a tests DSn, as it is by serology that most animals are identified and subsequently slaughtered for cultural examination. Although the same negative samples were not used to determine DSp for each of the tests, the selection was far larger than for the culture positive samples. As a result, the confidence intervals are smaller, yet these too must be interpreted with a degree of caution due to potential sampling bias such as the time of year the samples were collected.

There is a significant positive correlation between the continuous data from the iELISA and FPA results on the culture positive serum samples. Similarly, it was possible to discriminate between samples of high titre and lower titre positives. The strong correlation was also evident in the data from the tests on samples from Irish *Brucella*-infected herds where the FPA and the iELISA results were insufficiently different to be significant. The data from the cELISA test was sufficiently different from the iELISA to be able to say with 95% confidence that the two tests possessed different diagnostic properties. Without knowing the infection status of each animal sampled, it was not possible to say if each test was more sensitive or less specific, but judging from the validation data, the pattern of the field trial data was likely to be a result of both.

The data presented here suggests that the FPA is a significantly superior diagnostic test to the SAT, but despite having the highest combined DSp and DSn total, it was not conclusively superior in sensitivity and specificity over the CFT or the ELISAs. The advantages of the FPA in terms of the simplicity of the method compared to the other tests are clear. As such, further validation work on the FPA is warranted in order to evaluate its performance against known false positive reactors and during different stages of the disease.

It may not be possible to get real DSp and DSn values due to the bias of culture positive serum samples used as a 'Gold Standard', unless validation is performed using methods of analysis such as maximum likelihood (Hui and Walter, 1980). This method requires at least two diagnostic tests which are condi-

tionally independent such as one of the described serological tests and a diagnostic PCR. A satisfactory diagnostic PCR method, one that does not require preculture, is a current research goal (Nakkas et al., 2002). Maximum likelihood analysis would be an ideal method to validate such a test. These problems are true for all diagnostic tests as well as for the FPA. It has also been claimed that the FPA can discriminate between serum from vaccinated and nonvaccinated animals (Neilsen et al., 1996), and further investigation here is also warranted. It is clear that the methodology of the FPA offers clear advantages due to its ease of use. Full implementation and acceptance of FPA methods for the diagnosis of brucellosis will likely necessitate the use of an International Standard Serum panel containing at least a low titre positive sample and a negative. This would be similar to those used to standardise the ELISA which allowed it to be formally introduced into EU legislation.

Acknowledgements

The authors would like to thank Kevin Kenny (Key Laboratories, Dublin, Ireland) for his technical expertise and for his help in the preparation of this manuscript.

References

Caroff, M., Bundle, D.R., Perry, M.B., Cherwonogrodzky, J.W., Duncan, J.R., 1984. Antigenic S-type lipopolysaccharide of *Brucella abortus* 1119-3. Infect. Immun. 46, 384.

Corbel, M.J., MacMillan, A.P., 1996. Bovine brucellosis. In: Reichard, R. (Ed.), Office International Des Epizooties Manual of Standards for Diagnostic Tests and Vaccines, Paris. Office International Des Epizooties, 12 rue de Prony, 75017 Paris, France, p. 248.

Dalrymple-Champneys, W., 1960. *Brucella* Infection and Undulant fever in Man, Oxford Univ. Press, London.

Emmerzaal, A., de Wit, J.J., Dijkstra, Th., Bakker, D., van Zijderveld, F.G., 2002. The Dutch *Brucella abortus* monitoring programme for cattle: the impact of false-positive serological reactions and comparison of serological tests. Vet. Q. 24, 40.

Fekete, A., Bantle, J.A., Halling, S.M., Sanborn, M.R., 1990. Preliminary development of a diagnostic test for *Brucella* using polymerase chain reaction. J. Appl. Bacteriol. 69, 216.

Greiner, A., Dorit, S., Göbel, P., 1995. A modified ROC analysis for the selection of cut-off values and the definition of intermediate results of serodiagnostic tests. J. Immunol. Methods 185, 123.

Hui, S.L., Walter, S.D., 1980. Estimating the error rates of diagnostic tests. Biometrics 36, 167.

Jacobson, R.H., 1998. Validation of serological assays for diagnosis of infectious diseases. Rev. Sci. Tech.-Off. Int. Epizoot. 17, 469.

Manthei, C., Carter, R., 1950. Persistence of *Brucella abortus* infection in cattle. Am. J. Vet. Res. 11, 173–180.

McGuire, T.C., Musoke, A.J., Kurtti, T., 1979. Functional properties of bovine IgG1 and IgG2: interaction with complement, macrophages, neutrophils and skin. Immunology 38, 249.

Morgan, W.J.B., 1977. The national brucellosis programme of Britain. In: Crawford, R.P., Hidalgo, R.J. (Eds.), Brucellosis: an International Symposium. A and M Univ. Press, Austin, TX, p. 378.

Nakkas, A.F., Wright, S.G., Mustafa, A.S., Wilson, S., 2002. Single-tube, nested PCR for the diagnosis of human brucellosis in Kuwait. Ann. Trop. Med. Parasitol. 96, 397.

Nasir, M.S., Jolley, E., 1999. Fluorescence polarisation: an analytical tool for immunoassay and drug discovery. Comb. Chem. High Throughput Screen. 2, 177.

Nielsen, K., Duncan, J.R., 1988. Further evidence that bovine IgM does not fix guinea pig complement. Vet. Immunol. Immunopathol. 19, 197.

Nielsen, K., Gall, D., Jolley, M., Leishman, G., Balsevicius, S., Smith, P., Nicoletti, P., Thomas, F., 1996. A homogeneous fluorescence polarisation assay for detection of antibody to *Brucella abortus*. J. Immunol. Methods 195, 161.

Nielsen, K., Gall, D., Smith, P., Kelly, W., Yeo, J., Kenny, K., Heneghan, T., McNamara, S., Maher, P., O'Connor, J., Walsh, B., Carrol, J., Rojas, X., Rojas, F., Perez, B., Wulff, O., Buffoni, L., Salustio, E., Gregoret, R., Samartino, L., Dajer, A., Luna-Martinez, E., 2001a. Fluorescence polarisation assay for the diagnosis of bovine brucellosis: adaptation to field use. Vet. Microbiol. 80, 163.

Nielsen, K., Smith, P., Gall, D., Perez, B., Samartino, L., Nicoletti, P., Dajer, A., Rojas, X., Keyy, W., 2001b. Validation of the fluorescence polarisation assay for detection of milk antibody to *Brucella abortus*. J. Immunoassay. Immunochem. 22, 203.

Perrin, M.F., 1926. Polarization de la lumiere de fluorescence. Vie moyenne de molcules dans l'etat excite. J. Phys. Radium 7, 390.

Stack, J.A., Perrett, L.L., Brew, S.D., MacMillan, A.P., 1999. Competitive ELISA for bovine brucellosis suitable for testing poor quality samples. Vet. Rec. 145, 735.