Validation of an indirect enzyme-linked immunosorbent assay for the detection of antibody against *Brucella abortus* in cattle sera using an automated ELISA workstation

J.T. PAWESKA[1], A.D. POTTS[5], H.J. HARRIS[5], S.J. SMITH[5], G.J. VILJOEN[5]*, B. DUNGU[5], O.L. BRETT[2], M. BUBB[3] and L. PROZESKY[4]

ABSTRACT

PAWESKA, J.T., POTTS, A.D., HARRIS, H.J., SMITH, S.J., VILJOEN, G.J. DUNGU. B, BRETT, O.L., BUBB, M. & PROZESKY, L. 2002. Validation of an indirect enzyme-linked immunosorbent assay for the detection of antibody against *Brucella abortus* in cattle sera using an automated ELISA workstation. *Onderstepoort Journal of Veterinary Research,*69:61–77

An automated indirect enzyme-linked immunosorbent assay (I-ELISA) for the serological diagnosis of bovine brucellosis was developed and validated in-house. A total of 4 803 cattle sera from South Africa (*n* = 3 643), Canada (*n* = 652), Germany (*n* = 240), France (*n* = 73) and the USA (*n* = 195) was used. The South African panel of sera represented 834 sera known to be positive by the Rose Bengal test (RBT), serum agglutination test (SAT) and complement fixation test (CFT), 2709 sera that were negative by CFT, and 100 sera from animals vaccinated with a standard dose of *Brucella abortus* strain 19. Overseas sera were obtained from reference non-vaccinated brucella-free cattle (*n* = 834), naturally infected (*n* = 72), experimentally infected (*n* = 71), and vaccinated animals (*n* = 83). Also 100 sera collected from cattle in Canada and known to be positive by competitive ELISA (C-ELISA) were used.

The intermediate ranges ("borderline" range for the interpretation of test results) were derived from two-graph receiver operating characteristics analysis. The lowest values of the misclassification cost-term analysis obtained from testing overseas panels, covered lower I-ELISA cut-off PP values (0.02–3.0) than those from local panels (1.5–5.0). The relatively low cut-off PP values selected for I-ELISA were due to the fact that the positive control used represents a very strong standard compared to other reference positive sera. The greater overlap found between negative and positive cattle sera from South Africa than that between reference overseas panels was probably due to the different criteria used in classifying these panels as negative (sera from true non-diseased/non-infected animals) or positive (sera from true diseased/infected animals). The diagnostic sensitivity of the I-ELISA (at the optimum cut-off value) was 100 % and of the CFT 83.3 %. The diagnostic specificity of I-ELISA was 99.8 % and of the CFT 100 %. Estimate of Youden's index was higher for the I-ELISA (0.998) than that for the CFT (0.833). Analysis of distribution of PP values in sera from vaccinated and naturally infected cattle shows that in vaccinated animals all readings were below 31 PP where in infected ones these values represented 43 %. Therefore, it appears that I-ELISA could be of use in identifying some naturally infected animals (with values > 31 PP), but more sera from reference vaccinated and infected animals need to be tested to further substantiate this statistically. Of 834 sera positive by RBT, SAT and CFT, 825 (98.9 %) were positive in the I-ELISA. Compared to C-ELISA the relative diagnostic sensitivity of the I-ELISA was 94 % and of the CFT 88 % when testing 100 Canadian cattle sera. Of 258 South African cattle sera, of which 183 (70.9 %) were positive

* Author to whom correspondence is to be directed. E-mail address: gerrit@moon.ovi.ac.za

[1] Onderstepoort Veterinary Institute, Private Bag X05, Onderstepoort, 0110 South Africa. Current address: National Institute for Communicable Diseases, Special Pathogens Unit, Private Bag X4, Sandringham, 2131 South Africa

[2] Onderstepoort Biological Products, Private Bag X07, Onderstepoort, 0110 South Africa

[3] Natal Institute of Immunology, Private Bag X9044, Pinetown, 3600 South Africa

[4] Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort, 0110 South Africa

[5] Onderstepoort Veterinary institute, Private Bag X05, Onderstepoort, 0110 South africa

by the I-ELISA and 148 (57.4 %) by the CFT, 197 (76.4 %) were positive by C-ELISA when re-tested in Canada. One has to stress, however, that Canadian C-ELISA has not been optimised locally. Thus, the C-ELISA was probably not used at the best diagnostic threshold for testing South African cattle sera. This study shows that the I-ELISA performed on an automated ELISA workstation provides a rapid, simple, highly sensitive and specific diagnostic system for large-scale detection of antibodies against *B. abortus*. Based on the diagnostic accuracy of this assay reported here, the authors suggest that it could replace not only the currently used confirmatory CFT test, but also the two in-use screening tests, namely the RBT and SAT.

Keywords: Antibodies to *Brucella abortus*, cattle sera, diagnostic accuracy, indirect ELISA, validation

INTRODUCTION

Serological detection of antibodies is the usual method of choice for the control and eradication of bovine brucellosis caused by *Brucella abortus*. Several conventional serological tests have been used either singly or in combination, e.g. agglutination, activation of complement or precipitation assays. Since these measure only certain antibody isotypes but not others, the use of test-panels for more accurate diagnosis is required. This led to the development of serological screening tests of high sensitivity but lower specificity. A positive reaction in the screening test would lead to further testing with a number of confirmatory tests. This arrangement is costly and the time between submission and output of results is lengthy (Nielsen, Kelly, Gall, Balsevicius, Bosse & Kelly 1998).

In South Africa serological diagnosis of bovine brucellosis is usually accomplished using the Rose Bengal test (RBT) and/or standard agglutination test (SAT) as the screening tests followed by the complement fixation test (CFT) for confirmation. Where heifer vaccination with *B. abortus* strain 19 (S-19) vaccine is practiced, a titre of 18–24 IU mℓ^{-1} is regarded as suspect and 30 IU mℓ^{-1} or higher as positive in CFT. When heifers are tested at 18 months of age or more, CFt-positive reactors are removed from farms and slaughtered (Krige 1981).

Agglutination techniques have limitations in sensitivity and specificity due to prozone phenomena and frequent non-specific reactions due to antibodies binding with antigenic determinants in common with *B. abortus*, such as *Yersinia enterocolitica*, *Escherichia coli*, *Salmonella urbana* and *Campylobacter fetus* (Alton 1978).

Traditionally, the CFT has been used as a definitive, highly specific test for those reactors testing positive in agglutination assays (Sutherland 1980). The preparation of reagents for the CFT, however, is time consuming and the test is also difficult to standardize. Additionally, false positive and false

negative results may occur for a variety of reasons with the CFT. For example, infection with *Yersinia enterocolitica*, serotype 9, can produce antibodies that cross-react with *B. abortus* antigen (Thrusfield 1995). Improper timing may result in the test's failure to detect infection. For instance, the CFT may not detect antibodies against *B. abortus* after sampling cows prior to abortion, because detectable antibody levels may not appear until after abortion (Robertson 1971).

Some sera, notably from contaminated and haemolysed specimens, are anti-complementary, and will therefore, prevent complement fixation in the CFT. This test will thus be falsely presumed to be negative for the presence of brucella-specific antibodies (Worthington 1982). Occasionally blocking antibodies prevent antigen-antibody specific binding. This occurs due to excess IgG$_2$ blocking IgG$_1$, (responsible for complement fixation) at low concentrations (Plackett & Alton 1975). A serological test may be insensitive at detecting antibody.

It has been demonstrated that the relative analytical (Stites & Rodgers 1991) and diagnostic sensitivities (Nielsen *et al.* 1998) of enzyme-linked immunosorbent assay (ELISA) techniques are higher than that of CFT.

Finally, as S-19 is a live vaccine, persistent antibodies may be produced which may make the interpretation of serological tests difficult (Nagy, Hignet & Ironside1967; Herr, Ehret, Ribeiro & Chaparro 1990; Abalos, Ibarara, Pinochet, Navia & Boisier 1996).

The introduction of immunoenzymatic techniques in the diagnosis of bovine brucellosis has allowed for the attainment of higher sensitivity and specificity levels than most commonly used conventional techniques (Nielsen, Kelly, Gall, Balsevicius, Bosse, Nicoletti, Kelly & Ehenning 1996). Macmillan (1990) says the reasons for using immunoenzymatic techniques are:

- To replace conventional serological tests that in

many ways did not perform well and that frequently required a panel of tests for diagnosis.

- To introduce a primary bindig assay that could be well standardised, quality controlled and automated (MacMillan 1990)

A large number of indirect enzyme-linked immunosorbent assay (I-ELISA) formats have been described in the literature but in spite of the numerous modifications, the specificity of this assay was less than expected. The reason for this is partly because antibody resulting from S-19 vaccination or from exposure to cross-reacting antigens will be detected by this procedure (Nielsen *et al.* 1996; Wright, Nielsen & Kelly 1990; Wright, Nilsson, Van Rooij, Lelenta & Jeggo 1993).

To increase specificity, competitive enzyme-linked immunosorbent assays (C-ELISA) were developed (Macmillan, Greiser-Wilke, Moenning & Mathiadis 1990; Nielsen, Kelly, Gall, Nicoletti & Kelly 1995; Nielsen *et al.* 1998), which may better eliminate such reactions. One of the most widely validated C-ELISAs, also developed in an attempt to differentiate S-19-vaccinated from naturally *B. abortus*-infected animals is the FAO/IAEA Brucellosis C-ELISA kit (IAEA 1998). This assay was shown to have a very high diagnostic performance (Nielsen *et al.* 1998). It does, however, occasionally lack specificity (Gall, Colling, Marino, Moreno, Nielsen, Perez & Samartino 1998) due to cross-reacting antibodies. It also sometimes fails to differentiate infected from vaccinated animals (Carrasco, Uzal & Echaide 1998).

Both indirect and competitive ELISAs have been approved by the Office Internationale des Epizooties as prescribed tests for international trade in addition to the RBT, buffered plate agglutination, and CFT (MacMillan & Stack 2000). The I-ELISA may be used as a screening or as a confirmatory test. The test performance of the I-ELISA depends on the immunoglobulin specificity of the conjugate. Conjugates with broad specificity and the use of lower test dilutions will result in a screening assay of higher diagnostic sensitivity. Conjugates with a narrow specificity (e.g. anti-IgG$_1$) and the use of higher test dilutions will result in a confirmatory assay of greater diagnostic specificity (Corbel & MacMillan 1996).

This report presents data on the development and validation of an in-house I-ELISA for the large-scale diagnosis of bovine brucellosis in South Africa using an automated ELISA workstation.

MATERIAL AND METHODS

Sera

A total of 4 803 sera collected from cattle in South Africa (*n* = 3 643), Canada (*n* = 652), Germany (*n* = 240), USA (*n* = 195) and France (*n* = 73) was used. South African sera were collected mostly from cattle of unknown infection or vaccination history against brucellosis. Of these, 834 sera were known to be positive in the RBT, SAT and CFT, and 2 709 were negative in the CFT. Of 2 709 sera negative in the CFT, 237 and 165 were known to be positive in the RBT and SAT, respectively. Additionally, 100 sera from animals vaccinated at 4–6 months of age with a standard dose of *B. abortus* strain S-19 taken between 1–39 months post vaccination were used.

Canadian sera represented 500 negative sera collected from non-vaccinated herds, which had been brucellosis-free for 15 years. Of 37 Canadian positive sera, 32 were from cattle that tested positive for a field strain of *B. abortus* where biovar identity was unknown, and five tested positive for strain S-19; 15 individual samples were collected 5 or 7 months after immunization. Additionally, 100 sera that tested positive in Canada by C-ELISA were used.

Of the 240 samples from Germany, 221 were obtained from brucella-free areas and certified as negative (vaccination against brucellosis has been never exercised in this country), 12 were from naturally *B. abortus*-infected, and seven from experimentally *B. abortus* infected animals.

French sera were taken from 50 brucellosis-free and 23 from brucellosis-infected cattle.

USA sera represented 63 samples from non-vaccinated brucellosis-free animals, 64 individual specimens collected between 4–5, 12–13 and 12–22 weeks post challenge with *B. abortus* strain S2308, and 68 sera from calves 4–10 weeks after immunisation with *B. abortus* strain 19.

These sera were analysed in the following different reference panel formats:

- Overseas sera from non-vaccinated, brucellosis-free animals (*n* = 834)
- Overseas sera from animals naturally infected with *B. abortus* (*n* = 72)
- Overseas sera from animals experimentally infected with *B. abortus* (*n* = 71)
- Canadian sera known to be *B. abortus*-positive in C-ELISA (*n* = 100)

- Local diagnostic sera known to be *B. abortus*-negative in CFT (*n* = 2709)

- Local diagnostic sera known to be *B. abortus*-positive in CFT (*n* = 834)

- Local sera tested at the OVI by I-ELISA and the CFT (selected from panels V and VI) and re-tested in Canada by C-ELISA (*n* = 258)

- Local and overseas sera from vaccinated animals (*n* = 183)

Serological tests

Rose Bengal Test

The RBT was conducted according to the Australian method as recommended by the Sub-committee of Principal Veterinary Laboratory Officers of the Animal Health Committee (1980).

Serum Agglutination Test

The SAT was conducted according to Herr, Te Brugge & Guiney (1982).

Complement Fixation Test

The CFT was done using a microtitration procedure. *Brucella abortus* CFT antigen was prepared at Onderstepoort Biological Products (OBP), Onderstepoort, South Africa. CFT end-point reactions were converted and recorded as International Units (IU/mℓ) according to the interpretation of CFT end-point reading given by Herr, Huchzermeyer, Te Brugge, Wiliamson, Roos & Schiele (1985). In this study a test serum was considered CFT-positive when it had a titre $\geq$ 20 IU/mℓ (MacMillan & Stack 2000)

Indirect ELISA

PRODUCTION OF ANTIGEN

ELISA antigen was produced by OBP. *B abortus* strain-99 (Weybridge) was activated from freeze-dried culture on serum dextrose agar Petri dishes and then seeded on serum dextrose agar in Mason tubes. Material from one of the Mason tubes was inoculated into a 2 000 mℓ seeding flask containing 500 mℓ of liquid medium (2 % casamino acids, 1 % yeast extract 1 % glucose) and incubated at 37 °C for 2 days while aerating with a magnetic stirrer. The material from the seeding flask was then used to inoculate a 42 ℓ fermentor containing 20 ℓ of the same liquid medium as used for the seeding flasks. The incubation temperature was maintained at 37 °C and airflow was controlled automatically to maintain the dissolved oxygen concentration at 80 %. After 2 days of incubation, the culture was harvested using 2 ℓ of sodium carboxymethyl cellulose and overnight sedimentation. About 18 ℓ of the supernatant above the sediment were removed and the remaining material was inactivated at 60 $\pm$ 2 °C for 30 min in a water bath. This suspension was diluted with an equal volume of phenol saline (0.5 % phenol, 0.85 % sodium chloride) and centrifuged at 6 000 x g for 60 min. The sediment was then re-suspended in a minimum amount of phenol saline using a Polytron homogenizer with a 35/2 aggregate (Kinematica AG, Lucerne) and mixed overnight on a magnetic stirrer. The following day the packed cell volume was set at 20 % (1 200 x g for 60 min) using Fitch modified Hopkins tubes (Kimax code 45225, Kimble USA). The suspension was then stored for up 2 months. Hereafter, it was centrifuged at 6 000 x g for 120 min and the resulting supernatant was decanted and filtered (Seal-kleen filter cartridge, posidyne N66 0.2 µm, Code SLK7002NFZP, Pall Process Filtration). The resultant undiluted filtrate was freeze-dried in 1 mℓ aliquots and stored at 4 °C before being used to coat the immunoplates.

PRE-COATED PLATES

Flat-bottom, 96-wells immunoplates (NUNC C96 Polysorp, Cat # 4-46140), pre-coated with 50 µℓ of antigen (0.025 mg/mℓ) in 0.05 M carbonate buffer, pH 9.6, individually sealed in aluminium foil bags (LOT C-812) containing silica gel sachets were supplied by the Natal Institute of Immunology, Durban, South Africa.

TEST PROCEDURE

The method described by MacMillan & Stack (2000) was followed with minor modifications. Control and test sera were diluted 1/200 in dried milk diluent containing 3 % fat-free milk powder ("Elite") in TST buffer (Tris saline, Tween, pH 8 $\pm$ 0.2) and 50 µℓ of each serum were added to two wells of pre-coated microplate using a robotic system (Multiprobe 204DT, Packhard). Plates with diluted sera were then processed in an automated ELISA processor (Omni, Bio-Tek Instruments) as follows:

- After incubation at 37 °C for 30 min unbound antibody was removed by washing the plates three times with diluting buffer (100 µℓ/well)

- A volume of 50 µℓ recombinant protein G conjugated with HRP (Cat No. 10-1223, Zymed) diluted 1/10 000 in diluting buffer was added to each well and the plates incubated at 37°C for 15 min

- Unbound conjugate was removed by three washing steps

- A volume of 50 µℓ substrate/chromogen (TMB Single Solution, Cat No. 00-2023, Zymed) was added to each plate and the plates were incubated in the dark for 10 min at room temperature (20–25 °C)

- Reactions were stopped by adding 50 µℓ/well of 1M H_2SO_4 and colour development was immediately assessed in a spectrophotometer (EL 340, Bio-Tek Instruments) using 450 nm and 690 nm reference filters

- Absorbance readings were converted to PP values (percentage of strong positive control) using the following equation:

$$\% \ PP = \frac{\text{Mean absorbance of test sample}}{\text{Mean absorbance of strong positive control}} \times 100$$

where % PP = Percentage positivity.

Competitive ELISA

The C-ELISA was carried out according to the method of Nielsen *et al.* (1995). A cut-off of 30 PI (percentage inhibition of strong positive control) was used for discrimination between negative and positive sera.

Repeatability

Repeatability was estimated using five reference positive control sera containing different levels of antibodies against *B. abortus* (from high to low), and five reference negative control sera. These were obtained from the Serodiagnostic Unit of the Onderstepoort Veterinary Institute. Each serum was tested on four or five plates each with eight repeats on five separate occasions (No. samples x No. replicates x No. plates x No. occasions = No. determinations). Means, ranges and standard deviations of ELISA optical density (OD) values were calculated from replicates of all samples in each plate and runs of the assay to assess intra- and inter-plate variation. Additionally, coefficients of variation (CV) were calculated for positive samples as follows:

$$CV = \frac{\text{Standard deviation of replicates}}{\text{Mean of replicates}} \times 100$$

Quality control (QC)

The accuracy of the assay was evaluated by analysing mean OD and CV values of the high control serum (four repeats/plate) from a total of 83 plate runs on 15 separate occasions over a period of 7 weeks. The high positive control serum (Batch OPNS2) was provided by OBP. This standard serum constitutes a pooled mixture of sera collected from two cows that aborted following field infection with *B. abortus* biotype 1. Data obtained from this analysis was used to calculate the upper (UCL) and lower control (LCL) limits:

UCL = Mean values of duplicates + 2 SD

LCL = Mean values of duplicates – 2 SD

and CV values for the OPNS2. UCL and LCL together with CV values ($< 15\%$) were applied as QC rules for further analysis.

Characterisation of OPNS2

This high positive control serum was compared with eight other reference positive sera using dose/response profiles. Three of them were obtained from Bundesinstitut für Gesündheitlichen Verbraucherschutz und Veterinärmedizin, Berlin, four from the Canadian Food Inspection Agency, Ontario, and one from the FAO/IAEA Animal Production and Health Sub-programme. Prior to testing, each of these nine sera was serially diluted using the negative control reference serum as the primary diluent. The titration series consisted of undiluted serum plus 11 two-fold dilutions from 1/2 to 1/2048. At the time of testing, each sample in the series was further diluted 1/200 in dried milk diluent buffer containing 3 g of milk powder per 100 ℓ of TST buffer. Each dilution was tested in duplicate on two separate occasions. The mean OD values were used to plot dose/response curves.

Frequency distributions of I-ELISA absorbance readings in brucella-negative cattle from geographically distinct origins

Statistical analysis of the distribution of I-ELISA OD values in non-vaccinated, brucellosis-free cattle from Canada ($n = 200$), Germany ($n = 200$) and in South African sera known to be negative by the CFT, was done using the parametric ANOVA and the non-parametric Kruskal-Wallis one-way analysis of variance. Results of this analysis are reported here as interquartile ranges for each panel tested. The South African CFT-negative panel (panel No. V) included also sera positive by the RBT and/or SAT. To ensure the most accurate assessment of the true negative I-ELISA OD background in South African brucella-negative cattle, only sera, which tested negative in all the three traditional tests, were used ($n = 2446$) for this analysis.

Selection of the cut-off

A combination of statistical approaches was used to select an optimum cut-off for I-ELISA. The data from defined negative and positive sera were sorted in ascending order and then plotted using a frequency histogram. The cut-off PP values were approximated by visual inspection of the frequency distribution graphs as the point that gives maximum distinction between positive and negative samples (Jacobson 1996). Cut-off values, at 95 % accuracy level, were optimised using two-graph receiver operating characteristics analysis (TG-ROC) (Greiner 1995; Greiner, Sohr & Göbel 1995; Gardner & Greiner 2000).

In addition, optimisation of I-ELISA cut-off was done by the misclassification cost term (MCT) analysis (Greiner 1996) at p (prevalence) value of 0.5, and r (equal costs of false-positive and false-negative results) value of 1.0, and also by comparison of diagnostic sensitivity (D-Sn) and diagnostic specificity (D-Sp) of I-ELISA using different cut-off values with the other serological assays.

Analysis of diagnostic accuracy (validity)

Diagnostic sensitivity, diagnostic specificity, and Youden's index (J) were calculated according to methods described by Gardner & Greiner (2000) and are summarised in Table 4.

Relative sensitivity and specificity

The relative D-Sn and D-Sp of the I-ELISA in relation to other tests was calculated as follows:

Relative diagnostic sensitivty
$$= \frac{\text{No. of comparative test positive}}{\text{No. of relative test positive}} \times 100$$

Relative diagnostic specificity
$$= \frac{\text{No. of comparative test negative}}{\text{No. of relative test negative}} \times 100$$

RESULTS

Repeatability

There was no evidence of excessive variation in OD values in any of the 960 replicates of positive and in any of 1 000 replicates of negative control samples within and/or between runs of the assay. The average CV for the total of 960 replicates of the five positive sera was 10.2 % ± 4.8 standard deviation (SD).

Test accuracy

The ELISA OD values for a high positive control serum did not exceed upper and lower limits on 79 (95.2 %) out of 83 plates evaluated during a period of 7 weeks. A slight upward trend in accuracy was observed in four plates (4.8 %) on run three of the assay (Fig. 1). The average CV for quadruplicate samples was 8.92 % ± 3.2 SD.

Characterisation of OPNS2 serum

The dose/response profile of OPNS2 serum in comparison to 8 other positive reference sera is shown in Fig. 2. The OPNS2 serum showed the highest level of antibody against *B. abortus*.

Distribution of ELISA OD values in negative sera

The geometric mean OD value for the Canadian negative panel was 0.013 ± 0.009 SD, with 50 % sera positioned within a range from 0.008–0.015. For the German negative panel it was 0.0164 ± 0.011 SD, with 50 % sera positioned within a range from 0.010–0.019. For the South African panel it was 0.030 ± 0.0346 SD with 50 % sera positioned within a range of OD values from 0.013–0.035. The non-parametric Kruskal-Wallis one-way analysis of variance and the parametric ANOVA indicated that Canadian and German panels differed significantly ($P < 0.001$) from the RSA panel.

Cut-off

The visual inspection of frequency distributions of PP values in serum panels I & II (Fig. 3), V (Fig. 4), and VI (Fig. 5) shows that 1 PP value was the most accurate cut-off point. When analysing data from panels I & II and V & VI by TG-ROC, an intermediate range (IR) of PP values ranged from 0.023–2.67 PP (Fig. 6) and from 1.23–4.33 PP (Fig. 7), respectively. Thus, at the selected accuracy level of 95 %, only results outside IR would be considered valid (Greiner et al. 1995). Optimisation of I-ELISA cut-off by the MCT analysis shows that the MCT value is minimal at cut-off of 0.1–3.0 PP when used results from panel I & II used (Fig. 8). The MCT value is minimal at cut-off of about 1.5–5.0 PP when used results from panel V and VI (Fig. 9).

Comparison of I-ELISA with CFT based on 2 x 2 table calculations (Jacobson 2000) shows that the most similar level of D-Sn or D-Sp between the two tests was at 1 PP for panel III and VI (Table 1), at 5 PP for panel I (Table 1) and VII (Table 3), and at
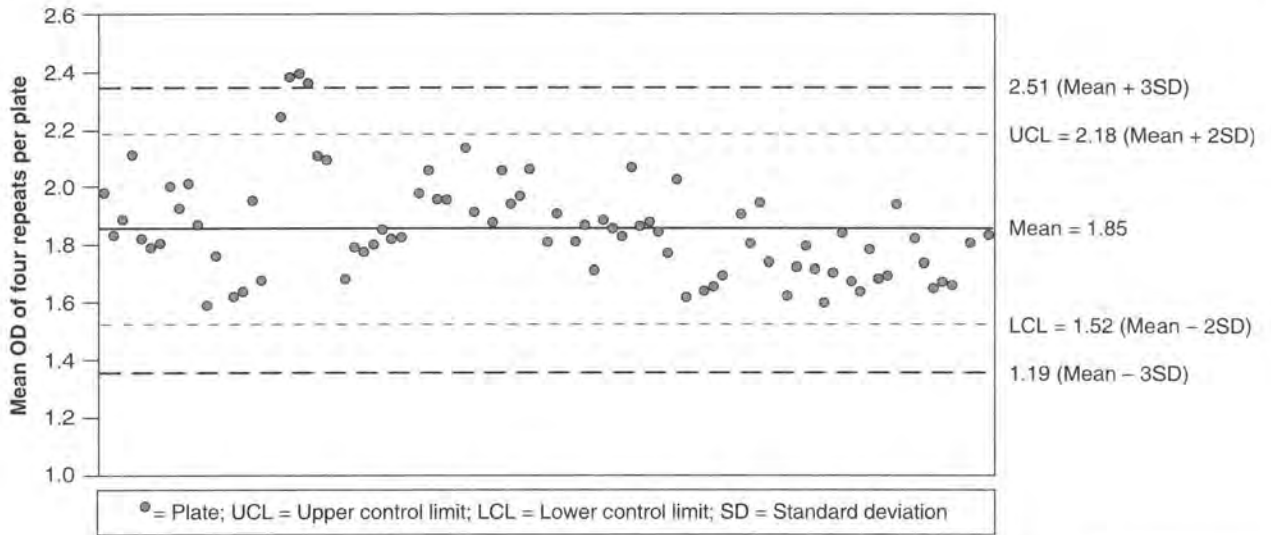
FIG. 1   Mean optical density (OD) values of control positive serum (OPNS2) tested on 83 plates during a period of 7 weeks on 15 separate runs
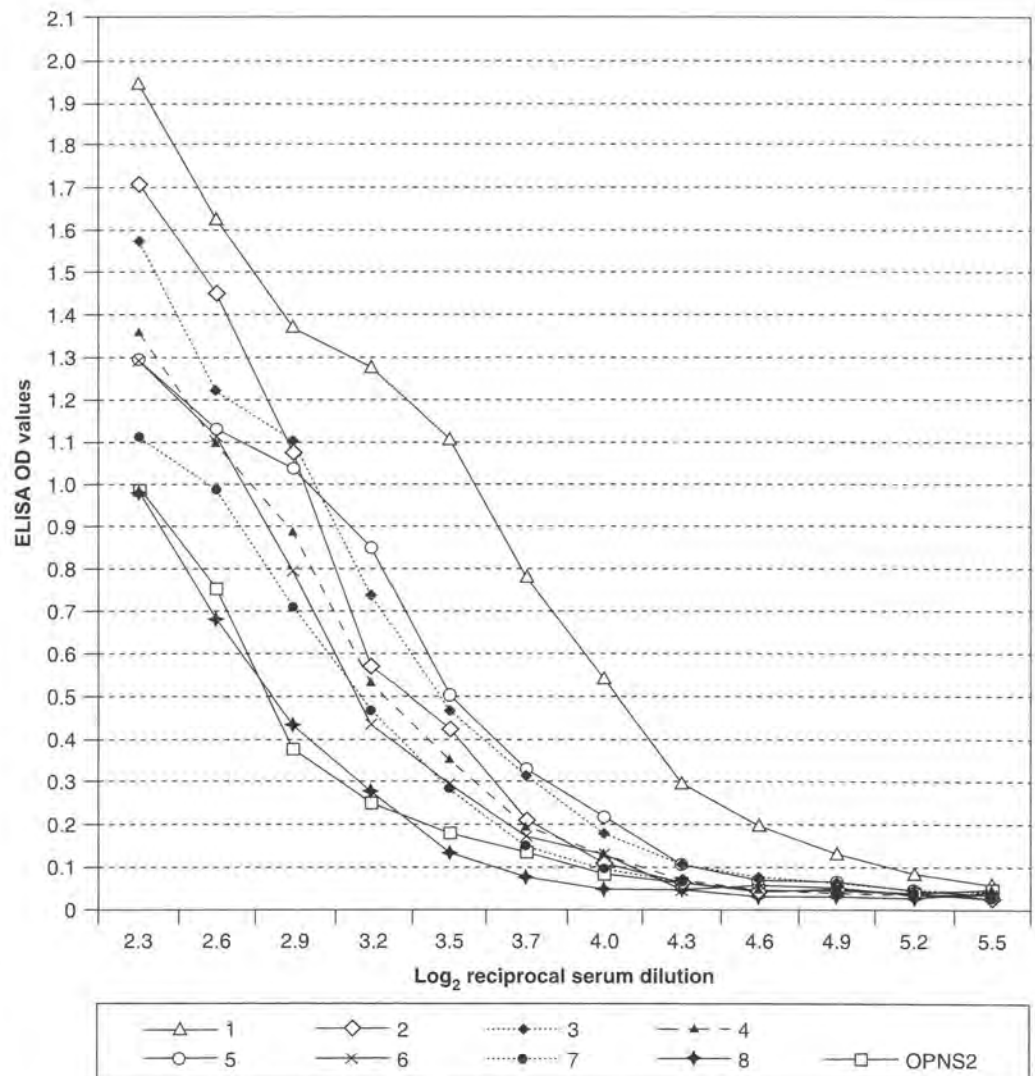

FIG. 2

Dose-response profiles of nine reference sera positive for antibodies to *Brucella abortus*
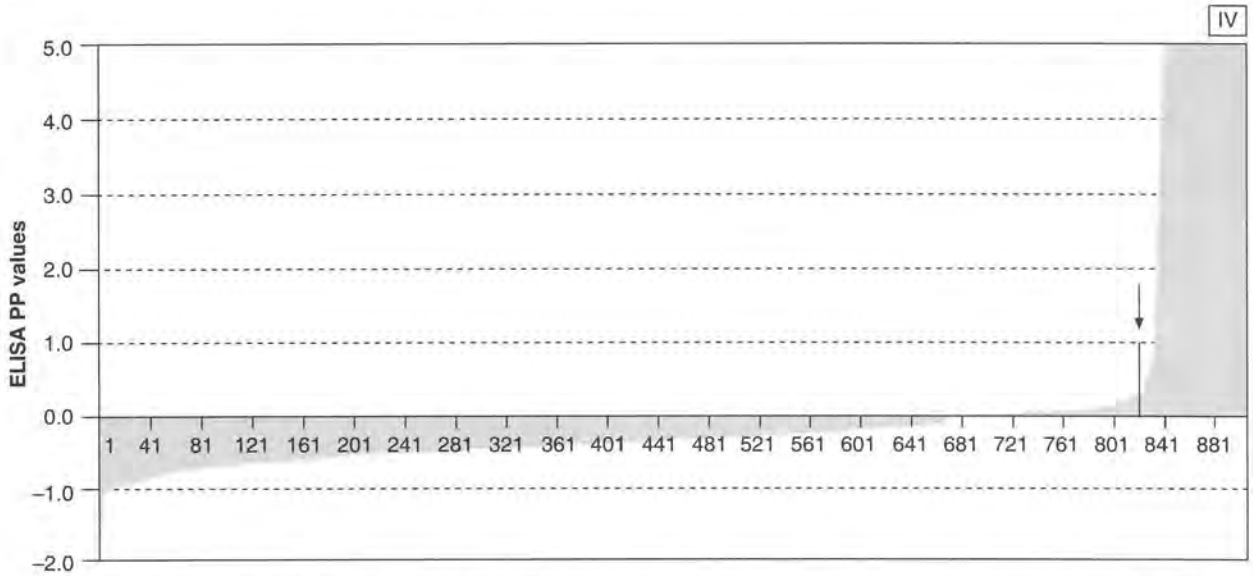
Log$_2$ reciprocal serum dilution

IV

FIG. 3   Distribution of ELISA PP values in 906 individual sera collected overseas from reference cattle of known brucella infection status

Sera ordered accordingly to ELISA PP values: Nos. 1– 834 from known non-vaccinated, brucella-free animals (panel I, $n = 834$); No. 835–906 from known brucella-naturally-infected animals (panel II, $n = 72$)

A vertical line on extreme right crossing the shaded area indicates the optimal ELISA cut off selected as 1 % of high positive control serum
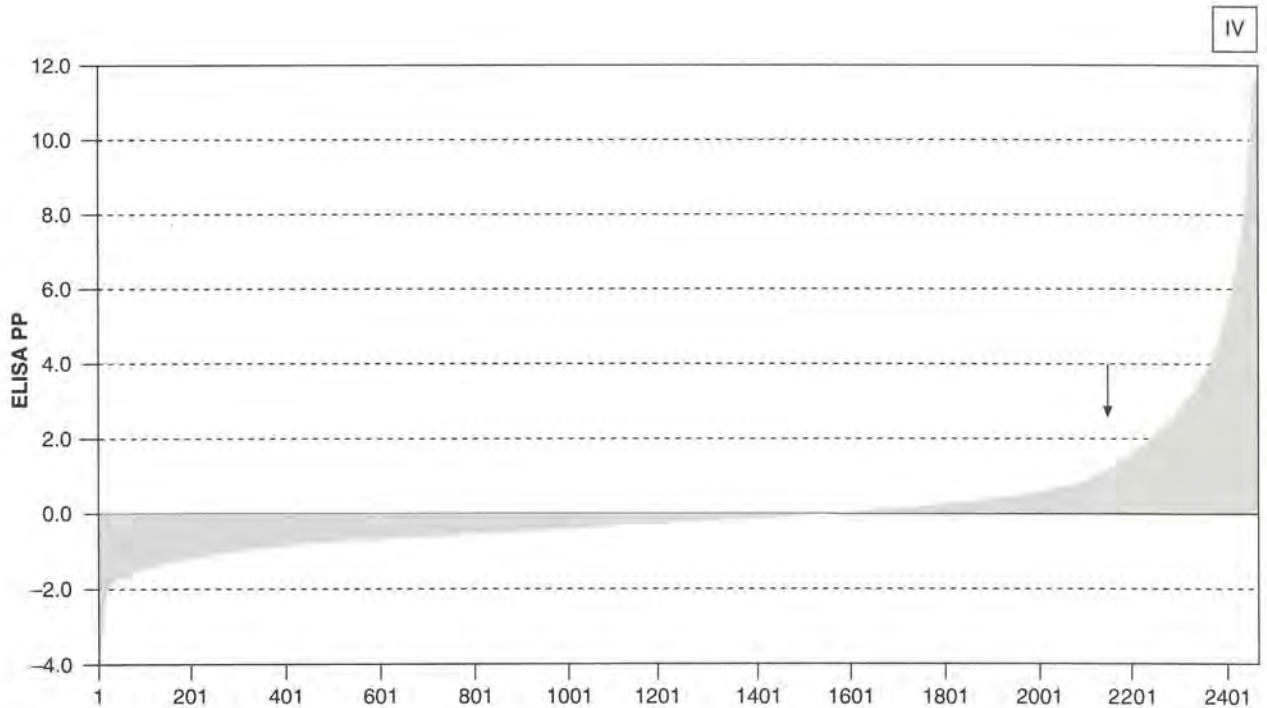
IV

FIG. 4   Distribution of ELISA PP values in 2466 individual South African cattle sera known to be negative in RBT, SAT and CFT (sera selected from panel V) for antibodies against *Brucella abortus*. Sere were taken from animals of unknown brucellosis vaccination or infection status

Sera ordered according to ELISA PP values animals. A vertical line crossing the shaded area indicates the optimal ELISA cut off determined as 1 % of high positive control serum when testing overseas reference animals
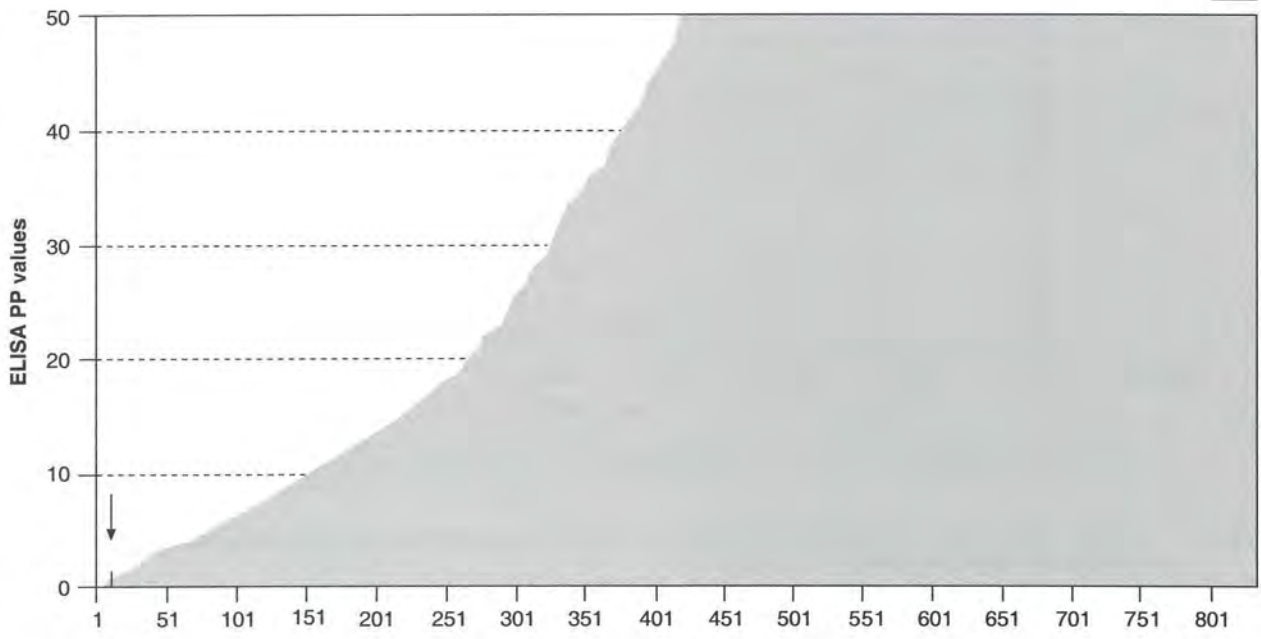
IV

FIG. 5 Distribution of ELISA PP values in 834 individual sera collected from cattle in South Africa that tested positive in CFT for antibodies against *Brucella abortus* (panel VI). Sera were taken from animals of unknown brucellosis infection or vaccination status

Sera ordered according to ELISA PP values. A vertical line on extreme left crossing the shaded area indicates the optimal ELISA cut off value determined as 1% of high positive (PP value) control serum when testing overseas reference sera collected from known *B. abortus*-negative and *B. abortus*-infected animals
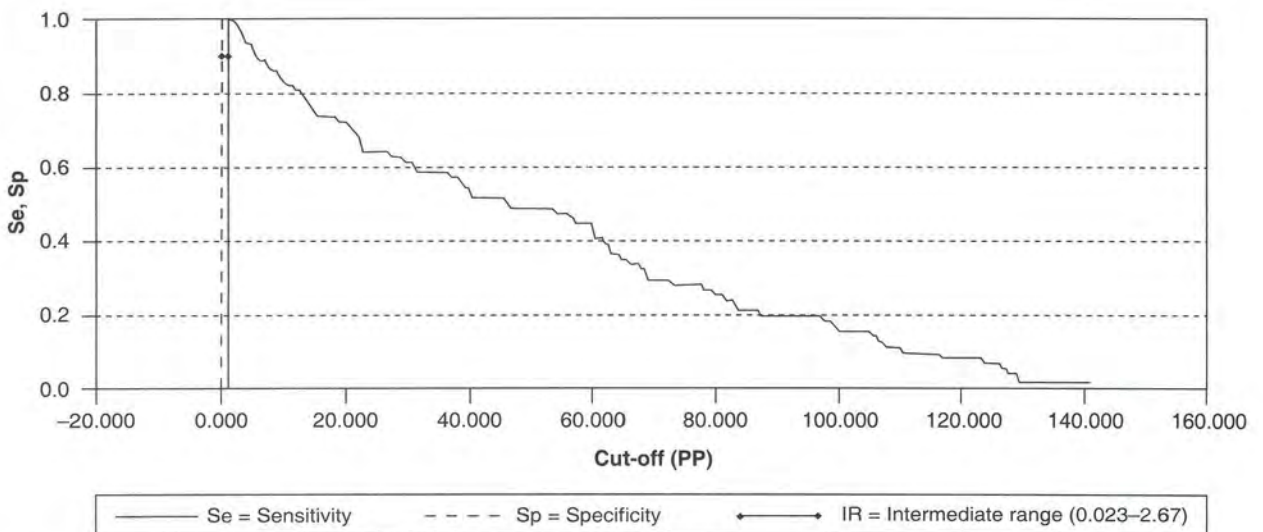

FIG. 6 Selection of cut-off by TG-ROC analysis for the I-ELISA based on results from overseas reference brucellosis uninfected (panel 1) and infected (panel II) animals

The insertion point of the sensitivity (Se) and specificity (Sp) graphs represents a cut-off PP value at which equivalent test parameters (Se = Sp) are achieved. Using two cut-off values (solid vertical lines) as limits of an intermediate range (IR), 95% Se and Sp is achieved if results to the right of IR were considered positive and those to the left were considered negative
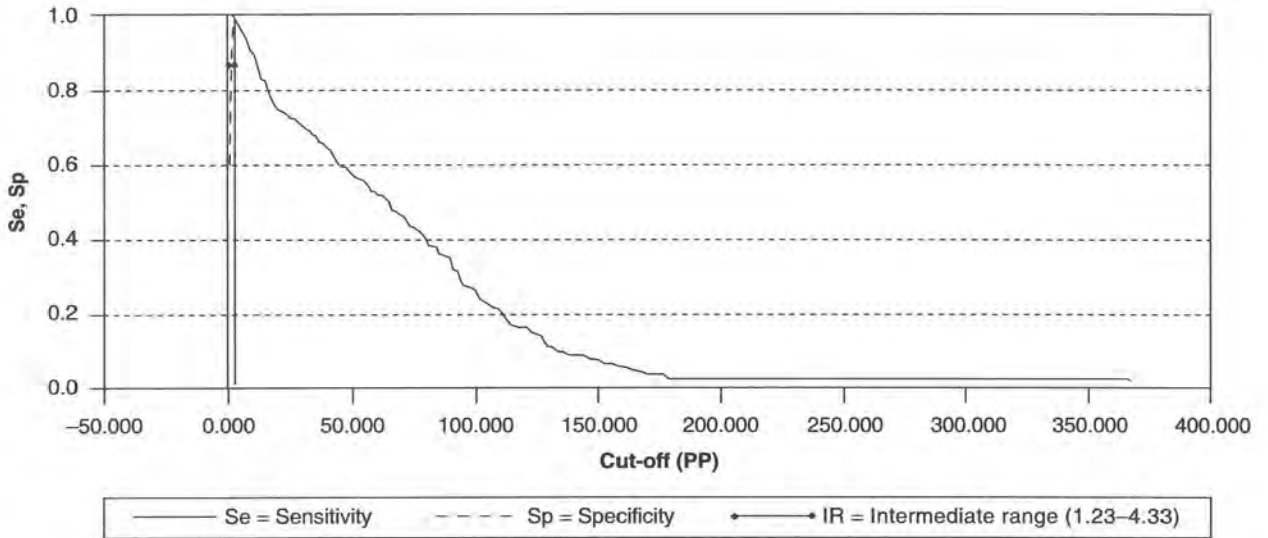
FIG. 7   Selection of cut-off by TG-ROC analysis for the I-ELISA based on results from South African cattle tested negative (panel V) and positive (panel VI) in the RBT, SAT and CFT

The insertion point of the sensitivity (Se) and specificity (Sp) graphs represents a cut-off PP value at which equivalent test parameters (Se = Sp) are achieved. Using two cut-off values (solid vertical lines) as limits of an intermediate range (IR), 95 % Se and Sp is achieved if results to the right of IR were considered positive and those to the left were considered negative
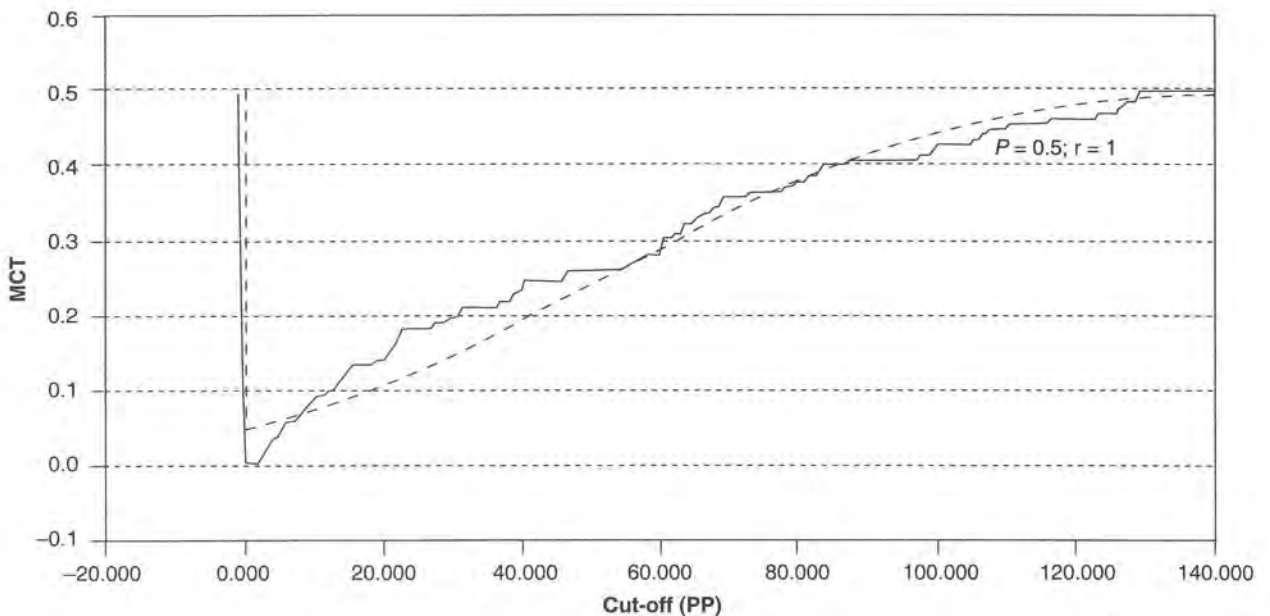

FIG. 8   Optimisation of cut-off for the I-ELISA using the misclassification cost term (MCT). Sensitivity (Se) and specificity (Sp) estimates were calculated from results in reference overseas cattle known to be brucellosis uninfected (panel I) and infected (panel II)

Cut-off values of approximately 0.1–3.0 are threshold points at which MCT becomes minimal under assumption of 50 % prevalence ($P = 0.5$) and equal costs of false-positive and false-negative test results ($r = 1$). The two curves represent MCT values based on non-parametric (empirical graph) or parametric (function graph) estimates of sensitivity and specificity, respectively
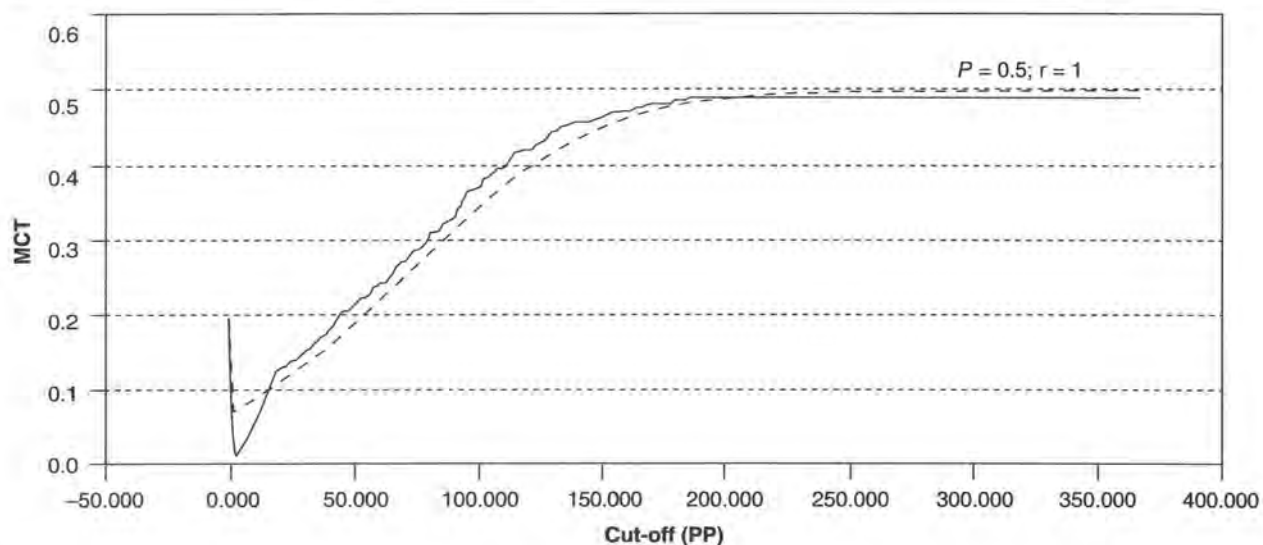
FIG. 9   Optimisation of cut-off for the I-ELISA using the misclassification cost term (MCT). Sensitivity (Se) and specificity (Sp) estimates were calculated from results in South African cattle tested negative (panel V) and positive (panel VI) in the RBT, SAT and CFT

Cut-off values of approximately 1.5-4.5 are threshold points at which MCT becomes minimal under assumption of 50 % prevalence ($P = 0.5$) and equal costs of false-positive and false-negative test results ($r = 1$). The two curves represent MCT values based on non-parametric (empirical graph) or parametric (function graph) estimates of sensitivity and specificity, respectively

TABLE 1 Comparison between the I-ELISA using different cut-off values and the CFT on overseas sera from *Brucella*-free (panel I), *Brucella*-infected (II), cattle experimentally infected with *B. abortus* (III), and from South African cattle of unknown brucellosis infection or vaccination status that tested (V) negative (V) or positive (VI) for antibodies against *Brucella abortus* in the CFT

Serum panel			I-ELISA cut-off value[1]			CFT
			10 PP	5 PP	1 PP	
I	$n = 834$	D-Sp[2] (%)	100	100	99.8	100
II	$n = 72$	D-Sn[2] (%)	81.9	91.7	100	83.3
III	$n = 71$	D-Sn	54.9	64.8	88.7	85.9
V	$n = 2\,709$	D-Sp	98.0	94.6	83.1	100
VI	$n = 834$	D-Sn	81.9	89.8	98.9	100
Total D-Sp			198.0	194.6	182.9	200
Total D-Sn			218.7	246.3	287.6	269.2
Total sum of D-Sp & D-Sn			416.7	440.9	470.5	469.2

[1]   ELISA cut-off expressed as a percentage of high positive (PP) control serum
[2]   Calculation of diagnostic specificity (D-Sp) and diagnostic sensitivity (D-Sn) aided by 2 x 2 table

10 PP for panel V (Table 1). Of the total of 2709 sera negative in CFT (panel V), 457 (16.9 %), 145 (5.4 %) and 55 (2 %) were positive by I-ELISA at 10, 5 and 1 PP cut-off values, respectively. Of these sera 237 (8.7 %) were positive in the RBT and 165 (6.1 %) in the SAT (Table 2). Of 100 Canadian sera positive in C-ELISA (panel IV) 73, 81, and 94 were also positive by I-ELISA at 10, 5, and 1 PP cut-off

values, respectively. Of these sera, 88 were positive in the CFT (Table 3). When using panel VII, the best estimate of the relative D-Sn of I-ELISA compared to C-ELISA was at 1 PP, and compared to the CFT it was at 5 PP (Table 3).

Regardless of the statistical approaches used, the data on cut-off selection suggest that 1 PP value

would provide the most optimum decision limit for I-ELISA as well as the highest level of agreement especially with the C-ELISA. The relatively very low cut-off for I-ELISA is most possibly due to the fact that a very strong positive control standard (OPNS2 serum) was used for data normalisation (Fig. 2).

Relative sensitivity and specificity

Estimates of the relative D-Sn and D-Sp of I-ELISA compared to CFT based on results from panel V & VI are presented in Table 1. When using the 1 PP cut-off value the relative D-Sn of the I-ELISA was the highest (98.9 %) but the D-Sp the lowest (83.1 %); at 10 PP the D-Sn was the lowest (81.9 %) but the D-Sp the highest (98 %). The relative D-Sn of I-ELISA compared to C-ELISA (panel IV) was the highest at 1 PP (94 %). The relative D-Sn of the CFT based on results from the same panel was 88 % (Table 3).

Validity

The best estimates of D-Sn (100 %) and D-Sp (99.8 %) for I-ELISA were obtained when the 1 PP value was applied to assess its diagnostic performance in a true negative (panel I) and a true positive (panel II) population of cattle. When analysing results from the same panels the D-Sn of the CFT was 83.3 % and D-Sp was 100 % (Table 4). Also estimates of Youden's index, which were calculated on reactivity of panel I and II, indicate better diagnostic performance of I-ELISA than the CFT (Table 4)

Vaccinated cattle

Of the total of 183 sera from vaccinated cattle, 8 (3.5 %), 18 (7.9 %) and 58 (25.9 %) tested positive in I-ELISA at 10, 5 and 1 PP cut-off value, respectively. Of these, 63 (27.6 %) were positive in the CFT (Table 5). The I-ELISA PP values in panel VIII (Fig. 10B) were in general low or very low compared to that of panel II (Fig. 10A); most sera were below 10 PP and none was > 31 PP. Although, a limited number of reference sera was tested, present data suggest that I-ELISA could be of use in identifying some cattle naturally infected with *B. abortus*. In contrast, no efficient differentiation is possible between serum panels II (Fig. 11A) and VIII (Fig. 11B) based on CFT titres.

TABLE 2 Comparison between the I-ELISA using different cut-off values, RBT and SAT on 2709 sera collected from South African cattle of unknown brucellosis infection or vaccination status that tested negative for antibodies against *Brucella abortus* in the CFT (panel V, n = 2 709)

Test	No. tested		Relative diagnostic specificity (%)
	Positive	Negative	
I ELISA			
10 PP[1]	55	2654	97.96
5 PP	145	2564	94.65
1 PP	457	2252	83.13
RBT	237	2472	91.36
SAT	165	2544	93.9

[1] ELISA cut-off expressed as a percentage of high positive (PP) control serum

TABLE 3 Comparison between the I-ELISA, CFT, and C-ELISA on Canadian cattle sera know to be positive in the C-ELISA (panel IV) and sera from South African cattle of unknown brucellosis infection or vaccination status re-tested in Canada in the C-ELISA (panel VII)

Serum panel	I-ELISA Cut-off[1]			CFT	C-ELISA
	10 PP1	5 PP	1 PP		
IV $n = 100$					
No. positive	73	81	94	88	100
No. negative	27	19	6	12	0
Percentage positive	73	81	94	88	100
Percentage negative	27	19	6	12	0
Relative D-Sn[2] (%)	73	81	94	88	
VII $n = 258$					
No. positive	102	136	183	148	197
No. negative	156	122	75	110	61
Percentage positive	39.5	52.7	70.9	57.4	76.4
Percentage negative	60.5	47.3	29.1	42.6	23.6
Relative D-Sn[2] (%)	51.5	69.0	92.9	75.1	

[1] ELISA cut-off expressed as a percentage of high positive (PP) control serum
[2] Diagnostic sensitivity

Note: Shaded area of table   Tests done at Onderstepoort Veterinary Institute, South Africa

Unshaded area of table   Test done at Canadian Food Inspection Agency, Ontario, Canada

DISCUSSION

It is necessary to first determine the diagnostic threshold or cut-off value that will separate the positive from the negative animals for the specific test to be used. Sera from true non-infected and true infected animals are ideally required to calculate

the real diagnostic sensitivity and specificity of a serological test in a specific area. The first should be from animals that have never had contact with the pathogen, and the second from animals with proven infection based on isolation of the aetiological agent of the disease of interest (Jacobson 1998; 2000).

Since such panels of South African cattle sera were unavailable during this study, overseas sera collected from both non-vaccinated brucellosis-free and from *B. abortus*-infected cattle were used. Additionally, relative standards of comparison, including results from other serological assays, from experimentally infected and from vaccinated animals were used.

TABLE 4 Comparison of diagnostic accuracy of the I-ELISA and CFT for the detection of antibodies against *Brucella abortus* in cattle sera. Validation data were generated from testing of 906 overseas reference sera collected from non-vaccinated, brucellosis- free (panel I, *n* = 834) and naturally infected animals with *B. abortus* (panel II, *n* = 72)

Test	Measures of diagnostic accuracy		
	D-Sn[a]	D-Sp[b]	J[c]
I-ELISA	100 %	99.8 %	0.998
CFT	83.3 %	100 %	0.833

Formulas applied for statistical approaches (Gardner & Greiner, 2000):

[a] D-Sn (Diagnostic sensitivity) = $TP^1/(TP + FN^4)$
[b] D-Sp (Diagnostic specificity) = $TN^3/(TN + FP^2)$
[c] J (Youden's index) = D-Sn + (D-Sp-1)

Where TP[1] = True positive
FP[2] = False positive
TN[3] = True negative
FN[4] = False negative

Visual inspection of the frequency distribution graphs and comparison of I-ELISA with the other tests suggests that the cut-off between positivity and negativity be 1 PP. To obtain a more accurate selection of the cut-off values for this assay, TG-ROC and MCT analyses were performed. These analyses clearly demonstrated that depending on the serum panel used, different cut-off values should be applied to maximise test sensitivity and specificity, and to minimise the costs of false positive results. The intermediate ranges as well as the most minimal MCT values derived from overseas panels covered lower PP values than those derived

TABLE 5 Comparison between the I-ELISA and CFT on 183 sera (panel VIII) collected from cattle vaccinated with *Brucella abortus* strain-19

Months post vaccination and No. tested	Test result	ELISA PP values			CFT
		10 PP[1]	5 PP	1 PP	
1–2 *n* = 70	Positive	4 (5.7 %)	11 (15.7 %)	40 (57.1 %)	50 (71.4 %)
	Negative	66 (94.3 %)	59 (84.3 %)	30 (42.9 %)	20 (28.6 %)
3–4 *n* = 43	Positive	0	0	7 (16.3 %)	2 (4.7 %)
	Negative	43 (100 %)	43 (100 %)	36 (83.7 %)	41 (95.3 %)
5–8 *n* = 28	Positive	2 (7.1 %)	5 (17.9 %)	7 (25.0 %)	9 (32.1 %)
	Negative	26 (92.9 %)	23 (82.1 %)	21 (75.0 %)	19 (67.9 %)
9–12 *n* = 16	Positive	1 (6.3 %)	1 (6.3 %)	3 (18.7 %)	1 (6.3 %)
	Negative	15 (93.7 %)	15 (93.7 %)	13 (81.3 %)	15 (93.7 %)
14–18 *n* = 16	Positive	1 (6.3 %)	1 (6.3 %)	1 (6.3 %)	1 (6.3 %)
	Negative	15 (93.7 %)	15 (93.7 %)	15 (93.7 %)	15 (93.7 %)
19–39 *n* = 10	Positive	0	0	0	0
	Negative	0	0	0	0
Total tested *n* = 183	Positive	8 (4.4 %)	18 (9.8 %)	58 (31.7 %)	63 (34.4 %)
	Negative	175 (95.6 %)	165 (90.2 %)	125 (68.3 %)	120 (65.6 %)

[1] ELISA cut-off expressed as a percentage of high positive (PP) control serum
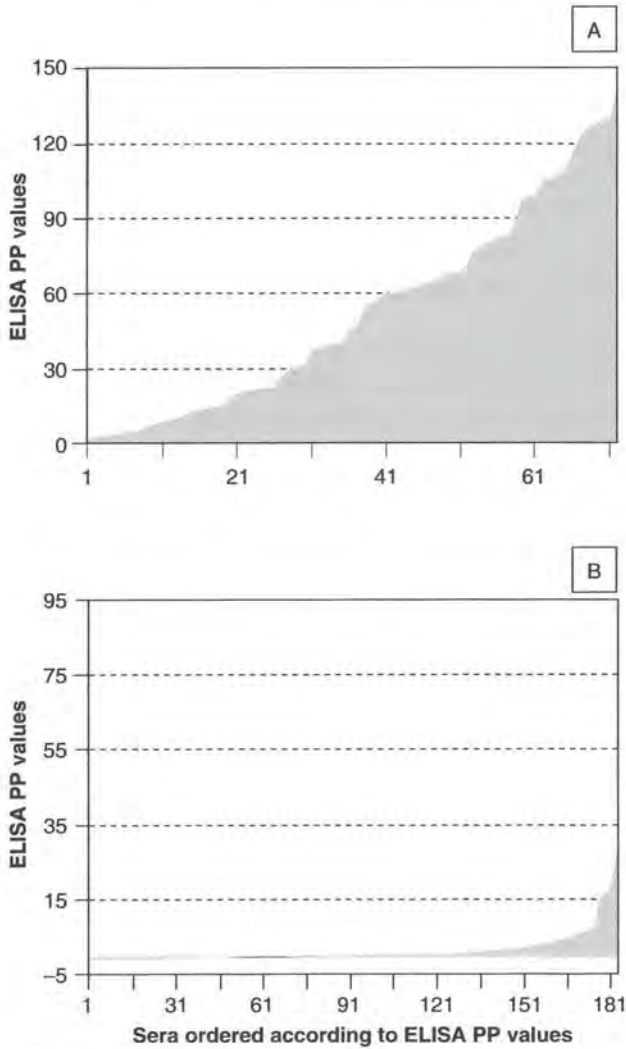
FIG. 10 Distribution of the I-ELISA PP values in (A) sera from cattle naturally Infected with *Brucella abortus* (panel II, *n* = 72) and (B) in sera from cattle vaccinated with *B. abortus* strain 19 (panel VIII, *n* = 183)

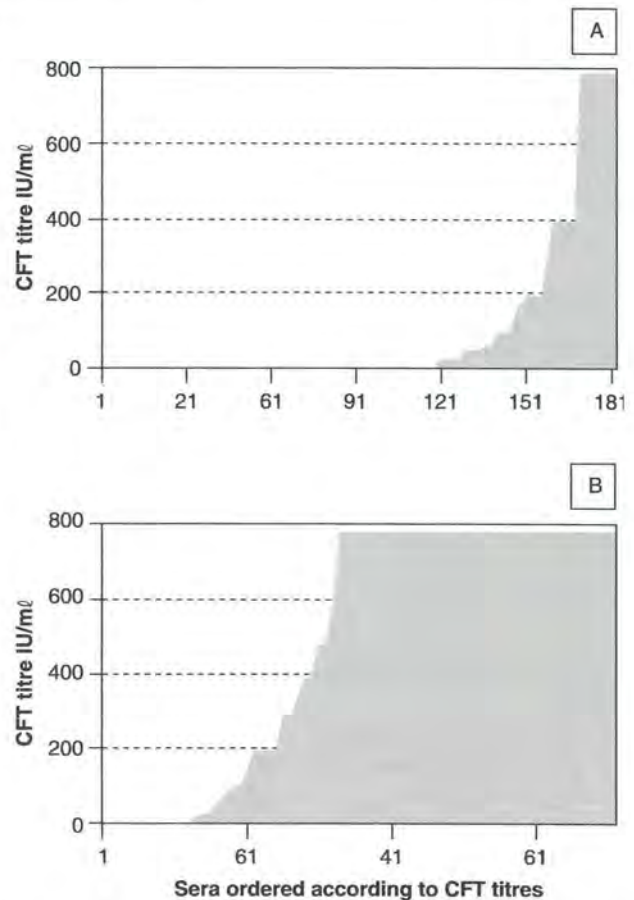Sera ordered according to ELISA PP values

FIG. 11 Distribution of the CFT titers in (A) sera from cattle naturally infected with *Brucella abortus* (panel II, *n* = 72), and (B) in sera from cattle vaccinated with *B. abortus* strain 19 (panel VII, *n* = 183)

Sera ordered according to CFT titres

from local panels. The statistically significant difference found in the distribution of I-ELISA absorbance values in the defined negative populations of sera tested also indicates that different cut-off values should be used when animals from geographically distinct origins are tested. The greater overlap between South African negative and positive sera than that found between overseas panels most probably was due to different criteria used in classifying these sera as negative and positive. The South African sera were mostly from animals of unknown *B. abortus* vaccination and infection status and were classified according to the RBT, SAT and CFT reactions. It has been shown that these

tests are less sensitive and specific than I-ELISA (Nielsen *et al.* 1998). Therefore, these tests when used to define sera can affect the optimisation of the cut-off values of the assay being validated. A better separation between the negative and positive sera by I-ELISA was demonstrated for overseas panels. This is not surprising, as the serum samples were selected based on true negativity or proven exposure to *B. abortus*.

In this study the CFT (current gold standard) gave an unacceptably high false negative rate (12 %) when testing sera from animals with proven infection status against *B. abortus*. When using 1 PP

value as a cut-off, the I-ELISA correctly classified all the sera as positive. When 1 PP value was applied to the overseas negative panel, the ELISA produced a very low rate of false positives (0.2 %) but all the sera would test negative when a higher cut-off value was applied. Nielsen *et al.* (1998) reported the same estimates of diagnostic sensitivity (100%) and specificity (98.8%) for I-ELISA. By lowering PP cut-off values similar results, i.e. higher estimates of sensitivity but lower estimates of specificity, were also obtained when testing other panels.

Regardless of the statistical approaches used in this study, the optimum cut-off values established for I-ELISA ranged from 0.11–5.5 PP. The cut-off values chosen for an ELISA are flexible and may vary not only between target populations but also for different diagnostic purposes (Jacobson 1996). Intuitively, one would be cautious with a diagnostic decision if a test result falls very close to a cut-off value. From a clinical viewpoint, it is reasonable (and a common practice) to refer to such results as "borderline" or "intermediate". Confirmation of the diagnosis by re-testing after a certain time interval will often clarify the matter. Selection of the actual cut-off will also be mandated by practical realities. For example, the cut-off may need to be lowered to assure that the test sensitivity approaches 100% so as not to misclassify any infected animals because false negative results for a disease with devastating economic and animal health consequences are unacceptable.

The following considerations may explain why such relatively low cut-off PP values were established for the I-ELISA:

- Hyperimmune animals are not considered to be typical, and should be avoided for use as positive control sera. Exceptionally, naturally infected animals may be used as the source of the standard.

- A positive reference standard for I-ELISA should be selected from animals experimentally infected or vaccinated.

- Animals exhibiting a typical humoral immune response to the organism in question should be used.

- The strong reference standard should demonstrate typical sigmoidal dose/response and should represent a binding antibody concentration (absorbance value) mid-way between the upper and central points of the linear portion of the curve (OIE 1998; Wright *et al.* 1993).

The high positive serum control used in the I-ELISA is a pooled mixture of sera collected from two cows, which aborted following field infection with *B. abortus*. It was originally selected and standardised for use in the CFT. This standard represented the strongest positive serum compared to other reference sera tested. At the dilution used in the test (upper point of the dose/response curve), it represents a very high concentration of binding-antibody. Due to inherent differences amongst assay systems, binding-antibody levels should be expressed in relative rather than absolute terms. It is recommended that for the indirect ELISA the OD value of each sample be expressed as a percentage of a high positive reference standard. This relative measurement based on the inclusion of a single positive reference standard, results in a uniform and continuous scale of 0–100 % positivity (OIE 1998; Wright *et al.* 1993). Thus, using too strong positive control for normalisation of OD values will result in relatively low PP values of test samples.

Very high estimates of the I-ELISA D-Sn and D-Sp and Youden 's index were established when 1 PP value was used in discriminating between reference negative and positive overseas sera. While this cut-off may not necessarily be optimal for local use. This study clearly demonstrates the potential superiority of this ELISAs' diagnostic accuracy over that of the CFT.

The estimates of relative D-Sn and D-Sp, calculated for the various tests used in this study, varied depending on serum panels analysed. The I-ELISA was more sensitive than the CFT when compared to the C-ELISA but less sensitive compared to C-ELISA. As mentioned above when relative standards of comparison are used, estimates of D-Sn and D-Sp for the new assay may be compromised because the error in the estimates of diagnostic accuracy for relative standards is carried over into those estimates for the new assay. Although the C-ELISA was shown to have very high estimates of D-Sn and D-Sp (Nielsen *et al.* 1998), the cut-off for this test as used in Canada may not necessarily provide the optimum diagnostic threshold for local conditions.

From the I-ELISA data it is clear that there is an increased number of reactors compared to the CFT. This may be explained by the use of Protein G conjugate, which measures both immunoglobulin isotypes IgG1 and IgG2 instead of only IgG1 detected by the CFT. In the same way, the I-ELISA detected a higher number of positive samples in comparison to the CFT due to the fact that the

I-ELISA detects IgG1 in lower amounts than the CFT. This could be very important in areas where this immunoglobulin can be related to infection because earlier stages of infection can be detected. However, in herds vaccinated with *B. abortus* S-19 strain, IgG1 could be the remainder of antibody due to immunisation.

In this study the cut-off value of the I-ELISA optimised for the discrimination between non-infected and infected animals did not separate vaccinated from naturally infected animals. Only a limited number of post-vaccination sera were, however, tested. Their reactivity, as measured by the I-ELISA, was always below 31 PP value. This may indicate differing responses by cattle to live attenuated vaccine versus field infection. It would be worthwhile to investigate whether this cut-off value would not provide a rapid screening tool for identifying some naturally infected animals. It has been demonstrated in other countries where S-19 vaccination is practiced that a different cut-off value for I-ELISA should be used depending on whether the population to be tested has been vaccinated or not (Abalos, Pinochet, Ibarra & Navia 1998; Peraza, Valdes, Fonseca, Izquierdo, Garcia & Alvarez 1998). Currently available PCR technology for the detection of *B. abortus* could be of great help in further validating this assumption.

CONCLUSIONS AND RECOMMENDATIONS

- The automated format of I-ELISA provides a rapid, simple, highly sensitive and specific diagnostic system for the large-scale detection of antibodies against *B. abortus*. It requires only small amounts of reagents, sera need not be heat inactivated, it is much easier to standardise than the CFT, and finally it measures reactivity objectively, which reduces reading errors.

- Considering present validation data, the following interpretation of results is recommended for use in South Africa:

  - When no detailed information about epidemiological status (e.g. vaccination, disease prevalence in a herd) is available, results between 1–5 PP values should be regarded as suspect and the animals in question subjected to re-testing

  - Results of $\geq$ 5 PP value should be regarded as positive

  - Results of < 1 PP should be regarded as negative.

- While the diagnostic accuracy of the I-ELISA appears to be very high, it cannot definitely distinguish between the antibody response induced by vaccination with *B. abortus* strain 19 and that resulting from natural infection. Although, only limited sera from reference vaccinated and infected cattle were tested, results of this study suggest that a cut-off value of >31 PP may indicate a natural exposure to *B. abortus*.

- Based on the specificity and sensitivity reported here, there is a strong indication that the I-ELISA may replace the traditional serological assays, CFT, RBT and SAT currently in use. It will be of particular use in areas where little epidemiological information is available about the disease and where large numbers of sera need to be tested to obtain this information.

- The validation of the in-house developed I-ELISA should be further expanded to achieve fully validated status, and especially more reference sera should be tested to clarify its possible use in identifying cattle naturally infected with *B. abortus*.

REFERENCES

ABALOS, P., PINOCHET, L., IBARRA, L. & NAVIA, F. 1998. Use of an indirect enzyme linked immunosorbent assay for the diagnosis and epidemiology studies of *Brucella abortus* in Chile, in *Diagnosis and epidemiology of animal disease in Latin America*, IAEA-TECDOC-1055: 49–54.

ABALOS, P., IBARARA, L., PINOCHET, L., NAVIA, F. & BOISIER, X. 1996. Residual anti-*Brucella* strain 19 antibodies detected in adult cattle by two indirect-ELISA tests. *The Veterinary Record*, 138:140–142.

ALTON, G.G. 1978. Recent development in vaccination against bovine brucellosis. *Australian Veterinary Journal*, 54:551–557.

CARRASCO, E.A., UZAL, F.A. & ECHAIDE, S. 1998. Serological response to an indirect and a competitive ELISA in

heifers vaccinated with *Brucella abortus* strain 19, in *Diagnosis and epidemiology of animal disease in Latin America*, IAEA-TECDOC-1055: 83–88.

CORBEL, M.J. & MACMILLAN, A.P. 1996. Bovine brucellosis, in *OIE Manual of standards for diagnostic tests and vaccines*, 3rd ed.

GALL, D., COLLING, A., MARINO, O., MORENO, E., NIELSEN, K., PEREZ, B. & SAMARTINO, L. 1998. Enzyme immunoassays for the diagnosis of bovine brucellosis, in *Diagnosis and epidemiology of animal disease in Latin America*, IAEA-TECDOC-1055: 113–129.

GREINER, M. 1995. Two-graph receiver operating characteristics (TG-ROC)—a Microsoft-Excel template for the selection of cut-off values in diagnostic tests. *Journal of Immunological Methods*, 185:145–146.

GREINER, M. 1996. Two-graph operating characteristics (TG-ROC): update version supports optimisation of cut-off values that minimise overall misclassification costs. *Journal of Immunological Methods*, 191:93–94.

GREINER, M. & GARDNER, I. A. 2000. Epidemiological issues in the validation of veterinary diagnostic tests. *Preventive Veterinary Medicine*, 45: 3–22.

GREINER, M., SOHR, D. & GÖBEL, P. 1995. A modified ROC analysis for the selection of cut-off values and the definition of intermediate results of serodiagnostic tests. *Journal of Immunological Methods*, 185:123–132.

HERR, S., TE BRUGGE, L.A. & GUINEY, M.C.M. 1982. The value of the microtitre serum agglutination test as a second screening test in bovine brucellosis. *Onderstepoort Journal of Veterinary Research*, 49:23–28.

HERR, S., EHRET, W.J., RIBEIRO, L.M.M. & CHAPARRO, F. 1990. *Journal of the South African Veterinary Association*, 6: 11–13.

HERR, S., HUCHZERMEYER, H.F.K.A., TE BRUGGE, L.A., WILIAMSON, C.C., ROOS, J.A. & SCHIELE, G.J. 1985. The use of a single complement fixation test technique in bovine brucellosis, Johne's disease, dourine, equine piroplasmosis and Q fever serology. *Onderstepoort Journal of Veterinary Research*, 52:279–282.

IAEA-TECDOC-1055. 1988. *Diagnosis and epidemiology of animal disease in Latin America*, November 1998.

JACOBSON, R.H. 1998. Factors in selecting serum samples for use in determining the positive/negative threshold (cut-off) in ELISA, in *Diagnosis and epidemiology of animal disease in Latin America*, IAEA-TECDOC-1055:25–28.

JACOBSON, R.H. 2000. Principles of validation of diagnostic assays for infectious diseases, in *OIE Manual of standards for diagnostic tests and vaccines*, 4th ed.

KRIGE, J. 1981. Department of Agriculture and Fisheries, SA, Memorandum 12/1/8/6/B of 1981/07/30.

MACMILLAN, A. 1990. Conventional serological tests, in *Animal brucellosis*, edited by K. Nielsen & J.R. Duncan. Boca Raton: CRC Press: 153–197.

MACMILLAN, A.P. & STACK, J. 2000. Bovine brucellosis, in *OIE Manual of standards for diagnostic tests and vaccines*, 4th ed.

MACMILLAN, A., GREISER-WILKE, I., MOENNING, V. & MATHIADIS, L.A. 1990. A competitive enzyme immuno-

assay for brucellosis diagnosis. *Deutche Tierärztliche Wochenschrift*, 97:83–85.

NAGY, L.K., HIGNET, P.G. & IRONSIDE, C.J.T. 1967. Bovine brucellosis; a study of an adult vaccinated, *Brucella* infected herd. *The Veterinary Record*, 81:140–144.

NIELSEN, K., KELLY, L., GALL, D., BALSEVICIUS, S., BOSSE, J. & KELLY, W. 1998. Comparison of enzyme immunoassays for the diagnosis of bovine brucellosis, in *Diagnosis and epidemiology of animal disease in Latin America*, IAEA-TECDOC-1055: 31–44.

NIELSEN, K., KELLY, W., GALL, D., BALSEVICIUS, S., BOSSE, J., NICOLETTI, P., KELLY, W. & EHENNING, D. 1996. Comparison of enzyme immunoassays for diagnosis of bovine brucellosis. *Preventive Veterinary Medicine* 26:17–32.

NIELSEN, K., KELLY, L., GALL, D., NICOLETTI, P. & KELLY, W. 1995. Improved competitive enzyme immunoassay for the diagnosis of bovine brucellosis. *Veterinary Immunology and Immunopathology*, 46:285–291.

OIE 1998. OIE guidelines for international reference standards for antibody assays, in *Report of the meeting of the OIE Standards Commission*, Paris, 18–20 February 1998: 15–19.

PERAZA, C., VALDES, O., FONSECA, N., IZQUIERDO, D.L., GARCIA, M. & ALVAREZ, M. 1998. Use of an indirect ELISA for *Brucella abortus* diagnosis in Cuba, in *Diagnosis and epidemiology of animal disease in Latin America*, IAEA-TECDOC-1055: 89–94.

PLACKETT, P. & ALTON, G.G. 1975. A mechanism for prozone formation in the complement fixation test for bovine brucellosis. *Australian Veterinary Journal*, 52:136–140.

ROBERTSON, F.J. 1971. Brucellosis: a possible symptomless carrier. *The Veterinary Record*, 88:313–314.

AUSTRALIAN SUB-COMMITTEE OF PRINCIPAL VETERINARY LABORATORY OFFICERS TO THE ANIMAL HEALTH COMMITTEE. Standardised Rose Bengal test for bovine brucellosis. *Australian Veterinary Journal*, 56:555.

STITES, D.P. & RODGERS, R.P.C. 1991. Clinical laboratory methods for detection of antigen and antibodies, in *Basic and clinical immunology*, edited by D.P Stites & A.I. Terr, East Norwalk. Appleton & Lange.

SUTHERLAND, S.S. 1980. Immunology of bovine brucellosis. *The Veterinary Record*, 50:359–368.

THRUSFIELD, M. 1995. Diagnostic testing, in *Veterinary epidemiology*, 2nd ed. Blackwell Science Ltd.

WORTHINGTON, R.W. 1982. Serology as an aid to diagnosis: uses and abuses. *New Zealand Veterinary Journal*, 30:93–97.

WRIGHT, P.F., NIELSEN, K. & KELLY, W. 1990. Primary binding assay techniques for the serodiagnosis of bovine brucellosis – enzyme immunoassay, in *Animal brucellosis*, edited by K. Nielsen & J.R. Duncan Boca Raton: CRC Press.

WRIGHT, P.F., NILSSON, E., VAN ROOIJ, E.M.A., LELENTA, M. & JEGGO, M.H. 1993. Standardisation and validation of enzyme-linked immunosorbent assay techniques for the detection of antibody in infectious disease diagnosis. *Revue Scientifique et technique Office International des Epizooties*, 12:435–450.