

COMPARISON OF ENZYME IMMUNOASSAYS FOR THE DIAGNOSIS OF BOVINE BRUCELLOSIS



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Abstract

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The indirect enzyme immunoassay for measurement of bovine antibody to *Brucella abortus* was tested on 15,716 Canadian sera to assess the specificity. These sera were also tested by the buffered plate antigen test. Two ELISA formats were used for assessment of data: the targeting procedure using a positive control serum allowed to develop to an optical density of 1.0 and the use of a positive control serum to determine relative positivity at a set time. Two different cut-off values were also assessed for each assay.

A total of 763 sera gave reactions above established cut-off values in the ELISA while 216 were positive in the buffered plate antigen test (BPAT). A modification of the indirect ELISA employed divalent cation chelating agents (EDTA/EGTA) incorporated into the serum incubation stage to eliminate some non-specific reactions. This method was applied only to the 763 indirect ELISA reactor sera and it eliminated all but 93 or 37, depending on the cut-off selected, of the reactions. Sensitivity was assessed by testing 424 sera from *Brucella abortus* culture positive cattle. The indirect ELISA classified all 424 sera as positive by either method of data handling and with or without addition of EDTA/EGTA for a specificity estimate of 100%. In the BPAT, 412 sera gave a positive agglutination reaction. Ten percent of the 15,716 sera were randomly selected and tested by two different competitive ELISAs and by the complement fixation test (CFT). One competitive ELISA used *Brucella abortus* O-polysaccharide as the antigen and an enzyme conjugated monoclonal antibody to the O-polysaccharide for competition and detection. Of the sera tested, 34 gave false positive reactions. On a retest, the false positive reactions were reduced to 2. The second competitive ELISA used lipopolysaccharide as the antigen, a different monoclonal antibody but also specific for the O-polysaccharide for competition and commercially available goat anti-mouse IgG enzyme conjugate for detection. In the initial assessment, this test gave rise to 5 false positive reactions. This number was reduced to 2 when retesting the sera. The CFT used was a micro format test and 3 sera gave false positive reactions. A total of 654 sera from animals from which *Brucella abortus* was isolated were tested by the first competitive ELISA. Of these, 9 sera were negative on the initial test. This number was reduced to 3 on repeat testing. All 636 sera tested by the second competitive ELISA were positive. Fifteen of 636 sera gave sufficient prozoning in the complement fixation test to be considered diagnostically negative and 59 sera were anticomplementary. Sensitivity and specificity are summarized in Table I. Cut-off values for each assay were initially established by visual observation of frequency distributions of positive and negative serum samples. These cut-off values were confirmed by receiver operating characteristics (ROC) analysis. In addition, an index of performance (accuracy) was established for each assay in order to allow direct comparison. Accuracy estimates were based on the sensitivity, specificity and disease prevalence for the data.

TABLE I. SENSITIVITY AND SPECIFICITY FOR DIFFERENT TESTS

Test	Sensitivity %	Specificity %
BPAT	97.9	98.6
CFT (AC reactors +)	97.1	93.1
CFT (AC reactors -)	87.9	99.8
I-ELISA-T 0.460 cut-off	100	96.0
I-ELISA-T 0.607 cut-off	100	98.5
I-ELISA-%P 46% cut-off	100	95.9
I-ELISA-%P 62% cut-off	100	98.5
I-ELISA EDTA/EGTA 0.460 cut-off	100	99.4
I-ELISA EDTA/EGTA 0.607 cut-off	100	99.8
C-ELISA (O-polysac)	98.6	97.7
C-ELISA (LPS)	100	99.7

1. INTRODUCTION

A number of enzyme immunoassays have been applied to the diagnosis of bovine brucellosis. Two main types of immunoassays used for these purposes, the indirect and the competitive formats, have recently been reviewed [1, 2]. The most commonly used methods of controlling bovine brucellosis has been vaccination with *Brucella abortus* strain 19 [3] and the serological detection of exposure to the organism, followed by removal of the animal from the herd or depopulation of the herd. In a number of eradication programmes, conventional serological tests were used for detection of infection. In these assays, the antibody to be assessed must be capable of a secondary function, such as agglutination, activation of complement or precipitation, to be measured.

Therefore these assays measure some of the isotypes of antibody and not others [2] leading to the use of panels of tests for more accurate diagnosis. This led to the development of serological screening tests of high sensitivity and lower specificity, such as the buffered plate antigen test - BPAT [4]. A positive reaction in the screening test led to the serum being tested in a number of confirmatory tests, such as the complement fixation tests [5] or the 2-mercaptoethanol modification of the tube agglutination test [6]. This arrangement is costly and the time between submission and output of results is long. An additional problem with these assays is that with the exception of an immunodiffusion test using a polysaccharide antigen [7], none could distinguish the antibody response due to vaccination from that resulting from infection. It was hoped that introduction of the indirect enzyme immunoassay would overcome some of these problems but in reality, until recently [8], the indirect ELISA, while more sensitive than the conventional test, has been less specific, even using highly specific monoclonal antibodies to bovine immunoglobulins as detection reagents. Similarly, the indirect ELISA could not distinguish vaccinal antibody from that arising from infection. For this reason there has been reluctance in accepting the indirect ELISA as a diagnostic test.

An additional problem has been the cost per test which was considerably higher than that of the BPAT. A competitive ELISA was developed [9] which was more specific than the indirect ELISA and which could discriminate vaccinal antibody from antibody induced by infection. This assay used O-polysaccharide prepared from lipopolysaccharide from *Brucella abortus* as the antigen and a monoclonal antibody specific for an O-polysaccharide epitope for competition. A problem with this assay was the difficulties in preparing and standardizing the antigen. This led to modifications of the antigen to make its passive absorption more uniform to the polystyrene matrix [10]. A second approach to improving the competitive ELISA has been to attempt to use lipopolysaccharide as the antigen. Lipopolysaccharide, because of the hydrophobic lipid A portion of the core region, attaches readily to polystyrene. Such a test was developed [11]. This communication compares the sensitivities and specificities of two conventional tests, the BPAT (screening test) and the complement fixation test (confirmatory test) and the indirect ELISA, with or without addition of divalent chelating agent and with data expressed by two methods and two competitive ELISAs, one using O-polysaccharide as the antigen and the other using lipopolysaccharide.

2. MATERIAL AND METHODS

2.1. Sera

The following serum samples were selected for testing as shown in Table II: Fifteen thousand seven hundred sixteen Canadian serum samples submitted for routine diagnostic testing for brucellosis were tested by the two indirect ELISA formats and by the BPAT. Of these sera, 763 gave positive reactions in the indirect ELISAs and these sera were retested using added divalent cation chelators. From the 15,716 sera, some were randomly selected for testing by the competitive ELISAs and by the complement fixation test. A number of sera from cattle from which *Brucella abortus* had been isolated from secretions or tissues were tested in all assays. Sera tested in each assay are shown in Table II. Sera that were depleted or became contaminated with bacteria resulted in different numbers of sera being tested in some of the assays.

TABLE II. NUMBER OF SERA TESTED AND RESULTS PER TEST

Test	Negative sera	Positive sera
Indirect ELISAs	15.716	424
Indirect ELISA (EDTA/EGTA)	763	636
BPAT	15.716	424
Competitive ELISA (O-polysac)	1508	654
Competitive ELISA (LPS)	1446	636
Complement fixation test	1508	654

2.2. Serological test

The BPAT was performed as described by Anderson et al., 1994 [6]. Briefly, 30 μ l of antigen was mixed with 80 μ l of serum and incubated at 37°C for 8 min. Agglutination was read by observation. The complement fixation test was described by Samagh and Boulanger [12].

All ELISA procedures used NUNC 2-69620 polystyrene plates (CIBCO-BRL, Burlington, Ontario, Canada) and unless otherwise indicated, horseradish peroxidase (type VI, Sigma Chem. Corp., St. Louis, Missouri, USA) was used to conjugate to the monoclonal antibodies by the method of Henning et al. [13]. All spectrophotometer readings were at 414 nm. The indirect ELISA format was described by Nielsen [2]. This format is a rapid screening procedure that used lipopolysaccharide [14] as the antigen, 1 μ g/ml in carbonate buffer, pH 9.6. An amount of 200 μ l of antigen was dispensed into each well of a 96 well polystyrene plate and incubated for 18 hours at 20°C. The plates were then frozen at -20°C. After thawing for 30 min. at 37°C, the plates were washed four times in 0.01M phosphate buffer, pH 7.2 and containing 0.15M NaCl and 0.05% tween 20 (PBS/tween buffer).

Two hundred microlitre of test serum, diluted 1:50 in PBS/tween buffer was added to individual wells. Control sera including serum from an infected animal (prediluted to give an optical density value of approximately 1.0 after 10 min. of development); serum that gave an optical density near the cut-off value after 10 min. of development; a negative serum and a buffer control, all set up in quadruplicate. After 30 min. of incubation at 20°C, the plates were again washed four times in PBS/tween and 200 μ l of a monoclonal antibody to bovine IgG1, conjugated with horseradish peroxidase, was added. After 30 min. of incubation at 20°C, the plates were washed as above and 200 μ l substrate (1mM H₂O₂) and chromogen (4mM ABTS) in 0.05 M citrate buffer, pH 4.5, was added to each well. Optical density readings are taken after 10 min. of incubation with the substrate/chromogen and data were presented as a percentage of the positive control serum included in each plate and 46% or 62% positivity were considered a positive reaction. A second method of data assessment used the targeting procedure [15] in which the optical density of the positive control serum was measured after 4 min. of substrate/chromogen interaction. The length of time required for the positive control serum to achieve an optical density value of 1.0 was calculated and the plate was re-evaluated for color development at that time. Cut-off values of 0.460 and 0.607 optical density values were used for data interpretation. A modification of the indirect ELISA (EDTA/EGTA ELISA) used PBS/tween to which 7.5mM of each of ethylene diaminetetra-acetic acid disodium salt (EGTA) and ethylene glycol-bis-(B-aminoethyl ether) N,N,N',N'-tetraacetic acid (EDTA) [both from Sigma Chem. Corp., St. Louis, Missouri, USA] were added as the serum diluent buffer. At all other stages, PBS/tween buffer was used. One competitive ELISA [9] used 100 μ l purified O-polysaccharide at 2.0 μ g/ml, covalently linked to poly-L-lysine [10] and dissolved in 0.06M carbonate buffer, pH 9.6 as the antigen, passively attached to polystyrene 96-well plates for 18 hours at 20°C. The sealed plates were then frozen at -20°C. After thawing at 37°C for 30 min., the plate was washed four times in 0.1M tris buffer pH 8.0 containing 0.15M NaCl and 0.05% tween 20 (tris/tween buffer).

Test serum (50 μ l) diluted 1:50 in tris/tween buffer was added to the wells in duplicate followed by appropriately diluted mouse monoclonal antibody YsT9-2 (specific for an O-polysaccharide epitope), conjugated with horseradish peroxidase (50 μ l). After mixing on a rotary shaker for 3 min., the plate was incubated at 20°C for 2 hours. After four further washes in tris/tween buffer, bound enzyme was measured by the targeting procedure using H₂O₂ and ABTS. Results were expressed as percent

inhibition of an uninhibited control to which tris/tween buffer was added instead of serum. Additional controls included serum from a *Brucella abortus* infected cow, serum from a *Brucella abortus* immunized animal and a negative serum. The second competitive ELISA [11] used 1.0 µg/ml lipopolysaccharide as the antigen at 100 µl per well, incubated for 18 hours at 20°C. The plates were then frozen at -20°C. After thawing at 37°C, the plates were washed with tris/tween buffer. Fifty microliter of serum, diluted 1:10 in tris/tween buffer containing 7.5 mM of each of EDTA and EGTA followed by 50 µl of monoclonal antibody, M84, also diluted in tris/tween buffer with EDTA/EGTA were added to duplicate wells. Controls (in quadruplicate) included a high titered serum from an infected cow, a low titered serum from an immunized cow, serum from a negative animal and a buffer control (only monoclonal antibody and buffer). The plate was incubated for 30 min. at 20°C and washed four times in tris/tween buffer. Goat anti-mouse IgG (H & L chain specific) affinity purified antibody conjugated with horseradish peroxidase (Jackson ImmunoResearch Labs. Inc., West Grove, Pennsylvania 19390, USA) was diluted in tris/tween and 100 µl was added to each well for 30 min. After four additional washes, substrate/chromogen (1.0 mM H₂O₂ and 4 mM ABTS) was added for 10 min. with continuous orbital shaking. Optical density readings were obtained after 10 min. and the data presented as % inhibition relative to the buffer (uninhibited control). Sera that gave an value of 30% inhibition or greater were considered positive and were retested.

2.3. Statistical analysis

A combination of statistical approaches were used to compare the data from the various assays. The initial estimates of cut-off values were derived using negative serum samples from the population of animals under study [2]. The data was sorted in ascending order and divided into 100 equal percentiles. The mean of the 99th percentile was calculated and used as the initial cut-off value. The diagnostic sensitivity and specificity could then be optimized by plotting the sorted data for defined negative and positive sera using a frequency histogram. The initial cut-off values were used in 2x2 tables to calculate specificity and sensitivity. Confidence limits (95%) were calculated.

The data was also analyzed using signal detection analysis (receiver operating characteristics ROC, analysis) [16, 17]. This analysis confirmed the proximity of the initial cut-off estimate by comparing sensitivities and specificities using a range of cut-off values. An index of comparison (accuracy) was used to compare the performance of different assays with respect to actual positive and negative data [17]. Accuracy estimates were obtained using the relationships summarized below:

Definitions:

TPF = Sensitivity

TNF = Specificity

P(D+) = Disease prevalence from data

P(D-) = 1-P(D+)

Relationships:

Accuracy = TPF x P(D+) + TNF x P(D-)

3. RESULTS

Of the 15,716 sera tested, 216 agglutinated the BPAT antigen, resulting in a specificity estimate of 98.6%. Nine of the sera from the *Brucella abortus* infected animals did not agglutinate the BPAT antigen resulting in a sensitivity estimate of 97.9%. These data are included in Table III along with similar calculations for the various data manipulations of the indirect ELISA data. There was no significant difference between data obtained for the indirect ELISA by the targeting procedure or by the percent positivity method and clearly the data indicates that the higher the cut-off value used, the higher the specificity of the indirect ELISA, however, even at the higher cut-off values, the specificity was marginally less than that of the BPAT.

The frequency distributions and the ROC analysis for the targeted and the percent positivity methods of expressing data are depicted in Figures 1 and 2, respectively. The accuracy estimates are presented in Table IV. As expected, increasing the cut-off values increased the accuracy from 0.96 to 0.98. If cut-off values of 0.460 or 46% positivity were used, 763 of the negative sera gave positive earlier study using approximately 1000 serum samples. The cut-off represents the 99th percentile of the mean of the values obtained with negative sera (unpublished data). The higher cut-off values, 0.607 or 62% positivity were derived by the same calculation from the current study.

The 763 serum samples that gave false positive reactions were tested in a modified indirect ELISA (targeted) in which 7.5 mM EDTA/EGTA were included in the serum diluent. This resulted in 93 sera remaining positive at the 0.460 cut-off value and 37 giving optical density values over 0.607. In both instances, the specificity of the assay was increased over that of the BPAT (Table III). The 424 sera from infected cattle gave positive reactions when either cut-off was used and addition of chelating agents did not alter these results. These data are summarized in Table III. Because 91 of the 1508 negative sera and 59 of the sera from confirmed positive animals (an additional 15 positive sera prozoned sufficiently to be diagnostically negative and 6 sera gave incomplete hemolysis) selected for testing by the complement fixation test gave anticomplementary reactions, one set of calculations considered these sera as negative and one set as positive.

If the anticomplementary reactions were considered as positive, the specificity of the complement fixation test was 93.1%, the sensitivity was 97.1% and the accuracy 0.95. A specificity value of 99.8%, test sensitivity of 87.9% and an accuracy estimate of 0.96 were obtained if the sera causing anticomplementary reactions were considered as negative. These data are presented in Tables III and II and frequency distributions and ROC analysis are presented in Figure 3. In testing 1508 negative sera, selected randomly, in the competitive ELISA with O-polysaccharide antigen, the initial specificity was 97.7%. When the 34 false positive reactor sera were retested, the specificity increased to 99.9%.

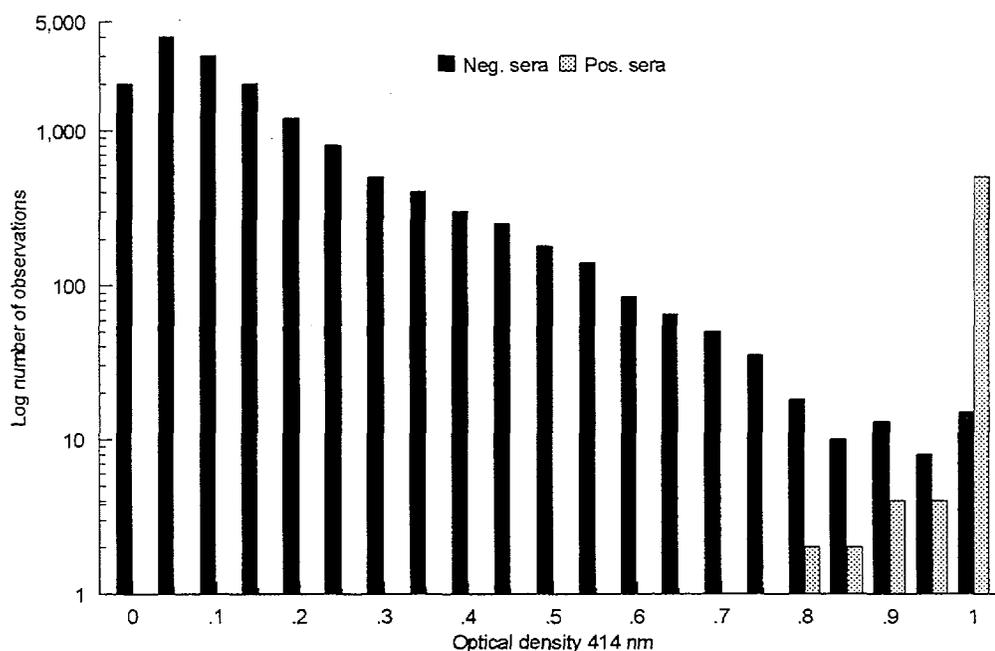


FIG.1. Frequency distribution of negative and positive sera selected for testing in the targeted indirect ELISA. The Y-axis represents the log₁₀ number of sera in each 0.05 optical density interval on the X-axis. The open bars represent negative sera and the closed bars positive sera. From this graph, cut-off values of 0.460 and 0.607 were selected for data analysis. The former cut-off represents 99th percentile of the mean of the negative samples tested previously (small sample) while the latter cut-off represents the 99th percentile of the mean of the current data. Theoretically the cut-off could be set as high as 0.700 optical density units and still retain a sensitivity estimate of 100%.

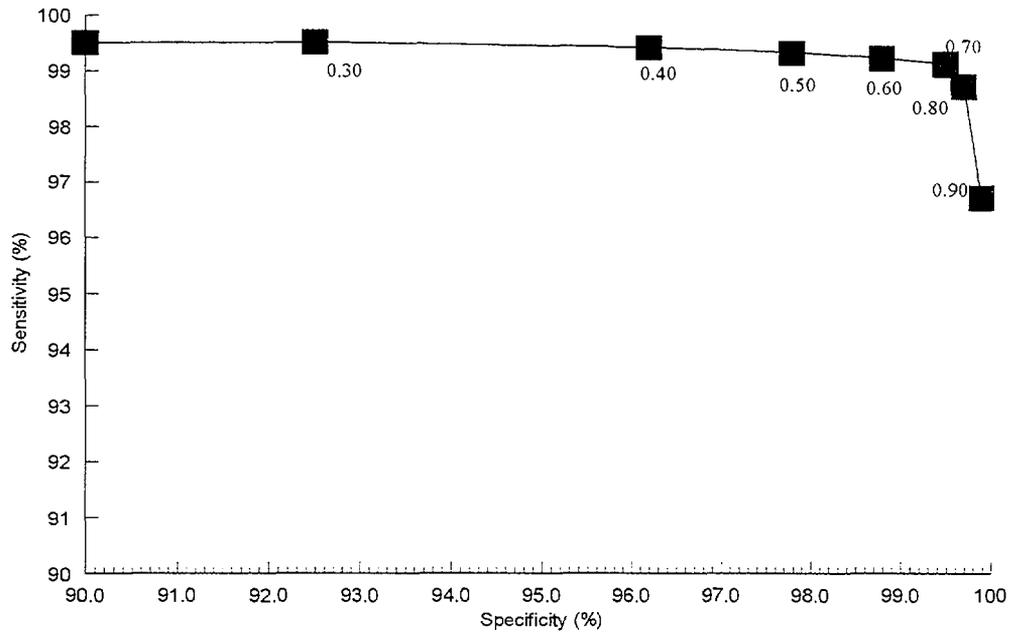


FIG.2. Receiver operating characteristic (ROC) analysis of the targeted indirect ELISA data (optical density values are included with the graph). The assay sensitivity in percent is plotted on the Y-axis and the specificity (%) is plotted on the X-axis. From this plot, a sensitivity may be selected to reflect a desired specificity value. For instance, if a sensitivity value of 99% is selected, the specificity will be 99.5% and a cut-off value of 0.700 would be used.

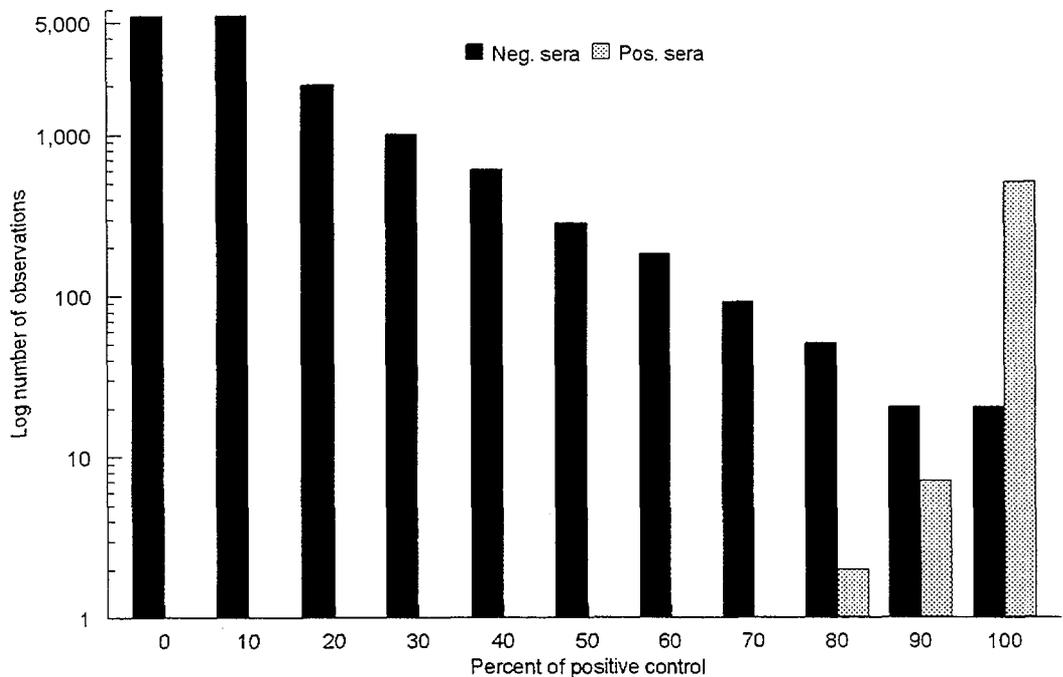


FIG.3. Frequency distribution for the same sera represented in Figure 1 in an indirect ELISA but using percent positivity of a positive control serum for data handling (plotted along the X-axis). Cut-offs of 46% (old value) and 62% (from the current data), representing the 99th percentile of the mean were used for data analysis.

The sensitivity of this assay of 98.6% was enhanced to 99.5% by repeat testing of false negative samples (Table III). The accuracies before and after retesting were 0.98 and 0.998, respectively (Table IV). Graphs representing the frequency distributions and the ROC analysis before and after retesting are presented in Figures 5 and 6. Using lipopolysaccharide antigen and M84

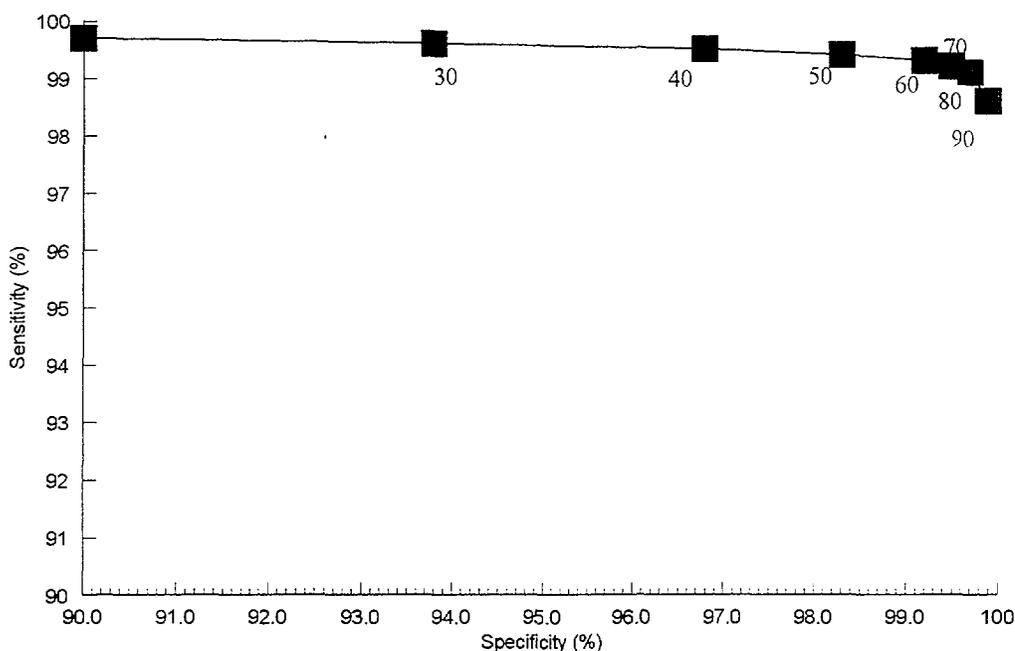


FIG. 4. ROC analysis of the data from the indirect ELISA using percent positivity (numbers included in graph) for data analysis. Based on this analysis, the optimum cut-off value would be approximately 70% which corresponds with the value of 0.700 optical density units established in Figure 2.

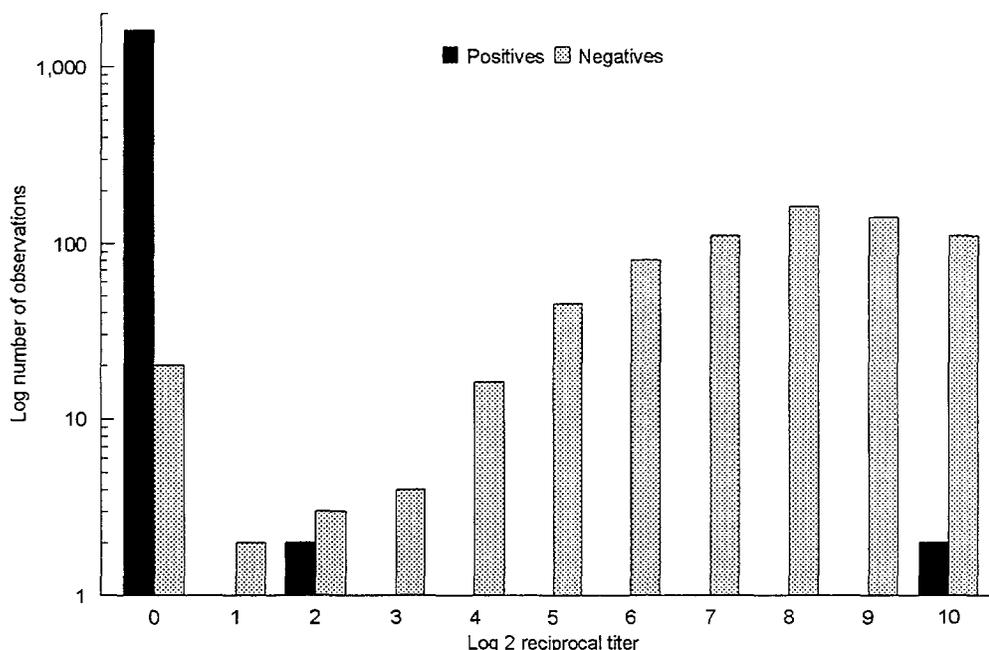


FIG. 5. Frequency distribution of sera tested by the complement fixation test for antibody to *Brucella abortus*. The \log_{10} number of observations are plotted on the Y-axis and reciprocal titers (\log_2) are plotted on the X-axis. Three sera gave false positive reaction (open bars) and 19 sera gave false negative reactions. Anticomplementary serum samples were assigned to their respective status. From this graph, it would appear that the cut-off for the complement fixation test should be a titer of zero.

monoclonal antibody in a competitive ELISA enabled detection of significant antibody in all 629 serum samples from proven infected animals giving an assay sensitivity of 100%. The specificity before testing false positive sera was 99.6% and this increased to 99.9% after retesting (Table III). The accuracy of this assay was 1.00 before and after retesting (Table IV). Frequency distributions and ROC analysis for data before retesting are presented in Figure 7. Data after retesting was not included due to the minor changes (the number of false positive reactions were reduced from 5 to 2. There were no false negative reactions).

TABLE III. SENSITIVITY AND SPECIFICITY ESTIMATES FOR THE BUFFERED PLATE ANTIGEN TEST, THE COMPLEMENT FIXATION TEST, THE VARIOUS FORMATS OF THE INDIRECT ELISA AND THE COMPETITIVE ELISAS, BEFORE AND AFTER RETESTING SERA THAT GAVE UNEXPECTED RESULTS (CONFIDENCE LIMITS OF 95% ARE INCLUDED)

Test	Sensitivity %	CL ¹ %	Specificity %	CL %
BPAT ²	97.9	1.4	98.6	0.18
CFT (AC reactors +) ³	97.1	1.3	93.1	1.2
CFT (AC reactors -)	87.9	2.5	99.8	0.20
I-ELISA-T (0.460 cut-off) ⁴	100		96.0	0.31
I-ELISA-T (0.607 cut-off)	100		98.5	0.19
I-ELISA-%P (46% cut-off) ⁵	100		95.9	0.31
I-ELISA-%P (62% cut-off)	100		98.5	0.19
I-ELISA EDTA (0.460 cut-off) ⁶	100		99.4	NA
I-ELISA EDTA (0.607 cut-off)	100		99.8	NA
C-ELISA C-ELISA (O-poly-before) ⁷	98.6	0.90	97.7	0.76
C-ELISA (O-poly-after)	99.5	0.50	99.9	0.10
C-ELISA (LPS-before) ⁸	100		99.7	0.18
C-ELISA (LPS-after)	100		99.9	0.10

1. confidence limits
2. buffered plate antigen test
3. complement fixation test with anticomplementary sera considered as positive or negative
4. targeted indirect ELISA using cut-off values of 0.460 or 0.607 optical density units
5. indirect ELISA data based on percent positivity relative to a positive serum control. Cut-off values of 46% or 62% positivity were selected
6. targeted indirect ELISA into which divalent cation chelating agents (EDTA and EGTA) were incorporated into the serum dilution tested. Two cut-off values, 0.460 and 0.607, were selected. Confidence limits for this modification were not calculated.
7. competitive ELISA format using O-polysaccharide from *Brucella abortus* as the antigen and an enzyme conjugated monoclonal antibody for competition and detection. Results before and after retesting sera that gave aberrant results are included
8. competitive ELISA format using lipopolysaccharide from *Brucella abortus* as the antigen, a monoclonal antibody for competition and enzyme conjugated anti mouse IgG antibody for detection. Results before and after retesting sera that gave aberrant results are included

4. DISCUSSION

It is of interest to note that the screening test for bovine antibody to *Brucella abortus* used in Canada identified 216 sera positive of the 15,716 sera from negative herds tested in this study. The specificity, 98.6%, found in this study agrees with that observed in other Canadian studies [18] of 98.7% and 98.9% found by Stemshorn et al. [19]. The sensitivity of the BPAT was 97.9%, a little higher than the figures reported previously [18] and Stemshorn et al. [19] of 95.4 and 96.9%, respectively. The accuracy of the BPAT was 0.98.

TABLE IV. ACCURACY ESTIMATES FOR EACH TEST WERE BASED ON THE SENSITIVITY, THE SPECIFICITY AND THE DISEASE PREVALENCE FOR THE DATA USED BY THE EQUATION BELOW

Test	Accuracy ¹
BPAT ²	0.98
CFT (AC reactors +) ³	0.95
CFT (AC reactors -)	0.96
I-ELISA-T (0.460 cut-off)	0.96
I-ELISA-T (0.607 cut-off)	0.98
I-ELISA-%P (46% cut-off)	0.96
I-ELISA-%P (62% cut-off)	0.98
C-ELISA (O-poly-before)	0.98
C-ELISA (O-poly-after)	0.998
C-ELISA (LPS-before)	1.00
C-ELISA (LPS-after)	1.00

1. Accuracy = Test sensitivity x disease prevalence for data + test specificity x (1-disease prevalence for data)
2. the legend is as for Table III
3. the legend is as for Table III

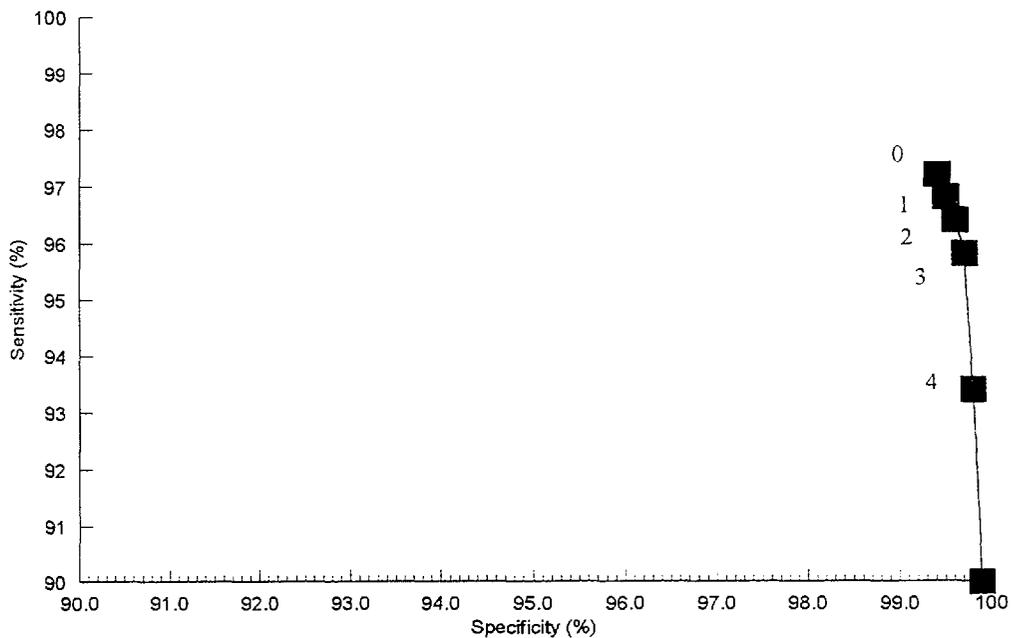


FIG. 6. ROC analysis of the complement fixation test data. The cut-off value of 0 gives the highest sensitivity (97%) while the specificity did not change a great deal irregardless of the titer selected for the cut-off.

These data are summarized in Tables III and IV. Sera from proven *Brucella abortus* infected cattle that were negative in the BPAT were unable to agglutinate at dilutions of 1:10, 1:50 and 1:100 eliminating prozoning as an explanation for the negative results. Since all sera reacted in the indirect ELISAs, an assay that measures only IgG1, lack of agglutination could not be a result of lack of antibody capable of reacting, as IgG1 has been shown to agglutinate efficiently at the lower pH [2]. Therefore, the inability of the antibody of the 9 negative serum samples to agglutinate the BPAT antigen remains unexplained.

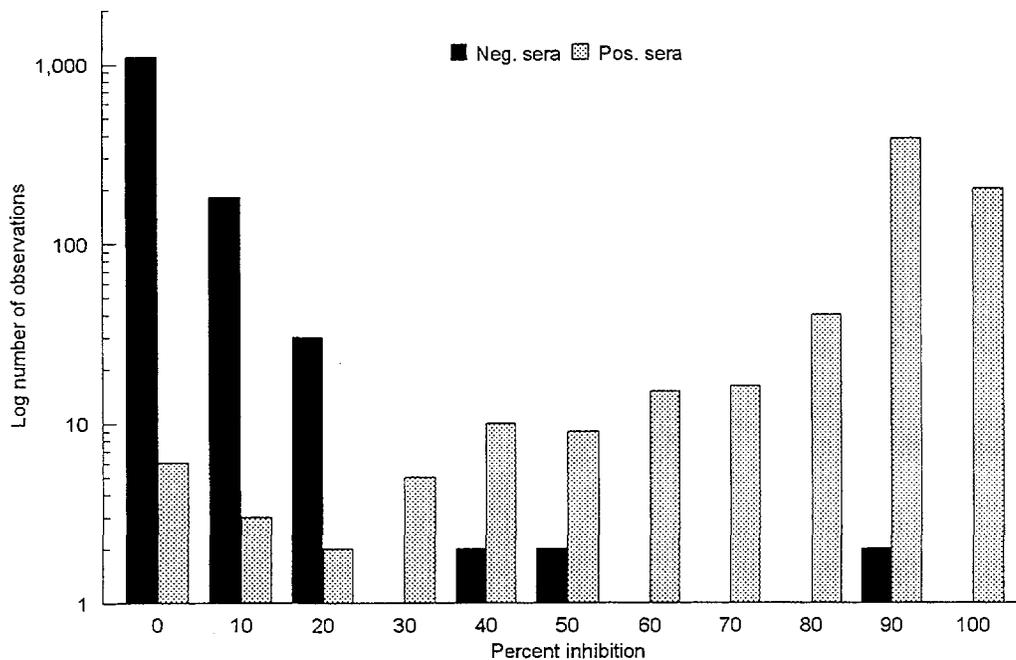


FIG. 7. Frequency distribution of negative and positive sera randomly selected for testing in a competitive ELISA before sample that gave unexpected results were retested in the assay. This competitive ELISA used the O-polysaccharide of *Brucella abortus* as the antigen and an enzyme conjugated monoclonal antibody, specific for the O-polysaccharide, as the competing and detection antibody. The Y-axis represents the log₁₀ number of observations and the X-axis indicates the percent inhibition in increments of 10%. From the graph it appears that the most suitable cut-off value for this assay is 20% inhibition.

The complement fixation test, the current Canadian confirmatory test, cannot be used to make a definite diagnosis if the serum sample activates the complement cascade in the absence of antigen. Such sera are deemed anticomplementary. In the current study, a number sera selected from negative herds and from the proven infected animals were found to be anticomplementary and therefore, the data obtained was analyzed twice, considering all anticomplementary reactions as positive reactions or as negative reactions. If the anticomplementary sera are considered as positive, the specificity of the test was 93.1%, the sensitivity was 97.1% and the accuracy was 0.95. If the anticomplementary reactions were considered negative, the specificity was 99.8%, the sensitivity 87.9 and the accuracy was 0.96. Dohoo et al. [18] found the specificity of the complement fixation test to be 100% and the sensitivity to be 92.9%, very similar to the latter interpretation of the current data. The data from the current study are presented in Tables III and IV.

The indirect ELISA detected antibody in all sera from infected animals regardless of the data interpretation used in this study. The specificity was 96% using a cut-off of 46% or a targeted cut-off value of 0.460 optical density units. If the cut-off values were increased to 62% or 0.602, the specificity increased to 98.5%. Again, the former values are similar to those published earlier [2], demonstrating the consistency of this test. The accuracy was determined to be 0.96 and 0.98 for the lower and higher threshold values, respectively. Since the specificity estimates for the indirect ELISA were lower than those observed for the BPAT, a modification utilizing divalent cation chelating agents as part of the serum diluent buffer was attempted. This modification was based on an earlier observation in which non-specific interaction in the tube agglutination test was reduced by addition of ethylenediaminetetraacetic acid disodium salt (EDTA) and ethylene glycol-bis-(B-aminoethyl ether) N,N,N',N' tetraacetic acid (EGTA) [20]. It was found that addition of EDTA and EGTA decreased the number of false positive reactions in the indirect ELISA from 763 to 93 or 37, using the 0.460 or the 0.602 cut-offs, respectively. This increased the specificity to 99.4% or 99.8%, both of which exceed that of the BPAT, without any reduction in sensitivity.

These data are summarized in Tables III and IV. The higher cut-off value used in this study may not be useful in a diagnostic context as it would only be suitable in surveillance situations where brucellosis is not expected to be found, as is the case in Canada. A high cut-off value in the indirect ELISA would not be suitable for testing imported animals, especially from areas where bovine brucellosis was in evidence as two test criteria are not advantageous. However, it would markedly decrease the number of confirmatory tests or trace-backs required. While the sensitivity and the specificity of the indirect ELISA appear to be excellent, this type of assay cannot distinguish between the antibody response induced by vaccination with *Brucella abortus* strain 19 and that resulting from infection with pathogenic strains.

This led to the development of competitive ELISAs [9, 21] which could differentiate between the induced antibody responses. The initial competitive ELISA developed at Agriculture Canada utilized purified O-polysaccharide as the antigen and a mouse monoclonal antibody, produced with O-polysaccharide from *Yersinia enterocolitica* serotype 0:9 and conjugated with horseradish peroxidase as the competing antibody and detection reagent [9]. The O-polysaccharide was subsequently modified by covalently linking it with poly-L-lysine to improve its binding characteristics to polystyrene [10]. This assay was also used to test the 1508 randomly selected sera from Canadian herds and 654 sera from animals from which *Brucella abortus* was isolated.

Two sets of data are included for this assay. Firstly, the data obtained on the initial test and then data obtained after serum samples that gave unexpected results were retested. The sensitivity of the test with the initial data and based on a cut-off value of 20% inhibition was 98.6%, the specificity was 97.7% and the accuracy was 0.98. After repeat testing of false positive and false negative samples, the overall sensitivity increased to 99.5%, the specificity increased to 99.9% and the accuracy was 0.998. These data are presented in Tables III and IV. A second version of the competitive ELISA [11], using lipopolysaccharide as the antigen and a different monoclonal antibody for competition in a buffer containing EDTA and EGTA and goat anti-mouse IgG antibody-horseradish peroxidase as the detection reagent was also used on the same sera.

Again, the data is presented before retesting of serum samples giving aberrant results. Initial test sensitivity, based on a cut-off value of 30% inhibition, was 100% sensitivity, test specificity was 99.7% and the accuracy was 1.00. After retesting five false positive samples, two were eliminated raising the specificity to 99.9%. It is of interest to note that one of the three samples that gave false positive reactions was obtained from a recently vaccinated animals and this sample was positive on all the tests. A second sample was contaminated with bacteria and was positive on both competitive ELISAs and anticomplementary in the complement fixation test. The third positive sample was different for the two competitive ELISAs and the complement fixation test and its reactivity remain unexplained.

These data are summarized in Tables III and IV. Initially, the cut-off values may be approximated by visual inspection of the frequency distribution graphs (Figures 1,3,5,7,9) as the point that gives maximum distinction between positive and negative samples. For example, in Figure 9, it would appear that the cut-off between positivity and negativity should be 20% to accommodate the most negative samples and the least number of positive samples. To obtain a more accurate estimate of the cut-off values for the assay, ROC analysis (Figures 2,4,6,8,10) allowed the determination of sensitivity and specificity values at different points and therefore a decision could be made to maximize the sensitivity or the specificity and the reciprocated decrease in the other. Thus, for instance in Figure 10, if an acceptable false positive rate was 0.36% (a cut-off value of 20% inhibition), then the specificity will be 99.64% and the sensitivity will be 98.47%.

Alternately, if a specificity value of 99.80 is required (at a cut-off of 50% inhibition), the sensitivity will decrease to 94.80%. Accuracy estimates allow direct comparison of the performance of each assay in that the false positive and the false negatives rates are considered in the context of disease prevalence for the specific data set. From Table IV, it is clear that the competitive ELISA using LPS antigen is the most accurate (1.0) but the older version of the competitive ELISA after repeat testing comes very close (0.998). A number of technical improvements have been made to the current competitive ELISA compared to that reported previously [9]. Thus lipopolysaccharide antigen is used rather than the O-polysaccharide, simplifying antigen production. A serum dilution of 1:10 was chosen to allow dilution directly into the wells of a 96-well plate, obviating the requirement for a tube for preparing the dilution.

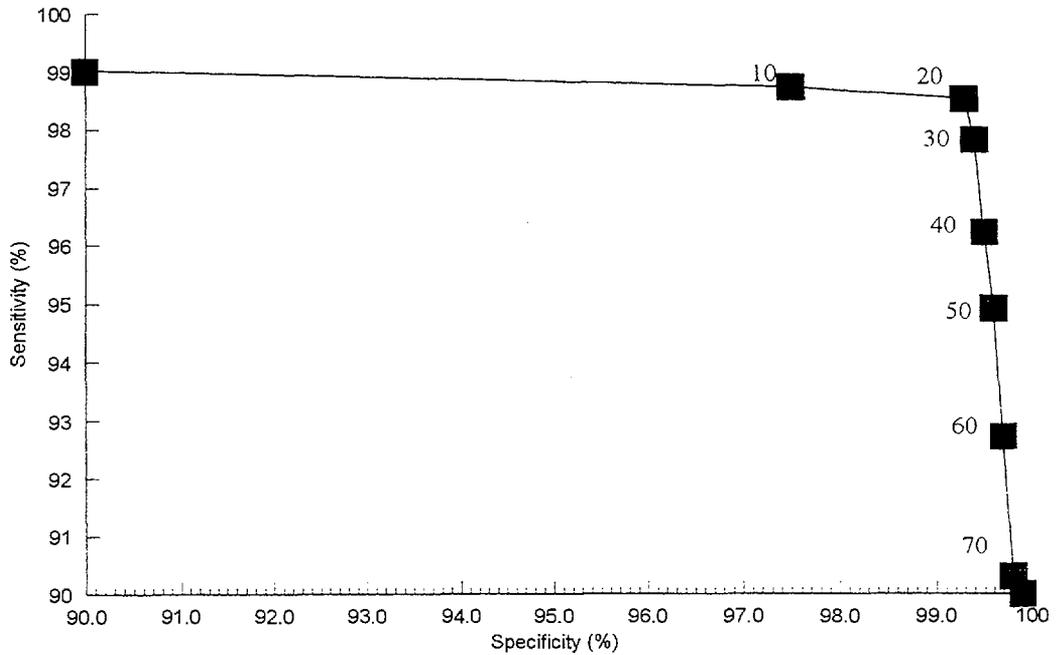


FIG. 8. ROC analysis of the competitive ELISA data. The most suitable cut-off value is 20% inhibition, giving a sensitivity of 98.6% and a specificity estimate of 99.6%.

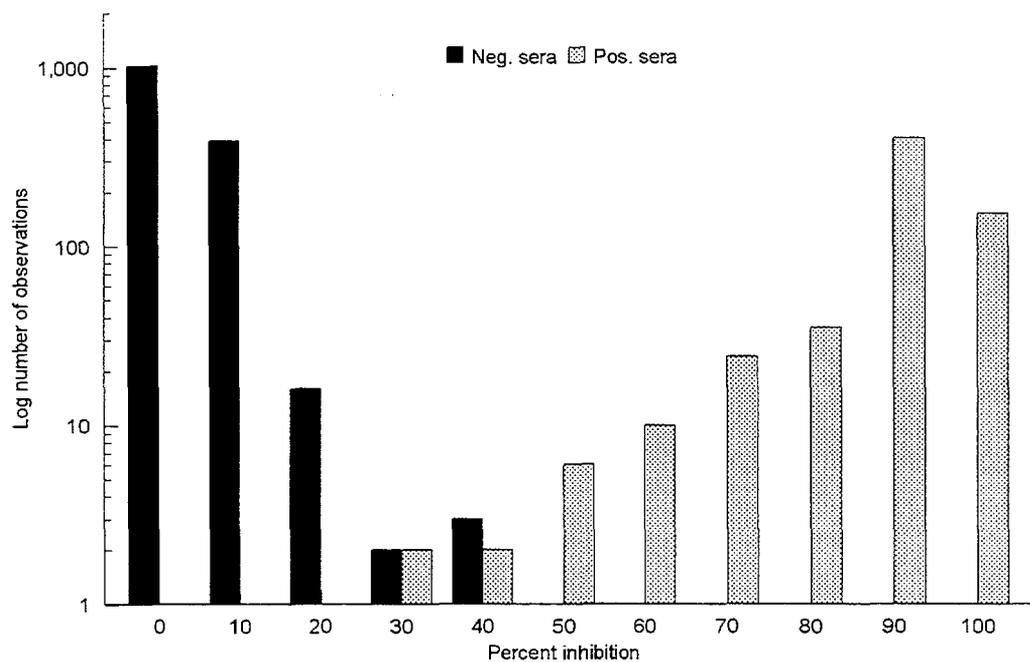


FIG. 9. Same as Figure 7 but data after aberrant samples were retested in the competitive ELISA. It is clear that the cut-off value did not change with repeat testing. Data obtained when testing randomly selected negative and positive sera in a second competitive ELISA after initial testing. This assay used *Brucella abortus* lipopolysaccharide as the antigen, a different monoclonal antibody (but also specific for the O-polysaccharide) for competition and an enzyme conjugated anti-mouse IgG antibody for detection. The log₁₀ number of observations are plotted along the Y-axis and the percent inhibition in increments of 10% is plotted on the X-axis. The cut-off value selected from this graph was 30% inhibition. Retesting sera that gave unexpected results eliminated all but 3 false positive reactions (data not shown).

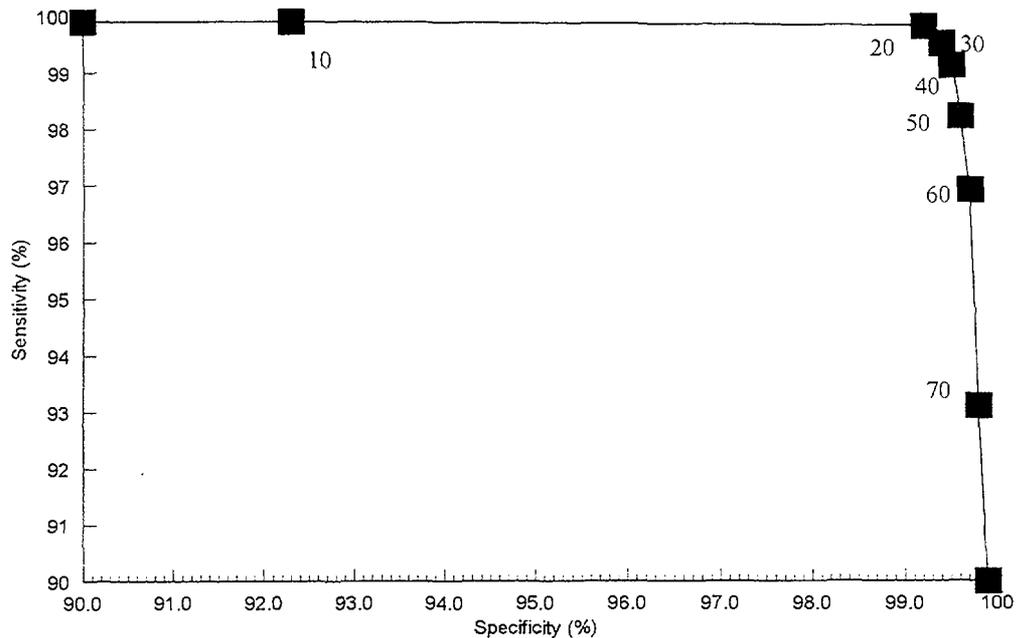


FIG.10. ROC analysis of the data from the second competitive ELISA. If a cut-off of 30% inhibition is selected, the sensitivity estimate is 99.9% and the specificity is 99.8%. Retesting of samples that gave unexpected reactions increased the specificity to 99.9% (data not shown). The ROC analysis revealed an increase in sensitivity to 99.5% and an increase in specificity 99.8%.

Addition of divalent cation chelating agents reduced the non-specific serum protein interactions, however, because EDTA/EGTA are incompatible with horseradish peroxidase, the additional step of using a goat anti-mouse IgG antibody reagent conjugated with enzyme was included. This inclusion also allowed for minor adjustments in optical density without altering the assay sensitivity as was the case when the competing monoclonal antibody was conjugated directly with enzyme. Both incubation periods have been decreased to 30 min. each, compared to a single stage of two hours previously and each sample may be set up once rather than in duplicate. Based on the specificity and sensitivity of the LPS-based competitive ELISA reported here, it would appear that this procedure may replace not only the currently used confirmatory test, the complement fixation test, but also the in-use screening test, the BPAT. Little or no extra cost is involved since although that it may be slightly more expensive to perform, there would be no requirement for additional expensive tests and 90% of the trace-backs would be eliminated. An additional saving can be made by reusing the antigen coated polystyrene plates for as many as five assays [22], making it possible to perform 400 assays per plate.

REFERENCES

- [1] WRIGHT, P.F., NIELSEN, K.H., KELLY, W., Primary binding techniques for the serodiagnosis of bovine brucellosis: enzyme immunoassay in animal brucellosis. Ed. Nielsen, K.H., Duncan, J.R. CRC Press, Boca Raton, Florida (1990) 199-235.
- [2] NIELSEN, K.H., GALL, D., KELLY, W., HENNING, D., GARCIA, M.M., Enzyme immunoassay: Application to diagnosis of bovine brucellosis, Agriculture Canada monograph, ISBN 0-662-19838-7, (1992).
- [3] NICOLETTI, P., Vaccination. in Animal Brucellosis, Eds Nielsen, K.H., Duncan, J.R., CRC Press, Boca Raton, Florida, (1990) 283-300.

- [4] ANGUS, R.D., BARTON, C.E., The production and evaluation of a buffered plate antigen for use in the presumptive test for brucellosis, *Dev. Biol. Standard* **56** (1984) 349-356.
- [5] HILL, W.K., Standardization of the complement fixation test for brucellosis, *Bull. Off. Internat. Epizool.* **60** (1963) 401-419.
- [6] ANDERSON, R.K, JENNESS, R, BRUMFIELD, H.P., GOUGH, P., Brucella agglutination antibodies, Relationship of mercaptoethanol stability to complement fixation, *Science* **143** (1964) 1334-1335.
- [7] DIAZ, R., GARATEA, P., JONES, L.M., MORIYON, I, Radial immunodiffusion test with *Brucella* polysaccharide antigen for differentiating vaccinated from infected cattle, *J. Clin. Microbiol.* **10** (1979) 37-41.
- [8] NIELSEN, K.H., KELLY, L., GALL, D., SMITH, P., BOSSE, J., NICOLETTI, P., KELLY, W., Use of divalent cation chelating agents (EDTA/EGTA) for reduction of non-specific serum protein interaction in enzyme immunoassay, *Vet Res Comm* **18** (1994) 433-437.
- [9] NIELSEN, K.H., CHERWONOGRODZKY, J., DUNCAN, J.R., BUNDLE, D.R., Enzyme linked immunosorbent assay for differentiation of the antibody response of cattle naturally infected with *Brucella abortus* or vaccinated with strain 19, *Am J Vet Res* **50** (1989) 5-9.
- [10] GALL, D., NIELSEN, K.H., Improvements to the competitive ELISA for detection of antibody to *Brucella abortus* in cattle sera, *J. Immunoassay* **15** (1994) 277-291.
- [11] NIELSEN, K.H., KELLY, L., GALL, D., BALSEVICIUS, S., NICOLETTI, P., KELLY, W., Improved competitive enzyme immunoassay for the diagnosis of bovine brucellosis, *Vet Immunol Immunopathol* **46** (1995) 285-291.
- [12] SAMAGH, B.S., BOULANGER, P., Adaptation of the Technicon Autoanalyzer II for an automated complement fixation test for detection of antibodies to *Brucella abortus* in bovine serums. *Proc. 21st Ann. Meet. Am. Assoc. Vet. Lab. Diagnost.* (1978) 347-366.
- [13] HENNING, D.M., NIELSEN, K.H., Peroxidase labeled monoclonal antibody for use in enzyme immunoassay, *J. Immunoassay* **8** (1987) 297-305.
- [14] BAKER, P.J., WILSON, J.B., Hypoferremia in mice and its application to the bioassay for endotoxin, *J Bacteriol* **90** (1965) 903-910.
- [15] WRIGHT, P.F., KELLY, W., GALL, D.E., Application of a timing protocol to the reduction of inter-plate variability in the indirect enzyme immunoassay for detection of anti-Brucella antibody, *J. Immunoassay* **6** (1985) 189-199.
- [16] STENSON, H., SIGNAL, A., Supplementary module for SYSTAT and SYSGRAPH, SYSTAT Inc. Evanston IL. (1988).
- [17] METZ, C.E., Basic principles of ROC analysis, *Seminars in Nuclear Medicine* **8** (1978) 283.
- [18] DOHOO, I.R., WRIGHT, P.F., RUCKERBAUER, G.M., SAMAGH, B.S., ROBERTSON, F.J., FORBES, L.B., A comparison of five serological tests for brucellosis, *Can. J. Vet. Res.* **50** (1986) 485-493.
- [19] STEMSHORN, B.W., FORBES, L.B., EAGLESOME, M.D., NIELSEN, K.H., ROBERTSON, F.J., SAMAGH, B.S., A comparison of standard serological tests for the diagnosis of bovine brucellosis in Canada, *Can. J. Comp. Med.* **49** (1985) 391-394.
- [20] NIELSEN, K.H., SAMAGH, B.S., SPECKMANN, G., STEMSHORN, B.W., The bovine immune response to *Brucella abortus*, Elimination of some sporadic serological reactions by the chelation of divalent cations, *Can. J. Comp. Med.* **43** (1979) 40-47.
- [21] MACMILLAN, A.P., GREISER-WILKE, I., MOENNIG, V., MATHIAS, L.A., A competitive enzyme immunoassay for brucellosis diagnosis. *Dtsch. Tierärztl. Wochenschr.* **97** (1990) 83-85.
- [22] NIELSEN, K.H., KWOK, A., Reuse of polystyrene 96 well plates for indirect enzyme immunoassay for detection of antibody to *Brucella abortus*, *Arch Med Vet* **27** (1995) 39.