# COMPARATIVE EVALUATION OF COMPETITIVE ELISA TEST IN COLOMBIAN CATTLE

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**Abstract**

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In order to contribute to the definition of the best ELISA test for screening and differential diagnosis of *Brucella abortus* to be applied for control programmes, a total of 2971 sera from Colombian cattle were tested for brucellosis. Conventional agglutination tests, Buffered Plate antigen test (BPAT) and Rose Bengal (RB) as well as Complement Fixation test (CFT) (Alton, et al. 1988) were used comparatively. Radial immunodiffusion test (RID) was also performed to all sera. The sera were also tested using four different ELISAs: indirect ELISA from FAO/IAEA and the indirect ELISA modified by Nielsen, et al. 1992 as well as two competitive ELISAs: one competitive ELISA used *B. abortus* O-polysaccharide antigen and an enzyme conjugated monoclonal to the O-polysaccharide for competition and detection. The second competitive ELISA used lipopolysaccharide (sLPS) antigen, a different monoclonal antibody for competition but also specific for the O-polysaccharide and a commercially available goat anti-mouse IgG enzyme conjugate for detection. The sera were analyzed based on its population status, 987 positive obtained from *Brucella abortus* infected herds based on clinical and/or bacteriological evidence and a high prevalence of brucellosis, CFT percentage of positive animals in the herd was greater than 5%. Eight hundred sixty six (866) negative sera from non-vaccinated cattle from a brucellosis free area and 1118 negative sera obtained from reglamentary vaccinated areas under a free herd program. Initial cut-off values were derived using negative serum samples. The diagnostic sensitivity and specificity was defined from frequency histograms based on this cut-off values and using 2x2 tables, corresponding confidence limits (95%) were calculated. The data were also analysed using signal detection analysis (ROC). Kappa statistics was determined for all tests and populations, accuracy was used as index of comparison to evaluate different assays. The data support the initial hypothesis that the ELISA methodology designed for brucellosis will provide more precise and standardised method for diagnosis and for the support of control and eradication campaigns.

## 1. INTRODUCTION

Animal diseases affect directly health and economy in all the countries of the world. In order to eliminate them it is important to develop control programs based on specific and opportune diagnosis [1]. To obtain this goal very precise diagnostic tests have been developed. Enzyme linked immunosorbent assays (ELISA) have the characteristics of high sensitivity and specificity, they are quick and economic. In the vast majority of its applications they are comparable and superior to most of the conventional diagnostic tests. They are versatile, permit mass screening of livestock, have become simple and objective results are obtained based on computerised programs [2,3,]. Since 1987, the Joint FAO/IAEA Division of the International Atomic Energy Agency has initiated a Coordinated Research Programme to evaluate the use of ELISA as diagnostic support for diseases of mayor importance in livestock production in Latin America [4,5].

In Brucellosis diagnosis, basically two main types of immunoassays are used for these purposes, the indirect and the competitive formats [6,7]. It was hoped that the introduction of the indirect ELISA for brucelosis [8] would overcome some of the problems with conventional tests, but the indirect ELISA while more sensitive than the conventional tests, has been less specific than expected, even using highly specific monoclonal antibodies as detection reagent for bovine IgG1. Similarly the indirect ELISA can not distinguish vaccinal antibody from that arising from infection [9,10].

The competitive ELISA, O-chain [11,12] proved more specificity than the indirect [13] and apparently discriminates between vaccinal and infection antibodies. The sLPS antigen modification of the competitive assay attempts to reduce cost and time to obtain results with similar sensibility and specificity [14,15].

The purpose of this study is to contribute to the validation of immunoassays for brucellosis diagnosis and control programs.

## 2. MATERIALS AND METHODS

### 2.1. Sera

Three different kind of animal populations were selected to provide validation:

*2.1.1.* Negative population: 866 sera collected from an area of the country in which no clinical disease have been detected.

*2.1.2.* Vaccinated negative: In an area of low prevalence, 1,76% (ICA, 1994), 1118 sera were obtained from randomly selected herds under a free herd control program established by the Animal Health authorities. The animals are vaccinated between 3 to 9 months of age and the herd maintained under control and considered free after two consecutive years of negative serology by the conventional tests.

*2.1.3.* Positive population: A total of 987 sera were obtained from herds with clinical signs of the disease and from which isolation of *Brucella abortus* biotype 1 was performed or herds with clinical signs and greater than 5% percent of CFT positive serology. The whole herd was bled after the positive isolation was confirmed and considered as positive

### 2.2. Serological Tests

Buffered plate antigen test (BPAT), Rose Bengal (RBT) and Complement Fixation Test (CFT) were performed as described by Alton et al., 1988. CFT was considered as reference test for comparative studies of sensibility and specificity. Radial immunodiffusion using O-chain from *Brucella mellitensis* antigen prepared as reported by Díaz et al., 1976, was used.

Four different ELISA tests were used in order to make the validation of the competitive assay. Indirect ELISA both FAO/IAEA kit [18] as well as ADRI modification [13] were performed on all sera collected.

Briefly the test uses *B. abortus* purified sLPS [13] antigen and monoclonal anti-bovine-IgG1 conjugate labelled with Horseradish peroxidase to detect the reactive sera. The difference between the two versions is essentially sera concentration and volume of reagents. The FAO/IAEA tests is stopped while ADRI is read at 10 minutes without stopping and the buffer uses EDTA/EGTA to reduce non-specific binding [19].

A targeted competitive ELISA [11], which was more specific than the indirect ELISA and which discriminate vaccinal from infection antibodies using O-chain as antigen [20] was performed. A second approach using sLPS as antigen and competition between the sera and the non-labelled anti-O-chain monoclonal [15] was also used to run all the sera.

All the ELISA plates were read at 405-414nm, using Multiskan Mark Plus II, under the computer control of the respective FAO/IAEA BRELISA and C-ELISA programs. Only plates which were accepted by the program were considered for analysis.

### 2.3. Statistical Analysis

Different statistical analysis were used to compare the data from the various assays [21]. Based on previously defined cut-off values for each ELISA, calculated on the negative population [13], 2x2 tables were used to evaluate diagnostic relative specificity and sensitivity using CFT as reference test. Sorted data were plotted for defined negative and positive sera for frequency histograms. Confidence limits (95%) were calculated. ROC analysis was performed by statistics program, to confirm and optimise the cut-off point definition. Kappa statistics was determined for all the tests and populations, index of comparison, accuracy, were used to compare the different assays [22,23].

## 3. RESULTS AND DISCUSSION

From the programmed 3000 sera to be tested, a total of 2971 were evaluated for the final report. All ELISA tests, as well as the conventional BPAT, RB and CFT were performed to this sera. Table I shows the relative sensitivity and specificity obtained for the infected herds confirming the higher sensitivity of the ELISA tests to detect the infection as compared with the other conventional tests used in the analysis [24,25]. Low values of specificity observed for ADRI ELISA indirect and Competitive ELISA version II were not expected based on the improved characteristics of this tests regarding non-specific reactions [15], but Complement fixation test could not detect positive sera with low level of antibodies [26].

TABLE I. RELATIVE SENSITIVITY AND SPECIFICITY AS COMPARED WITH COMPLEMENT FIXATION TEST IN POSITIVE POPULATION , N=987

| Test | Sensitivity % | CL* % | Specificity % | CL* % |
|---|---|---|---|---|
| Rose Bengal, AT | 87.00 | 2.0 | 87.00 | 2.0 |
| BPAT | 78.17 | 2.5 | 82.10 | 2.3 |
| I-ELISA-IAEA | 95.81 | 1.2 | 81.80 | 2.4 |
| I-ELISA-ADRI | 98.07 | 0.8 | 71.16 | 2.8 |
| C-ELISA-1 | 95.81 | 1.2 | 83.57 | 2.3 |
| C-ELISA-2 | 93.24 | 1.5 | 77.07 | 2.6 |
| RID | 85.40 | 1.4 | 99.90 | 0.2 |

* CL= 95%Confidence limits

When the analysis included the negative population, (Table II) higher specificity was observed and the capacity of the ELISA tests to detect the positive animals in regard to Complement Fixation Test could be evaluated more clearly. From Table II, it is also important to consider that competitive ELISAs versions 1 (O-chain) and 2, appears to be good assays for the differentiation between vaccinated and infected animals due to their high sensitivity and specificity compared with the low sensitivity of the differential RID test [17, 19].

TABLE II. RELATIVE SENSITIVITY AND SPECIFICITY AS COMPARED WITH COMPLEMENT FIXATION TEST IN POSITIVE AND NEGATIVE POPULATION , N=2971

| Test | Sensitivity % | CL* % | Specificity % | CL* % |
|---|---|---|---|---|
| Rose Bengal, AT | 86.83 | 1.2 | 96.56 | 0.65 |
| BPAT | 78.13 | 1.4 | 83.97 | 1.31 |
| I-ELISA-IAEA | 95.81 | 0.72 | 94.62 | 0.81 |
| I-ELISA-ADRI | 98.07 | 0.50 | 89.28 | 1.1 |
| C-ELISA-1 | 95.81 | 0.72 | 93.87 | 0.86 |
| C-ELISA-2 | 93024 | 0.90 | 90.82 | 1.03 |
| RID | 85.40 | 1.4 | 99.90 | 0.2 |

* CL= 95%Confidence limits

When the analysis was done on population selected based on the conventional tests, (Table III) the competitive ELISA tests proved to have higher performance [15].

TABLE III. RELATIVE SENSITIVITY AND SPECIFICITY AS COMPARED WITH COMPLEMENT FIXATION TEST IN POSITIVE AND NEGATIVE SELECTED SERA, N=2137

| Test | Sensitivity % | CL* % | Specificity % | CL* % |
|---|---|---|---|---|
| I-ELISA-IAEA | 100 | | 99.11 | 0.3 |
| I-ELISA-ADRI | 100 | | 96.08 | 0.8 |
| C-ELISA-1 | 99.55 | 0.3 | 97.39 | 0.6 |
| C-ELISA-2 | 99.54 | 0.3 | 95.56 | 0.8 |
| RID | 93.4 | 0.5 | 99.9 | 0.2 |

* CL= 95%Confidence limits

Table IV shows the different cut-off values [27] calculated and defined using different approaches and no significant differences could be seen for the different tests except for the CELISA2.

TABLE IV. CUT-OFF VALUES OR DECISION LIMITS (POSITIVE/NEGATIVE THRESHOLD) BASED ON NEGATIVE "FREE AREA" POPULATION

| ELISA Test | Pre-established [1] | Local [2] | ADRI selected [3] | ROC [4] |
|---|---|---|---|---|
| IELISA-IAEA | 35 | 30 | 30 | 20 |
| IELISA-ADRI | 46 | 44 | 50 | 50 |
| CELISA1 | 30 | 33 | 30 | 30 |
| CELISA2 | 30 | 37 | 29 | 29 |

1 Defined by the IAEA and ADRI
2 Defined based on the entire uninfected population from San Andrés. Colombia, N=866
3 Defined based on selected (BPTA/RB/CFT (-)) negative population, N=842
4 Defined based on Received Operation Characteristic (ROC) analysis, N=1064

Figures 1, 3, 5 and 7 show the frequency distribution of the selected sera evaluated for each of the ELISA tests under study. In Figures 2,4,6 and 8 values for optimisation of the cut-off are plotted as ROC analysis.

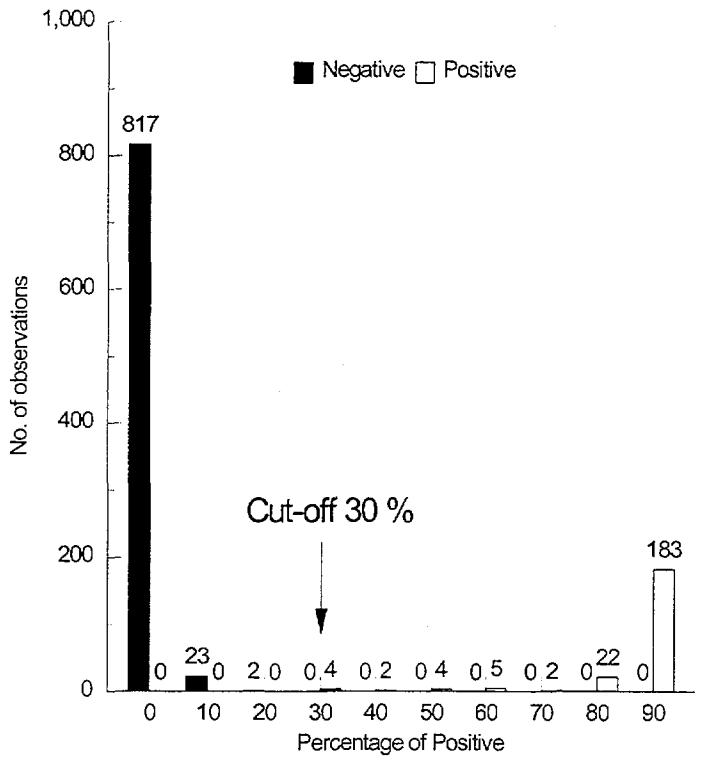


*FIG. 1. Frequency distribution Brucella abortus I-ELISA-IAEA (BPRT/RB/CFT N=1064).*

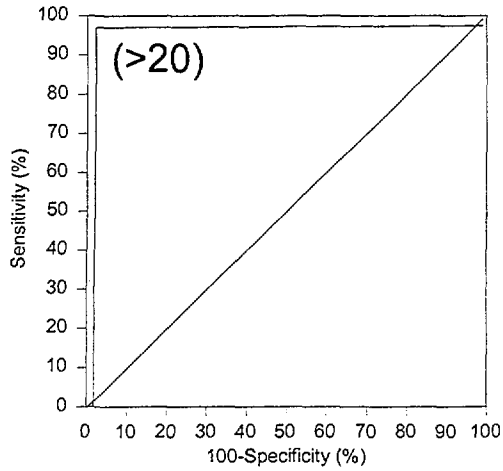


*FIG. 2. ROC curve I-ELISA-IAEA.*

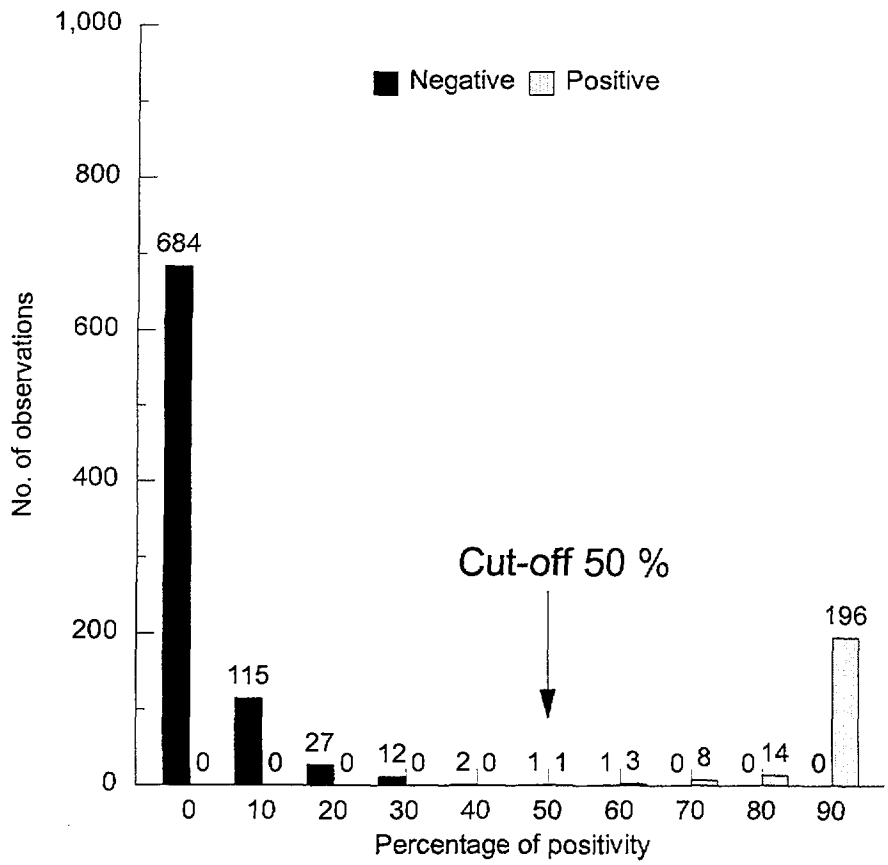


*FIG. 3. Frequency distribution Brucella abortus I-ELISA-ADRI (BPRT/RB/CFT N=1064).*

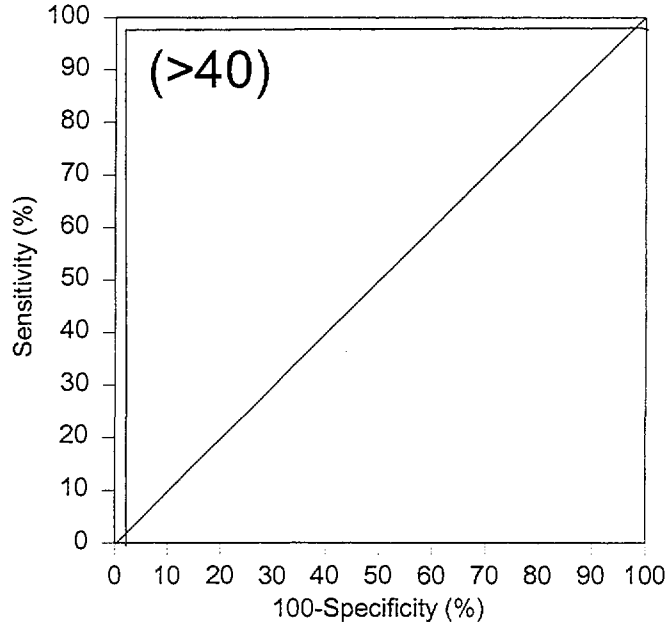


*FIG.4. ROC curve I-ELISA-ADRI.*

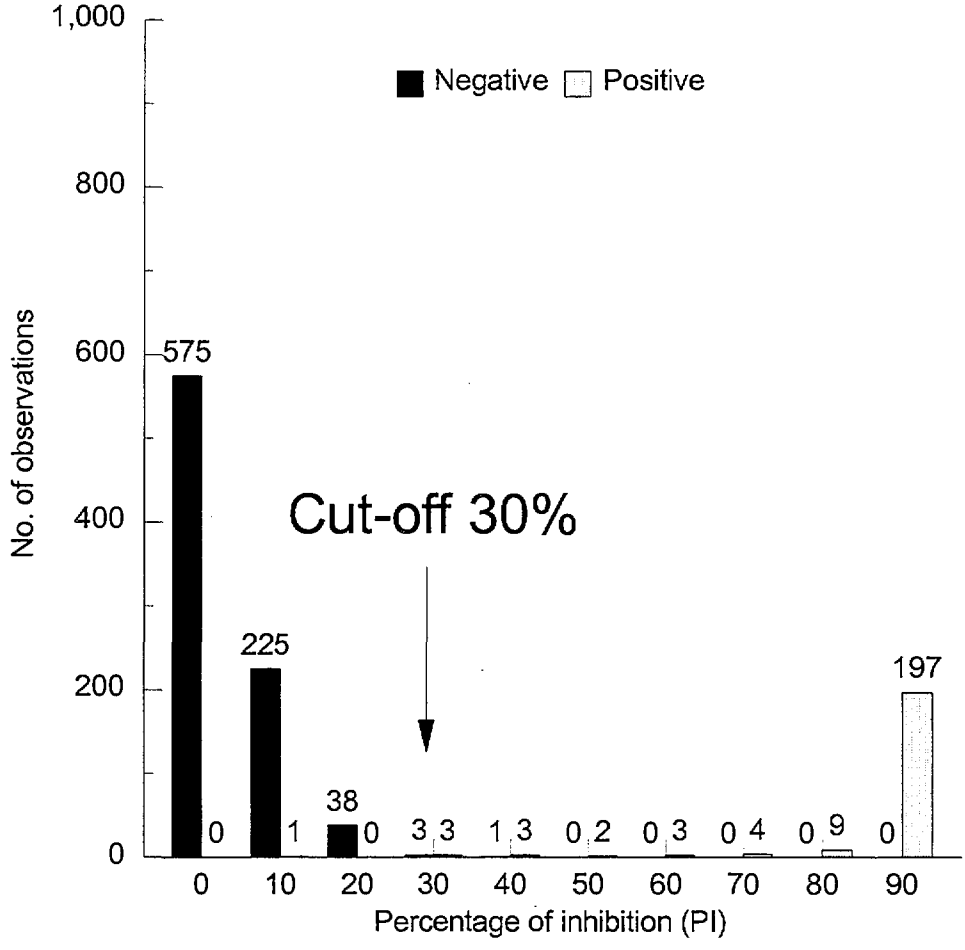


*FIG. 5. Frequency distribution Brucella abortus C-ELISA-1 (BPRT/RB/CFT N=1064).*

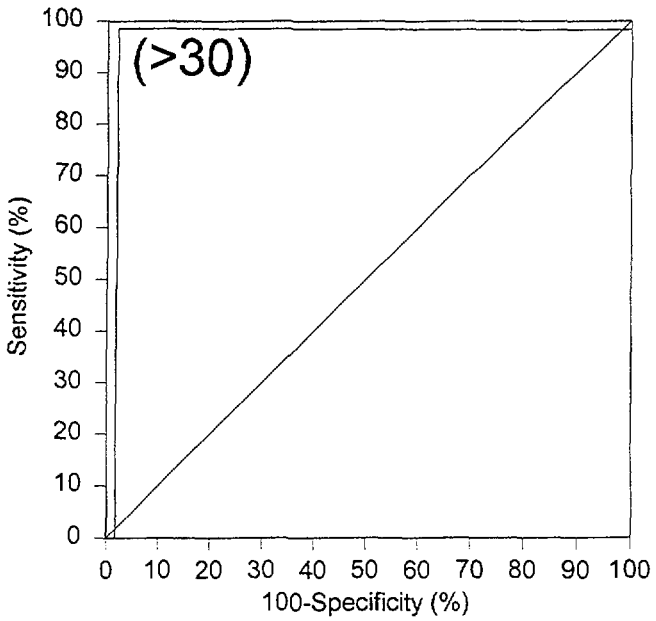


*FIG. 6. ROC curve C-ELISA-1.*

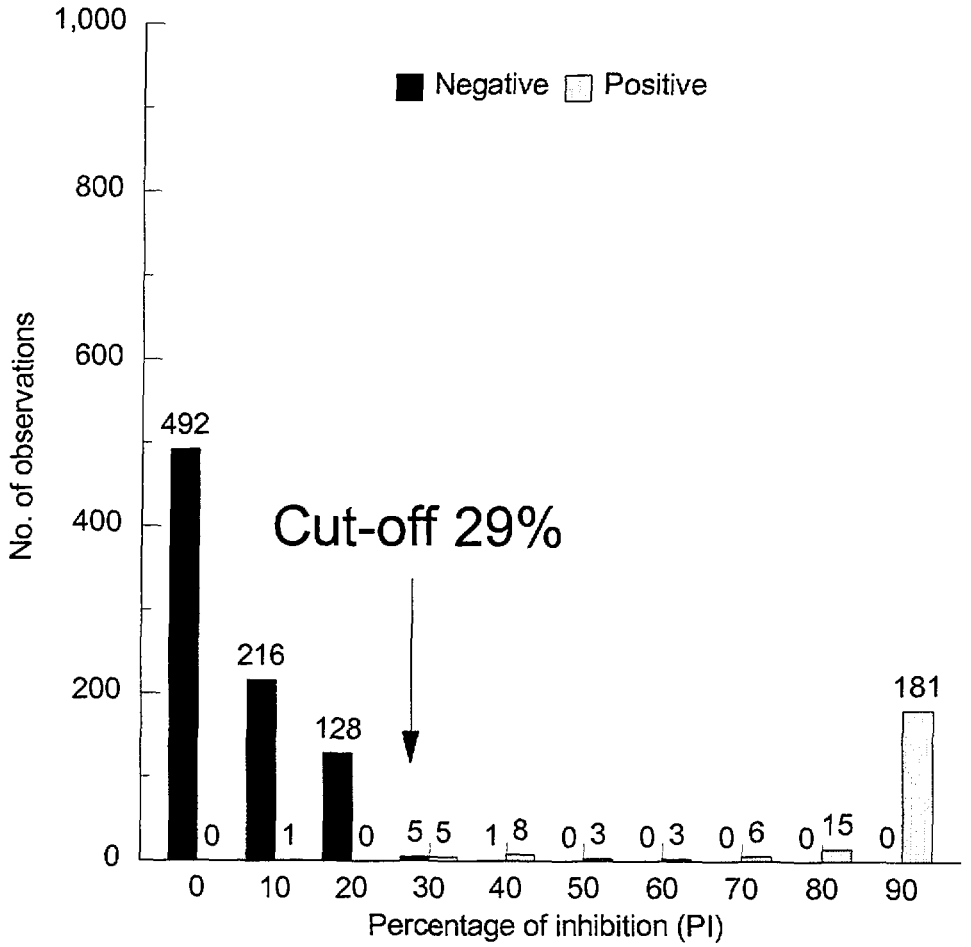


*FIG. 7. Frequency distribution Brucella abortus C-ELISA-2 (BRT/RB/CFT N=1064).*

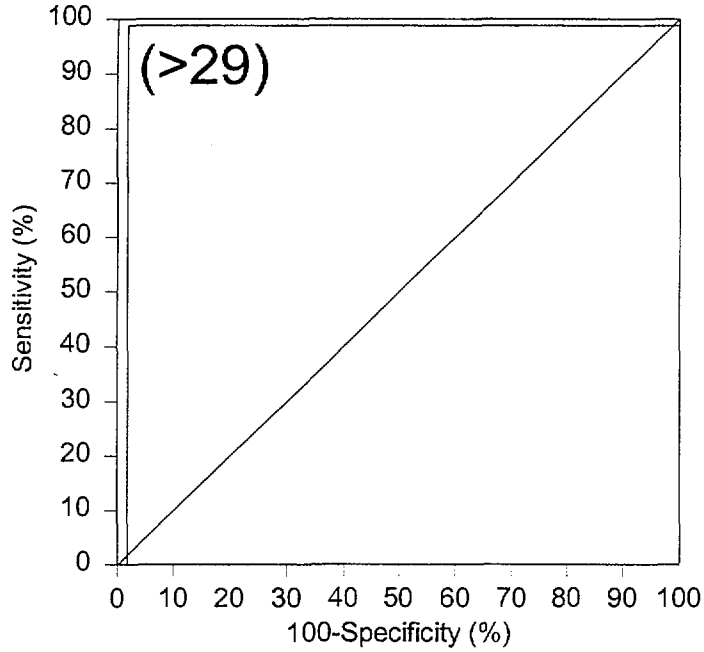


*FIG. 8. ROC curve C-ELISA-2.*

In our hands the indirect ELISAs performed as well as conventional screening tests but with a higher specificity for FAO/IAEA indirect ELISA. Regarding competitive tests the cut-off values were 30 and 29% for CELISA1 and CELISA2 respectively. As expected for vaccinated population (Table V) in areas were vaccination is commonplace or mandatory the specificity values were lower for all tests, since ELISA detects more positives than CFT. CELISA1, demonstrated higher specificity than CELISA2 and the local cut-off for this test was higher than the one obtained by the ROC analysis. The differences were reduced significantly when the analysis was performed on selected populations. Comparison between ELISAs gave high Kappa agreement and acceptable confidence limits as shown in Table VI . Accuracy estimates were over 0.95 for all ELISA tests Table VII confirming the quality of the assays.

TABLE V. RELATIVE DIAGNOSTIC VALUES FOR SENSITIVITY AND SPECIFICITY IN SELECTED POPULATION

| ELISA (cut-off) | Sensitivity[1] | Specificity[2] | Specificity vacc.[3] |
|---|---|---|---|
| IELISA IAEA (30%) | 100% (97.84 to 100) | 100% (99.11 to 100) | 98.70% +/-0.76% |
| IELISA ADRI (50%) | 100% (97.89 to 100) | 99.76% (99.46 to 100) | 95.51% +/-1.39 |
| CELISA1 (30%) | 99.08% (96.37-99.84) | 99.81% (98.79-99.99) | 96.34% +/-1.26% |
| CELISA2 (29%) | 98.62% +/-1.55% | 97.00% +/-1.45% | 94.08% +/-1.59% |

1 ELISA positives defined (BPTA/RB/CFT(+)) N=222 RID positives N=103
2 ELISA negatives defined (BPTA/RB/CFT(-)) N=842
3 ELISA negatives defined (BPTA/RB/CFT (-)) N=1039

TABLE VI. MEASURE OF AGREEMENT BETWEEN ELISAs: KAPPA VALUES

| Test | IAEA | C.L.* % | ADRI | C.L. % | C-ELISA1 | C.L. % |
|---|---|---|---|---|---|---|
| I-IAEA | | | | | | |
| I-ADRI | 0.9954 | 0.3 | | | | |
| C-ELISA1 | 0.8707 | 0.9 | 0.7766 | 1.7 | | |
| C-ELISA2 | 0.8031 | 0.9 | 0.7454 | 1.7 | 0.9475 | 1.4 |

Kappa=observed proportion agreement-total chance proportion agreement /1-total chance proportion agreement
*C.L. 95% Confidence limits
BPTA/RB/CFT selected sera, N=2137

TABLE VII. ACCURACY ESTIMATES BASED ON SENSITIVITY, SPECIFICITY AND DISEASE PREVALENCE FOR THE DATA

| TEST | (Cut-off) | Accuracy* |
|---|---|---|
| I-ELISA IAEA | (30%) | 0.9920 |
| I-ELISA ADRI | (50%) | 0.9648 |
| I-ELISA ADRI | (46%) | 0.9632 |
| C-ELISA1 | (30%) | 0.9761 |
| C-ELISA2 | (29%) | 0.9597 |
| C-ELISA2 | (30%) | 0.9636 |

*Accuracy TPF x P(D +) + TNF x p(D -) Where: TPF= sensitivity, TNF= specificity, P(D+)= disease prevalence for data and P(D-)= 1-P(D+), BPTA/RB/CFT selected sera, N=2137

## 4. CONCLUSIONS

The ELISA tests were standardised and validated, and the cut-off values defined for the local conditions. The indirect ELISA demonstrated a higher diagnostic specificity than does the BPAT, RB and CFT without compromising diagnostic sensitivity. The test offer a distinct diagnostic advantage as a laboratory based screening assay.

The competitive ELISA is capable of discriminate between infected cattle and those who have been vaccinated or exposed to a cross reactive organism. Lower values than those observed were expected for CELISA2. This could be explained based on aberrant results from sera of animals from recently vaccinated animals from areas in which adult vaccination could not be excluded.

The data presented continue to support the initial hypothesis that the ELISA methodology designed for Brucellosis will provide more precise and standardised method for diagnosis, and for the support of the control and eradication campaigns.

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