USE OF AN INDIRECT ELISA FOR *BRUCELLA ABORTUS* DIAGNOSIS IN CUBA

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Abstract

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Introducing immunoassays in *Brucella* diagnosis requires a comparative study with reference techniques such as the complemente fixation reaction (CFR). Sensitivity and relative specificity studies allowed us to observe the behaviour of this immunoassay, using samples from free of disease, free by vaccination and affected areas. Sensitivity results for a cut-off point of 40PP and a confidence interval of 95% ranged from 94.8 to 99.5% and the specificity between 94.1 and 97.5%. For free of disease areas a cut-off point of 22PP was calculated that reached a 99% specificity. This immunoassay for the detection of antibodies against *Brucella abortus* must be used with two different cut-off points, depending on the epidemiologic conditions of the country, with CFR in affected or vaccinated areas as a confirmative method.

1. INTRODUCTION

Serological diagnosis of brucellosis is used in many countries as the criteria for control and eradication of this disease. Several conventional techniques are used for this, and although each one characteristically detects different antibody isotypes, to determine an animal seropositive to brucellosis it is necessary to use techniques such as Bengal Rose (BR), agglutination in buffered plate (ABP) or Slow tube agglutination (SAT). The 2-mercaptoethanol (2-Me) and Complement fixation test (CFT) are used as confirmatory techniques. [1].

The introduction of indirect immunoenzymatic techniques (ELISA) in serological diagnosis has allowed the achievement of higher sensitivity and specificity levels than most commonly used conventional techniques [2,3]. These assays have already been approved by the Office Internationale des Epizootics (OIE) [4]. Nevertheless a correct diagnosis requires adjusting the technique according to the epidemiological situations existent in each region [3,5,6,7]. These studies require negative samples from disease free animals and positive samples from animals with clinical, serological or epidemiological evidences of the disease [6,8].

In Cuba, for more than 30 years, a programme for the control and eradication of brucellosis in cattle has been carried out by slaughter of all serologically positive animals. A decrease of 0.045% in the incidence of the disease has been achieved. Presently to assure an efficient diagnosis in the affected areas, as well as an adequate monitoring of disease-free areas it is necessary to use a highly sensitive and specific diagnostic method.

This study details the results obtained with an indirect ELISA provided by the Joint FAO/IAEA Division of the International Atomic Energy Agency (IAEA), for the serological diagnosis of brucellosis in sera of disease-free, vaccinated disease-free and affected animals, using the complement fixation test as a reference technique.

2. MATERIALS AND METHODS

2.1. Sera

2.1.1. Brucellosis-free area

Sera samples from 2125 non-vaccinated animals from areas free of brucellosis were collected. The studied herds had not presented clinical, bacteriological or serological evidences of disease during the last 20 years.

2.1.2. Vaccinated area

Sera samples from 1313 animals vaccinated with *Brucella abortus* strain 19, from areas without clinical, bacteriological or serological evidences of the disease for the last 2 years.

2.1.3. Brucellosis affected area

Sera samples from 1278 animals from brucellosis affected areas. These herds were vaccinated with strain 19 and animals with clinical, bacteriological and serological evidences of brucellosis infection have been found. In these areas the incidence is low thanks to the adequate control and eradication program carried out in the country.

2.2. Serological test

2.2.1. Complement Fixation Test

The antigen used in the CFT was produced in Cuba by Laboratorios Biológicos Farmaceúticos (LABIOFAM, Biological Pharmaceutical Laboratories). All sera were evaluated by 50% hemolysis CFT according to the protocol of Alton and co-workers (1988).

2.2.2. Indirect ELISA

This ELISA was carried out using an indirect ELISA kit for the diagnosis of brucellosis, following the instructionsdescribed elsewhere [10]. In NUNC polystyrene microplates 100 µl of 1 µ g/ml concentrated smooth lipopolysaccharide (SLPS) in 0.05M pH 9.6 carbonate buffer were added, and incubated overnight at 4°C. The plates were washed three times in PBS-Tween-20 and samples or controls diluted 1:200 were added and incubated for 1 hour at 37°C. All samples were tested in duplicate, while the control sera were tested four times. Controls: strongly positive, weakly positive, negative and a conjugate control, where no serum was added. After another washing, a peroxidase conjugated anti bovine IgG monoclonal antibody was added. While using the kit, conjugate dilutions varied; they were adjusted to an O.D. values of 1.000 The incubation time was 1 hour at 37°C. Finally, after another washing, 100 µl of 3% hydrogen peroxide plus 1 mM [2,2 azinobis (3-ethyl-benzthiazoline sulfonic acid)] ABTS dissolved in 0.05M citric acid/sodium citrate buffer (PH-4,5) were added and incubated 10 min. at 37°C. The reaction was stopped by adding 100 µl of 4% solution of sodium dodecyl sulphate and the plate was read at 405 nm using the software supplied with the kit.

All the data were stored in Microsoft Excel and the cut-off point calculations, as well as the analysis of the samples were carried out using EPI INFO-6.0 and the Statistical Package Program.

The cut-off point for areas free of disease was calculated organizing all the results in increasing order and dividing them in one hundred equal percentiles. The average of the 99 percentile for each population was calculated and this value used as the cut-off point for areas free of disease. For the affected areas the Receiver Operating Characteristics Analysis (ROC-analysis) was used [2,7]. To select the cut-off value, the point where the specificity of the assay assures a minimum of false positive samples and a higher positive predictive value without affecting the sensitivity of the technique was determined.

3. RESULTS AND DISCUSSION

Of the 2125 samples tested in areas free of disease, 37 reacted positively when the cut-off point was established at a 22 positivity percent (PP) reaching a specificity of 99 %. This sera were reevaluated and negative results obtained. In the samples from vaccinated disease free areas only 25 sera were positive at a 44 PP cut-off point with specificity ranging between 97.3 and 98.8 %. Among the sera from affected areas the results for 58 samples were not in accordance with the CFT. In this population sensitivity values reached 99 to 99.5 % with a positive predictive value between 66 and 78 % while specificity reached 94 to 96.3 % and the negative predictive value was 99 - 100 %. All these data are shown in Table I.

TABLE I. SENSITIVITY, SPECIFICITY, POSITIVE PREDICTIVE AND NEGATIVE PREDICTIVE VALUES OF INDIRECT ELISA USING CFT AS A REFERENCE FOR A 95% CONFIDENCE INTERVAL

Area	Sensitivity	PPV	Specificity	NPV
Free areas 35PP	-	-	99.5%	-
Free areas 22PP	-	-	99%	-
Vaccinated areas 35PP	-	-	96.5-98.2%	-
Vaccinated areas 44PP	-	-	97.3-98.8%	-
Affected areas 35PP	95-99.8%	59-71%	92-94%	99-100%
Affected areas 44PP	94-99.5%	66-78%	94-96.3%	99-100%

PPV Positive predictive values
NPV Negative predictive values
PP Percent positivity

Figure 1 shows the behaviour of the sensitivity and specificity using different cut-off points in animals from affected areas. It can be observed that when the specificity of the assay increases, the sensitivity decreases [2,3]. The 44 PP cut-off point results in a specificity of 95.2 % with a sensitivity of 98 %.

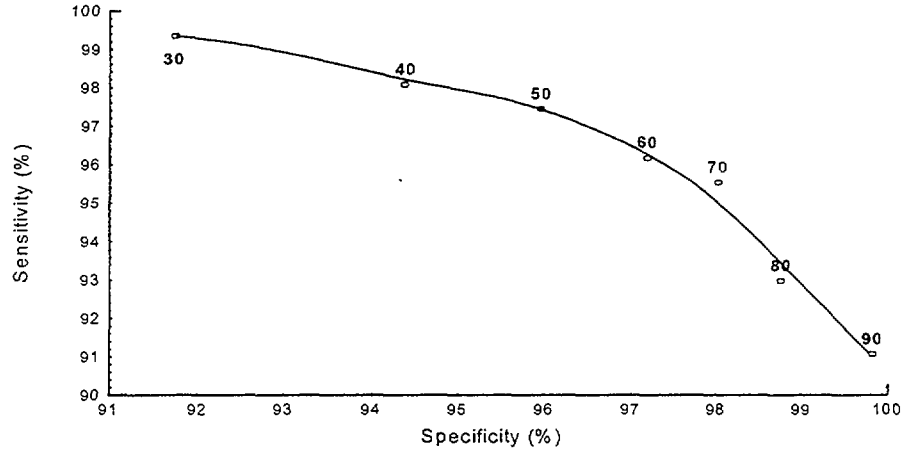


FIG. 1. Analysis of the sensitivity and specificity using different cut-off points in animal from affected areas.

The frequency distribution of the samples from areas free of disease (Figure 2) shows that the maximum dispersion of samples is found between 0 and 20 PP, which proves that there are no specific antibodies against *brucella sp.* Samples over 22 PP in a first analysis showed non-specific reactions and resulted negative upon reevaluation, as expected. This indicates that samples in these areas showing unexpected results should be retested.

In vaccinated free-of-disease and affected areas the frequency distribution for negative samples (Figures 3 and 4) showed dispersion in a higher PP range than the one observed for disease free areas, because the levels of antibodies in healthy animals in these areas are higher due to circulation of the vaccine strain [2,5]. In the affected areas non-specific reactions were found in 58 samples, since the assay can not distinguish vaccinated from infected animals and because beyond the selected cut-off point (44 PP) there are samples with positive and negative results for the CFT. For better diagnostic assurance Jacobson (1990) recommends confirmation of the ELISA positive results by a reference technique (CFT) when the disease prevalence is low [6].

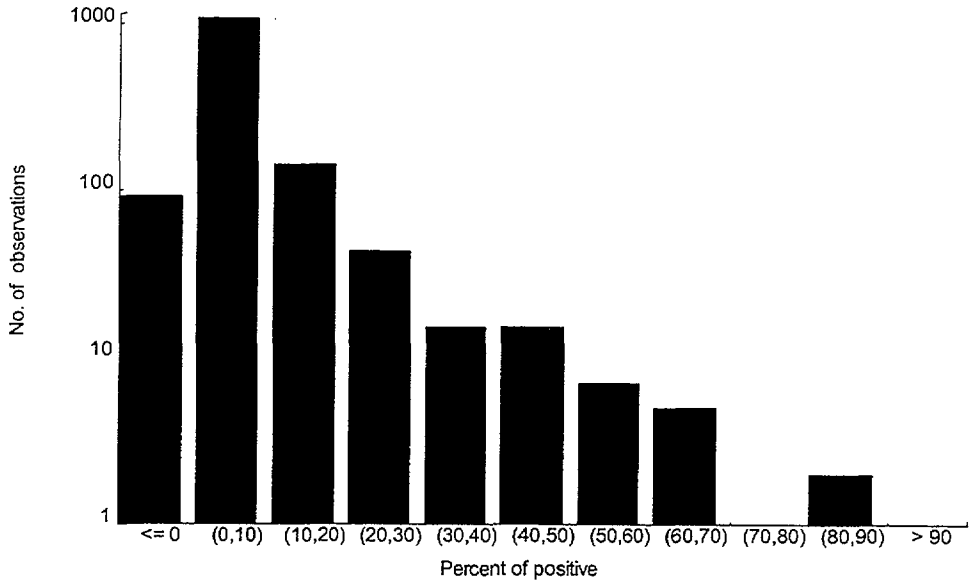


FIG. 2. Frequency distribution of the samples from areas free of disease.

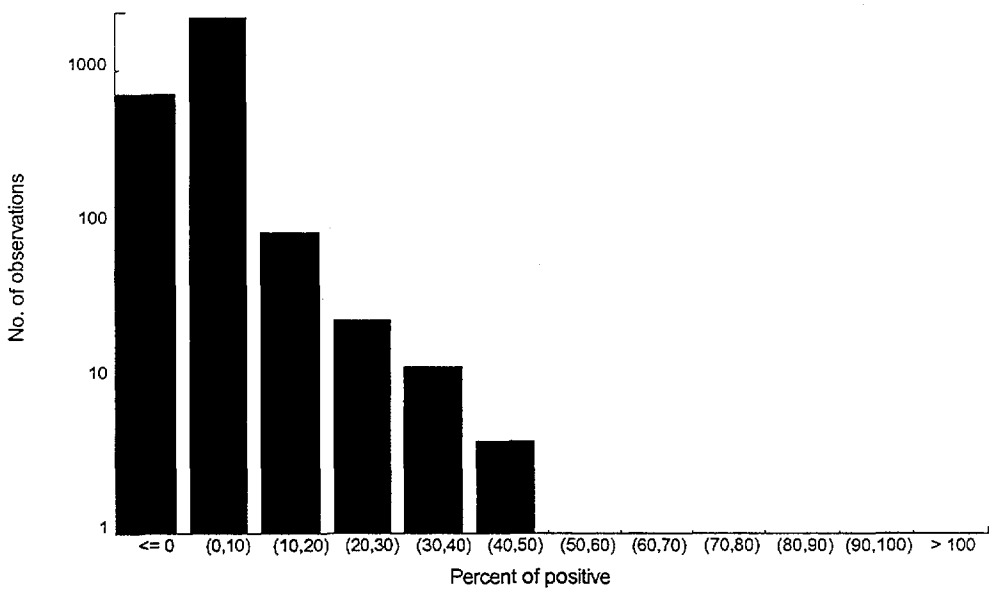


FIG. 3. Frequency distribution of the samples from vaccinated free of disease areas.

As can be appreciated in Figure 4, for a cut-off point of 44 PP the positive predictive value of this immunoassay is affected by false positive samples. Jacobson [7] establishes the need for increasing the specificity of the assay in these cases, nevertheless in our conditions this is not posible because we would miss some of the true positives. In Cuba, thanks to the control and eradication program, there is a low incidence of this disease. At present it is considered preferable to slaughter an animal suspicious of being positive than missing a seropositive animal. The use of two cut-off points depending on the epidemiological situation found in different area has been established before by Jacobson [7] and Uzal and co-workers [11]. In Cuba this allows using same assay for the serological study of any herd by just changing the cut-off value. Due to the disease control in the country these areas are well defined geographically, which makes laboratory diagnosis easier.

An analysis of the sample distribution in the three evaluated categories is shown in Figure 5. It can be appreciated that for negative samples from the affected areas the maximum PP values are higher than those obtained in vaccinated areas and both, taken separately, are higher than those found in free of disease areas. These results are justified by the circulation of the vaccine strain in those areas, that maintains detectable antibody titers [4]. It is also observed that most of the negative samples from these populations are under the 44 PP cut-off point for affected and free by vaccination areas and 22 PP for disease free areas.

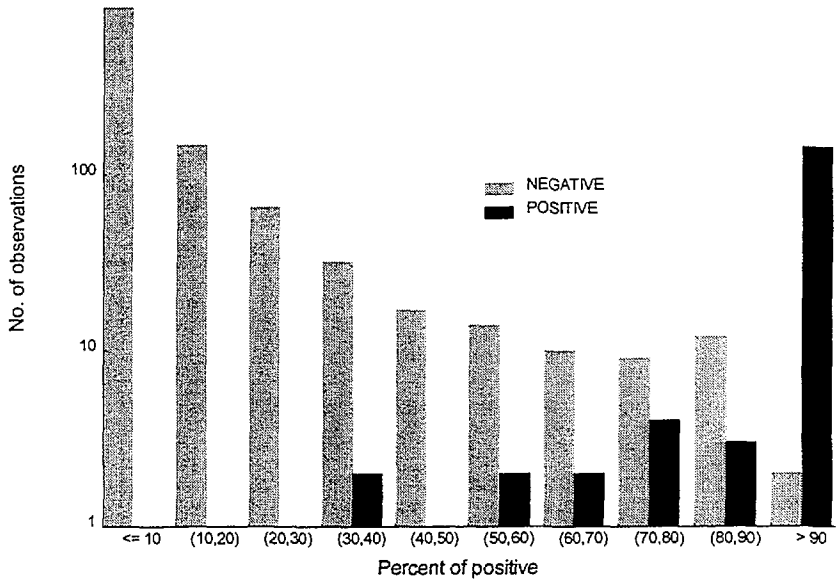
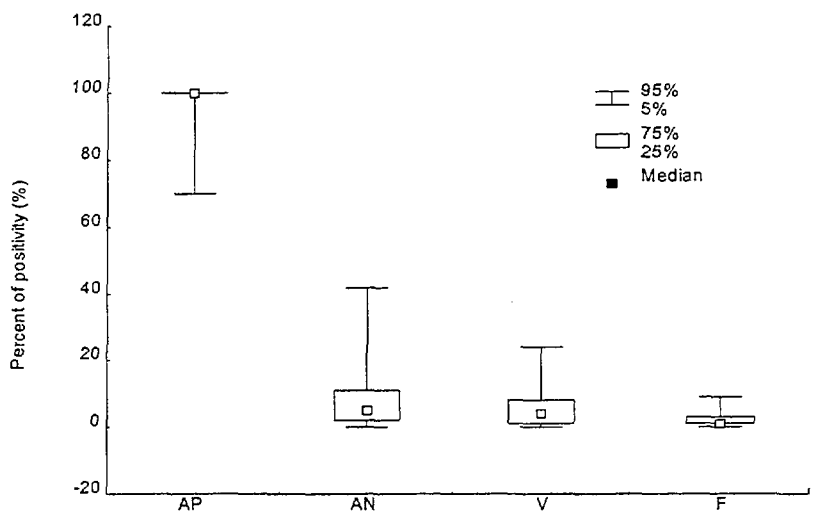


FIG. 4. Frequency distribution of samples from affected areas.



AP Positive animals from affected areas
AN Negative animals from affected areas
V Animals from vaccinated areas
F Animals from free areas

FIG. 5. Analysis of the samples dispersion in the three evaluated categories.

4. CONCLUSIONS

In the indirect ELISA, changing the cut-off point to 22 PP for free-of-disease areas does not affect the specificity of the technique and allows a much more strict control for monitoring these areas, where antibody titres are low.

Using a 44 PP cut-off point instead of the 35 PP recommended by the IAEA gives a better assay specificity in affected areas, as well as a better positive predictive value. Although this assay does not distinguish vaccinated from infected animals, it can also be used for monitoring free by vaccination areas and for diagnosing affected areas where animals were vaccinated more than one year before. For that purpose an appropriate cut-off has to be determined.

Due to its high sensitivity CFT can be used as a confirmatory diagnostic test to eliminate false positive samples.

ELISA technique is a fast diagnostic method that enables a large number of samples to be tested at a relatively low cost.

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