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Determining a Diagnostic Cut-Off on Fluorescence Polarization Assay (FPA) for Bovine Brucellosis in Carchi, Ecuador

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Abstract

Serology is the foundation of any brucellosis control and eradication program worldwide, thus it is important to define accuracy diagnostics assays and cut-off of those assays, due to variations from country to country and even among specific areas in the country. The variation of cutoff values depended on: prevalence of disease, vaccination status, animal management, and control and eradication programs. Therefore, a cut-off for the diagnosis of bovine brucellosis through fluorescence polarization assay (FPA) in Carchi—Ecuador was determined. The survey has been carried out in Carchi province of Ecuador, who is considered a province of high prevalence of brucellosis and the vaccination status is unknown due to the lack of registers. Sera samples (n = 200) were obtained from individual cows from randomly selected herds. Blood sera were tested through Fluoresce Polarization Assay (FPA) and competitive enzyme-linked inmunosorbent assay (cELISA) as confirmatory test, and then receiver operating characteristic (ROC) analysis was done. The sensitivity and specificity values of FPA were 88.7% and 92.50% respectively using a cut-off of 89.90 mP. Moreover, the area under the curve showed that 92.2% is the probability accuracy of the test. The advantage of the FPA is that it is a test with good characteristics of sensitivity and specificity as well as a simple and quick test.

Keywords

Brucella, Diagnosis, Fluorescence Polarization, Sensitivity, Specificity

1. Introduction

Brucellosis is a bacterial disease caused by species of the genus Brucella [1]. It is

considered the most persistent zoonosis worldwide by the World Health Organization (WHO), the International Organization for Animal Health (OIE), and the Food and Agriculture Organization of the United Nations (FAO) [2]. Animal brucellosis causes important productive and reproductive losses, where the most common consequence is abortions [2]. The human can be accidentally infected through direct contact with bacteria from infected animals or by ingestion of contaminated products without proper cooking, causing symptoms ranging from asymptomatic to debilitating [2].

Although brucellosis has been controlled and eradicated in many countries of the world, Ecuador continues to be an endemic country with high prevalence rates; so a project that seeks to reduce the number of infected animals in the different epidemiological areas was developed by "Agencia Ecuatoriana para el Aseguramiento de la Calidad del Agro" (AGROCALIDAD) [3], using as strategies: vaccination, epidemiological surveillance with serology and slaughter of seropositive animals.

Serology is the basis of any program for the control and eradication of brucellosis worldwide, so it is important to define accurate and precise diagnostic tests based specifically on the epidemiological situation of the country [4]. In addition, once the diagnostic tests are defined, it is also important to define the "cut-off", which is a value that allows to categorize from the area of medicine or epidemiology a disease as "presence" or "absence" [4]. The cut-off of diagnostic tests varies in each country and even between localities within the country, depending on: disease prevalence, vaccination status, animal management and the implementation or non-implementation of control and eradication programs [4]. The aim of this study was to determine "cut-off" of the fluorescence polarization assay (FPA) for the diagnosis of bovine brucellosis in the province of Carchi, Ecuador, to improve sensitivity and specificity of the test to be used in the area, since it constitutes a region of high prevalence of brucellosis in the northern part of Ecuador, with prevalence ranging from 4% to 10.62% [5].

2. Methodology

This study was carried out in the province of Carchi, located in the northern Andean region of Ecuador, whose main activity is agriculture, representing 8.22% of the total of Agricultural Productive Units (APUs) in the country. It also concentrates 8.74% of the total dairy cattle of the Sierra region and contributes with 5% of the milk production of Ecuador [6].

2.1. Study Design

Among the epidemiological characteristics of the study area are: estimated prevalence of brucellosis from 4% to 10.62% [5], the vaccination status is unknown due to lack of registers, and the use of different types of vaccines [7].

Blood serum samples (n = 200) were obtained by calculating the sample considering a population (n = 15,536) of bovine females older than two years, a

sample error of 7%, and a 95% confidence interval, because the population is considered homogenous thanks to its sanitary status, and the similar animal husbandry [8].

The serum was analyzed by the fluorescence polarization assay (FPA) and a competitive enzymatic test (cELISA) as confirmatory test, due to the cELISA is prescribed as confirmatory test in the National Control Program for Brucellosis in Ecuador [3]. Moreover the cELISA is an accuracy test that presents sensitivity and specificity of 99.02% and 99.96% respectively [10].

FPA. The fluorescence polarization assay was performed as described in the Ellie Brucella Antibody Test Kit (Milwaukee, USA, Lot B1001). Briefly, the FPA kit uses a polysaccharide-O (OPS) extract of *Brucella abortus* bacterium conjugated with fluorescein. Sera and controls (20 µl) were placed into boron-silicate tubes plus a diluent (1 ml) and incubated for three minutes at room temperature to perform a white reading of all samples and controls. Then, 10 µl of the antigen with fluorescein was added in all tubes and incubated for three minutes at room temperature, in order to get repeated reading and obtaining the mili-polarization (mP) values of all samples and controls.

cELISA. The competitive ELISA test was performed in an external laboratory certified by AGROCALIDAD, as described by the SVANOVIR® Brucella-Ab c-ELISA commercial kit from SVANOVA. Briefly, the c-ELISA kit uses mouse monoclonal antibodies specific for the polysaccharide-O (OPS) of the bacterium *Brucella abortus* as a solid base. Sera samples and controls (5 µl) plus diluent (45 µl) were placed in each well of the flat-bottomed microplate in duplicate. Then 50 µl of mouse monoclonal antibodies were placed in both sera and controls, after that the reagents were mixed for an approximate time of 5 minutes and then incubated for 30 minutes at room temperature, thereafter performing 4 consecutive washings using the wash buffer. Then, 100 µl of the conjugate solution was added and incubated again for 30 minutes at room temperature, thereafter repeating 4 consecutive washings using the wash buffer. After that, 100 µl of the substrate solution was added and incubated for 10 minutes at room temperature in order to apply the braking solution (50 µl) in each well. The measurement through optical densities (OD) uses a wavelength of 450 nm, which is performed after 15 minutes of applying the braking solution. The interpretation of results was performed considering the percentage of inhibition (PI), obtained by subtracting 100 for the division of the average of OD of the samples with the OD of the conjugate, as shown in Table 1.

Table 1. Interpretation of competitive ELISA results (cELISA).

Percent inhibition results (PI)	
Negative	**Positive**
<30%	≥30%

2.2. Statistical Analysis

In order to determine the cut off, a Receiver Operational Characteristics (ROC) analysis was performed, which is a graphical representation of the sensitivity and specificity of a binary system according the variation of discrimination threshold. In addition, the area under the curve was calculated, which is indicative of the accuracy of the test, the closer it is to 1.00, the more accurate it is [4].

3. Results and Discussion

The OIE mentions that for the case of brucellosis there is no appropriate diagnostic test that can be applied uniquely within a control and eradication program, due to the epidemiological variants of the area and the animal species affected by this disease. Because of this, the OIE proposes a series of tests that can be applied for the control of brucellosis at the national or local level, such as: Bengal Rose (RB), Plate or Tube Agglutination (BPA), Enzyme Testing (ELISA) and fluorescence polarization (FPA) [1].

In the case of Ecuador, there are no reported studies of the use of the fluorescence polarization assay for the diagnosis of bovine brucellosis, which the OIE says is an accurate, simple and fast technique, but it is important to adapt it to the conditions where it is going to be applied according to the epidemiological situation [1].

Once the coordinates of the curve (**Table 2**) were revised, a cut off of the fluorescence polarization assay for the province of Carchi-Ecuador could be defined as 89.90 mP, thus achieving values of 88.7% of sensitivity and 92.50% specificity, using cELISA as the confirmatory test.

Based on our results the FPA test is acceptable for the diagnosis of bovine brucellosis in Carchi - Ecuador, since it shows characteristics of sensitivity and specificity of 88.7% and 92.50% respectively with a cut off of 89.90 mP. Similar results were reported by Nielsen & Gall [9] with values of sensitivity and specificity of 88.1% and 94.6%, respectively. In the same way Nielsen *et al.* [10] obtained values of sensitivity and specificity of 99.02% and 99.96% respectively with a cut off of 90.0 mP.

Despite the acceptability of the FPA test for the diagnosis of bovine brucellosis in Carchi - Ecuador, the characteristics of sensitivity and specificity obtained are not very encouraging, attributed to the fact that the sampling was performed on animals where the sanitary status was unknown as mentioned by Nielsen *et al.* [11].

This outcome was obtained due to the cattle from which the sampling was done was vaccinated with *Brucella abortus* strain 19 (S19). This is despite the FPA in most cases does not detect S19 vaccine antibodies, as indicated by Nielsen *et al.* [10].

The knowledge of the vaccination status for brucellosis is an epidemiological key for control and eradication programs, but in the study area there is a very

Table 2. Coordinates of the curve, and sensitivity and specificity values of the fluorescence polarization assay.

Coordinates of the curve	Sensitivity	1-Specificity
38.9000	1.000	1.000
53.7000	1.000	0.981
69.0500	0.991	0.981
70.7000	0.981	0.981
72.0500	0.981	0.962
74.5500	0.972	0.962
78.1000	0.962	0.962
81.0000	0.962	0.943
81.6500	0.962	0.925
81.7500	0.962	0.906
81.9500	0.962	0.868
82.5500	0.962	0.849
83.1500	0.962	,830
83.5000	0.962	0.811
83.7500	0.962	0.792
83.9000	0.953	0.792
84.0500	0.953	0.774
84.1500	0.953	0.736
84.3000	0.953	0.717
84.4500	0.943	0.698
84.5500	0.943	0.679
84.7000	0.943	0.642
84.9500	0.943	0.585
85.2000	0.943	0.566
85.3500	0.943	0.547
85.5000	0.934	0.528
85.6500	0.934	0.472
85.7500	0.934	0.453
85.8500	0.934	0.415
85.9500	0.934	0.377
86.0500	0.934	0.358
86.1500	0.934	0.340
86.3500	0.934	302
86.6000	0.934	0.264
86.8000	0.934	0.245
89.0500	0.896	0.113

Continued

89.0500	0.896	0.113
89.5500	0.896	0.094
89.7500	0.887	0.094
89.9000	0.887	0.075
90.2500	0.877	0.075
90.6000	0.868	0.075
90.8500	0.858	0.075
91.1000	0.755	0.075
91.4500	0.726	0.075
91.9000	0.726	0.038
92.3500	0.717	0.038
93.0000	708	0.038
93.9000	0.698	0.038
95.8000	0.698	0.019
98.5000	0.689	019
100.8500	0.679	0.019
103.3500	0.670	0.019
105.2000	0.660	0.019
107.8000	0.651	0.019
110.0500	0.651	0.000
113.4000	0.642	0.000

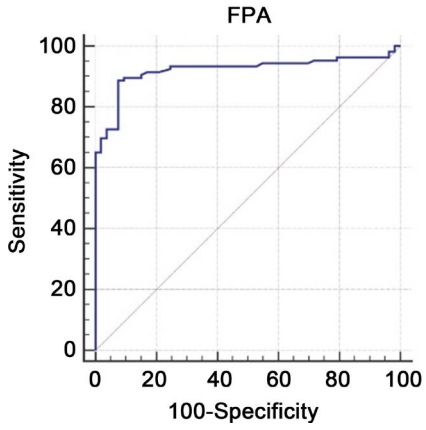

Figure 1. Curve and area under the curve for the fluorescence polarization assay.

low or no culture to use registers, making it difficult to analyze this variable. Similar results were described by Ron J. [12]; Corbel A. [2]; Ibarra M. [7].

The area under the curve, indicative of the accuracy of the test, shows that there is a probability of 92.2% that the test yields correct results (**Figure 1**). Results that are similar to those described by McGiven *et al.* [13], show a ratio of

91.5% between the FPA and cELISA.

In addition, the area under the curve shows values close to 1.00 that denote the feasibility of using FPA for the diagnosis of bovine brucellosis in Carchi - Ecuador, as to say of Hanley J. & McNeil B. [14] the area under the curve of a receiver operational characteristics analysis (ROC) allows to define the capacity of a diagnostic test to discriminate between healthy and sick individuals.

4. Conclusions

Our research indicates that the cut-off for the diagnosis of bovine brucellosis through the fluorescence polarization assay in the province of Carchi, Ecuador is 89.90 mP, with characteristics of sensitivity and specificity of 88.7% and 92.50% respectively, considering the cELISA test as confirmatory.

In addition, this cut-off is a beginning to work on vaccinated animals by evaluating the specificity of this test and to propose it as a confirmatory diagnosis, replacing cELISA, since it is a quick and simple test.

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