FIELD TRIAL OF A BRUCELLOSIS COMPETITIVE ENZYME LINKED IMMUNOABSORBENT ASSAY (ELISA)

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Abstract

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The purpose of this study was to evaluate the performance of a competitive ELISA system for the diagnosis of bovine brucellosis in comparison to conventional serological tests routinely used in Argentina. A total of 2,500 serum samples, comprising Brucella-free herds, vaccinated cattle and naturally infected animals, was tested by the following tests: buffered plate agglutination, Rose Bengal, 2-mercaptoethanol, complement fixation, and indirect and competitive ELISAs. Specificity and relative sensitivity at each test were determined. The competitive ELISA was considered suitable for detection of vaccinated animals and had higher specificity than the other tests. The results point to the potential use of the test as a complementary assay in the brucellosis control programme in Argentina.

1. INTRODUCTION

Bovine brucellosis has been a major disease for many years in Argentina. Although considerable efforts have been undertaken to control it, in some regions of the country, prevalence rates are still high ranging from 30 to 35% [1]. As an action of the National Control and Eradication Program, massive vaccination of female calves, at an age between 3 and 10 months, using the S-19 vaccine strain, has been carried out during the last two years. Vaccine coverage raised from 45% in 1994 to 85% in 1996 [2,3].

The Buffer Plate Agglutination Test (BPAT) is considered the screening diagnostic assay in Argentina. Negative animals are not further tested and positive samples are tested by Tube Agglutination (TA), 2-mercaptoethanol (2-ME) and Complement Fixation (CF) as complementary tests [3]. However, many animals have vaccine-induced antibodies over a long period, especially those vaccinated around 10 months of age. Therefore, in infected herds it is difficult to distinguish antibody titres generated after vaccination from those produced due to natural field infections. None of the usual serological tests is able to distinguish antibodies produced against S-19 vaccine strain from natural infections [4]. A specific and reliable test will be desirable in any control and eradication program to enable to distinguish vaccinated from infected animals.

The purpose of this study was to evaluate the performance of a Competitive ELISA (C-ELISA) for the diagnosis of bovine brucellosis in the differentiation of Brucella-infected from Brucella-vaccinated cattle, in comparison to the Indirect ELISA (I-ELISA) and to conventional serological techniques used in Argentina.

2. MATERIALS AND METHODS

2.1. Experimental Design

Sera from 2,500 animals from three different groups were analysed:

Group A: 500 serum samples collected from non-vaccinated cattle from brucellosis-free herds. A herd was considered free if no clinical and/or bacteriological evidence of brucellosis was registered and CF or 2-ME or Rivanol tests were negative for at least 2 years prior sampling.

Group B: 1,000 serum samples collected from S-19 calfhood-vaccinated cattle from brucellosis-free herds. Vaccinated herds had no clinical and/or bacteriological evidence of brucellosis for at least 2 years prior sampling, but had low prevalence rates (< 1%) of CF or 2-ME or Rivanol positive results during the last 2 years prior sampling.

Group C: 1,000 serum samples collected from vaccinated cattle from Brucella-infected herds, in which field strains of B. abortus had been isolated. In infected herds some animals had clinical and/or bacteriological evidence of brucellosis and high prevalence rates (> 5%) of CF or 2-
ME or RIV positive results in animals older than 18 months were observed at the time of sampling. Samples were collected in a total of 12 farms, being 4 for each group.

2.2. Serological Tests

The BPA and RB results were expressed as positive or negative. Classification of cattle as seronegative or seropositive was set at a titre of 1:25 for the 2-ME and at a titre of 1:10 for the CF. These conventional tests were performed as described elsewhere [5,6].

Four ELISA systems were used:

a) an indirect ELISA (IE-1) using smooth lipopolysaccharide (SLPS) antigen and a mouse monoclonal anti-bovine IgG1 (MabM23) conjugated to horseradish peroxidase (HRPO) [9];

b) a modification of the IE-1 (IE-2) using SLPS and Mab M23, specific for bovine IgG1, conjugated with HRPO. In this technique, the sera is previously treated with EDTA/EGTA (dilution 1/50) [10];

c) a competitive ELISA (CE-1) using O-polysaccharide of B. abortus S1119-3 as antigen and YsT9 monoclonal antibody conjugate for competition;

d) a competitive ELISA (CE-2) using SLPS of B. abortus 1119/3 as antigen and a mouse monoclonal antibody (Mab M84) conjugate.

The local cut-off values were set at 40% positivity (%P) for IE and 40% inhibition (%I) for CE. The cut-off were obtained by testing negative serum samples [4]. The results were recorded as the mean of the optical density (O.D.) value of duplicates in all ELISA systems.

The BPA (12% Cel. Vol.) and RB (4,2% Cel. Vol.) antigens and all ELISA reagents and plates were provided by the FAO/IAEA. The antigens for 2-ME and CF tests were produced at INTA following standardised procedures [7].

All ELISAs were performed using polystyrene microplates (NUNC 269620,) and O.D. values were measured in a Multiskan II microplate reader linked to a 486 personal computer, using the FAO/IAEA software program BREIA 1.02.

2.3. Estimation of assay performance [8]

Diagnostic Specificity = \( \frac{\text{number of test negative cattle}}{\text{number of true negative cattle}} \times 100 \)

Relative Sensitivity = \( \frac{\text{number of comparative test positive}}{\text{number of relative test positive}} \times 100 \)

3. RESULTS

Table I shows the diagnostic specificity (D.S.) of each test in relation to negative serum samples (group A). The D.S. was 100% for CF, 100% for 2-ME, 98.6% for I-ELISA, 99.8% for C-ELISA, 99.8% for BPA and 97.7% for RB. Table II shows the D.S. of the sera from vaccinated animals (group B). D.S. for CF was 96.4%, 2-ME 93.6%, I-ELISA 95.8%, C-ELISA 97.5%, BPA 35.4% and for RB 37.6%. Table III shows the relative sensitivity (R.S.) for the CF test considering sera from infected animals (group C). The R.S. for 2-ME was 99.8%, I-ELISA 98.2%, C-ELISA 97.3%, BPA 99% and RB 96%. Tables IV and V show the kappa agreement and the ROC analyses respectively.
TABLE I. DIAGNOSTIC SPECIFICITY (DS) OF SEROLOGICAL TESTS FOR BOVINE BRUCELLOSIS CONSIDERING THE NEGATIVE HERDS (Group A, n = 500)

<table>
<thead>
<tr>
<th></th>
<th>BPA</th>
<th>RB</th>
<th>CF</th>
<th>2-ME</th>
<th>IE-1</th>
<th>IE-2</th>
<th>CE-1</th>
<th>CE-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>1</td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>9</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>-</td>
<td>499</td>
<td>489</td>
<td>500</td>
<td>500</td>
<td>493</td>
<td>491</td>
<td>496</td>
<td>499</td>
</tr>
<tr>
<td>DS%</td>
<td>99.8</td>
<td>97.8</td>
<td>100</td>
<td>100</td>
<td>98.6</td>
<td>98.2</td>
<td>99.2</td>
<td>99.8</td>
</tr>
</tbody>
</table>

2-ME: Positive = 1:25 or more. CF: Positive = 1:10 or more
CE-1: LPS + O-Chain, cut-off: 40%
CE-2: LPS + M84 (EDTA/EGTA) cut-off: 40%
IE1: LPS + M23 (EDTA/EGTA) IE-2: LPS + M23, cut-off: 40%

TABLE III. RELATIVE SENSITIVITY (R.S.) OF CF TEST CONSIDERING SERA FROM INFECTED ANIMALS (Group C, n = 1,000)

<table>
<thead>
<tr>
<th></th>
<th>BPA</th>
<th>RB</th>
<th>2-ME</th>
<th>IE-1</th>
<th>IE-2</th>
<th>CE-1</th>
<th>CE-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>999</td>
<td>971</td>
<td>998</td>
<td>982</td>
<td>969</td>
<td>965</td>
<td>975</td>
</tr>
<tr>
<td>-</td>
<td>1</td>
<td>29</td>
<td>2</td>
<td>18</td>
<td>31</td>
<td>35</td>
<td>25</td>
</tr>
<tr>
<td>RS%</td>
<td>99.9</td>
<td>97.1</td>
<td>99.8</td>
<td>98.2</td>
<td>96.9</td>
<td>96.5</td>
<td>97.5</td>
</tr>
</tbody>
</table>

RS: Relative Sensitivity (CF= 1:10 or more)
2-ME: Positive = 1:25 or more
CE-1: LPS + O-Chain. Cut-off: 40%
CE-2: LPS + M84 (EDTA/EGTA) Cut-off: 40%
IE-1: LPS + M23 (EDTA/EGTA) IE-2: LPS + M23, Cut-off: 40%

TABLE IV. KAPPA AGREEMENT

<table>
<thead>
<tr>
<th></th>
<th>C-ELISA-1-OC</th>
<th>C-ELISA-2-M84</th>
<th>I-ELISA-1-ADRI</th>
<th>I-ELISA-2-IAEA</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-ELISA1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C-ELISA2</td>
<td>0.931 (0.903-0.958)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>I-ELISA1</td>
<td>0.812 (0.769-0.855)</td>
<td>0.855 (0.817-0.893)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>I-ELISA2</td>
<td>0.910 (0.879-0.941)</td>
<td>0.927 (0.899-0.955)</td>
<td>0.824 (0.782-0.866)</td>
<td>-</td>
</tr>
</tbody>
</table>

95% Confidence Limits in brackets

TABLE V. ROC ANALYSIS (Area under the ROC Curve)

<table>
<thead>
<tr>
<th>ELISA</th>
<th>Area under ROC curve</th>
</tr>
</thead>
<tbody>
<tr>
<td>IE-1ADRI</td>
<td>0.983 (95% CI = 0.978 to 0.995)</td>
</tr>
<tr>
<td>IE-2 (IAEA)</td>
<td>0.983 (95% CI = 0.978 to 0.995)</td>
</tr>
<tr>
<td>CI-1 (O-Ch)</td>
<td>0.991 (95% CI = 0.979 to 0.996)</td>
</tr>
<tr>
<td>CE-2 (M-84)</td>
<td>0.992 (95% CI = 0.980 to 0.998)</td>
</tr>
</tbody>
</table>

4. DISCUSSION AND CONCLUSIONS
In Argentina the bovine brucellosis control and eradication campaign was initiated through a national resolution in November of 1993 [2] and considerable efforts have been undertaken to achieve its goals. An insufficient vaccination coverage has substantially contributed to the spread of the disease. In Argentina, where the use of the S-19 vaccine is compulsory since 1982, vaccine coverage of female calves has never been more than 50% until recently. Due to an animal health information campaign during the last 3 years vaccination coverage raised to approximately 90% in 1996 [2]. The use of a whole set of diagnostic serological techniques is mandatory. Performance of these tests is expensive and has created a certain reluctance in the farmer community. Thus, a unique and standardised technique could have an important impact in our brucellosis program. Additionally, none of the conventional tests used for diagnosis is able to differentiate Brucella-vaccinated from Brucella-infected animals.

Enzyme immunoassays for diagnosis of brucellosis have been studied for many years. In this study it could be demonstrated that the diagnostic specificity and relative sensitivity of ELISA is comparable to CF and 2-ME, which are the official complementary tests in Argentina. Validation of the I-ELISA some years ago corroborated its high sensitivity but its major drawback was the low specificity and its incapacity to differentiate Brucella-vaccinated from infected animals [4,8,9,1]. The competitive ELISA proved to be the most suitable test to identify vaccinated animals. The specificity of the test was higher than other tests including CF. Although no significant differences between both competitive ELISAs could be observed, the C-ELISA using SLPS antigen is preferred because the antigen can be easily produced at low costs. Most of the false negative animals were common to all complementary tests. Interestingly, most of them are corresponding to Brangus breeds (cross breeding between Bos angus and Bos indicus).

Application of ELISA is feasible on large scale due to its reproducibility and easy standardisation. Complement fixation has a great performance but its application is cumbersome. The 2-ME test takes 48 hrs. to be completed and the reagent is toxic. Due to our conditions at farm level e.g. brucellosis prevalence and compulsory vaccination of female calves the C-ELISA could be applied, replacing the conventional tests as a complementary test of the official screening test (BPA). This study demonstrates that the ELISA test would be very useful to the Control and Eradication Program of Bovine Brucellosis in Argentina.

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REFERENCES
