APPLICATION OF ENZYME-LINKED IMMUNOSORBENT ASSAYS FOR THE DIAGNOSIS OF BOVINE BRUCELLOSIS IN RIO GRANDE DO SUL, BRAZIL

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Abstract

APPLICATION OF ENZYME-LINKED IMMUNOSORBENT ASSAYS FOR THE DIAGNOSIS OF BOVINE BRUCELLOSIS IN RIO GRANDE DO SUL, BRAZIL.

The results of an indirect ELISA (I-ELISA) and a competitive ELISA (C-ELISA) for detection of antibody to Brucella abortus in cattle were compared with those of conventional serological tests. The sensitivity of I-ELISA using 230 sera from infected animals was 98.6% and the specificity in 720 sera from brucellosis-free animals was 98.2%. The C-ELISA when tested in 94 positive sera and 91 negative sera was 100% sensitive and specific. The relative sensitivity and specificity of I-ELISA compared to C-ELISA in 582 sera from groups of animals vaccinated with S19 by different routes and doses was respectively 64% and 100%. When comparing five serological tests on sera from vaccinated animals, the specificity was 94% for C-ELISA, 93% for 2-mercaptoethanol (2ME), 88% for Rose Bengal Plate test (RBPT), 84% for Slow Tube Agglutination test (SAT) and 34% for I-ELISA. It can be concluded from this study that the RBPT gave less false positive results than the I-ELISA in vaccinated animals and the C-ELISA is a good confirmatory test with the advantage of distinguishing the antibody response due to vaccination from that resulting from infection with Brucella abortus.

1. INTRODUCTION

The diagnosis of brucellosis in cattle is frequently complicated particularly when live vaccines such as strain 19 of Brucella abortus are used on a large scale. The antibody response induced by these vaccines is difficult to distinguish from that of natural infection by conventional serological tests [1].

Several supplementary serological tests such the agglutination with 2 mercaptoethanol, complement fixation and agar-gel immunodiffusion containing a soluble polysaccharide antigen have been shown to differentiate to some extent the antibody response of vaccinated from infected animals [2,3].

In recent years, the enzyme-linked immunosorbent assay (ELISA) using well characterized smooth lipopolysacharide of Brucella abortus in an indirect ELISA or the O-polysacharide in a competitive ELISA, has been shown to be a very sensitive and specific method for measuring antibody responses [4,5]. On the other hand, brucellosis can be controlled by the use of a reduced dose of S19 either by subcutaneous or conjunctival routes in young or adult females [6,7]. In controlled conditions, these methods resulted in good protection against exposure with a minimum of serological responses [8]. In order to minimize the problems of agglutinin titers that complicate the diagnosis, the use of reduced doses of S19 vaccine and more specific serological tests such enzyme immunoassays have been investigated [5, 9,10].

In the State of Rio Grande do Sul, Brazil, after a period of 20 years of vaccination with Brucella abortus S19, the prevalence of brucellosis decreased from 5.2% in 1965 [11] to 0.33% in 1986 [12]. In this situation, the possibility of starting a program of eradication of the disease is envisaged. The objective of the present study was to compare ELISA tests (indirect and competitive) with conventional tests on sera from negative, infected and S19 vaccinated cattle.

2. MATERIAL AND METHODS

2.1. Test sera

Negative sera: a total of 720 sera were obtained from herds in an area without recent history of infection or vaccination against brucellosis.

Positive sera: a total of 230 sera from three herds were collected. These herds presented reproductive problems, abortions, several positive bacteriological and serological results for Brucella abortus.
2.2. Vaccinated animals

**Group 1**: 16 heifers (3-6 months) were vaccinated with standard dose \((6 \times 10^{10})\) of *Brucella abortus* S19 by subcutaneous route.

**Group 2**: 20 adult females (over 2 years) were vaccinated with reduced dose \((3-5 \times 10^9)\) of *Brucella abortus* by conjunctival route and revaccinated four months later.

**Group 3**: 21 heifers (3-6 month) were vaccinated with reduced dose \((3-5 \times 10^9)\) of *Brucella abortus* by conjunctival route and revaccinated four months later.

All animals were tested in all serological tests before vaccination and bled monthly for up to 300 days after vaccination.

2.3. Serological tests

2.3.1. Conventional tests

The tests used were the Rose Bengal Plate Test (RBPT), the Slow Tube agglutination test (SAT) and the 2 mercaptoethanol (2ME) as described by Alton et al. [2]. In the RBPT any degree of agglutination was considered to be positive. For the SAT, visible agglutination at the dilution of 1/100 was considered to be positive and for the 2ME, visible agglutination at the dilution of 1/25 was considered to be positive.

2.3.2. Indirect ELISA

An ELISA kit provided by the FAO/IAEA, which contained all the necessary reagents was used. The test was performed according the manual which accompanied the kit [13]. Briefly, a 1 µg/ml dilution of smooth lipopolysaccharide (S-LPS) was prepared in 0.05M carbonate buffer (pH 9.6) and 100 µl were added to wells of 96-well polystyrene plates (NUNC 2-69620) and incubated covered overnight at 4°C.

After three wash cycles (Handiwasher, BDSL) with 0.01M phosphate buffered saline (pH 7.4) containing 0.05% Tween 20 (PBST), 100 µl of test sera (in duplicate) and control sera (in quadruplicate) diluted 1/200 in PBST were added to the wells and incubated for 1h. at 37°C with continuous shaking on an orbital shaker.

Four controls were included: a strong positive serum, a weak positive serum, a negative serum and a buffer control. After three more wash cycles with PBST, 100 µl of a horseradish peroxidase (HRPO) conjugated with rabbit anti-bovine IgG (H+L), diluted in PBST, were added to each well and the plates incubated again as described above.

After three final wash cycles, 100 µl of 4mM hydrogen peroxide (H₂O₂) and 1mM [2,2-azinobis (3-ethyl-benzthiazoline sulfuric acid)] (ABTS) dissolved in 0.05M sodium citrate/citric acid (pH 4.5) were added to all wells. The plates were incubated for 10 min. at 37°C with continuous shaking for color development and to stop the reaction 100 µl of sodium dodecyl sulphate (SDS) were added to all wells.

Optical density readings were obtained using a spectrophotometer (Titertek Multiskan Plus) at 405 nm. The reader was linked to a computer and the results expressed as percent positivity (PP), calculated by means of the software BREIA 1.02, supplied by the FAO/IAEA with the kit. Any serum which gave 35% or higher positivity (PP) was considered as positive.

2.3.3. Competitive ELISA

The technique adopted was described by Nielsen et al. [14]. Briefly, the O-polysaccharide (PS) was diluted in coating buffer (carbonate/bicarbonate, pH 9.6), and used at 2µg/ml. The plates were coated with 100 µl/well of diluted antigen and incubated at 25°C overnight and again for 18-20 hours at -20°C. After thawing at 37°C in water bath, the plates were washed 5 times as described for I-ELISA and test sera and control: a buffer control, a serum from vaccinated animal, a negative serum and a strongly positive serum were added.

All sera were diluted 1/50 in PBST and 50 µl of each dispensed in duplicate and the controls in quadruplicate. Immediately, 50 µl of prediluted horseradish peroxidase conjugated monoclonal antibody was dispensed into all wells.
The plates were sealed and incubated for 2 hours at 25°C. After 5 wash cycles, H₂O₂-ABTS was added as described for I-ELISA and color was developed for 10 minutes, when 100 µl of stopping solution were added to all wells.

The readings were performed on the same equipment as for I-ELISA and the results expressed as percent inhibition, calculated by mean of the software ELISA 2.11. Any serum which gave over 20% inhibition was considered as positive.

2.4. Data analysis

**Indirect ELISA** - provided the controls were within acceptable limits, the results were expressed in percent positivity (PP) which was calculated as follows [13]:

\[
PP = \frac{\text{Mean OD of duplicate tests}}{\text{Mean OD of C++ control}} \times 100
\]

**Competitive ELISA** - the results were expressed as percent inhibition (PI) of the monoclonal antibody activity against O-polysaccharide antigen (buffer control) and was calculated by the formula [14]:

\[
PI = 1 - \frac{\text{Mean OD of replicate tests}}{\text{Mean OD of buffer control}} \times 100
\]

**Sensitivity and Specificity** - the sensitivity of tests used on sera from infected cattle and the specificity in brucellosis free animals was calculated according to Thrusfield [15] and followed the formulae for each test:

\[
\text{Sensitivity} = \frac{\text{Total N° of positive results}}{\text{Total N° of positive animals sampled}}
\]

\[
\text{Specificity} = \frac{\text{Total N° of negative results}}{\text{Total N° of negative animals sampled}}
\]

3. RESULTS

3.1. Negative sera

The specificities of the four tests performed on serum from 720 brucellosis-free animals are presented in Table I. Additionally the competitive ELISA was done on 91 sera from the negative group.

3.2. Positive sera

The sensitivities of the four serologic tests performed on 230 Brucella-infected animals are presented in Table II. Additionally competitive ELISA data for 94 sera are included.

3.3. Vaccinated animals

The specificities of the serologic tests on serum from 452 vaccinated animals are presented in Table III. Additionally competitive ELISA results on 291 sera are also included.

The comparison between C-ELISA and I-ELISA on 582 sera from S19 vaccinated animals is shown in Table IV.
TABLE I. THE SPECIFICITY OF DIFFERENT TESTS ON SERA FROM BRUCELLOSIS-FREE ANIMALS

<table>
<thead>
<tr>
<th>Test</th>
<th>Neg</th>
<th>Pos</th>
<th>Total</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBPT</td>
<td>720</td>
<td>0</td>
<td>720</td>
<td>100.0</td>
</tr>
<tr>
<td>SAT</td>
<td>719</td>
<td>1</td>
<td>720</td>
<td>99.8</td>
</tr>
<tr>
<td>2ME</td>
<td>720</td>
<td>0</td>
<td>720</td>
<td>100.0</td>
</tr>
<tr>
<td>I-ELISA</td>
<td>707</td>
<td>13</td>
<td>720</td>
<td>98.2</td>
</tr>
<tr>
<td>C-ELISA</td>
<td>91</td>
<td>0</td>
<td>91</td>
<td>100.0</td>
</tr>
</tbody>
</table>

RBPT—Rose Bengal Plate Test  
SAT—Serum Agglutination Test in tubes  
2ME—2 Mercaptoethanol  
I-ELISA—Indirect Elisa  
C-ELISA—Competitive ELISA

TABLE II. THE SENSITIVITY OF DIFFERENT SEROLOGIC TESTS ON SERA FROM BRUCELLA-INFECTED ANIMALS

<table>
<thead>
<tr>
<th>Test</th>
<th>Pos</th>
<th>Neg</th>
<th>Total</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBPT</td>
<td>230</td>
<td>0</td>
<td>230</td>
<td>100.0</td>
</tr>
<tr>
<td>SAT</td>
<td>195</td>
<td>35</td>
<td>230</td>
<td>84.8</td>
</tr>
<tr>
<td>2ME</td>
<td>206</td>
<td>24</td>
<td>230</td>
<td>89.6</td>
</tr>
<tr>
<td>I-ELISA</td>
<td>227</td>
<td>3</td>
<td>230</td>
<td>98.6</td>
</tr>
<tr>
<td>C-ELISA</td>
<td>94</td>
<td>0</td>
<td>94</td>
<td>100.0</td>
</tr>
</tbody>
</table>

(See Table 1 for key)

TABLE III. THE SPECIFICITY OF DIFFERENT SEROLOGICAL TESTS IN SERA FROM S19-VACCINATED ANIMALS

<table>
<thead>
<tr>
<th>Test</th>
<th>Pos</th>
<th>Neg</th>
<th>Total</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBPT</td>
<td>54</td>
<td>398</td>
<td>452</td>
<td>88.0</td>
</tr>
<tr>
<td>SAT</td>
<td>72</td>
<td>380</td>
<td>452</td>
<td>84.0</td>
</tr>
<tr>
<td>2ME</td>
<td>31</td>
<td>421</td>
<td>452</td>
<td>93.1</td>
</tr>
<tr>
<td>I-ELISA</td>
<td>299</td>
<td>153.0</td>
<td>452</td>
<td>34</td>
</tr>
<tr>
<td>C-ELISA</td>
<td>18</td>
<td>273</td>
<td>291</td>
<td>94.0</td>
</tr>
</tbody>
</table>

(See Table 1 for key)

TABLE IV. COMPARISON BETWEEN C-ELISA AND I-ELISA ON 582 SERA FROM S19-VACCINATED ANIMALS

<table>
<thead>
<tr>
<th>Competitive ELISA</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indirect ELISA</td>
<td>18</td>
<td>191</td>
<td>209</td>
</tr>
<tr>
<td>Positive</td>
<td>0</td>
<td>373</td>
<td>373</td>
</tr>
<tr>
<td>Total</td>
<td>18</td>
<td>564</td>
<td>582</td>
</tr>
</tbody>
</table>

Relative sensitivity = 100%, Relative specificity = 64%

The serological response of heifers and adult cattle vaccinated with a standard dose subcutaneously and reduced doses conjunctivally of S19 vaccine in five serological tests is shown in
Figures 1 - 3. All animals were bled at an interval of approximately 1 month until 300 days after vaccination.

**FIG. 1.** Serological response of heifers vaccinated with subcutaneous standard dose of S-19 in five serological tests (n=16).

**FIG. 2.** Serological response in adult cattle vaccinated with reduced dose of S 19 in five serological tests (n=20).
4. DISCUSSION

A simple, rapid and inexpensive serological test that will detect infected animals early in the incubation period and at all stages of the disease and that does not detect antibody in vaccinated animals is still to be found. Nevertheless, a great deal of improvement was achieved recently either by the introduction of enzyme immunoassays [16] or by vaccination of animals with reduced dose of S19 *Brucella abortus* vaccine [8].

In the present study two different enzyme immunoassays were compared with conventional tests on sera of negative, infected and vaccinated animals. The specificity of the enzyme immunoassays was high, 98.2% for the I-ELISA and 100% for the C-ELISA on sera from brucellosis-free animals. In this situation, the I-ELISA was the test which gave the highest rate of false positive reactions. Those positive sera gave a PP just over the threshold value and the sera could be classified as suspicious.

The sensitivities of the ELISAs were 98.6% for the indirect and 100% for the competitive. Except for the RBPT, the two ELISAs were more sensitive than the other tests. These findings are in accordance with others [17,18] in that RBPT was found to be a good screening test, although some authors [19] have found an unacceptable false negative rate with the RBPT.

In the vaccinated groups, the animals vaccinated with a standard dose subcutaneously (Figure 1) gave 100% of positive results in RBPT, I-ELISA and SAT, 90% in 2ME and less than 40% in C-ELISA at 50 days after vaccination. Except for the SAT and I-ELISA, most sera were negative 6 months after vaccination, which is in accordance with the literature [10-20]. The I-ELISA was the most sensitive test, giving a high percentage of positive results until the end of the experiment (300 days). This may be explained by the use of a polyclonal anti bovine IgG (H+L) conjugate which measures all
isotypes present in the sera. This stresses the usefulness of the test as a screening test among vaccinated animals [14].

The competitive ELISA gave fewer positive results in the vaccinated groups. It is known that this test may be unable to differentiate about 15% of sera from S19 vaccinated animals, and it is not uncommon to observe positive results at the peak of the antibody response [16]. Furthermore, one animal remained positive in all tests throughout the experiment. This could be due to a transient S19 infection making this serum behave as sera from infected animals [5].

In the groups of animals vaccinated with a reduced conjunctival dose (Figures 2-3) except for I-ELISA, most animals became negative to all tests in less than 3 months after vaccination, with a peak of antibodies just after revaccinations. These rapid decreases in the antibody suggest that this practice could be adopted in campaigns against brucellosis. The I-ELISA gave a high percentage of positive results which could be explained by the different antibody affinity of the test. [4].

The degree of immunity induced by a reduced dose of S19 dose was not determined. Nevertheless protective immunity is well documented in the literature [8,21].

When the specificity of all tests was determined for vaccinated (Table III), the C-ELISA was the most specific (94%), followed by 2ME (93%), RBPT (88%), SAT (84%) and I-ELISA (34%). The relative sensitivity and specificity of I-ELISA when compared with C-ELISA (Table IV) was 64% and 100% respectively. This low specificity of I-ELISA may account by the fact that only vaccinated animals were compared in both tests.

From these results it can be concluded that C-ELISA can be used as confirmatory test. The actual mechanism by which C-ELISA can differentiate vaccinated from infected animals is not fully understood. Some authors speculate that it can be a result of antigen presentation on the polystyrene matrix, duration of exposure to the bacterium and antibody affinity [14-16].

In spite of the low specificity of the I-ELISA and the relatively high cost of this test, it has the advantage of being highly sensitive, that only small amounts of reagents are required and that it can be readily adapted to large scale screening.

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