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SENSITIVITY AND SPECIFICITY OF A COMPETITIVE ENZYME IMMUNOASSAY IN THE SERODIAGNOSIS OF BOVINE BRUCELLOSIS

SENSIBILIDADE E ESPECIFICIDADE DE UM TESTE IMUNOENZIMÁTICO COMPETITIVO NO DIAGNÓSTICO SOROLÓGICO DA BRUCELOSE BOVINA

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SUMMARY

The purpose of this work was to evaluate the sensitivity and the specificity of a competitive enzyme immunoassay, using as conjugate the monoclonal antibodies BM-38 and BM-40, in the serodiagnosis of bovine brucellosis. Seventy-four sera from culture-positive cattle and 2,118 cattle sera from herds free from brucellosis and negative to the Rose Bengal plate test were examined. The competitive enzyme immunoassay, using any of the two conjugates, was able to reveal the presence of antibodies to Brucella lipopolysaccharide in all of the 74 sera of the infected cattle, resulting in a sensitivity of 100%. The specificity of the test using the conjugate BM-38 was 98.82% and using the conjugate BM-40 was 99.95%. These results indicated that the competitive enzyme immunoassay, mainly when using the conjugate BM-40, consists in a technique very useful in the confirmation of the serological diagnosis of bovine brucellosis.

UNITERMS : Brucellosis, bovina; Serological diagnosis; Competitive Enzyme Immunoassay; Cattle

INTRODUCION

Programs for the eradication or control of bovine brucellosis are based mainly on test and slaughter measures, and the diagnosis is usually performed by serological methods, due to their practicality. A great variety of serological tests have already been developed for brucellosis' diagnosis and some of them, as the Rose Bengal plate test and the complement fixation test have proved very useful for sanitary purposes. However, as advanced steps of an eradication program are reached, there is a need of more sensible and more specific tests.

Several different serological tests have been developed to detect antibodies to **Brucella**, including enzyme immunoassays. After the development of technology for monoclonal antibody production, a competitive enzyme assay was developed [11].

The results already published on the performance of the competitive enzyme immunoassay very according by to some details of the test, as the technique, the antigen, and mainly according to the monoclonal antibody used as conjugate. RYLATT et al.[11] (1985) found, that the test using monoclonal antibodies Bruce 3 revealed positive results in only seven out of twelve sera of infected cattle, whereas the test using monoclonal antibodies Brucel 1, 4 and 7 correctly identified the twelve infected animals, but incorrectly classified six (Bruce 1) and eight (Bruce 4 and 7) as positive among of the 18 animals which were free from the disease. SUTHERLAND[12], (1985), using two monoclonal antibodies, called "A" and "B" in this study, also prepared by BUNDESEN et al.[5] (1985) found that the competitive enzyme immunoassay was capable of providing a positive diagnosis earlier than the complement fixation test, but observed the occurrence of many false-positive reactions. In another study, using the some monoclonal antibodies and testing sera of cattle experimentally infected with **Brucella abortus** strain 544, SUTHERLAND; DEN HOLLANDER [13] (1986) observed that the results revealed by the competitive enzyme immunoassay were similar to those revealed by the complement fixation test and concluded that the competitive test showed few advantages for use as a supplementary test which was intended to identify infected animals but with negative results in the complement fixation test. CHIN et al.[7] (1989) when examining sera of 40 cattle from which

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Brucella abortus biovar 1 was isolated, found that the competitive enzyme immunoassay using monoclonal antibodies Bruce 1 classified ten of those animals as false-negative. YONG; EDWARDS[16] (1989) found that the competitive enzyme immunoassay with monoclonal antibodies Bruce 4 showed a sensitivity of, at most, 75%, being as, in this case, specificity of 94%. They also found that, when the test using Bruce 1 and Bruce 7 antibodies was set up so as to show a sensitivity of 100%, specificity diminished considerably, being 69% in the case of the test with Bruce 1 antibodies and 52% in the case of the test with Bruce 7 antibodies. These authors concluded that the competitive enzyme immunoassay, using the monoclonal antibodies studied, against the lipopolysaccharide of **Brucella abortus**, hardly would show the sensitivity and specificity required to detect the small percentage of infected animals remaining in the final stages of a brucellosis eradication campaign. In this paper, the results obtained in the determination of sensitivity and specificity of the competitive enzyme immunoassay, using as conjugates the monoclonal antibodies prepared by WILKE et al.[15] (1985), are presented.

MATERIAL AND METHOD

Sera examined

Seventy-four sera of cattle from which **Brucella abortus** biovar 1 was isolated and 2,118 bovine sera from herds free from brucellosis and showing a negative result to the Rose Bengal plate test were examined by the competitive enzyme immunoassay.

Competitive enzyme immunoassay

The test was carried out as described by MACMILLAN et al.[9] (1990). The conjugates were prepared with monoclonal antibodies produced by two clones of hybridomas, BM-38 and BM-40, resulting from immunisation of BALB/c mice with **Brucella melitensis** strain 16 M[8]. The monoclonal antibodies were purified by affinity chromatography and conjugated with horseradish peroxidase (Sigma Chemical Company - USA) according to the method of BOORSMA; STREEFKERK[4] (1979).

The antigen consisted of the lipopolysaccharide of **Brucella abortus** strain 99, extracted using the hot water - hot phenol method [3].

The substrate used was hydrogen peroxide and OPD (O-phenylenediamine, Sigma Chemical Company - USA) was used as chromogen.

Optical density was read in a Titertek Multiskan MCC/340 apparatus, in a wavelength of 450 nm.

Rose Bengal plate test

This test was carried out in accordance with the standard method used in the serological diagnosis of animal brucellosis [2].

Determination of specificity and sensitivity

Specificity and sensitivity of the competitive enzyme immunoassay were calculated on the basis of the following formulae[14].

$$\text{Specificity} = \frac{\text{Nondiseased animals negative to the test}}{\text{Nondiseased animals tested}} \times 100$$

$$\text{Sensitivity} = \frac{\text{Diseased animals detected by the test}}{\text{Diseased animals tested}} \times 100$$

To determine sensitivity and specificity, the serum which showed any antibody titre was considered positive.

Statistical analysis

An analysis was carried out to obtain measures of association[1], proportion tests and regression studies between scores by using STATGRAPHICS statistics software.

The Kappa coefficient , with the purpose of determining the reproducibility between the test using both of the conjugates was obtained according to LANDIS; KOCH[8] (1977).

RESULTS

The competitive enzyme immunoassay, using conjugate BM-38 or conjugate BM-40, revealed the presence of antibodies to **Brucella** in all 74 sera of cattle from which **Brucella abortus** biovar 1 was isolated. The lowest antibody titre revealed by the test using conjugate BM-38 was 1/4 and the lowest titre revealed by conjugate BM-40 was 1/2. The highest titre was 1/1,024, for the test using conjugate BM-38 or BM-40. Comparing the antibody titres obtained with each conjugate, it may be seen that, for a large proportion of sera, conjugate BM-38 revealed higher titres, although for some sera this situation was reversed (Tab. 1). Statistically, there is a significant/not null association between the results provided by the CEIA with conjugates BM-38 and BM-40, resulting in a chi-square of 270.9 (72 D.F.) and a contigency coefficient of 0.87, showing a relation of dependence between both of the conjugates. In the linear approximation, the values revealed

by the CEIA using conjugate BM-38 showed, on average, 46% higher than the results revealed by the test using conjugate BM-40, and this relation can explain 70% of the variation. In the diagonal, an agreement in 12 of the 74 sera (16.2%) can be observed and, under the indepence hypothesis the expected value would be 11.9%, resulting in a Kappa coefficient of 0.05, that means a low reproducibility. At the right side of the diagonal, 54 values can be observed, when the expected under the indepence hypothesis would be 32.6, confirming the capability of CEIA with conjugate BM-38 in showing higher titres.

TABLE 1

Number of culture-positive cattle distributed according to the antibody titres to **Brucella** revealed by the competitive enzyme immunoassay (CEIA) carried out with conjugate BM-38, compared with the titres revealed by the CEIA using conjugate BM-40.

CEIA (BM - 40)	CEIA (BM - 38)											TOTAL
	N	2	4	8	16	32	64	128	256	512	1024	
N	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	2	0	0	0	0	0	0	0	2
4	0	0	1	0	0	0	0	0	0	0	0	1
8	0	0	0	0	0	5	1	0	0	0	0	6
16	0	0	0	0	2	3	0	1	0	0	0	6
32	0	0	0	0	0	2	1	2	3	0	0	8
64	0	0	0	0	0	0	1	1	6	2	0	10
128	0	0	0	0	0	0	3	0	1	6	2	12
256	0	0	0	0	0	0	0	1	2	4	4	11
512	0	0	0	0	0	0	1	0	3	1	10	15
1024	0	0	0	0	0	0	0	0	0	0	3	3
TOTAL	0	0	1	2	2	10	7	5	15	13	19	74

N - No titre

Out of 2,118 sera of cattle originating from herds free from brucellosis, 2,093 were negative in the competitive enzyme immunoassay using conjugate BM-38 and 25 sera showed some titre, with the highest antibody titre of 1/8. When the test was carried out using conjugate BM-40, only one serum showed antibody titre, of 1/4 (Tab. 2). A significant association could be observed (chi-square = 110.5 , with 3 D.F.) but the contigency coefficient was only 0.22, showing that the relation between the test with both of the conjugates is not strong. Twenty-four out of 2,117 negative sera when tested with conjugate BM-40, white being positive by the showed positive conjugate BM-38. This value results in a low proportion, but it is significantly different from zero (Z = 4.93).

The competitive enzyme immunoassay using conjugate BM-38 showed a sensitivity of 100%, since it was capable of detecting the presence of antibodies in all of the 74 sera of cattle from which **Brucella abortus** was isolated. The specificity shown by the test was 98.82%, since it showed antibody titres in 25 of the 2,118 sera of cattle free from brucellosis (Tab. 3). The statistic Z was 5.03, a significant value, i.e., the specificity differs from 100%.

TABLE 2

Number of bovine sera from herds free from brucellosis, distributed according to the antibody titres to **Brucella** revealed by the competitive enzyme immunoassay (CEIA) carried out with conjugate BM-38, compared with the titres revealed by the CEIA using conjugate BM-40.

CEIA (BM - 40)	CEIA (BM - 38)				TOTAL
	N	2	4	8	
N	2,093	5	18	1	2,117
2	0	0	0	0	0
4	0	0	1	0	1
	2,093	5	19	1	2,118

TABLE 3

Sensitivity and specificity of the competitive enzyme immunoassay (CEIA), carried out with conjugate BM-38, when used in the serological diagnosis of brucellosis in culture-positive cattle and in cattle free from the disease.

CEIA (BM - 38)	CONDITION OF THE ANIMALS		TOTAL
	Diseased	Nondiseased	
Positive	74	25	99
Negative	0	2,093	2,093
TOTAL	74	2,118	2,192

$$\text{Sensitivity} = \frac{74}{74} \times 100 = 100.00\%$$

$$\text{Specificity} = \frac{2{,}093}{2{,}118} \times 100 = 98.82\%$$

When carried out using conjugate BM-40, the competitive enzyme immunoassay also showed a sensitivity of 100% and its specificity was 99.95%, as a negative result was shown in 2,117 of the 2,118 sera of cattle free from brucellosis (Tab. 4). The specificity of 99.95% did not significantly differ from 100% (Z = 1.00).

TABLE 4

Sensitivity and specificity of the competitive enzyme immunoassay (CEIA), carried out with conjugate BM-40, when used in the serological diagnosis of brucellosis in culture positive cattle and in cattle free from the disease.

CEIA (BM-40)	CONDITION OF THE ANIMALS		TOTAL
	Diseased	Nondiseased	
Positive	74	1	75
Negative	0	2,117	2,117
TOTAL	74	2,118	2,192

$$\text{Sensitivity} = \frac{74}{74} \times 100 = 100.00\%$$

$$\text{Specificity} = \frac{2,117}{2,118} \times 100 = 99.95\%$$

DISCUSSION

The results obtained by means of the competitive enzyme immunoassay revealed that this test, both when using conjugate BM-38 and when using conjugate BM-40, was capable of detecting the presence of antibodies to the lipopolysaccharide of **Brucella abortus** in the 74 sera of cattle from which this microorganism was isolated.

Since the test revealed antibody titres as low as 1/2 or 1/4 in sera from culture positive cattle, any antibody titre should be considered as positive. In this case, the sensitivity of the test, using either one of the two conjugates, was 100%. Although it must be considered that the value obtained in estimating the sensitivity and specificity cannot be regarded as being of universal application, since it relates only to the population studied and to the time at which the study was carried out[6], it is a significant fact that the competitive enzyme immunoassay should have revealed the presence of antibodies in all the sera of infected cattle, enven in sera in which the complement fixation test was not capable of providing a positive result. It is also important to point out that, even if we consider as positive any antibody titre revealed by means of the competitive enzyme immunoassay, when testing 2,118 sera of cattle free from brucellosis, only one was considered positive by the test with conjugate BM-40 and 25 were considered positive by the test using conjugate BM-38, resulting in a specificity of 99.95% and 98.82%, respectively.

These data differ a little from those obtained by other authors in evaluating a competitive enzyme immunoassay[9,10,11,12,16]. Comparing these results with the results obtained in the present work, it may be seen that the competitive enzyme immunoassay, performed with conjugate BM-38 or BM-40, proved able to provide a reliable test in the serodiagnosis of bovine brucellosis.

Although the differences between these results may reflect variations in the techniques used or in the parameters adopted for their interpretation, they also characterize differences between the behaviour of the monoclonal antibodies, wether as regards their specificity for a particular epitope of the antigen, or through a difference in affinity and capacity to compete with the antibodies present in the sera tested. This difference in behaviour shows the importance of evaluating the greatest possible number of monoclonal antibodies, so as to have available a serological test which allows the combination of the greatest possible specificity with the greatest possible sensitivity.

RESUMO

O trabalho teve por objetivo avaliar a sensibilidade e a especificidade de um teste imunoenzimático competitivo, empregando como conjugado os anticorpos monoclonais BM-38 e BM-40, no diagnóstico sorológico da brucelose bovina. Foram examinados 74 soros de bovinos dos quais havia sido isolada **Brucella abortus** e 2.118 soros de bovinos procedentes de rebanhos livres de brucelose e que apresentaram resultado negativo quando submetidos ao teste Rosa Bengala. O teste imunoenzimático competitivo, usando qualquer dos dois conjugados, foi capaz de revelar a presença de anticorpos contra o lipopolissacáride bacteriano em todos os soros de bovinos infectados, o que resulta em uma sensibilidade de 100%. A especificidade do teste usando o conjugado BM-38 foi de 98,82% e usando o conjugado BM-40 foi de 99,95%. Estes resultados indicam que o teste imunoenzimático competitivo, principalmente ao se empregar o conjugado BM-40, consiste em um método bastante útil para ser usado como teste confirmatório no diagnóstico sorológico da brucelose bovina.

UNITERMOS: Brucelose bovina; Diagnóstico sorológico; Teste Imunoenzimático Competitivo; Bovinos

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