Diagnostic efficiency of *Brucella* soluble antigens in immunodiffusion tests and ability to differentiate *Brucella abortus* strain 19 vaccinated cattle[*]

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Eficiência diagnóstica de antígenos solúveis de *Brucella* em testes de imunodifusão e capacidade para diferenciar bovinos vacinados com *Brucella abortus* CEPA 19

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SUMMARY

Three soluble antigens were compared by radial immunodiffusion (RID) and agar gel immunodiffusion (AGID) tests: a native haptene (NH) from *Brucella melitensis* 16M, and a polysaccharide (PS) from *B. abortus* 1119-3, both obtained by non-hydrolytic methods, and the (O-Chain) polysaccharide extracted also from *B. abortus* 1119-3 but using an hydrolytic method. Three groups of bovine sera were tested: a) Naturally infected (n = 76); b) Non-infected (n = 130) and c) S-19 vaccinated (n = 61); the sensitivity (Se), the specificity (Sp) and the ability to differentiate vaccinated (ADV) were determined in each group a, b and c respectively. The highest Se in the RID test (84.3%) was achieved by NH; while the three antigens gave 100% Sp. The O-Chain showed 100% ADV in this test. In the AGID test PS antigen showed the best Se (86.6%), and all antigens showed 100% of Sp and ADV. Finally, for its production qualities and efficiency the antigens PS and NH represent a promising alternative for complementary diagnosis of brucellosis.

UNITERMS: Brucellosis; Immunodiffusion tests; Vaccination; Cattle.

INTRODUCTION

The control of bovine brucellosis based on vaccination with *Brucella abortus* Strain 19 (S19), either in the traditional or non-traditional schemes, needs to have available tests with high specificity (Sp) and with the ability to differentiate vaccinal (ADV) responses, defined as the capacity to diagnose as negative, to those animals which were vaccinated with S19, and have given positive results to classical tests. The classic serological tests: standard agglutination (SAT), rose bengal (RBT), complement fixation (CFT) among others, have high sensitivity (Se), but due the use of whole *Brucella* cells as antigen do not differentiate vaccinated cattle[1,2].

Different tests have been reported for the The agar gel immunodiffusion (AGID) test has been used[3] mainly by its high Sp, and several authors[2, 8,9,10,11] have reported its special ability to differentiate between S-19 vaccinated and naturally infected cattle, when using soluble antigens.

On the other hand, the radial immunodiffusion (RID) test has been used with Poly-B and later with NH[4,5,6]. These reports always recorded higher Sp than SAT, RBT and CFT, and also an excellent ADV.

The AGID and RID tests lack on Se, hence did not have usefulness as screening tests in the bovine brucellosis diagnosis[1,13]. However, owing to their excellent Sp and ADV, they are usually requested as complementary diagnosis.

Comparative studies of these three antigens have not been done, thus the objective of this work is to compare them simultaneously against cattle sera with different epidemiological status.

MATERIAL AND METHOD

Bovine Sera[a]

The sample size was calculated as described in Wright; Nielsen[12] using a 95% confidence level. Sera from three groups of cattle were included:

a) **Naturally infected** (n = 76) cows from which *B. abortus* was isolated. All these sera were positive to SAT, RBT, CFT and indirect ELISA test;

b) **Non-infected** (n = 130) animals from brucellosis free areas and where S19 vaccination had never been practiced. All these sera were negative to SAT, RBT and CFT;

c) **S19 vaccinated** (n = 61) non-infected heifers, which sera were obtained 90 days after vaccination and all being positive to SAT, RBT and CFT.

ANTIGENS

a) **NH**[b]: is a native haptene obtained from *B. melitensis* 16M as described by Diaz *et al.*[5];

b) **O-Chain**[c]: is a polysaccharide obtained from *B. abortus* 1119-3 as described by Cherwonogrodzky; Nielsen[2];

c) **PS**[d]: is a polysaccharide obtained from *B. abortus* 1119-3 by the same method of Diaz *et al.*[5] with the modifications proposed by Pinochet *et al.*[9,10].

SEROLOGICAL TESTS

a) **AGID**: was performed following previous recommendations[2,6,11]. Briefly, the agarose gel was prepared in a 10% NaCl, 0.1 M TRIS HCl pH 7.2 buffer with 0.7% agarose. Polystyrene 100 mm diameter Petri dishes were filled with 9 ml of the agarose preparation and 3 mm wells punched in a circular pattern were filled with test and control sera, while in a central well the antigen was placed. Readings to detect precipitation lines were done at 24, 48 and 72 hours;

b) **RID:** was performed according to methods described previously[4,6]. Briefly, agarose at a final concentration of 0.8% and 10% NaCl was prepared using two buffers: glycine 0.1M, pH 7.8 and TRIS-HCl 0.1 M, pH 7.2, to determine the better for each antigen. Equal volumes of gel and antigen dilutions were mixed at 60°C and 5 ml were poured into 50 mm diameter polystyrene Petri dishes. Petri dishes were stored at 4°C by 24 hours and then 1 mm diameter wells were done and filled up with 5 μl of test and control sera.

Both agarose gel preparations were maintained in a moist chamber at room temperature and at 35°C without moisture to determine the best precipitation ring for each antigen. Readings was done at 3, 5, 8 and 24 hours.

OPTIMIZATION OF ANTIGEN USE FOR EACH IMMUNODIFFUSION TEST

Preliminary assays were performed because these antigens have never been used simultaneously in AGID and RID: for the AGID, 500 μg/ml of the PS antigen were used according to a previous report[9]; double dilutions from 12.5 μg/ml to 100 μg/ml of NH were tested; and O-Chain was tested in concentrations of 25, 50, 125, 250 and 500 μg/ml.

Table 1

Diagnostic performance of an agar gel immunodiffusion (AGID) test using three soluble *Brucella* antigens. Faculty of Veterinary and Animal Sciences, University of Chile. August to December, 1994.

Groups		NH *B. melitensis* 16M	Antigens O-CHAIN *B. abortus* 1119-3	PS *B. abortus* 1119-3
		POSITIVE SERA (N)		
Infected (n=76)	Se (%)	85.5 (65)	84.2 (64)	86.6 (66)
Non-infected (n=130)	Sp (%)	100 (0)	100 (0)	100 (0)
Vaccinated (n=61)	ADV (%)	100 (0)	100 (0)	100 (0)

[a]These sera are property of Infectous Diseases Laboratory, University of Chile
[b]Kindly provided by Dr. Ignacio Moviyón, University of Navarra, Pamplina, Spain.
[c]Kindly provided by Dr. Klaus Wilsin, Animal Diseases Research Institute, Nepean, Ontario, Canada
[d]This antigen is property of Infectious Diseases Laboratory, Faculty of Veterinary Sciences University of Chile

Table 2

Diagnostic performance of a radial immunodiffusion (RID) test using three soluble *Brucella* antigens. Faculty of Veterinary and Animal Sciences, University of Chile. August to December, 1994.

Groups		NH *B. melitensis* 16M	Antigens O-CHAIN *B. abortus* 1119-3	PS *B. abortus* 1119-3
		POSITIVE SERA (N)		
Infected (n=76)	Se (%)	84.2 (65)	60.5 (46)	81.6 (62)
Non-infected (n=130)	Sp (%)	100 (0)	100 (0)	100 (0)
Vaccinated (n=61)	ADV (%)	100 (0)	100 (0)	88.5 (7)

Se = sensitivity;
Sp = specificity;
ADV = ability to differentiate vaccinated animals.

For the RID test both in glycine and TRIS-HCl buffer agarose gels, different antigen dilutions were tested. The NH was used at 20 µg/ml according to previous reports[4,5]; the O-Chain was prepared in 2.5, 10, 20, 50 and 75 µg/ml concentrations; the PS antigen was also diluted in 20, 50, 100 and 200 µg/ml concentrations. In both tests the diluent was distilled water and all dilutions were tested against 10 control sera of each group, as a preliminary assay.

Analysis of results

Se and Sp for AGID and RID were calculated with a) and b) bovine sera group respectively, according to Martin *et al.*[7] The ADV for AGID and RID tests was calculated just with sera belonging to S19 vaccinated animals (group c), using the next proportion:

ADV = <u>N° of negative sera from vaccinated animals by AGID or RID x 100</u>

N° sera from vaccinated animals tested by AGID or RID

The results of the different antigens were compared using Mc Nemar's chi-squared test as a measure of statistical association, and the strength of this association was determined by the Odds Ratio (**OR**) method[7].

RESULTS

For the AGID test, the best antigen concentrations to obtain an optimal precipitation lines were: 50 µg/ml of NH antigen; 125 µg/ml O-Chain antigen; as expected 500 µg/ml of PS antigen gave a clear precipitation line.

For the RID test the best performance was obtained using the following concentrations of antigens and agarose buffered media: 20 µg/ml in glycine buffer for the NH antigen; 50 µg/ml of O-Chain antigen also in glycine buffer. Both antigens required incubation in moist chamber at room temperature; the PS showed the greatest precipitation ring at a 200 µg/ml in TRIS HCl buffer and required 35°C without moisture during the incubation.

The AGID test showed the highest sensitivity (86.6%) with the PS antigen, while the specificity and the ADV were of 100% for the three antigens. These results can be seen in Tab. 1.

The RID test gave the best sensitivity (84.2%) with the NH antigen, while the Sp was 100% with all the antigens; using the O-chain the ADV was 100%. Only seven sera of vaccinated heifers gave a very small precipitation ring with the PS and NH antigen. The RID test results can be seen in Tab. 2.

The Se in AGID test using the three antigens did not show significative differences (p>0.05) and they were strongly associated (OR = 0.33). Using RID test, Se significative differences were detected between NH and PS respect to O-Chain; while O-Chain showed significative differences (p<0.05) in ADV respect to the former antigens.

DISCUSSION

The simultaneous comparison of the three antigens represents an original study, because its fulfillment at the same time limited the effect of laboratory changes that potentially influence test performance.

With respect to the sera, we think that the samples of naturally infected cattle are representative of field conditions in Chile and allowed us to evaluate the Se performance of each antigen in front of different antibody levels. In the same way the Sp evaluation with sera obtained in free brucellosis area allowed us to have a condition that is difficult to achieve in brucellosis. When considering sera from S19 vaccinated

heifers we provided a better assessment of the tests, because these sera were obtained close after vaccination when antibodies were still in a high titer. The ADV proportion appeared to be a good way to assess the ability of the tests to correctly diagnose as negative animals those which have been vaccinated and resulted positive to classical tests, specially when the antigens were used in the AGID test.

About the antigen performances in AGID, the NH results are very important because this was the first time it was done. According to a previous report[2], the O-Chain showed an excellent precipitation line, perhaps due to its high purity and also its diagnostic performance was optimal. The PS antigen had a behavior according to former reports[9,10,11] showing an excellent Sp and ADV and also gave the fast diffusion rate at 24 hours. As to the Se values offered in AGID test by the PS, there is an agreement with a previous report[13], although in our work this Se value was higher than that given for the RID, which did not agree with values reported by other authors[4,5].

When RID test is considered, the NH antigen gave the highest Se (84.2%), although no significative differences can be established with the Se given for the PS antigen. Previous reports[4,5] showed similar Se with the NH antigen. Unexpectedly in RID, the Se offered for the O-Chain was very low. The results on Sp agreed with those reported previously for NH antigen[4,5,6,13], while for the O-Chain and PS antigens there are no data since they have never been used in RID test before. The best ADV (100%) was given by O-Chain, surely for its lower Se than the PS and NH antigens. The seven sera of vaccinated heifers that were reported as positives in RID test with both NH and PS could be explained by the same reason.

For both AGID and RID tests, ADV values ranged from 88.5% to 100%, which could be considered excellent performance for control programs involving S19 low-dose vaccination of adult cattle.

It is concluded that the antigens tested in the two tests considered showed excellent Sp and ADV values and their use can be recommended to define brucellosis vaccination status. Considering that the PS and NH are easily produced and have similar performances, they represent a safe and economic alternative for complementary diagnostic in brucellosis control programs.

RESUMO

Foram comparados três antígenos solúveis: um hapteno nativo (NH) de *B. melitensis* 16M, um polissacarídeo (PS) obtido de *B. abortus* 1119-3 e outro polissacarídeo de cadeia O (O-Chain) originado também da última *Brucella*. Os testes de imunodifusão radial (RID) e imunodifusão em gel de ágar (AGID) foram confrontados com as três classes de soros bovinos: a) infectados naturalmente (n = 76), b) não infectados (n = 130) e c) vacinados com B19 (n = 61) reagindo a testes sorológicos clássicos. Foram determinadas a sensibilidade (Se), a especificidade (Sp) e a capacidade para discriminar vacinados (ADV). A Se mais alta (84,3%) no teste RID foi demonstrada pelo antígeno NH, enquanto os três antígenos tiveram 100% de Sp. O antígeno O-Chain teve 100% de ADV nesse teste. O teste AGID com estes antígenos demonstrou 100% Sp e ADV, enquanto o antígeno PS mostrou uma melhor Se (86,6%). Finalmente, por sua qualidade de produção e eficiência, os antígenos PS e NH representam uma alternativa segura e econômica para o diagnóstico suplementar da brucelose.

UNITERMOS: Brucelose; Testes de imunodifusão; Vacinação; Bovinos.

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