ELISA FOR THE DIAGNOSIS AND EPIDEMIOLOGY OF *BRUCELLA ABORTUS* INFECTION IN CATTLE IN CHILE

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Abstract

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A serum bank of 1251 adult cows sera was prepared. The sera originated from animals of three different epidemiological groups: 1) 244 from infected cows, strain 19 vaccinated when calves; 2) 507 from herds free of infection but all cows were strain 19 vaccinated when calves and 3) the last group, 500 sera from cows free of infection and non-vaccinated.

All the sera where tested with the routine Rose Bengal (RB) Rivanol (RIV) and Complement Fixation (CF) tests and additionally three enzyme immunoassays were performed. They included two indirect ELISA both using the kit from the Joint FAO/IAEA Division, Vienna, Austria. One assay used a polyclonal conjugated antibody (I-ELISAp) and the other a monoclonal conjugated antibody (I-ELISAm). The third assay was a competitive ELISA (C-ELISA) performed with sLPS, plus monoclonal antibody, M84, and goat anti-mouse antibody-HRPO. Using the CFT as "gold standard" the sensitivities of all the methods were: RB 87.1%, RIV 87.1%, I-ELISAp 100%, I-ELISAm 100%. The calculated specificity was: RB 100%, RIV 100%, I-ELISAp 96.4% and I-ELISAm 100%.

In the group of infected animals (244) the following results were obtained: RB 13.5%, RIV 11.9%, CF 12.7%, I-ELISAp 50.8% and I-ELISAm 22.9%. Results for the non-vaccinated group were: RB 0.2%, RIV 0%, CFT 0.2%, I-ELISAp 6.9% and I-ELISAm 2.9%.

The C-ELISA was performed on samples from the positive group or with positivity values close to the cut-off value in the I-ELISAm. In the infected group 28 out of 63 animals were detected as infected and from the non-vaccinated herds none of 15 I-ELISAm positive samples were detected as infected in the C-ELISA.

1. INTRODUCTION

In Chile like in other Latin American countries brucellosis is one of the most important infectious diseases mainly due to the economic losses it causes. This is the main reason why animal health authorities wish to improve the diagnostic and vaccination schemes to decrease the prevalence of the disease.

For that purpose the following control plan was applied from 1968 until June 1997:

a) strain 19 vaccination, using doses of 10 to 20 x 10^9 cells/ml for female calves between 3 to 8 month of age

b) diagnosis using the Rose Bengal test as screening test and Rivanol and Complement Fixation tests as the confirmatory tests

c) to remove all positive reactors from the farm for slaughtering.

Unfortunately due to budget limitations this plan was not compulsory and farmers participated on a voluntary basis. As a result there are some brucellosis-free farms but brucellosis-infected farms may still exist. This aspect is closely related with the efficacy of the diagnostic methods for epidemiological surveillance. It is crucial to have a highly sensitive and specific test to detect animals recently infected and furthermore differentiate between infected and vaccinated animals as the risk of infection makes it necessary to keep the strain-19 vaccinated cattle in the herd.

Other farms with a high prevalence of infection could be included in the control plan by increasing the vaccine protection using strain-19 in reduced doses for adult cows. In this case it is very important to have a diagnostic method which is able to accurately differentiate between vaccinated and infected animals.

Based on the aspects mentioned above the purpose of this research was to compare the diagnostics methods included in the brucellosis control plan with two indirect ELISAs and the positive reactors in a competitive ELISA.

2. MATERIAL AND METHODS

2.1. Serum Bank

The serum bank included a total of 1251 adult cow sera divided in three categories according the farm status:

a. From positive herds, with *Brucella abortus* isolation from aborted fetuses and some level of strain 19 vaccination(N = 244).

b. From *Brucella* free cows, strain 19 vaccinated with complete dose when calves between 3 to 8 month of age and located in areas free of brucellosis(N = 507).

c. From free herds, located in non vaccinated areas(N = 500).

2.2. Diagnostic tests

All the sera were tested by the following methods:

2.2.1. The Rose Bengal test (R.B.T)

This was done according to Alton et al. [1]. The antigen was prepared at the Instituto de Microbiologia following the CEPANZO standardization procedure and officially accepted by the Chilean Department of Agriculture (S.A.G.).

2.2.2. The Rivanol (RIV) test

The antigen and Rivanol solution were produced in the Instituto de Microbiologia as mentioned above, and the test was performed according to Alton et al. [1].

2.2.3. The Complement Fixation test (CF)

The cold method was used [1] and the antigen prepared at the Institute.

2.2.4. Indirect ELISA (I-ELISA) with a polyclonal antibody

The brucellosis ELISA kit was provided by the Joint FAO/IAEA Division of the International Atomic Energy Agency (IAEA) and the procedure was in according to the manual included in the kit using a conjugated polyclonal antibody.

2.2.5. Indirect ELISA (I-ELISA) with a monoclonal antibody

Kit and methodology provided by IAEA with conjugated monoclonal antibody.

2.2.6. Competitive ELISA (C-ELISA)

The sLPS antigen, the M84 monoclonal antibody and the goat anti-mouse HRPO conjugate (Jackson Laboratories) were kindly provided by Dr. Klaus Nielsen from A.D.R.I., Canada. The methodology for this test, also provided by Dr. Nielsen was followed with some modification [2]. Briefly, NUNC polystyrene plates were coated with 100 µl per well of sLPS, 1ug/ml in carbonate buffer pH 9.6, at 20°C. overnight and frozen until used or used immediately. After 3 washes using washing buffer plus 0.05% tween 20 the control and sample sera plus the previous diluted M84 were added to each well, e.g. 95 µl diluted M84 plus 5 µl of undiluted serum. Each control was added to four wells and each sample separately only to one well. After shaking for 3 Min. the plate was incubated for 30 Min. at room temperature and after washing 3 times 100 µl of the previously titrated goat anti-mouse conjugate were added. The plate was incubated for 30 Min. at 20°C. and after 3 washes ABTS and H_2O_2 were added as in the I-ELISA method. After 10 minutes at 20°C. the plate was read at 405nm and percentage of inhibition (%I) was calculated using the conjugate control as 0 % inhibition (about OD=1.0) in the formula:

$$\%\,I = 100 - \frac{\text{OD sample}}{\text{OD conjugate control}} \times 100$$

Sera ranging from complete inhibition (no color) to 30% inhibition were considered as originating from infected cattle while less than 30% I the sera were thought to come from vaccinated or brucellosis free animals.

The modifications introduced to this technique were the antigen incubation temperature and the use of stopping solution (SDS). For the antigen coating the plates were coated and incubated at 4°C. overnight. Some of them were maintained at this temperature until further use and others were frozen at -20°C. For stopping 100 µl of SDS were added to each well after the substrate-chromogen incubation period.

3. RESULTS AND DISCUSSION

The sera tested by six methods previously mentioned and divided according to their serological status gave the results shown in Tables I , II and III.

TABLE I. ANTI-*BRUCELLA* ANTIBODIES DETECTED BY SIX DIAGNOSTIC METHODS IN SERA FROM A HERD WITH *B. ABORTUS* INFECTION AND STRAIN-19 VACCINATION

Tests	Number (+)	%	Number (-)	%
RB	33	13.5	211	86.5
RIV	29	11.9	215	88.1
CF	31	12.7	213	87.3
I-ELISAp	124	50.8	120	49.2
I-ELISAm	56	22.9	188	77.0
C-ELISA	28	44.4	35	55.5

Cut-off value for I-ELISAs was 35% of Positivity (P) and 30% of Inhibition (I) for the C-ELISA

TABLE II. ANTI-*BRUCELLA* ANTIBODIES DETECTED BY SIX DIAGNOSTIC METHODS USING SERA FROM A BRUCELLOSIS-FREE, STRAIN-19 VACCINATED HERD

Tests	N (+)	%	N (-)	%
RB	1	.2	506	99.8
RIV	0	0	507	100
CF	1	2	506	99.8
I-ELISAp	35	6.9	472	93.1
I-ELISAm	15	2.9	492	97.0
C-ELISA	0	0	15	100

Cut-off values as for Table I

TABLE III. ANTI-*BRUCELLA* ANTIBODIES DETECTED BY SIX DIAGNOSTIC METHODS, SERA FROM A BRUCELLOSIS-FREE, NON-VACCINATED HERD

Tests	N (+)	%	N (-)	%
RB	0	0	500	100
RIV	0	0	500	100
CF	0	0	500	100
I-ELISAp	8	1.6	492	98.4
	2	0.4	498	99.6
I-ELISAAm	0	0	500	100

I-ELISA cut-off as for Table I

The I-ELISA results were obtained using an FAO/IAEA defined cut-off of PP 35% being considered as positive. From an epidemiological point of view our samples could be considered as belonging to at least two categories: the first one including those samples from cattle free of infection and not vaccinated (non-exposed) and the second group made up of samples from a negative but calfhood vaccinated population. With these groups the threshold value was calculated for each non-infected group resulting from I-ELISAm and the data are presented in Table IV.

TABLE IV. THRESHOLD FOR THE NEGATIVE GROUPS

	(a)	(b)
2 x X	3%	15%
X + 3 S.D.	10%	26%
Median of 100 percentile	18%	30%

a) Brucellosis-free/not vaccinated
b) Brucellosis-free/vaccinated

If we consider, for example the threshold from (a) 10% (Mean + 3 S.D.), 148 (29.19%) serum samples will be positive from the samples belonging to a *Brucella*-free, vaccinated herd but the same threshold from (b) decreased the number of positive reactors to 27 (5.3%). Therefore, it seems advisable to calculate different thresholds for the different epidemiological states of the population.

From the I-ELISAp data it is clear that there is an increased number of reactors compared to the CF. This could be due to the detection of all four immunoglobulin isotypes instead of only IgG1 detected by the CF. In the same way, the I-ELISAm also increased the number of positive samples in comparison to CF but this could be due to the fact that I-ELISAm detects IgG1 in lower amounts than the CF. This is very important in areas where this immunoglobulin can be related to infection because earlier stages of infection can be detected. However, in herds were strain-19 vaccination is carried out this IgG1 antibody could be the remainder of the antibody due to vaccination. This may be the IgG1 antibody detected in the samples from cattle free of infection but strain 19 vaccinated, as is apparent in Table II. In this case all the sera detected in the I-ELISAm were negatives in the C-ELISA.

The C-ELISA appears to be promising because it is quite easy to perform less time consuming, repeatable and from a practical point of view can differentiate infected from vaccinated animals. The sensitivity and specificity estimates of the *B. abortus*-infected group (a) and the brucellosis free group (c) respectively using the CF test as the "gold standard" are shown in Table V.

TABLE V. SENSITIVITY AND SPECIFICITY OF FOUR DIAGNOSTIC TESTS IN THE DETECTION OF *Brucella abortus* ANTIBODIES USING THE COMPLEMENT FIXATION TEST AS THE "GOLD STANDARD" USING 244 SERA FROM *B. abortus* INFECTED AND STRAIN 19 VACCINATED HERDS AND 500 SERA FROM A BRUCELLOSIS FREE NON-VACCINATED HERD

Tests	(a) Sensitivity %	(c) Specificity %
RB	87.1	100
RIV	87.1	100
I-ELISA(p)	100	96.4
I-ELISA(m)	100	100

The correlation between the RB and Rivanol test in comparison to the CF test was in the range observed by others [3], who reported a sensitivity of 92% compared to *brucella* isolation. Dajer et al. [4] obtained 100% sensitivity and 83% specificity in a group of non vaccinated cattle with the RB test. For

infected cattle 80% and 100% sensitivity and specificity values respectively were obtained with the RIV test.

The sensitivity of both I-ELISAs were high but the specificity of I-ELISAp was relatively low. As may be seen in Table III, repeated testing of some of the positive samples in this test, when the result were close to the threshold gave some negative results, improving the specificity. Perhaps results near the cut-off value routinely should be considered for retesting to confirm that the serum was obtained from an infected cow.

The results presented above raise the question: Would it be advisable to use RB as screening test and C-ELISA a the confirmative test in those areas where strain 19 vaccination is routine ? On the other hand in areas free of vaccination perhaps the most advisable test as the confirmative one could be the I-ELISAm because of its ability to detect small amount of IgG1.

ACKNOWLEDGEMENTS

We would like to thank Dr. Klaus Nielsen, ADRI Canada, advisor of this research and provider of C-ELISA reagents, Dr. Benigna Perez from SAG Chile, who kindly ran the CF test and the Joint FAO/IAEA Division, especially the Biotechnology Laboratory in Seibersdorf for providing us with the I-ELISA kits.

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