EVALUATION OF AN INDIRECT ELISA FOR THE DIAGNOSIS OF BOVINE BRUCELLOSIS IN PATAGONIA, ARGENTINA

F.A. UZAL* , E.A. CARRASCO*, S. ECHAIDE** , C.A. ROBLES*

* Animal Health Unit,
National Institute of Agriculture Technology (INTA),
Bariloche

** Animal Health Unit,
National Institute of Agriculture Technology (INTA), Rafaela

Santa Fe, Argentina

XA9848647

Abstract

EVALUATION OF AN INDIRECT ELISA FOR THE DIAGNOSIS OF BOVINE BRUCELLOSIS IN PATAGONIA, ARGENTINA.

Control and eradication of bovine brucellosis is usually based on the serological detection of antibodies. In Argentina, the Rose Bengal test (RB) and the Buffered Plate antigen test (BPA) are the two screening test officially recognized, while the 2-mercaptoethanol test (2ME) and the Tube Agglutination test (SAT) are the confirmatory assays currently in use. In order to improve the serological diagnosis of bovine brucellosis in Patagonia, Argentina, an indirect ELISA kit produced by the Joint FAO/IAEA Division was evaluated. Sera from negative non-vaccinated, negative but vaccinated and positive animals were tested by all the above techniques. The specificity of the I-ELISA (99.6% and 99.7%) was similar to that of the BPA, RB, 2ME and Complement Fixation test (CF) when used to test sera from non-vaccinated, negative and vaccinated, negative animals, respectively. The sensitivity of the I-ELISA (98%) was higher than the BPA test (96%) and the CF test (95,2%). The I-ELISA kit evaluated in this study was thought to be a valuable tool for the diagnosis of bovine brucellosis in Patagonia region where little epidemiological information is available about this disease and where large numbers of sera should be tested to obtain such information.

1. INTRODUCTION

Serological detection of antibodies is usually the method of choice for control and eradication of bovine brucellosis. Several conventional serological tests have been used singly or in combination for the serological diagnosis of this disease [1].

Usually, a rapid screening test of high sensitivity is applied initially in testing of sera in control programmes. A positive reaction in the screening test would result in the serum being subjected to a confirmatory test of high specificity. In Argentina, the rose bengal test (RB) and the buffered plate antigen test (BPA) are the two screening tests officially recognized, with the 2-mercaptoethanol test (2ME) and the tube agglutination test (SAT) used as the confirmatory tests (Resolución 1269/93. Servicio Nacional de Sanidad Animal, 16-11-93). However, the agglutination techniques may have limitations in sensitivity due the to prozone phenomena and may result in non-specific agglutination reactions due to the presence of antibodies against bacteria with antigenic determinants common with *Brucella abortus* such as *Yersinia enterocolitica, Escherichia coli, Salmonella urbana* and *Campylobacter fetus* [2, 3]. The complement fixation test (CF) is a highly sensitive and specific technique, but is a cumbersome, time consuming and difficult to standardize test. The indirect enzyme linked immunosorbent assay (I-ELISA), on the other hand, has less of these problems, is highly sensitive and specific and detects all the isotypes of IgG and IgM in serum [4]. In addition, this test requires a minimum volume of serum and other reagents to be performed.

The aim of the present study was to evaluate an indirect enzyme linked immunosorbent assay for the diagnosis of bovine brucellosis in the Patagonia region, southern Argentina.

2. MATERIAL AND METHODS

2.1. Sera

The following groups of Patagonic sera were processed by the RB, BPA, 2ME, CF and I-ELISA tests.

2.1.1. Group 1

Sera from 286 cows, older than 24 months, from 13 herds free from bovine brucellosis. The herds were serologically negative (to RB and 2ME) for bovine brucellosis in two consecutive tests and had no history of abortions or other signs of this disease in at least the previous 5 years. These animals had never been vaccinated against bovine brucellosis.

2.1.2. Group 2

Sera from 459 cows, older than 24 months, vaccinated against bovine brucellosis (strain 19, standard dose) between 3 and 8 months of age that were negative to RB, BPA and 2ME originated from 11 farms.

2.1.3. Group 3

Sera from 156 cows, older than 24 months, that reacted positively in the RB and 2ME tests. These cows originated from 10 herds with at least 2% of animals positive to the RB and 2ME tests.

2.1.4. Group 4

1309 sera originated from 17 farms with at least 2% positive reactors to the RB and 2ME tests.

2.2. Serological techniques

Most of the sera were tested by the RB, BPA, 2ME and I-ELISA tests. A selected group of sera were also processed by the CF test. The antigens for the conventional tests were purchased from the Research Center on Veterinary Sciences, The National Institute of Agricultural Technology (INTA), Castelar, Argentina.

2.2.1. Rose Bengal test

This technique was performed as previously described [5,6]. Briefly, a dilution of serum was obtained by mixing 30 µl of serum and 30 µl of rose bengal antigen on a glass plate. The reaction was incubated for 4 minutes at room temperature applying rotatory movements to the plate (approximately 12 rotations per minute). The reaction was interpreted as positive when agglutination was visible at 4 minutes and negative when the mixture was homogeneous at this time.

2.2.2. Buffered Plate Agglutination test

This technique was also performed as previously described [5,7]. In brief, a dilution of serum was obtained mixing 80 µl of serum and 30 µl of antigen on a glass plate. The reaction was incubated for 8 minutes at room temperature. Four rotatory movements were applied to the plate after the first 4 minutes of incubation. The reaction was interpreted as positive when agglutination was visible at 8 minutes and negative if the mixture was homogeneous at this time.

2.2.3. 2-mercaptoethanol test

This technique was performed according to Alton et al. (1988). Dilutions of serum (1:25, 1:50, 1:100 and 1:200) were obtained by mixing 0.08 ml, 0.04 ml, 0.02 ml and 0.01 ml, respectively, with 1ml of a 1% solution of 2 mercaptoethanol followed by addition of 1ml of a 2% antigen suspension after 30-60 minutes of incubation at room temperature. The mixture was then incubated for 48 $\pm$ 6 h at 37 °C. The reaction was considered positive when the supernatant was transparent and there was an agglutinate in the bottom of the tubes and negative if the supernatant was turbid and no agglutinated cells were observed.

2.2.4. Complement Fixation test

This technique was performed according to Alton et al. (1975), using haemagglutination plates incubated at 37°C for 30 minutes. The antigen was standardized to give 50% fixation of complement with a dilution of 1/256 of the second international standard anti-*Brucella abortus* serum. The sera were

tested up to a 1/256 dilution. The sera that did not reach a final titer were retested at higher dilutions. Fifty percent fixation of complement at 1/8 dilution was considered as the positive threshold.

2.2.5. Indirect enzyme linked immunosorbent assay, ELISA

The I-ELISA was performed using an Indirect ELISA Brucellosis Kit provided by the Joint FAO/IAEA Division, International Atomic Energy Agency (IAEA, Vienna, Austria) and following the recommendations of the manual supplied with the kit. Briefly, medium binding capacity, 96 wells polystyrene plates (Flat bottom, Nunc, cat.#2-69620), were coated with 100 µl of hot water/hot phenol extracted *Brucella abortus* smooth lipopolysacharide at a dilution of 10 ug/ml in a 0.06 M carbonate buffer pH 9.6 and incubated overnight at 4°C. The plates were then washed three times and test and control sera were added to the wells of microplates at a dilution of 1:200. The plates were incubated for 1 hr at 37°C. All the test sera were tested in duplicate, while control sera were tested in quadruplicate. Controls consisted of a conjugated antiglobulin control with no sera being added to the wells, a strong positive control serum, a weak positive control serum and a negative control serum. After further washing cycles, 100 µl of a 1:12,000 dilution of a rabbit anti-bovine IgG (H+L) conjugated to horseradish peroxidase serum was added to all the wells followed by another 1 hr period of incubation at 37°C. Finally, and after another wash cycle, ABTS/H_2O_2/citrate buffer substrate/chromogen solution was added and incubated for 10 minutes at 37°C with shaking. The reaction was stopped by addition of 100 µl of 4% sodium dodecyl sulphate solution. Plates were read in a Multiskan Plus ELISA reader using the software provided with the kit. Optical density values were converted to percentages of the strong positive control serum (pp). The threshold was determined by adding 3 SD to the mean of the pp values of the negative non-vaccinated animals (Group 1). Values below this threshold were considered to be negative.

2.2.6. Specificity

Specificity was defined as the ability of a given technique to correctly identify negative cattle as negative. The diagnostic specificity of each test was calculated for both non-vaccinated negative (Group 1) and vaccinated negative animals (Group 2).

Diagnostic specificity was calculated as follows:

$$\frac{\text{No. of test negative}}{\text{No. of negative cattle tested}} \times 100$$

The relative specificity of each test relative to the 2 screening tests used (RB and BPA) was calculated for the sera from infected herds (Group 4), as follows:

$$\frac{\text{No. of comparative test negative}}{\text{No. of screening test negative}} \times 100$$

2.2.7. Sensitivity

Sensitivity was defined as the ability of a technique to correctly identify positive cattle as positive. It was calculated with sera from Group 3 for each test as:

$$\frac{\text{No. of test positive}}{\text{No. of positive cattle tested}} \times 100$$

The sensitivity of the test in relation to each other was calculated using sera from infected herds (Group 4) as:

$$\frac{\text{No. of comparative test positive}}{\text{No. of relative test positive}} \times 100$$

3. RESULTS

The pp threshold for the ELISA technique was 49% positivity. Therefore, for further calculations sera with pp higher than 49% was considered to be positive, while sera with pp below this value was considered as negative. The diagnostic specificity of all the tests used for non-vaccinated negative herds is shown in Table I. The distribution of I-ELISA pp values of the 286 non-vaccinated negative sera is shown in Figure 1. Only 1 out of the 286 sera tested was positive by I-ELISA.

The diagnostic specificity of all the tests used for vaccinated negative herds is depicted in Table II. The distribution of I-ELISA pp values of the 459 vaccinated negative sera is displayed in Figure 2. Only 1 of the sera gave a positive reaction in the I-ELISA.

The sensitivity of all tests estimated with sera positive to the RB and 2ME tests is shown in Table III. The distribution of I-ELISA pp values of the 156 positive sera from infected herds is plotted in Figure 3. Three of the 156 sera gave pp values below the threshold of 49.9% pp. The comparative distribution of I-ELISA pp values of sera from Groups 1 and 3 is plotted in Figure 4.

The specificity relative to the two screening tests used (BPA and RB) in sera from *Brucella* infected herds is shown in Table IV.

The sensitivity relative to both screening and confirmatory tests positive reactors in *Brucella* infected herds, is shown in Table V.

TABLE I. DIAGNOSTIC SPECIFICITY OF ALL SEROLOGICAL TESTS CALCULATED USING SERA FROM NON-VACCINATED, NEGATIVE HERDS

Test	No. of sera tested	Negative	Positive	Specificity
BPA	286	286	0	100.0%
RB	286	286	0	100.0%
2ME	286	286	0	100.0%
CF	70	70	0	100.0%
ELISA	286	285	1	99.6%

TABLE II. DIAGNOSTIC SPECIFICITY OF ALL SEROLOGICAL TESTS ESTIMATED WITH SERA FROM VACCINATED, NEGATIVE HERDS

Test	No. of sera tested	Negative	Positive	Specificity
BPA	459	448	11	97.6%
RB	459	459	0	100.0%
2ME	459	459	0	100.0%
CF	72	72	0	100.0%
ELISA	459	458	1	99.7%

TABLE III. SENSITIVITY OF ALL THE SEROLOGICAL TESTS ESTIMATED USING SERA POSITIVE TO THE RB AND THE 2ME

Test	No. of sera tested	Positive	Negative	Specificity
BPA	153	147	6	96.0%
RB	-	-	-	-
2ME	-	-	-	-
CF	42	40	2	95.2%
ELISA	156	153	3	98.0%

TABLE IV. SPECIFICITY RELATIVE TO THE BPA AND RB TESTS NEGATIVE SERA IN *Brucella abortus* INFECTED HERDS

Test	Specificity relative to BPA %	Specificity relative to RB %
BPA	-	83.91
RB	97.28	-
2ME	94.31	89.37
ELISA	93.82	91.54

TABLE V. SENSITIVITY OF THE BPA, RB, 2ME AND I-ELISA TESTS RELATIVE TO BOTH SCREENING AND CONFIRMATORY TEST

Test	BPA 279*	RB 208*	2ME 259*	ELISA 260*
BPA	-	84.6%	79.45%	73.84%
RB	63.08%	-	61.62%	70.38%
2ME	77.47%	76.44	-	70.38
ELISA	68.81%	87.98%	70.93%	-

* Positive reactors out of 1309 cattle tested

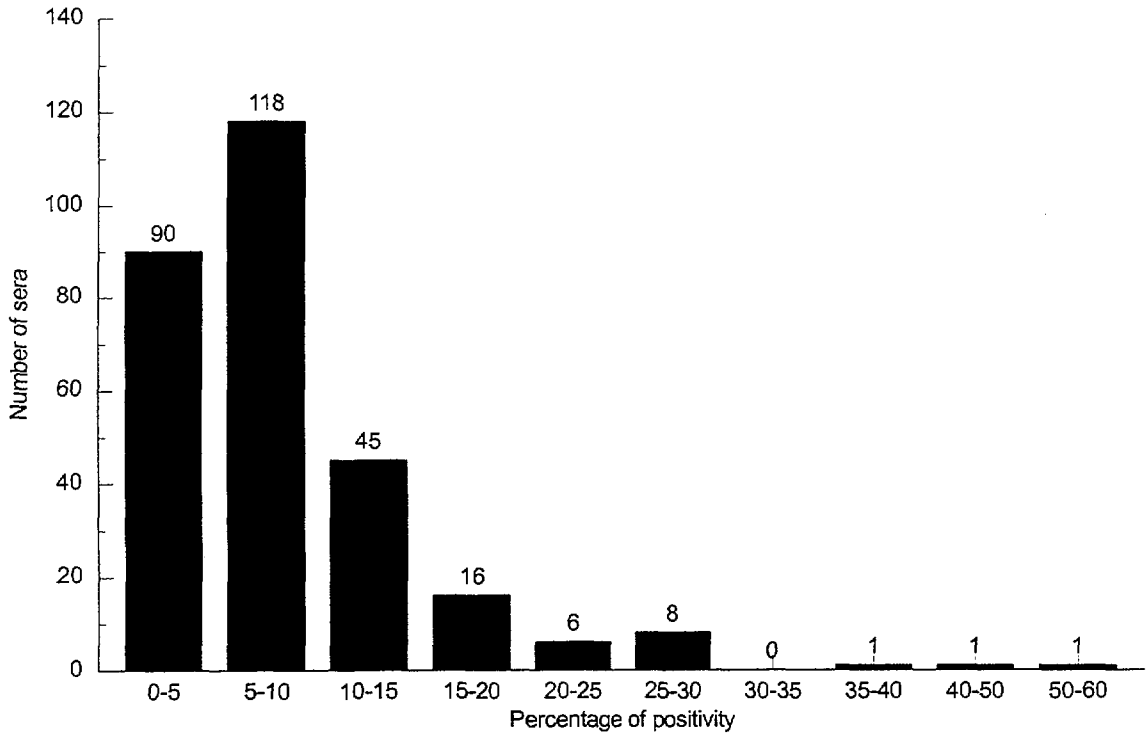

FIG. 1. Distribution of I-ELISA pp values of 286 non-vaccinated, negative sera.

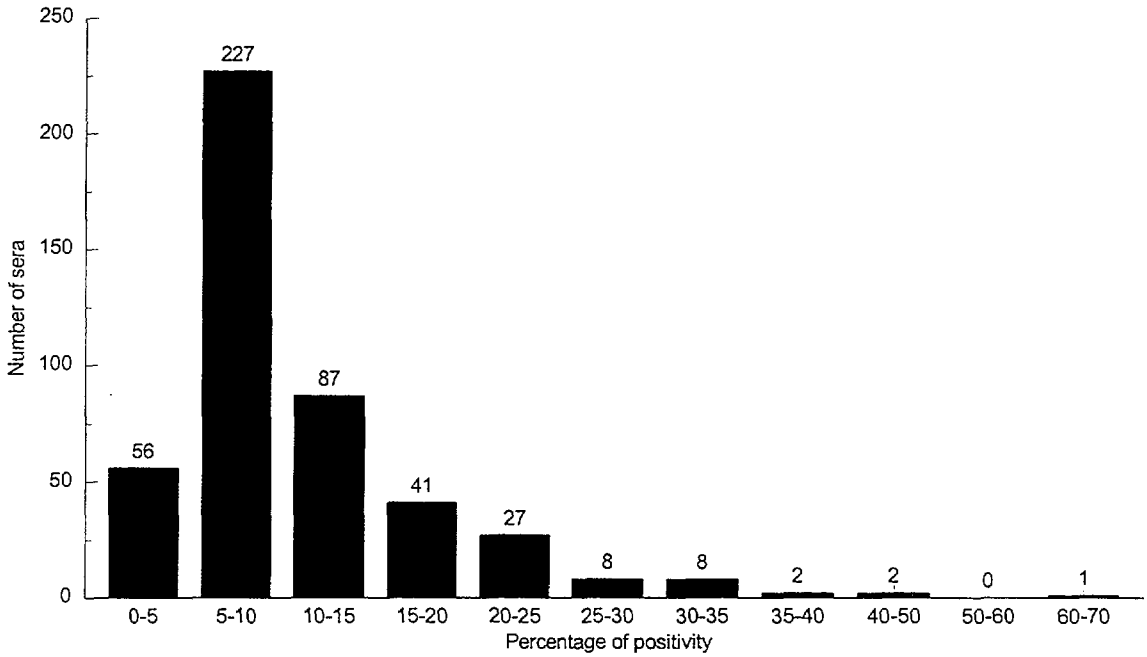

FIG. 2. Distribution of I-ELISA pp values of 459 vaccinated, negative sera.

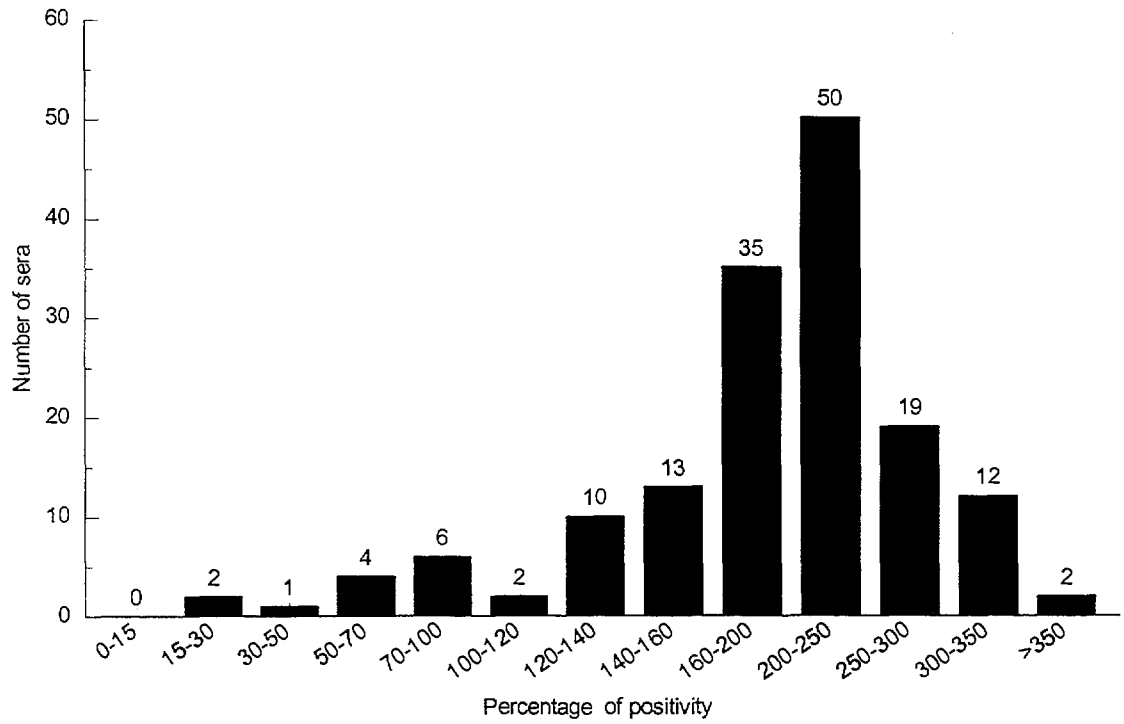

FIG. 3. Distribution of I-ELISA pp values of 156 sera positive to RB and 2ME.

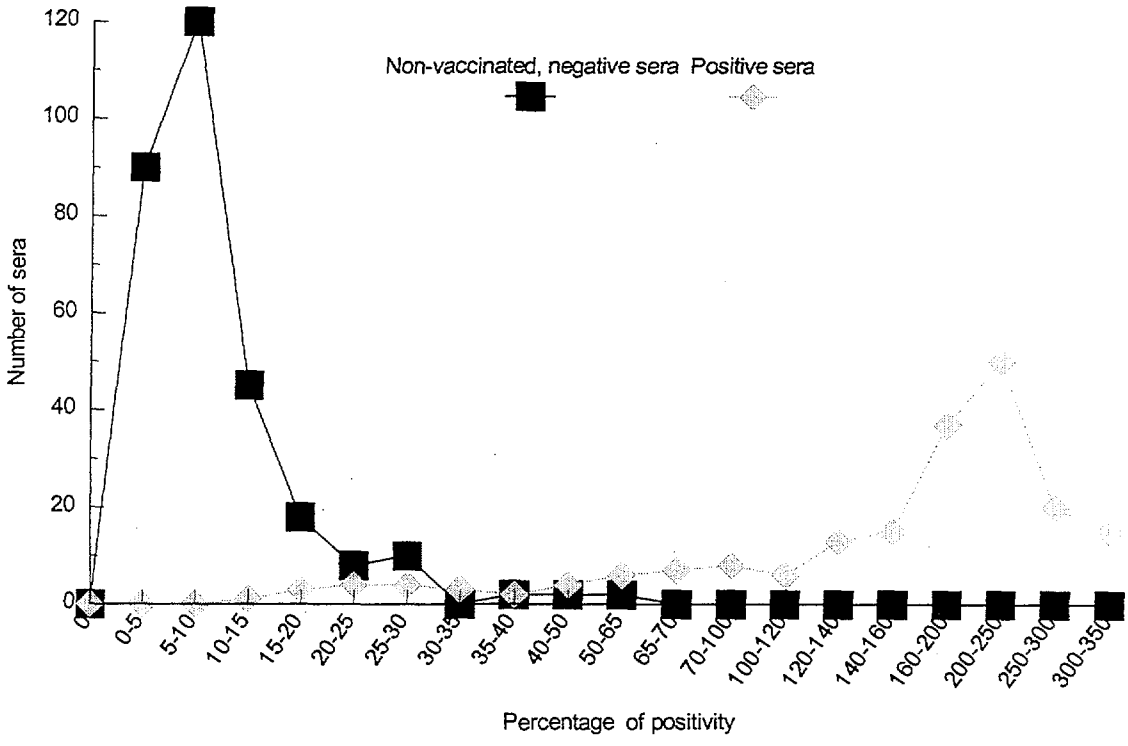

FIG. 4. Comparative distribution of I-ELISA pp-values of 286 non-vaccinated, negative and of 156 positive sera.

4. DISCUSSION

The objective of this study was to evaluate the diagnostic performance of an indirect ELISA kit produced by the Joint FAO/IAEA Division for diagnosis of bovine brucellosis, in Patagonia, Argentina.

The results presented in this communication for the conventional techniques are similar to those cited by the literature [9]. Stemshorn et al (1985), based on 1051 sera from brucellosis free herds (both vaccinated and non-vaccinated) found a specificity of 98.9% for the BPA, 99.8% for the 2ME and 100% for the RB and the CF. These results are quite similar to those presented here, however, Stemshorn et al. (1985) observed no improvement of BPA specificity when only non-vaccinated herds were considered in contrast with our results which show that BPA specificity was higher for non-vaccinated (100%) than for vaccinated (97.6%) cattle. Calfhood (3-8 months) vaccination with the standard dose of strain 19 is compulsory in Argentina. A possible explanation for this difference could be that in Patagonia, the age of vaccination for heifers (3-8 months) is not always observed and some animals may be vaccinated later than 8 months of age. This could result in production of antibodies that persist for a longer period.

The specificity of the I-ELISA was only slightly lower than the BPA, RB, 2ME, and CF tests with sera from non vaccinated animals. However for negative, vaccinated animals, I-ELISA specificity was higher than BPA. This result is encouraging as the most frequent situation in Patagonia is to ignore the vaccinal status of the cattle and therefore, a technique of high sensitivity with vaccinated animals is desirable. Nevertheless, a large sample would be required to estimate the specificity of the techniques with more precision.

In the study by Stemshorn et al (1985) only 82.0% of 167 culture positive cattle were detected by any of the serological methods used. The authors suggested that rapid spread of infection in the herds may have contributed to some of these failures, the cattle not having time to develop serological responses. In our case, the higher sensitivity demonstrated by all the techniques may be due to chronic infection with good antibody response in most infected animals. A difference in sensitivity was observed between RB and BPA. The later technique was more sensitive. However, the specificity of both

techniques was similar. The higher sensitivity of the BPA is in agreement with previous reports. The I-ELISA detected more infected cattle than any other test.

The I-ELISA resulted in specificity estimates approximating that of the other tests used when evaluated with sera from negative, non-vaccinated herds.

The I-ELISA showed a good diagnostic performance. In addition, this technique offers several major advantages, e.g. sera need not to be heat inactivated as for the CF test or treated as for the 2ME test. This technique also requires fewer complex standardization processes than the CF test. The I-ELISA measures reactivity objectively which reduces reading errors and it allows greater number of samples to be processed at one time.

From the results obtained, the I-ELISA kit appeared to be a very useful tool in the diagnosis of bovine brucellosis in the Patagonia region. The technique seems to be particularly useful for this region, where little epidemiological information is available about this disease and where large numbers of sera should be tested to obtain such information. The conventional diagnostic tests for bovine brucellosis are time consuming and not sensitive and reliable enough to be used in a large scale survey. The I-ELISA kit assay evaluated in our lab seemed to be rapid, simple, sensitive and specific for detecting antibodies to *Brucella abortus*.

The I-ELISA should be further evaluated as a diagnostic tool in control programmes in the Patagonia region.

ACKNOWLEDGEMENTS

We thank Dr. K. Nielsen for his help and guidance and for critical review of the manuscript. Dr. P. Wright and Dr. E. Nilsson are thanked for their guidance throughout this study. We also thank Dr. J. Moreno Lopez and Dr. A. Bengochea for their help and for providing some of the sera tested and Ms. T. Perez and S. Vannelli for typing the manuscript. This study was funded by the Joint FAO/IAEA Division, International Atomic Energy Agency (IAEA), Vienna, Austria (Research Contract No. 6518).

REFERENCES

[1] FAO/OMS, Comité Mixto de expertos en brucelosis 1986, Sexto informe, OMS, Geneve, pp 149.

[2] ALTON, G.G., Recent developments in vaccination against bovine brucellosis, Aust. Vet. J. **54** (1978) 551-557.

[3] MARIÑO, O., Gallego, M.M.I., de Leon, L.S., Almansa, M.J., Comparación de técnicas serológicas en la evaluacíon de los bovinos infectados naturalmente por *Brucella abortus*. In: J.F. Frank (Ed.), Networking in Brucellosis Research, The United Nations University, Tokyo (1991) 120-130.

[4] NIELSEN, K.H., WRIGHT, P.F., KELLY, W.A., CHERWONOGRODZKY, J.H., A review of enzyme immunoassay for detection of antibody to *Brucella abortus* in cattle, Vet. Immunol. Immunopath. **18** (1988) 331-347.

[5] ALTON, G.G., JONES, L.M., ANGUS, R.D., VERGER, J.M., Techniques for the Brucellosis Laboratory, INRA, Paris (1988) 190.

[6] MACMILLAN, A., Conventional Serological test, In: Nielsen, K. & Duncan, J.R. Animal brucellosis, CRC Press, Boca Raton, U.S.A. (1990) 153-197.

[7] ANGUS, R.D., BARTON, C.E., The production and evaluation of buffered plate antigen for use in a presumptive test for brucellosis, Develop. Biol. Standard. **56** (1984) 349-356.

[8] ALTON, G.G., JONES, L.M., PIETZ, D.E., Laboratory techniques in brucellosis, 2nd. edn. FAO-WHO, Geneve, Series de monografía **55** (1975).

[9] STEMSHORN, B.W., FORBES, L.B., EAGLESOME, M.D., NIELSEN, K.H., ROBERTSON, F.J., SAMAGH, B.S., A comparison of standard serological test for the diagnosis of bovine brucellosis in Canadá, Can. J. Comp. Med. **49** (1985) 391-394.