EVALUATION OF AN ENZYME LINKED IMMUNOSORBENT ASSAY KIT FOR THE DETECTION OF *BABESIA BOVIS* ANTIBODIES IN CATTLE IN ARGENTINA

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Abstract

EVALUATION OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY FOR THE DETECTION OF *BABESIA BOVIS*-ANTIBODIES IN CATTLE IN ARGENTINA.

An enzyme linked immunosorbent assay (ELISA) for the detection of antibodies to *Babesia bovis* was evaluated by using sera of 874 cattle carrying *B. bovis* antibodies, 700 sera of uninfected cattle, and 357 sera from calves from 16 herds subjected to different *B. bovis* inoculation rates. The seropositive/ seronegative cut-off point set as double the mean percent positivity of negative cattle sera (= 16%). The sensitivity of the ELISA (four trials) ranged from 97.1% to 100% and the specificity (three trials) varied from 92.0% to 97.0%. The agreement between ELISA and immunofluorescent antibody test was ≥ 90.0% in 18 of 23 evaluations and it ranged from 86.0% to 88.0% in the remainder. The correlation coefficient between percentage of sera positive to ELISA and IFA test in 16 herds was 0.9958 ($P < 0.001$). The ELISA has the advantages of a high sensitivity, objectivity and capacity to test large number of samples in short period of time and could replace the IFA test specially for epidemiological studies.

1. INTRODUCTION

Babesiosis caused by *Babesia bovis* is the most economically important tick-borne disease of cattle in the Southern Cone of America where it is transmitted only by larvae of *Boophilus microplus* ticks [1]. Clinical cases rarely occur in cattle younger than seven months [2,3]. Infection during this period induces a long-lasting immunity [4], while primary infection later in life can produce severe illness [5]. Therefore, the probability of the occurrence of babesiosis outbreaks in a cohort-herd can be estimated by detecting the proportion of infected cattle, 6-12 months-old using techniques suitable for the diagnosis of babesial antibodies.

The indirect immunofluorescent antibody (IFA) test is widely used to detect *B. bovis* antibodies for epidemiological or experimental studies. However this is a subjective test in which background fluorescence can cause difficulty to arrive at an endpoint serum titration. Microfluorometry can be used to partly solve this problem as demonstrated for a related cattle disease, anaplasmosis [6]. Nevertheless the major problem with the IFA test is the difficulty to process daily a large number of sera as is frequently required in surveys or to use the test to make decisions affecting the economics of vaccination or management of cattle on a regional basis.

Enzyme linked immunosorbent assays (ELISA) have been applied increasingly to detect antibodies against agents of many animal and human diseases. Moreover the ability to couple the detection system to computerized automatic readers makes ELISA a practical and powerful tool for experimental and epidemiological studies, particularly for those diseases in which high quality antigens for ELISA have been developed. The current study was designed to validate an indirect ELISA-kit for detection of antibodies to *B. bovis* under local conditions in Argentina, and to compare the results of ELISA with those of an IFA test. The ELISA data were then used to estimate the endemic stability of *B. bovis* in regions of Argentina.

2. MATERIALS AND METHODS

2.1. ELISA procedure

The antigen was an oxyhemoglobin-free distilled water lysate of *B. bovis* infected erythrocytes preserved in a freeze-dried state [7].

The guidelines provided by the manufacturer of the kit [8] were followed to perform the test. Briefly 100µl of antigen diluted 1:200 in carbonate/bicarbonate buffer pH 9.6 (coating buffer) was adsorbed onto the wells of 96-well microplates (Nunc) by overnight incubation at 4°C. After incubation, excess antigen was removed and 150 µl of 5% skimmed milk in coating buffer were added as blocking solution and incubated for 1 h at 37°C with continuous shaking (Micro Shaker II, Dynatech). Thereafter the plates were washed three times in phosphate buffer saline 0.002M, pH 7.2, containing 0.05% Tween 20 (PBS-T; wash buffer). Sera were added at the dilution 1:200 in 0.01M PBS, pH 7.2, containing 0.05%Tween 20 (diluent buffer). A weak positive (C+), a strong positive (C++), and a negative (C-) control serum, as well as a conjugate control were run in quadruplicate, while test sera were run in duplicate and incubated for 1 h at 37°C with shaking. After five washings, 100 µl of conjugate (rabbit anti-bovine IgG antibody conjugated to horseradish peroxidase), diluted 1:10000 in diluent buffer, was added and incubated for 1 h at 37°C with shaking. The plates were washed and 100 µl of substrate solution (H_2O_2 containing O-phenylenediamine as chromogen) was added and incubated 10 min at room temperature. The reaction was stopped with 100 µl of sulfuric acid 2N. The absorbance values were determined at 492 nm. A Multiskan Plus (type 314) ELISA reader linked to a personal computer (AT 386 IBM clone) and the BAEIA version 1.01 software programme (copyright Walter Kelly, Agriculture Canada 1991-1992) were used throughout. The results for each sample were calculated as a percent of the mean of the quadruplicated C++ sample for each plate and expressed as percent positive (PP) values. Quality control was maintained by assuring that the optical density (OD) readings for each control fell within limits set by the kit manufacturer.

2.2. IFA test procedure

The IFA test was performed as described by Ríos et al. [9] using an antigen prepared from *B. bovis* grown *in vitro*. The antigen was harvested from the cultures when infection of erythrocytes reached 8%. The antigen was kept at -20°C until use. Sera were diluted 1:60 in PBS solution for screening or for the first dilution when endpoint titration of the serum was done. A positive and a negative control serum were added on each smear. A conjugate (fluorescein-labeled rabbit anti-bovine IgG) was used at a 1:80 dilution. The fluorescent reactions were observed with a microscope (Leitz) equipped for epi-illumination using 50-W mercury vapor lamp. A 50X water immersion objective was used.

2.3. Determination of seropositive/seronegative threshold PP value for ELISA (cut-off point)

Five hundred sera from cattle negative to *B. bovis* (bornE and raised in an area of Argentina free of *B. microplus* ticks) were analyzed by ELISA. The threshold was set as double of the mean PP value of these sera from uninfected cattle. To determine the frequency distribution of PP values among uninfected and infected animals, 500 sera from cattle known to be infected with *B. bovis* were also analyzed.

2.4. Determination of the sensitivity and the specificity

The diagnostic sensitivity of the assay was expressed as the number of positive sera/number of sera tested from cattle inoculated with *B. bovis* antigens. The diagnostic specificity of the assay was defined as the number of negative sera/number of sera tested from cattle from a tick free-zone. In all cases a comparison with the IFA test was done, and the degree of agreement between the results of both techniques obtained.

The sensitivity of the ELISA for *B. bovis* antibodies was determined using 374 cattle sera in four trials as follows: Trial 1: Sera from 76 cattle experimentally infected with a pathogenic *B. bovis*

strain; Trial 2: Sera from 201 cattle inoculated with a commercial live immunogen containing vaccinal *B. bovis, B. bigemina* and *Anaplasma centrale* strains; Trial 3: Sera from 70 cattle inoculated with soluble *B. bovis* antigens derived from an *in vitro* culture of this protozoan; Trial 4: Sera from 26 cattle naturally infected with *B. bovis* detected by inspecting thick smears from peripheral blood under oil immersion microscopy.

The specificity was evaluated in three trials involving sera of cattle from an Argentinean region free of *B. microplus* as follows: Trial 1: Analysis of sera from 50 cattle experimentally infected with *B. bigemina*; Trial 2: Analysis of 50 sera from cattle naturally or experimentally infected with *Anaplasma marginale*; Trial 3: Analysis of 100 sera of cattle from the *B. microplus*-free zone with unknown history of haemoparasite infection.

2.5. Cross-sectional studies of cattle herds from areas with different prevalences of *B. bovis* infection

Sera from calves (9-12 months-old) from 16 herds subjected to different *B. bovis* inoculation rates were evaluated by using ELISA and IFA tests to detect antibodies against this protozoan. The degree of agreement between results of both techniques was also determined. The percentage of positive results of each herd tested was used to determine the correlation (r) between IFA and ELISA. Arbitrarily, IFA values were judged as the dependant variable.

3. RESULTS

No major problems were encountered in conducting the ELISA and the OD values were consistently within the acceptance ranges. The mean of the PP values of the negative sera was 8%. Therefore the cut-off point to define the seropositive/seronegative threshold was set at 16%, using published criteria for the ELISA kit. A small overlap in the PP values of positive and negative sera to *B. bovis* antibodies was found (Figure 1). Ten sera from cattle infected with *B. bovis* antigens (1% of all sera analyzed) showed PP values below the cut-off point, while 25 sera (5%) of the total analyzed from cattle not infected with *B. bovis* showed false positive reactions.

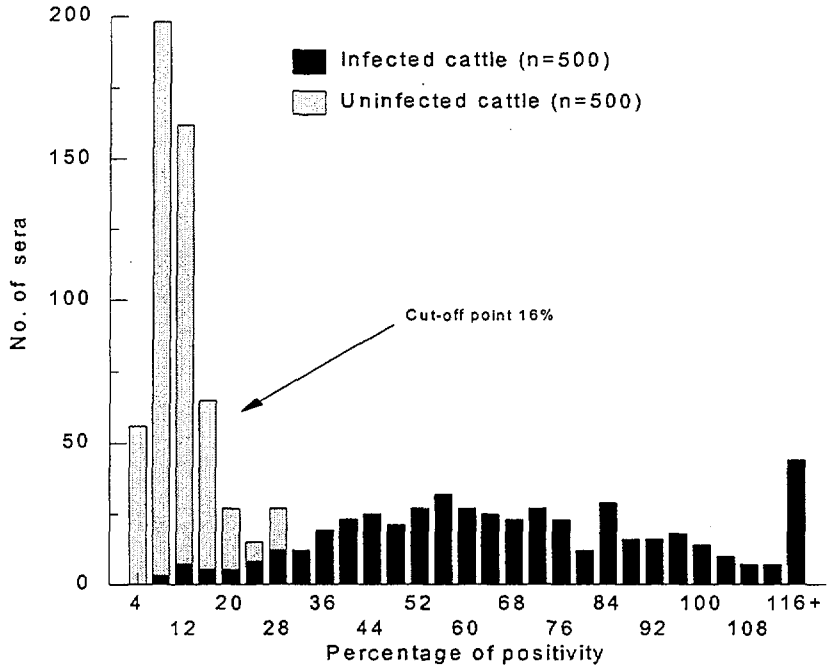

FIG. 1. Frequency distribution of percentage of positivity of sera from cattle infected or uninfected with Babesia Bovis analyzed to determine ELISA seropositive/seronegative cut-off point (twice the mean percentage positivity value of negative sera in relation to a strong positive control).

The sensitivity and specificity of IFA and ELISA along with the degree of agreement between the techniques are presented in Table I. Three false positive reactions to ELISA were found among 50 sera from cattle experimentally infected with *B. bigemina* (PP values 19%, 39% and 44%), four were found among 50 sera of cattle infected with *A. marginale* (26%, 21%, 18% and 16% of PP values) and three false positive reactions were detected in 100 sera of cattle from an area known to be free of *B. bovis* but of unknown history of other haemoparasite infection (22%, 17% and 44% PP values).

The results of the cross-sectional studies of herds are presented in Table II. The concordance value of ELISA and IFA was ≥ 90% in 18 of 23 estimations (results from Table I + Table II) which is consistent with the data presented by Barry et al (10). This resulted in a strong association between the percent of cattle positive to *B. bovis* antibodies using both techniques as shown by the r coefficient of 0.9958 ($P < 0.001$).

TABLE I. SENSITIVITY AND THE SPECIFICITY OF ELISA, ITS COMPARISON WITH AN IFA TEST, AND THE AGREEMENT BETWEEN BOTH TECHNIQUES

Trial	Origin of cattle sera	n	Percentage of positive results		Agreement (%)
			ELISA	IFA	
		SENSITIVITY			
1	Pathogenic B. bovis	77	100	96.1	96.1
2	Vaccinal B. bovis	201	99.5	99.5	98.5
3	Soluble antigens	70	97.1	92.8	91.4
4	B. bovis field strains	26	100.0	100.0	100.0
		SPECIFICITY*			
1	B. bigemina	50	94.0	96.0	90.0
2	A. marginale	50	92.0	96.0	88.0
3	Status unknown	100	97.0	97.0	94.0

* All sera used to test specificity were from cattle borne and raised in the *B. microplus* free area of Argentina

TABLE II. COMPARATIVE PREVALENCE OF ANTIBODIES TO *B. BOVIS* IN CATTLE HERDS NATURALLY INFEECTED USING ELISA AND IFA TEST, AND THE AGREEMENT BETWEEN BOTH TECHNIQUES

Herd	n	ELISA (%)	IFA(%)	Agreement (%)
1	25	4	0	96
2	21	14	24	86
3	15	20	20	87
4	25	100	100	100
5	25	8	0	92
6	25	4	4	100
7	25	24	17	88
8	16	100	100	100
9	25	80	80	92
10	25	68	72	88
11	20	100	100	100
12	20	100	100	100
13	17	100	100	100
14	25	8	4	96
15	23	9	9	100
16	25	4	4	100
TOTAL	418			

4. CONCLUSIONS

As expected, the sensitivity of the assay for detection of *B. bovis* antibodies was high. This is in concordance with the results presented by Barry et al [10] and Waltisbuhl et al. [7] testing cattle infected with vaccinal or pathogenic *B. bovis* strains using other ELISA systems. The assay showed a higher sensitivity than IFA in Trial 1 and 3 but no difference was found in Trials 2 and 4.

Cattle vaccinated with *B. bovis* soluble culture antigens (Trial 3), may be true negatives because they might not have been infected with *B. bovis* strains. Different antigens would be present in the immunizing culture extract vs the antigen used in ELISA thus accounting for the inability of ELISA to detect the antibodies to the immunizing antigens.

The specificity of the ELISA and IFA did not reach the same high level as the sensitivity estimates. The false positive reactions with sera of cattle infected with *A. marginale* or *B. bigemina* is disturbing since the geographical distributions of these haemoparasites match with the distribution of *B. bovis* in several American countries [1]. Waltisbuhl et al. [7] concluded that false positive reactions with an ELISA for *B. bovis* in cattle infected with *A. marginale* might be due to common changes in membrane isoantigens of infected red cells. They proposed further purification of the antigen or absorption of sera with a pool of lysate of normal erythrocytes to improve the specificity of the ELISA. The first option appears to be the best since the absorption of sera will add a time consuming step to the assay procedure. Böse et al. [11] dealing with sera of cattle free of *B. bovis* also found nonspecific reactions using an ELISA to diagnose antibodies to this protozoan. These reactions were probably due to contamination of the IgG conjugate with IgM. More research is needed to further improve the specificity of the assay. Alternatives like increasing the cut-off point are not proper because they will cause a commensurate drop in the sensitivity of the ELISA.

The agreement between both techniques (Table II) was good, ranging between 86 and 100%. Fifty percent of the herds showed 100% of agreement. In cases where the agreement between ELISA and IFA was the lowest (86-92%), the prevalence data indicated the same epidemiological status and obviously the same strategic measures were recommended.

Although the specificity of the ELISA needs to be improved this assay can replace the IFA test to detect *B. bovis* antibodies in cattle. The high degree of sensitivity, objectivity, and its capacity to be adapted to test large number of sera in short period of time are advantages not provided by the IFA test. This ELISA will permit an increase of epidemiological studies of babesiosis. This is especially important in developing countries where areas of enzootic instability to babesiosis are ill-defined, precluding the implementation of preventive measures with economical rationality unless the true infection status of the cattle is known.

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