VALIDATION AND USE OF AN ELISA KIT FOR THE DIAGNOSIS OF *BABESIA BOVIS* IN CUBA

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Abstract

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Babesia bovis, the most important etiological agent causing bovine babesiosis, is widely distributed in Cuba and affects mainly adult cattle. A survey of the prevalence of the disease in cattle using an ELISA kit (FAO/IAEA) revealed that 34.2% of the animals between 6 and 18 months of age were positive to *Babesia bovis*, whereas 69.9% of the cattle older than 18 months were positive. Antibodies to *Babesia bovis* were detected in 96.9% of calves vaccinated with an attenuated *Babesia bovis* vaccine. A good correlation was found between the results of ELISA kit with those from indirect immunofluorescence and immunoperoxidase tests developed in Cuba.

1. INTRODUCTION

Babesia bovis is the principal atiological agent of bovine babesiosis which is responsible for high morbidity and mortality of cattle in tropical areas. In Cuba this parasite is widely distributed and is responsible for a disease that is of considerable economic importance.

In order to detect the immune response of cattle to *B. bovis* infection, several tests, e.g. agglutination [1], fluorescence antibodies [2] and immunoenzymatic assays [3,4,5] have been developed.

The enzyme linked immunosorbent assay (ELISA) has been described as a very sensitive assay [6]. In this paper, an ELISA kit for detection of antibodies to *B. bovis* was used for a survey and a control programme in Havana province, and it was compared with other serological tests developed in our laboratory for the diagnosis of this disease.

2. MATERIAL AND METHODS

2.1. ELISA kit

An indirect ELISA test kit for detection of antibodies to *B. bovis* was provided by the Animal Production and Health Section of the Joint FAO/IAEA Division for evaluation in our country. The assay procedure was used as described in the FAO/IAEA manual.

Optical density (OD) readings, representing color development in ELISA, were expressed as a percent of the OD of the positive serum control (PP). The cut-off point, separating positive from negative animals, was determined using serum samples that were collected from 15 splenectomized calves negative to hemoparasites. The cut-off was calculated as twice the mean of the PP value of these sera.

One hundred thirty-three samples collected from calves 1 month after vaccination with an attenuated *B. bovis* vaccine, and sera negative to haemoparasites were used to calculate the sensitivity and specificity of ELISA test. For each plate the results were considered valid when the results of positive (C++), moderate positive (C+) and negative (C-) sera were between the control limits recommended in the manual of the ELISA kit.

2.2. Serological survey

Nine hundred and ninety-seven blood samples were collected from cattle from 4 farms in Havana Province. Three of the four farms investigated were randomly sampled. Approximately 20% of

cattle on each farm were sampled and the cattle placed into two groups according to age (6 - 18 months and > 18 months). All cattle on the fourth farm (> 18 months) were sampled. These cattle were breeding bulls known to have a very low prevalence of *B. bovis* by the indirect immunofluorescence test. All sera were analyzed using the ELISA kit to determine the seroprevalence of *B. bovis*.

2.3. Comparison of ELISA with indirect immunofluorescence test (IFAT) and immuno-peroxidase test (IPT)

The ELISA was compared with IFAT and IPT using 445 serum samples that included 60 sera from known infected animals, 15 sera from known uninfected animals, and 370 sera from animals of unknown infection status that were collected from the farms.

2.4. Immunofluorescence test

Slides coated with antigen and stored frozen were allowed to thaw for 10 minutes at 37°C. Each slide was divided into twelve rectangular compartments with a marker pen. Test sera were diluted 1:100 in phosphate buffered saline pH 7.2 (PBS), added to the slide, and incubated for 30 min at 37°C in a moist chamber. The slides were washed three times with PBS and then allowed to react for 45 min at 37°C with a rabbit anti-bovine IgG fluorescein conjugate diluted 1:400. The slides were washed three times with PBS, glycerol-PBS was added to the surface, and the slides were examined using an oil immersion × 100 objective of a fluorescence microscope.

2.5. Immunoperoxidase test

This test was performed as described for IFAT except the use of an horseradish peroxidase conjugate diluted 1:200 in 1% egg albumin in PBS. The slides were washed three times and the substrate solution (H_2O_2 and 4-chloro-1- naphtol) diluted in TRIS buffered saline (pH 7.4) was added. Finally the slides were examined with an oil immersion × 100 objective of a light microscope.

2.6. Control of *B. bovis* vaccine application

For two of the farms investigated, the efficacy of the control program was calculated using 1,335 calves vaccinated with an attenuated *B. bovis* vaccine. One month after vaccination, 10% of the animals were investigated for the presence of antibodies to *B. bovis*.

3. RESULTS

The cut-off point in the ELISA, based on 15 splenectomized calves, was determined to be 14.5% of the positive control serum (14.5% PP). The frequency distribution of PP values was obtained by the analysis of all samples (Figure 1). A distinct bimodal distribution was not observed but two peaks could be distinguished. The cut-off determined from the graph was estimated to be between 13 and 23%. Using the cutoff of 14.5% PP, the sensitivity of the ELISA for vaccinated calves was 96.9% at a specificity of 100%.

The results of the survey using the ELISA kit are summarized in Table I. These results show that for the 3 farms taken together, antibodies to *B. bovis* were detected in 32.4% of cattle 6 to 18-months of age, and in 69.9% of adult cattle. For the remaining farm (breeding bulls) the sero-prevalence was only 3.1%. A 92.8% correlation was found between the results obtained using the ELISA kit and IFAT (Table II). For the samples that were not in agreement between the two tests, 3.8% were positive by ELISA and negative using IFAT whereas 3.4% were IFAT positive but ELISA negative.

The comparison between the ELISA kit and the IPT is summarized in Table III. A 94% correlation was observed between the tests. Of the 27 serum samples that did not agree between the

tests, 3.3% were positive by ELISA but negative using IPT whereas 2.7% were IPT positive but ELISA negative.

TABLE I. RESULTS OF A SEROLOGICAL SURVEY OF *B.BOVIS* USING THE FAO/IAEA ELISA KIT

Farm	No. of samples	Age (months)	Positive sera	Percentage
1	263	6 - 18	98	37.2
	206	> 18	142	68.9
2	237	6 - 18	66	27.8
3*	129	> 18	4	3.1
4	102	6 - 18	42	41.1
	60	> 18	44	73.3

* breeding bull

TABLE II. CORRELATION OF ELISA AND IFAT FOR THE DETECTION OF *B. BOVIS* ANTIBODIES

Correlation between tests	Fraction from total	Percentage
Agreement		
188 positives 225 negatives	413/445	92.8%
Disagreement		
17 positives by ELISA and negatives by IFAT	17/445	3.8%
15 positives by IFAT and negatives by ELISA	15/445	3.5%

TABLE III. CORRELATION OF ELISA AND IPT FOR THE DETECTION OF *B.BOVIS* ANTIBODIES

Correction between tests	Fraction from total	Percentage
Agreement:		
190 positives 220 negatives	418/445	94%
Disagreement:		
15 positives by ELISA and negatives by IFAT	15/445	3.3%
15 positives by IPT and negatives by ELISA	12/445	2.7%

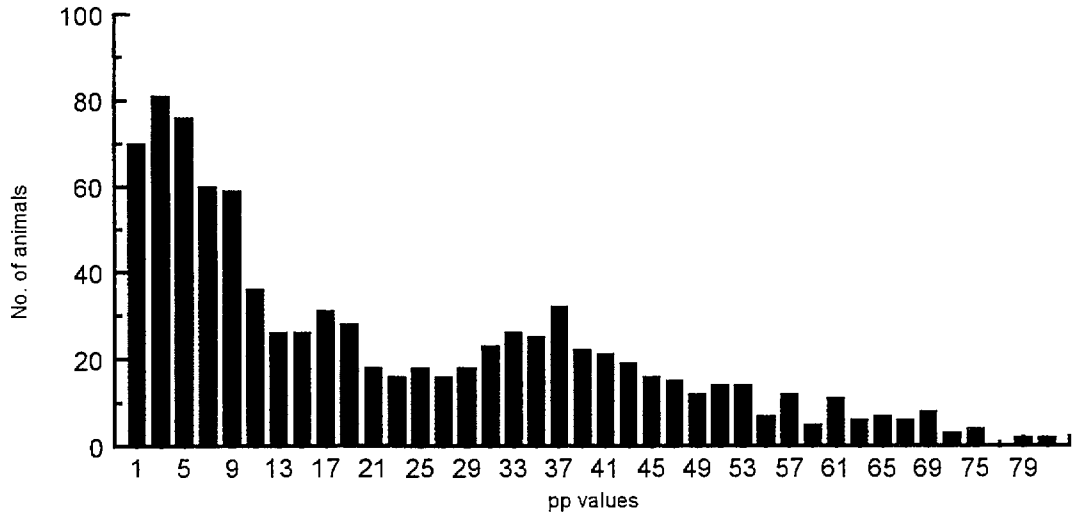

FIG. 1. Frequency distribution of pp-values.

4. CONCLUSIONS

The difference in the determination of cut-off point using negative sera from splenectomized calves and the frequency distribution method was probably due to the fact that a portion of the investigated population used to determine the frequency distribution was older than 18 months. The cut-off for Cuba is probably between 13% and 23 % PP.

The specificity of ELISA determined in this work was very high (100%). However, because a limited number of samples were used for making this determination, evaluation of more sera is needed to confirm this result.

The ELISA was successfully used in the survey for the detection of specific antibodies to *B. bovis* and in a babesiosis control program. Three of the four farms investigated would appear to be at risk from babesiosis because the percentage of seropositive cattle was lower than necessary to obtain endemic stability of the infection in the population. In the other farm the seroprevalence to *B. bovis* was very low and the situation is potentially unstable, but the risk of clinical babesiosis is minimal.

The high percentage vaccinated calves with antibodies to *B. bovis* indicated that the vaccine was successful in stimulating a response to *B. bovis;* the ELISA kit was useful in confirming this observation. The 3.1% of vaccinated calves that were negative by ELISA could be attributed to the interval of only one month between vaccination and collection of the serum samples. It is possible that some calves had not yet produced detectable antibodies. A longer time interval for the collection of sera after vaccination (2 months) could give a better estimation of an immune response to *B. bovis* vaccination.

The ELISA kit (FAO/IAEA) and IFAT had a good correlation for the detection of antibodies to *B. bovis*. The IFAT has probably been the most widely used test for the detection of antibodies to *Babesia sp.* [7]. It has been standardized in Cuba and is still being used routinely for this purpose. However, the problem with IFAT is that the test is subjective and vulnerable to technician bias in reading of the slides microscopically.

The immunoperoxidase test also had a good agreement with the ELISA kit. Blandino et al. [8] reported a relative sensitivity of 97% when this assay was compared to IFAT; this is similar to the results obtained by Kung [5] with the S-ELISA. This technique is simple to perform and did not require special equipment.

ELISA allows a far better standardization than other assays, its results are read and computed automatically. For this reason it is a very useful technique for mass screening.

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REFERENCES

[1] MONTENEGRO, S., JAMES, M. A., LEVY, M. G., PRESTON, W.D., ESPERANZA,. J., RISTIC, M., Utilization of culture derived soluble antigen in the latex agglutination test for bovine babesiosis and anaplasmosis, Rev. Parasit. **8** (1981) 291.

[2] ALONSO, M., BLANDINO, T., LARRAMENDI, R., JIMENEZ, T., MESA, J., Inmunofluorescencia indirecta en el diagnóstico de la babesiosis bovina en Cuba, Rev. Salud Anim. **10** (1988) 197.

[3] ROSE, R., JACOBSON, R. H., GALE, K. R., WALTISBUHL, D. J., WRIGHT, I.G., An improved ELISA for the detection of antibodies against *B. bovis* using either a native or a recombinant *B. bovis* antigen, Parasitol. Res. **76** (1990) 648.

[4] MONTENEGRO, S. Dot ELISA para el diagnóstico serológico de la babesiosis y anaplasmosis, XII Congreso Panamericano de Ciencias Veterinarias, La Habana, Cuba, 1990.

[5] KUNG, U. M. W., GOODGER, B. V., A slide enzyme-linked immuno-absorbent assay (SELISA) for the diagnosis of *Babesia bovis* infections and for screening of *Babesia*-specific monoclonal antibodies, Int. Jour. Parasit. **20** (1990) 341-345.

[6] WALTISBUHL, D.J., GOODGER, B.V., WRIGHT, I.G., COMMINS, M. A., MAHONEY, D.F., An enzyme linked immunosorbent assay to diagnose *Babesia bovis* infection in cattle. Parasitol. Res. **73** (1987) 126-131.

[7] PAYNE, R. C., OSORIO, O., Tick-borne diseases of cattle in Paraguay. I Seroepidemiological study on anaplasmosis and babesiosis. Trop. Anim. Hlth. Prod. **22** (1990) 53-60.

[8] BLANDINO, T., BARRERA, M., ALONSO, M., Prueba de inmunoperoxidasa para el diagnóstico serológico de *Babesia bovis*, Progreso de la Medicina Veterinaria **2** (1992) 11.