VALIDATION OF AN INDIRECT ELISA FOR THE DIAGNOSIS OF *BABESIA BOVIS* IN CATTLE IN YUCATAN, MEXICO

A.J.L. DOMÍNGUEZ, V.R.I. RODRÍGUEZ, C. OURA, G.L.A. COB
Departamento de Parasitología, Universidad Autónoma de Yucatán,
Mérida, Yucatan, México

XA9848673

Abstract

VALIDATION OF AN INDIRECT ELISA FOR THE DIAGNOSIS OF *BABESIA BOVIS* IN CATTLE IN YUCATAN, MEXICO.

The ELISA kit provided by the FAO/IAEA for the diagnosis of *Babesia bovis* was validated. In order to determine the appropriate ELISA cut-off point that would serve as the threshold between positive and negative samples, 119 serum samples from a Mexican *Babesia*-free zone were analyzed. The optimal cut-off point chosen was at 12% of the reactivity of the high positive control serum sample (PP) which resulted in a specificity of 97%. One hundred and ninety-six cattle from Wisconsin, USA, were introduced into Yucatan, Mexico, of which 181 were vaccinated with an attenuated live *Babesia bovis* vaccine; 15 animals remained as unvaccinated controls. Before and after vaccination all animals were bled and tested by enzyme lnked immunosorbent assay (ELISA) and indirect fluorescence antibody test (IFAT). Both tests showed a high degree of correlation in their results. To evaluate an immune response to vaccination the optimal cut-off point chosen was 12% PP resulting in a sensitivity 99% and a specificity 95%. We concluded that the ELISA test has proved to be useful in Yucatan, Mexico for serological surveys and monitoring the efficiency of vaccination programmes.

1. INTRODUCTION

More than one billion cattle in the world are estimated to be at risk of acquiring the tick-borne hemoprotozoan disease babesiosis [1]. Bovine babesiosis, caused by the protozoan parasite *Babesia* spp., continues as a major threat to livestock industries throughout the world. *Babesia bovis* and *Babesia bigemina* infect the red blood cells of cattle and area transmitted by the one host tick, *Boophilus microplus*.

The diagnosis of acute babesiosis in cattle is relatively straightforward when clinical signs are evident and supported by microscopic examination of stained blood films [2]. On the other hand, mild and subclinical infections are more difficult to recognize since peripheral blood parasitaemias fluctuate and frequently do not rise to levels detectable by microscopy. Although classical serological techniques of babesiosis such as the indirect fluorescence antibody test (IFAT) and complement fixation (CF), have proved useful, they suffer from a number of drawbacks. Generally, these drawbacks relate to a combination of inadequate diagnostic performance, lack of standardization, and/or poor cost efficiency. The enzyme-linked immunosorbent assay (ELISA) potentially could resolve these problems. In this survey we validated an ELISA kit provided by FAO/IAEA and used it in a serological survey in Yucatan, Mexico.

2. MATERIALS AND METHOD

2.1. ELISA Procedure

The ELISA kit used in this study was similar to that described by Waltisbuhl *et al.* [3] and was provided by FAO/IAEA [4]. The test was carried out using flat-bottomed 96-well micro-ELISA plates (Nunc-Immunoplate, Denmark). Briefly, the stock antigen was diluted 1:200 in carbonate-bicarbonate buffer pH 9.6 (coating buffer). One hundred microlitres of diluted antigen was added to each well of a micro-ELISA plate and incubated over night at 4°C. After incubation the antigen solution was discarded and 100 µl of a blocking solution (5% skimmed milk powder in coating buffer - w/v) was added to each well and incubated for 1 h at 37°C. After blocking, the plates were washed three times in phosphate buffered saline (PBS) pH 7.2 containing 0.001% Tween 20 (PBS-T). The positive reference sera (moderate and strong antibody activity to *B.*

bovis) and a negative serum were diluted 1:200 in PBS-T and added to the antigen coated wells in quadruplicate. Test sera at the same dilution were run in duplicate. The plates were incubated for 1 h at 37°C and then washed three times in PBS-T. Rabbit anti-bovine IgG horseradish peroxidase conjugate, diluted 1:11,000 in PBS-T, was then added and a further incubation for 1 hour carried out at 37°C. The plates were again washed three times, a substrate-chromogen solution of H_2O_2 containing O-phenylenediamine was added, and after ten minutes, the reaction was stopped by adding 100µl of 2M sulfuric acid. The intensity of color development was determined by measuring absorbance using a micro-ELISA reader equipped with a 492 nm filter. The results of were expressed as a percent of the high positive control serum sample (PP) [5].

2.2. Cut-off determination

The cut-off value separating positive from negative sera was calculated as 3 standard deviations above the mean of the PP values obtained from 119 adult cattle raised in Toluca in a high altitude tick free zone in central Mexico and 196 cattle imported from Wisconsin, USA, to Yucatan.

2.3. Serum sample

To compare the ELISA with IFAT, 196 cattle (*Bos taurus*) imported from Wisconsin, USA, were to Yucatan were used. The cattle were sent to two farms: 98 animals were placed on ranch 1 and the other 98 on ranch 2. All animals were bled 3 days after arriving in Yucatan. One week later, 181 of the animals received a dose (1ml) of 1 $x10^7$ of *Babesia bovis* vaccine (prepared by 54 rapid cell culture passages) intramuscularly in the neck and 15 control animals received vaccine diluent [6]. Sixty days after vaccination all animals were bled and tested by ELISA and IFAT as described by Todorovic and Long [7]. The pre-vaccination and post-vaccination sera served as known negative and known positive animals, respectively. The specificity and sensitivity of each test were calculated and compared using vaccination status as the gold standard.

3. RESULTS

3.1. ELISA results

The ELISA cut-off point for local conditions and indigenous cattle was determined from 119 negative samples from Toluca in a high altitude *Babesia*-free zone in central Mexico. Three cut-off points of 10%, 12% and 15% PP were calculated (Table I).

TABLE I. CALCULATION OF THE CUT-OFF SEPARATING NEGATIVE FROM POSITIVE SAMPLES BASED ON SERA FROM CATTLE IN THE TOLUCA REGION OF YUCATAN THAT ARE KNOWN TO NOT BE INFECTED WITH *B. BOVIS*

Cut-off (%)	Positives	Negatives	Retest	Spec. (%)
10	6	113	1	95
12	3	116	1	97
15	2	117	1	98

ELSIA results for the pre- and post-vaccinal samples taken from the cattle that were vaccinated at both ranches were used to determine specificity and sensitivity of the ELISA for detection of vaccinal antibody (Table II). All the post-vaccination samples from ranch 1 were

high, indicating an excellent antibody response to the vaccine, whereas, some of the cattle did not respond well on ranch 2. The calculated specificity and sensitivity of the ELISA test using the pre-vaccination samples from both ranches and the post-vaccination samples from only ranch 1 (controls removed), were then used to determine the three cut-off points 10%, 12% and 15% of the PP (Table III). This improved the efficiency of the test.

TABLE II. CALCULATION OF SENSITIVITY AND SPECIFICITY OF ELISA BASED UPON SERUM SAMPLES DERIVED FROM ALL ANIMALS THAT WERE VACCINATED AGAINST *B. BOVIS* ON RANCHES 1 AND 2

Cut-off (%)	Pre-vaccination samples	Post-vaccination samples	Sens. (%)	Spec. (%)
10	179 (-) 15 (+) 2 (r)	6 (1) 172 (+) 3 (r)	97	92
12	183 (-) 9 (+) 4 (r)	13 (-) 160 (+) 8 (r)	92	95
15	189 (-) 3 (+) 4 (r)	26 (-) 150 (+) 5 (r)	85	98

(-) = Test Negative, (+) = Test Positive, (r) = Retest

TABLE III. CALCULATION OF SENSITIVITY AND SPECIFICITY OF ELISA FOR DETECTION OF ANTIBODY TO *B. BOVIS* USING ALL PRE-VACCINATION SERUM SAMPLES FROM RANCHES 1 AND 2 (BUT ONLY POST-VACCINATION SAMPLES FROM RANCH 1)

Cut-off (%)	Pre-vaccination samples	Post-vaccination samples	Sens. (%)	Spec. (%)
10	179 (-) 15 (+) 2 (r)	0 (1) 81 (+) 1 (r)	100	92
12	183 (-) 9 (+) 4 (r)	1 (-) 79 (+) 2 (r)	99	95
15	189 (-) 3 (+) 4 (r)	6 (-) 72 (+) 4 (r)	92	98

The retest values were found to be very similar to the initial results suggesting that the antibody levels were in the equivocal range. The PP value used was an average of the two tests on repeated samples for calculation of the measurement of specificity and sensitivity.

3.2. IFAT results

All the pre and post-vaccination samples were run with the IFAT (Tables IV and V). A few samples from the pre-vaccination population gave a weak fluorescence which was deemed positive. Five samples collected post-vaccination were found to be negative for both ELISA and IFAT.

TABLE IV. SENSITIVITY AND SPECIFICITY OF IFAT BASED UPON ALL CATTLE FROM RANCHES 1 AND 2 THAT WERE VACCINATED WITH AN ATTENUATED *B. BOVIS* VACCINE

Pre-vaccination samples	192 (-) 4 (+)	
		Sensitivity 98% Specificity 98%
Post-vaccination samples	177 (+) 4 (-)	

TABLE V. CALCULATION OF SENSITIVITY AND SPECIFICITY OF IFAT FOR DETECTION OF ANTIBODY TO *B. BOVIS* USING ALL PRE-VACCINATION SERUM SAMPLES FROM RANCHES 1 AND 2 BUT ONLY POST-VACCINATION SAMPLES FROM RANCH 1

Pre-vaccination samples	192 (-) 4 (+)	
		Sensitivity 100% Specificity 98%
Post-vaccination samples	82 (+) 4 (-)	

4. CONCLUSIONS

The cut-off point of 12%, determined from the "local" Mexican population of cattle from Toluca, appeared to provide a satisfactory estimate of sensitivity and specificity for an ELISA that would be used for prevalence studies. Using 196 pre-vaccination samples from the herd that originated in the USA as a gold standard negative population, and 82 (ranch 1) post-vaccination samples as the gold standard positive population, a 12% PP cut-off for the ELISA kit provided a 99% sensitivity and a 95% specificity. A cut-off point of 10% PP resulted in a sensitivity of 100% but a commensurate drop in specificity to 92%, while a cut-off point of 15% gave a sensitivity of 92% and an increase in specificity to 98%. These cut-off points were calculated to illustrate how the selection of an appropriate cut-off point could be driven by the nature of projected studies. For example, if the ELISA were used to confirm results of a highly sensitive but non-specific screening test, a higher cut-off point would be appropriate to enhance specificity in the ELISA.

Running the same 196 pre-vaccination samples and 82 post-vaccination samples in the IFAT test resulted in a specificity of 98% at a sensitivity of 100%. Both the ELISA and IFAT test showed a high degree of correlation in results. This confirmed the observations of Ramirez [8] in a recent study in Yucatan. The 5 post-vaccination samples that were negative in both the ELISA

and IFAT suggests that either both tests were insufficiently sensitive to detect *babesia* antibodies or the vaccine did not induce detectable levels of antibodies. Alternatively these cattle may have been in the early stages of a developing antibody response to vaccination and could become sero-positive over time. This does not however prove that the vaccine failed to protect these animals since cell mediated immunity can play an important role in protection [9].

Our results demonstrate that the indirect ELISA can be used, not only for serological studies, but also to evaluate the ability of vaccinations to induce antibody. Thirty days after vaccination, ELISA was able to detect IgG-antibodies. Since the microtiter ELISA tests are easy to perform and are ideally suited to the processing of large number of test samples [10], the ELISA is an excellent candidate for assessment of antibody responses to *babesia* antigens or infections.

In conclusion the ELISA kit for the detection of *Babesia bovis* worked very well under Yucatan conditions. The kit methodology produced consistently acceptable plates with control values that fell well within the specified acceptable ranges. It is suitable for use in serological studies and vaccination programs.

REFERENCES

[1] KÖNIGSHOFER, H.O. In: PAO-WHO-OIE, Ed. Animal Health Yearbook (Food and Agriculture Organization), Rome, 1977.

[2] ROSS, J.PJ., LOHR, K.F., Serological diagnosis of Babesia bigemina infection in cattle by the indirect fluorescent antibody test, Res Vet Sci. **9** (1968) 557-562.

[3] 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.

[4] GOODGER, B.V., WRIGHT, I.G., WALTISBUHL, D.J., The lysate from bovine erythrocytes infected with Babesia bovis. Analysis of antigens and a report on their immunogenicity when polymerized with glutaraldehyde, Z Parasitenkd. **69** (1983) 473-482.

[5] WRIGHT, P.F, NILSSON, E., VAN ROOIJ, E.M.A., LELENTA, M., JEGGO, M.H., Standardization and validation of enzyme-linked immunosorbent assay techniques for the detection of antibody in infectious disease diagnosis, Rev. Sci. Tech. Off. Int. Epiz. **12** (1993) 435-450.

[6] WAGNER, G., HOLMAN, P., CRUZ, D., WAGHELA, S., SAHAGUN, A., SNYDELAAR, A. MEDELLIN, J., DOMINGUEZ, J., RODRIGUEZ, I., Proceedings of the Ninth International Veterinary Haemoparasite Disease Conference, Merida, Yucatan, Mexico, 1993.

[7] TODOROVIC, R.A., LONG, R.F., Comparison of indirect fluorescence antibody (IFA) with complement fixation (CF) test for diagnosis of *Babesia spp.* infections in Colombian cattle, Tropenmed. Parasitol. **27** (1976) 169-181.

[8] RAMIREZ, C.G., Epidemiology of bovine babesiosis in the state of Yucatan, Mexico, Central for Tropical Veterinary Medicine, Mphil. Thesis, Scotland, U.K., 1993.

[9] VALENTIN, A., PRECIGOUT, E., L'HOSTIS, M. CARCY, B., GORENFLOT, A., SCHREVEL, J., Cellular and humoral immune response induced in cattle by vaccination with *Babesia divergens* cultured derived exoantigens correlate with protection, Infec. Immunol **61** (1993) 734-741.

[10] O'DONOGHUE, P.J., FRIEDHOFF, K.T., VIZCAINO, O.G., WEYRETER, H., The detection of IgM and IgG antibodies *Babesia bigemina* in bovine sera using semi-defined antigens in enzyme immunoassays, Vet. Parasitol. **18** (1985) 1-12.