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Validation and Field Evaluation of a Competitive Enzyme-Linked Immunosorbent Assay for Diagnosis of *Babesia bovis* Infections in Argentina

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Infections by *Babesia bovis* limit cattle production and cause important economic losses in tropical and subtropical areas around the world. Monitoring of calf sera can be used to detect unprotected cattle herds and to decide on strategic control measures, as well as for epidemiological studies. Merozoite surface antigen 2c (MSA-2c) is an immunodominant surface protein expressed in *B. bovis* merozoites and sporozoites and contains B-cell epitopes that are conserved among geographic isolates. A monoclonal antibody against recombinant MSA-2c (rMSA-2c) was previously shown to inhibit the binding of anti-*B. bovis* antibodies to a parasite B-cell epitope in a competitive enzyme-linked immunosorbent assay (cELISA) format. In the work at hand, the parameters of this cELISA were reevaluated and adjusted when necessary, and a cutoff value was determined by receiver operator characteristic (ROC) curve analysis of a total of 357 bovine sera of known reactivity, as assessed by indirect immunofluorescence assay (IFAT). The established rMSA-2c cELISA demonstrated a specificity of 98% and a sensitivity of 96.2%. An additional set of 303 field bovine sera from regions where ticks are endemic and tick-free regions of Argentina was tested by both rMSA-2c cELISA and IFAT, and the results were shown to be in very good agreement (kappa index, 0.8325). The performance shown by rMSA-2c cELISA in the detection of *B. bovis*-specific antibodies and its suitability for standardization and large-scale production, as well as the possibility of its application in most veterinary diagnostic laboratories, make the assay a powerful tool for the surveillance of herd immunity as a strategic measure for the control of bovine babesiosis.

The hemoprotozooan *Babesia bovis* severely limits cattle breeding in vast tropical and subtropical areas of the world, where its tick vectors, belonging to the family *Ixodidae*, are endemic (5, 25). Economic losses caused by the parasite are due to a decrease in meat and milk production, treatment of clinical cases, abortions, and death, as well as losses of potential production and cattle trade restrictions (5, 30, 36). *B. bovis*-infected bovines that survive the infection before 10 months of age acquire long-lasting protective immunity, while previously unexposed adult animals often succumb to the infection (28). Accordingly, the presence of *B. bovis*-specific circulating antibodies identifies previously exposed immune animals that will not develop clinical disease upon reinfection. The risk of a babesiosis outbreak can thus be assessed by analyzing the immunological status of a herd (18, 28). In Argentina, monitoring of calves for the presence of anti-*B. bovis* antibodies is periodically performed in regions of enzootic instability to decide the application of control measures, such as vaccination with live attenuated vaccines (2, 24, 25).

Merozoite surface antigen 2c (MSA-2c) is one of the five variable merozoite surface antigens (VMSAs) that are encoded in the same genomic region (17, 34). Antibodies recognizing recombinant forms of all VMSA members (MSA-1, MSA-2a$_1$, MSA-2a$_2$, MSA-2b, and MSA-2c) have been demonstrated in calves infected with a homologous Mexican strain of *B. bovis* (17, 34). MSA-2c is a species-specific, immunodominant antigen and the most conserved member of this family, showing very high amino acid sequence identity among *B. bovis* strains from Argentina, the United States, Mexico, and Australia (12, 19, 38). These features encouraged the use of MSA-2c for the development of serological tests, like an indirect enzyme-linked immunosorbent assay (ELISA) and a rapid immunochromatographic diagnostic test (6, 26). A competitive ELISA (cELISA) is an adequate serological tool for the epidemiological surveillance of the spread of bovine babesiosis, as it can be easily standardized, is less laborious and less time-consuming than the traditionally used indirect immunofluorescence assay (IFAT) (immunofluorescence antibody test), and, in addition, has the potential to display higher specificity than an indirect ELISA. In a previous work, a monoclonal antibody (MAb) against recombinant MSA-2c (rMSA-2c) was generated which showed competitive binding for this antigen with antisera of *B. bovis*-infected bovines in a cELISA format (13). In this work, the conditions and parameters of this rMSA-2c cELISA were optimized, and its cutoff, sensitivity, and specificity were established. In addition, using field samples, its performance was compared with that of the currently accepted gold standard, IFAT. The results demonstrate the applicability of rMSA-2c cELISA in the field for the diagnosis of cattle naturally infected with *B. bovis* in Argentina (22, 32).

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MATERIALS AND METHODS

Production and purification of recombinant antigen and monoclonal antibody. Recombinant expression of MSA-2c with an N-terminal histidine tag and subsequent purification by affinity chromatography in Ni-agarose was carried out as described previously (13, 38). Validation and quality assessment of expression were analyzed by Western blotting. To this end, a sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis (SDS-PAGE) was run, protein transfer was carried out, and the resulting blot was probed using either an anti-histidine antibody (GE Healthcare, Chalfont, United Kingdom) or the MAb H9P2C2 (20 μg/ml) as the primary antibody (see below). Anti-mouse alkaline phosphatase-conjugated IgG (KPL, Gaithersburg, MD; 1/1,500) was used as the secondary antibody, and immunodetection was carried out using nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) (Promega, Fitchburg, WI) as the substrate. Quantity assessment of rMSA-2c expression was carried out by comparison of band sizes with known amounts of bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO) after SDS-PAGE and Coomassie blue staining.

The H9P2C2 hybridoma cell line producing the anti-rMSA-2c MAb H9P2C2 was cultured (13). Subsequently, the culture supernatant was collected, and the MAb was purified by affinity chromatography using the Affi-Gel Protein A MAPS II Kit (Bio-Rad, Hercules, CA). After protein quantification with a BCA colorimetric kit (Pierce, Rockford, IL), the MAb was aliquoted and stored at −20°C until it was used.

Serum samples. Bovine blood samples were aseptically collected without anticoagulants from different geographical regions of Argentina as indicated below. Serum was separated by centrifugation, aliquoted, and stored at −20°C until it was used. For calculation of the cutoff value by receiver operator characteristic (ROC) analysis, a set of known-positive and known-negative sera was used. The known-positive sera ($n = 104$) originated from (i) animals from regions of endemicity in the provinces of Salta and Chaco that tested positive by diagnostic nested PCR, as reported by Figueroa et al. (15) ($n = 27$), and (ii) experimentally *B. bovis*-infected bovines after inoculation with either the BboR1A or the BboS2P strain ($n = 77$). In each case, establishment of infection was verified by observation of *B. bovis*-infected erythrocytes in Giemsa-stained smears. Known-negative sera ($n = 253$) originated from (i) animals from tick-free regions ($n = 200$), (ii) animals that had been experimentally infected with *Babesia bigemina* ($n = 28$) after confirmation of their hemoparasite-free status by IFAT and nested PCR (18), and (iii) animals from tick-free regions that were naturally infected with *Anaplasma marginale* ($n = 25$). An additional set of field serum samples ($n = 303$) was evaluated in a blind test by IFAT and rMSA-2c cELISA to estimate Cohen's kappa value. These samples were obtained from bovines from areas where ticks are enzootic in the provinces of Salta ($n = 91$) and Santiago del Estero ($n = 120$) and tick-free areas in the province of Santa Fe ($n = 92$).

IFAT. Diagnostic IFAT was carried out essentially as described by Rios et al. (32) with minor modifications. Briefly, smears were prepared using a suspension of *in vitro*-cultured *B. bovis*-parasitized erythrocytes (8% infection) diluted 1:3 in phosphate-buffered saline (PBS)-1% BSA. Sera were tested in a 1/60 dilution in PBS, and a 1/2,500 fluorescein isothiocyanate (FITC)-labeled rabbit anti-bovine IgG (Sigma-Aldrich, St. Louis, MO) was used. Microscopic detection of positive reactions was carried out using a Leitz microscope (500×) equipped for epifluorescence with a 50-W mercury vapor lamp after mounting cover slides with glycerol-PBS, pH 7.5 (1:2 [vol/vol]). Positive and negative reference sera were included in each slide. Recognition of *B. bovis* merozoites of the BboS2P (Argentina) and RAD (Mexico) strains by MAb H9P2C2 (20 μg/ml) was carried out as previously described (12).

cELISA conditions. The format of the cELISA as reported by Dominguez et al. (13) was reevaluated in order to further optimize the conditions and test parameters (data not shown). Based on this thorough assessment, only minor modifications of the serum dilution (1:5 instead of 1:20) and the composition of the blocking solution (0.5% gelatin-0.1% Tween 20-PBS instead of 0.5% gelatin-0.051% Tween 20-PBS) as indicated were found to slightly increase its performance. In brief, the rMSA-2c cELISA protocol was as follows. Immulon 2HB plates (Thermo Fisher Scientific, Waltham, MA) were incubated overnight with rMSA-2c (8.5 ng/well) in a final volume of 50 μl in 50 mM carbonate/bicarbonate buffer, pH 9.6, at 8°C. After three washings with PBS-0.1% Tween 20 (PBS-T), the plates were blocked with 200 μl of PBS-0.05% gelatin-0.1% Tween 20 (blocking buffer) for 1 h at 37°C with shaking. Then, the wells were sequentially incubated with (i) a 1/5 dilution of either control or test sera, (ii) MAb H9P2C2 (25 ng/well), and (iii) a 1/2,000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD). In all cases, dilutions were made in blocking buffer; incubations were carried out in a final volume of 50 μl for 1 h at 37°C with shaking and were followed by extensive washing with PBS-T. Finally, 50 μl of the orthophenylenediamine/hydroperoxidase (OPD/H_2O_2) colorimetric substrate were added, which contains 0.4 mg/ml OPD (Sigma-Aldrich St. Louis, MO) in citrate-phosphate buffer (0.035 M citric acid–0.067 M disodic-phosphate, pH 5.0) and 0.024% (vol/vol) H_2O_2. After 10 min at room temperature, the reactions were stopped by the addition of 50 μl sulfuric acid (2 N H_2SO_4) in water. Absorbance at 490 nm (A_{490}) was recorded in an ELISA plate reader (Multiskan EX; Thermo Fisher Scientific, Waltham, MA). All samples were tested in triplicate. Two known-positive and two known-negative control sera were included in each plate as reference controls. rMSA-2c cELISA results were expressed as inhibition percentages (PI) of the negative control and calculated with the following formula: PI = 100 − [(average A_{490} of test serum × 100)/average A_{490} of negative-control sera].

Calculations and statistical analysis. A frequency distribution graph was plotted using the MedCalc 8.1.0.0 program, and the optimal cutoff was established by ROC analysis. This allowed an accurate estimation of the diagnostic specificity and sensitivity of the established cELISA. Concordance between IFAT and cELISA results was estimated by Cohen's kappa value using the same program (1, 16). The sensitivity (ss) and specificity (sp) of rMSA-2c cELISA applied to field samples, considering IFAT the gold standard, were calculated according to the method of Martin et al. (29) from a two-entry table of cELISA and IFAT positive and negative results, using the following formula: $ss = (a/[a + c]) \times 100$ and $sp = (d/[b + d]) \times 100$, where a and d are the number of positive and negative results, respectively, by both methods; c is the number of cELISA-positive and IFAT-negative results; and b is the number of cELISA-negative and IFAT-positive results.

RESULTS AND DISCUSSION

The availability of suitable, reliable, and specific diagnostic tools is imperative for efficient epidemiological surveillance of *B. bovis* infection and the rational use of control measures. Serological assays are currently most likely to meet these requirements, as *B. bovis* infection finally leads to an asymptomatic carrier animal state in which the parasite commonly escapes direct detection by PCR, reverse line blot hybridization (RLB), or Giemsa-stained blood smears (10, 23). A number of serological methods have been established for the detection of *B. bovis*-specific antibodies, of which ELISA is considered the most advantageous for epidemiological investigations, as it offers greater sensitivity and objectivity and can be easily adapted to test large numbers of serum samples (3, 4, 6, 7, 8, 11, 20, 21, 27, 31, 35, 37). Furthermore, this format is amenable to the use of recombinant antigens, which makes it independent of the necessity to produce large amounts of parasites *in vivo* or *in vitro*, facilitates standardization, and has the potential to overcome limitations caused by cross-reactivity (22, 32). The use of rMSA-2c as a diagnostic antigen in an indirect-ELISA format has been reported, and it has proved useful in the study of the development of antibody titers after experimental infection of bovines (6). However, the indirect-ELISA formats based on

rMSA-2c suffer from low specificity and sensitivity and have not been recommended for field studies (6, 35).

A cELISA format based on an epitope located in the C-terminal region of the *B. bovis* rhoptry-associated protein 1 (CT-RAP-1) has been shown to have high specificity and sensitivity (20, 21). However, the first version of this test has been applied to only 130 field serum samples from Morocco, Bolivia, and Puerto Rico, while its validity for Argentina or any other geographic region with large cattle herds has not been assessed (20). In its second version, this cELISA features a different cutoff than the first version (21% versus 40%) and was exclusively applied to a fixed set of known-positive and -negative samples in different laboratories in order to validate the reproducibility of its results (21). It is noteworthy that the rMSA-2c and CT-RAP-1 antigens have both recently been directly compared in an indirect-ELISA format, and it was demonstrated that rMSA-2c clearly outperformed CT-RAP-1 with respect to specificity, sensitivity, and concordance with IFAT (35). This strongly suggests that a cELISA based on an MSA-2c epitope may have the potential to result in further improved test parameters.

In the present work, we evaluated the cELISA format using an MSA-2c epitope. The rMSA-2c cELISA format was applied to 357 bovine sera composed of a panel of known-positive ($n = 104$) and known-negative ($n = 253$) samples, as described in Materials and Methods, and PI values were calculated. By ROC analysis, the optimal cutoff was determined to be a PI of 29.5 (Fig. 1A). A scatter plot of the frequency distribution of the known-positive and -negative sera is shown in Fig. 1B. The area under the curve (AUC) was assessed to be 0.994, which corresponds to an excellent ability of the assay to discriminate truly infected from truly uninfected animals. At the established cutoff, the cELISA yields a salient specificity and sensitivity of 98% and 96.2%, respectively, resulting in only 5 false-positive and 4 false-negative test results. Importantly, all sera that originated from *B. bigemina*-infected ($n = 28$) or *A. marginale*-infected ($n = 25$) animals scored below the established cutoff value, and thus, the rMSA-2c cELISA did not show any cross-reactivity with antibodies against *B. bigemina* and *A. marginale*, the main *B. bovis*-coinfecting hemoparasites of cattle in Argentina.

As 100 of the 104 known-positive sera reacted positively, the rMSA-2c cELISA results provide evidence that the target epitope is strongly conserved and immunodominant. This coincides with previously reported findings of the immunodominance and high sequence conservation of MSA-2c (>90%) (12, 19, 38). The expression of the epitope recognized by MAb H9C2P2 has been demonstrated by immunofluorescence assay of merozoites of the BoS2P (Argentine) and RAD (Mexican) strains, yielding a homogeneously stained parasite surface (data not shown). The presence of an immunodominant B-cell epitope in surface-exposed antigens, such as MSA-2c, agrees well with the "smoke screen" theory, in which elicitation of a humoral response to this kind of epitope may distract the host immune system, allowing other parasite epitopes to remain unnoticed and available to play important functional roles (33). Alternatively, the possibility that the target epitope of MAb H9C2P2 may be involved in erythrocyte invasion of the parasite, as has been reported for MSA-2c (38), cannot be excluded.

The predictive positive and negative values of the rMSA-2c cELISA at a supposed prevalence of 10% were estimated as 84% and 100%, respectively. In turn, a prevalence of 90% resulted in positive and negative predictive values of 100% and 74%, respectively. These results show that the rMSA-2c cELISA is a suitable tool in various epidemiological scenarios. The rMSA-2c cELISA identified specific antibodies in bovines experimentally infected with *B. bovis* for up to 3 ($n = 10$) and even 5 ($n = 16$) months, demonstrating that circulating epitope-specific antibodies are detected for a prolonged period. This result corresponds well to the detection of anti-MSA-2c antibodies up to at least month 4 postinfection (p.i.), as has been reported for an indirect-ELISA format based on the antigen (6).

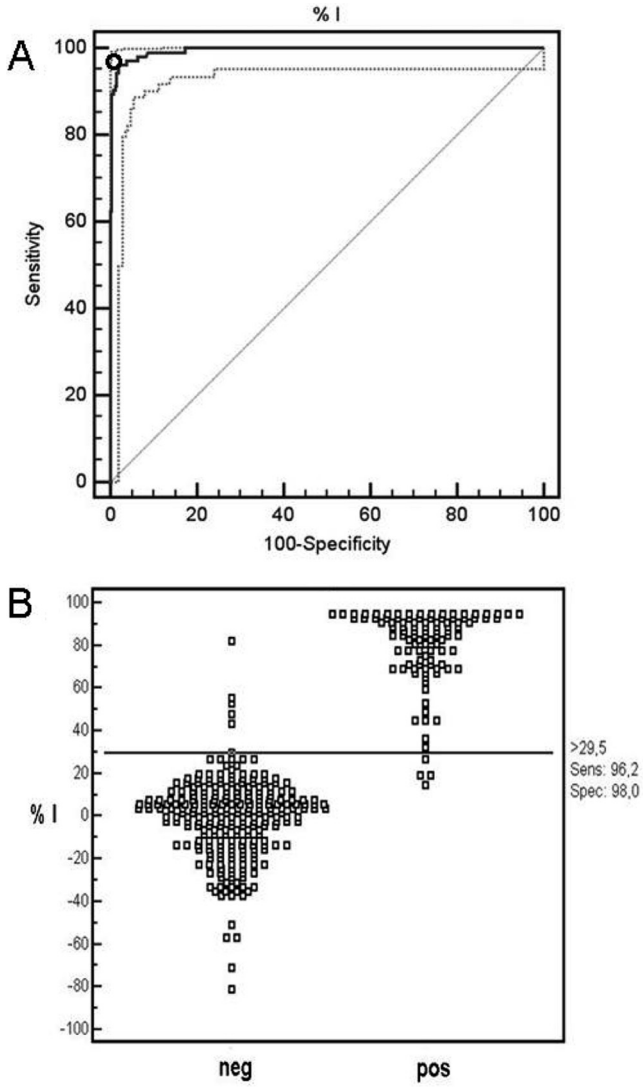

FIG 1 ROC analysis of rMSA2c cELISA. (A) Optimal cutoff point (o) providing the highest sensitivity and specificity. Solid lines indicate the fitted ROC curve (thick) and line of no discrimination (thin), and dotted lines indicate the 95% confidence interval of the fitted ROC curve. (B) Frequency distribution based on 253 known-negative (neg) and 104 known-positive (pos) *B. bovis*-infected bovine serum samples.

In a blind trial, the concordance of cELISA with IFAT was thoroughly assessed using a panel of an additional set of 303 bovine sera, collected in the field from areas of endemicity and nonendemicity. Cross-tabulation of cELISA and IFAT results (Table 1) demonstrated a high level of agreement between the two tests,

TABLE 1 Serological results of a blind test of IFAT and rMSA2c-cELISA on bovine field samples from Argentina[a]

IFAT result	No. with cELISA result:		Total
	Positive	Negative	
Positive	159	20	179
Negative	5	119	124
Total	164	139	303

[a] A total of 303 samples, 211 of which originated from areas where *B. bovis* is endemic and 92 from areas where it is not endemic.

resulting in a kappa value of 0.833 ($0.80 < \kappa \leq 1.00$; very good agreement) (1). A total of 278 of 303 sera (91.7%) tested either positive ($n = 159$) or negative ($n = 119$) by both methods, while 25 samples showed discrepant test results. In five of these samples (1.6%), the cELISA result was positive while that of IFAT was negative, and for 20 samples (6.7%), the cELISA result was negative while that of IFAT was positive. Using the formulas described by Martin et al. (29), the specificity and sensitivity of the rMSA-2c cELISA when applied to field samples were estimated at 96% and 89%, respectively, compared with IFAT (Table 1). Thus, the specificity was very similar to that based on known-infected and -uninfected sera (96% versus 98.5%). On the other hand, the sensitivity value obtained (89% versus 96.2%) was considerably lower when using samples of unknown serological status, which is consistent with the notion that IFAT produces a number of false-positive results.

Competitive ELISAs potentially have interesting advantages over indirect ELISAs. Since they are based on competition for a single B-cell epitope present in the antigen between host antibodies and a custom-designed monoclonal antibody, opposite to what happens in an indirect ELISA, the presence of *E. coli* antigens in the protein preparation does not lead to a decrease in assay specificity due to cross-reactivity reactions. In addition, since the target for detection is the MAb and not the antibodies present in the test serum samples, cELISAs can be directly applied to different host species. Accordingly, a preliminary version of the rMSA-2c cELISA presented proved useful for the detection of anti-*B. bovis* antibodies in water buffaloes, a type of cattle with increasing popularity in tick-infested regions of Argentina and Brazil (9, 14).

In summary, our results validate the developed rMSA-2c cELISA as a useful method for the detection of anti-*B. bovis* antibodies in Argentine cattle. This assay can contribute to future efforts to improve control of bovine babesiosis in the country. Studies to evaluate its applicability in other geographic regions are under way.

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