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Detection of Equine Antibody to *Babesia equi* Merozoite Proteins by a Monoclonal Antibody-Based Competitive Inhibition Enzyme-Linked Immunosorbent Assay

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A competitive inhibition enzyme-linked immunosorbent assay (CI ELISA) was developed to detect antibody to *Babesia equi*. One hundred fifty-four equine serum samples from 19 countries were tested for antibody to *B. equi* by the complement fixation test and by CI ELISA. The CI ELISA and complement fixation test results agreed in 94% (144) of the serum samples tested. The 10 discrepant serum samples were retested and analyzed for ability to immunoprecipitate in vitro translation products from *B. equi* merozoite mRNA. Five discrepant results were clearly resolved in favor of the CI ELISA, and the remaining five discrepancies were not definitively resolved.

Equine babesiosis, caused by *Babesia equi* and *Babesia caballi*, is a tick-borne hemoprotozoan disease affecting horses worldwide (6, 8, 10). Clinical disease is characterized by fever, anemia, and icterus (6, 10, 11). *B. equi*, which is diagnosed more frequently than *B. caballi*, causes a persistent infection for which drug therapy or vaccination is not available (4, 10).

The complement fixation test (CFT) (2, 3, 5) is the official United States Department of Agriculture test for detecting antibody to *B. equi* and *B. caballi*. Horses with antibody to either parasite are restricted from importation into the United States (4). Three problems with the CFT are that (i) sera with anticomplement activity are not testable by the CFT; (ii) sera which react with CFT control erythrocyte antigen cannot be evaluated by the CFT; and (iii) sera containing specific immunoglobulin G(T) [IgG(T)] antibody may yield false-negative results because IgG(T) does not fix complement by the classical pathway (9). In this report, we describe a competitive inhibition enzyme-linked immunosorbent assay (CI ELISA) (1) for detection of antibody to *B. equi*. The formatting of the CI ELISA overcomes the above three problems. Furthermore, data presented indicate a high concordance between the CI ELISA and CFT in detecting antibody to *B. equi* and demonstrate that 5 of 10 discrepant results are resolved in favor of the CI ELISA.

One hundred fifty-four equine serum samples from 19 countries in North America (6 samples), South America (113 samples), Europe (28 samples), and the Middle East (7 samples) were obtained from the National Veterinary Services Laboratory, USDA-APHIS, Ames, Iowa. Each serum was tested for antibody to *B. equi* by the CFT as described previously (2, 3). Three anticomplement serum samples and one serum sample reactive with the CFT erythrocyte antigen control were also obtained from the National Veterinary Services Laboratory. H5 serum is from a horse experimentally infected with stabilate of a Florida *B. equi* isolate (7), and SN76N8401 is a CFT-negative control serum obtained from the National Veterinary Services Laboratory. One hundred and four equine serum samples submitted to Washington State University for equine infectious anemia testing were used as control sera.

The CI ELISA used an IgG1 monoclonal antibody (MAb) (36/133.97) which reacts with a protein epitope on the surface of *B. equi* merozoites (7). The CI ELISA was performed as described previously (7). Briefly, 0.8 μg of *B. equi* (Florida isolate) merozoite proteins in phosphate-buffered saline (PBS) with 20 mM $MgCl_2$ were coated on individual wells of flat-bottomed plates (Immulon 2; Dynatech Laboratories, Chantilly, Va.). All incubations were done at room temperature. Coated wells were incubated for 16 h and blocked for 2 h with buffer A (PBS, 0.2% Tween 20, 20% milk), and equine serum samples diluted in buffer A were added. After a 30-min incubation, 0.125 μg of MAb 36/133.97 in buffer A was added. A 1-h incubation was followed by washing three times with buffer B (PBS, 0.2% Tween 20). Biotinylated equine anti-murine IgG in buffer A was added and incubated for 30 min, and the wells were washed three times with buffer B. Addition of avidin-conjugated alkaline phosphatase in buffer B (Vector Laboratories, Burlingame, Calif.) was followed by a 30-min incubation. Wells were washed three times with buffer B, and 100 μl of 1.0-g/μl *p*-nitrophenyl phosphate in 100 mM $NaHCO_3$ (pH 9.5)–10 mM $MgCl_2$ (Sigma Chemical Co., St. Louis, Mo.) was added to each well. After a 30-min incubation, reactions were stopped with 50 μl of 0.2 M EDTA and the optical density (OD) was read at 405 nm on an MR-5000 Dynatech ELISA plate reader.

Serum samples were tested by CI ELISA in groups of 5 to 15 per day without knowledge of the CFT results. Duplicates of each serum sample were tested at dilutions of 1:2 and 1:10. Five to 10 different control serum samples were tested at a 1:2 dilution in duplicate each day. A mean and standard deviation of the OD for the control serum samples was calculated following each test day. A serum sample was considered positive for antibody to *B. equi* if it inhibited the binding of MAb 36/133.97 such that the mean duplicate OD value for that dilution of test serum was at least 3 standard

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TABLE 1. Sample data from CI ELISA and CFT[a]

Serum	CI ELISA OD[b]		CFT titer[c]	
	1:2	1:10	*B. equi*	*B. caballi*
224	0.381, 0.389	0.382, 0.441	1:5	1:40
225	0.471, 0.486	0.732, 0.721	1:5	Negative
226	1.489, 1.470	1.717, 1.672	Negative	1:5
227	1.337, 1.369	1.146, 1.619	Negative	1:40
228	0.217, 0.156	0.229, 0.236	1:40	1:20
229	0.301, 0.298	0.336, 0.363	1:5	1:40
230	1.374, 1.362	1.560, 1.528	Negative	1:5
231	0.356, 0.356	0.439, 0.426	1:40	1:5
232	0.219, 0.254	0.334, 0.313	1:5	Negative
233	0.246, 0.260	0.351, 0.389	1:5	1:10
234	0.521, 0.486	0.761, 0.736	1:10	Negative
235	0.189, 0.198	0.314, 0.383	1:40	1:10
236	1.380, 1.351	1.535, 1.384	Negative	1:40
237	0.347, 0.277	0.465, 0.345	1:5	1:40
238	0.314, 0.308	0.461, 0.470	*	*
H5	0.293, 0.303	ND[d]	Negative	Negative

[a] CI ELISA and CFT were performed as described in the text.
[b] Serum samples reducing mean of duplicate OD values to less than 3 SD below mean of control horses (<1.17) were considered positive. OD for control horses at a 1:2 dilution on this test day = 1.47 ± 0.10 (SD) (n = 9). OD for isotype control MAb = 0.145, 0.142.
[c] CFT titers are presented at the highest dilution yielding a positive result. *, serum sample which reacted with CFT erythrocyte control antigen.
[d] ND, not done.

deviations below the mean OD value of the control serum samples for that test day. Sample data from the CI ELISA and CFT for a test day are given in Table 1.

Of the 154 serum samples testable by CFT, 126 were both CFT and CI ELISA positive [CFT(+) CI ELISA(+)] for antibody to *B. equi*. Eighteen serum samples were negative in both tests, and CFT and CI ELISA results differed in the remaining 10 serum samples. Sixteen of the 18 serum samples negative by both the CFT and CI ELISA for antibody to *B. equi* were CFT(+) for *B. caballi*.

The 10 serum samples in which the CI ELISA and CFT results differed were retested in both assays and analyzed by immunoprecipitation. CI ELISA, CFT, and immunoprecipitation results for the 10 discrepant serum samples are summarized in Table 2. Upon retesting, four of the CFT(+) CI ELISA(−) serum samples had decreased CFT titers. Two of these serum samples which were originally CFT(+) were

TABLE 2. CI ELISA, CFT, and immunoprecipitation results of sera differing on initial testing[a]

Serum	CFT		CI ELISA		Immunoprecipitation
	Orignial	Repeat	Original	Repeat	
8	Negative	Negative	Positive	Positive	Positive
17	Negative	Negative	Positive	Positive	Positive
113	Negative	Negative	Positive	Positive	Positive
175	Negative	Negative	Positive	Positive	Positive
H5	Negative	Negative	Positive	Positive	Positive
18	1:10	Trace	Negative	Negative	Inconclusive
22	1:10	1:5	Negative	Negative	Inconclusive
126	1:5	Negative	Negative	Negative	Inconclusive
167	1:40	1:40	Negative	Negative	Inconclusive
171	1:5	Negative	Negative	Negative	Inconclusive

[a] CI ELISA, CFT, and immunoprecipitation results were determined as described in the text. Serum samples which differed in the CI ELISA and CFT at original testing were retested by CI ELISA, CFT, and immunoprecipitation. CFT titers are presented as the highest dilution yielding a positive result.

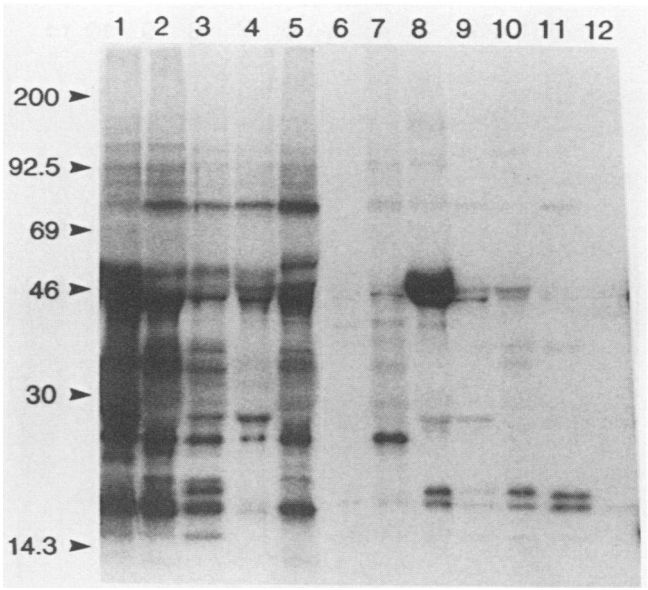

FIG. 1. Immunoprecipitation of [^{35}S]methionine-labeled in vitro translation products with 1:10 dilutions of sera yielding discrepant results in the CFT and CI ELISA. Lanes 1 to 5 represent sera which tested CFT(−) and CI ELISA(+), H5, 8, 17, 113, and 175, respectively. Lane 6, SN76N8401, CFT(−) control serum. Lanes 7 to 11 represent sera which tested CFT(+) and CI ELISA(−), 18, 22, 126, 167, and 171, respectively. Lane 12, serum 236, CFT(−) and CI ELISA(−). Numbers on left show size in kilodaltons.

negative in the repeat CFT. The decreasing CFT titers of these serum samples may reflect, at least in part, multiple freeze-thaw cycles.

Serum samples at a 1:10 dilution were evaluated for their ability to immunoprecipitate ^{35}S-labeled in vitro translation products of *B. equi* merozoite mRNA as described previously (7). Figure 1 displays immunoprecipitation data from the five serum samples which were CFT(−) CI ELISA(+), the five serum samples which were CFT(+) CI ELISA(−), and two serum samples which were negative in both tests. Five serum samples which were CI ELISA(+) CFT(−) clearly immunoprecipitated multiple *B. equi* proteins that comigrated with proteins immunoprecipitated by positive control serum H5 (7) (Fig. 1, lanes 1 to 5). Interestingly, serum H5, from a horse experimentally infected with *B. equi* and used as positive reference serum in the CI ELISA and immunoprecipitations, was one of the serum samples consistently negative by the CFT.

While *B. equi*-specific IgG(T) antibody was not measured in the five CI ELISA(+) CFT(−) serum samples, IgG(T) remains a likely explanation for the false-negative CFT results. It has been previously shown that IgG(T) specific for equine infectious anemia virus inhibits the CFT for detecting antibody to equine infectious anemia virus because IgG(T) does not fix complement by the classical pathway (9).

Immunoprecipitation results with the five serum samples which were CI ELISA(−) CFT(+) were inconclusive (Fig. 1, lanes 7 to 11). Fewer proteins were immunoprecipitated by these serum samples than by H5 serum. However, proteins not present in the negative control serum samples (Fig. 1, lanes 6 and 12) were immunoprecipitated by the CI ELISA(−) CFT(+) serum samples. The results obtained from the five CI ELISA(−) CFT(+) serum samples may represent false-positive CFT results; however, the immuno-

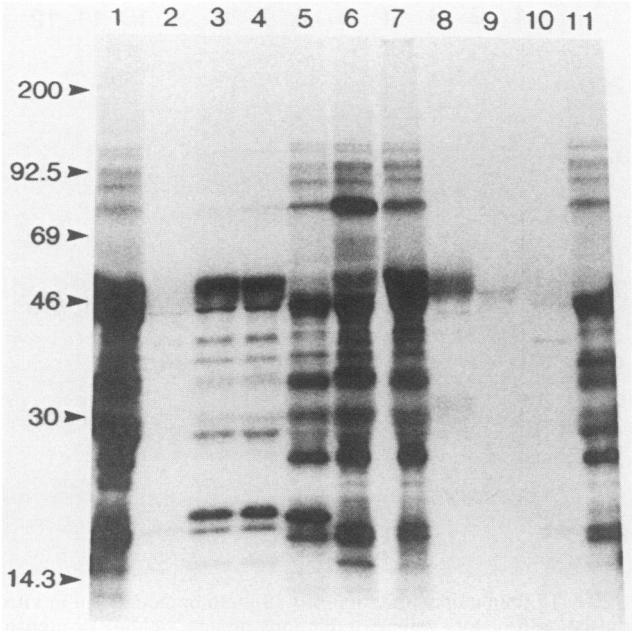

FIG. 2. Immunoprecipitation of [^{35}S]methionine-labeled in vitro translation products with 1:10 dilutions of equine sera with anticomplement activity or reactivity with CFT erythrocyte control antigen. Lanes: 1, serum H5; 2, serum 2, CFT(−) CI ELISA(−); 3 to 6, sera 215, 216, 140, and 146, respectively, CFT(+) CI ELISA(+); 7, serum 213, anticomplement, CI ELISA(+); 8 and 9, sera 240 and 245, respectively, anticomplement, CI ELISA(−); 10, SN76N8401, CFT(−) control serum; 11, serum 238, reactive with CFT erythrocyte control, CI ELISA(+). Numbers on left show size in kilodaltons.

precipitation results show reactivity with *B. equi* merozoite proteins (Fig. 1, lanes 7 to 11). Three of these serum samples (22, 126, 171) also had CFT titers to *B. caballi*, and the immunoprecipitation results with these serum samples may reflect serological cross-reactivity between *B. equi* and *B. caballi* merozoite proteins (3).

If the five CI ELISA(−) CFT(+) serum samples are true positives, there are at least three possible explanations: (i) a genetic inability of those horses to produce antibody to the epitope defined by MAb 36/133.97; (ii) absence of the epitope on *B. equi* isolates which infected those horses; and (iii) insufficient CI ELISA sensitivity. The third explanation does not seem likely since 32 of the CFT(+) CI ELISA(+) serum samples had CFT titers of only 1:5.

Three anticomplement serum samples and one serum sample which reacted with the CFT erythrocyte control antigen were tested by the CI ELISA and immunoprecipitation (Fig. 2). Immunoprecipitations with these serum samples were compared with immunoprecipitations with H5 serum, four randomly selected CI ELISA(+) CFT(+) serum samples, and two serum samples negative by both tests (Fig. 2). One of three anticomplement serum samples and the serum sample reactive with CFT erythrocyte control antigen were positive by both CI ELISA and immunoprecipitation (Fig. 2, lanes 7 and 11). Two anticomplement serum samples were CI ELISA(−), and one of these serum samples was clearly negative by immunoprecipitation (Fig. 2, lane 9). Lane 8 of Fig. 2 represents immunoprecipitation with the additional anticomplement serum which was CI ELISA(−). Data obtained from this immunoprecipitation were inconclusive. The proteins in lane 8 not found in the control serum samples (lanes 2 and 10) may signify cross-reactivity between antigens of *B. equi* and *B. caballi* (3). Also, this serum may represent a false CI ELISA(−).

The collective data of this report indicate a high (94%) concordance between the CI ELISA and CFT for detecting antibody to *B. equi*. Since 16 of the 18 serum samples in this study which were CI ELISA(−) CFT(−) for antibody to *B. equi* were CFT(+) for antibody to *B. caballi*, the CI ELISA is clearly specific for *B. equi*. Furthermore, the formatting of the CI ELISA overcomes the aforementioned limitations associated with the CFT, and as the data clearly indicate, the geographic conservation of the epitope recognized by MAb 36/133.97 allows reliable use of the CI ELISA to detect *B. equi* antibody in sera from horses worldwide.

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