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Serum Antibodies from a Subset of Horses Positive for *Babesia caballi* by Competitive Enzyme-Linked Immunosorbent Assay Demonstrate a Protein Recognition Pattern That Is Not Consistent with Infection

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Tick-borne pathogens that cause persistent infection are of major concern to the livestock industry because of transmission risk from persistently infected animals and the potential economic losses they pose. The recent reemergence of *Theileria equi* in the United States prompted a widespread national survey resulting in identification of limited distribution of equine piroplasmosis (EP) in the U.S. horse population. This program identified *Babesia caballi*-seropositive horses using rhoptry-associated protein 1 (RAP-1)–competitive enzyme-linked immunosorbent assay (cELISA), despite *B. caballi* being considered nonendemic on the U.S. mainland. The purpose of the present study was to evaluate the suitability of RAP-1–cELISA as a single serological test to determine the infection status of *B. caballi* in U.S. horses. Immunoblotting indicated that sera from U.S. horses reacted with *B. caballi* lysate and purified *B. caballi* RAP-1 protein. Antibody reactivity to *B. caballi* lysate was exclusively directed against a single ~50-kDa band corresponding to a native *B. caballi* RAP-1 protein. In contrast, sera from experimentally and naturally infected horses from regions where *B. caballi* is endemic bound multiple proteins ranging from 30 to 50 kDa. Dilutions of sera from U.S. horses positive by cELISA revealed low levels of antibodies, while sera from horses experimentally infected with *B. caballi* and from areas where *B. caballi* is endemic had comparatively high antibody levels. Finally, blood transfer from seropositive U.S. horses into naive horses demonstrated no evidence of *B. caballi* transmission, confirming that antibody reactivity in cELISA-positive U.S. horses was not consistent with infection. Therefore, we conclude that a combination of cELISA and immunoblotting is required for the accurate serodiagnosis of *B. caballi*.

Central to biosecurity strategies is preventing the spread of infectious animal diseases by strictly regulating movement of infected animals (1, 2). The introduction of infected animals into a naive population increases the risk of transmitting pathogens that could cause significant economic losses to the livestock industry (2, 3). Importation of animals and animal by-products from areas with a high prevalence of infectious agents is tightly regulated, and imported animals are screened and quarantined at all entry points for further testing and evaluation (4). Both direct and indirect diagnostic tests are performed to determine infection status, and the use of these tests to prohibit the entrance of infected animals into areas where pathogens are nonendemic has been critical in preventing disease outbreaks (5, 6).

Tick-borne pathogens that result in persistent infections represent significant disease control challenges (7, 8). Among these are the apicomplexan hemoprotozoan parasites *Babesia caballi* and *Theileria equi* that both cause equine piroplasmosis (EP) (9). The disease is characterized by fever, anemia, icterus, hemoglobinuria, and, in some cases, death (4, 9). Animals that recover from acute infection become persistently infected (7, 10, 11). These persistently infected animals pose a threat to naive populations, as they serve as reservoirs for iatrogenic or tick-borne transmission (10, 11). Due to country-imposed regulations, EP has resulted in a reduction in international trade and, subsequently, equestrian sporting events. Both *B. caballi* and *T. equi* are widespread in tropical and subtropical regions, where approximately 90% of the world's horse population is located (9), and few countries in these regions can be considered EP free, as reported by the World Organization for Animal Health (OIE). The United States is still considered EP free and has been reporting to the OIE on individual outbreaks identified since 2008 (2, 12). EP is designated a foreign animal disease in the United States, and any case identified initiates an immediate regulatory response, including quarantine and control measures.

Identifying and quarantining infected domestic horses and refusing entry of infected horses presented for importation are the methodologies being used to prevent further EP outbreaks in the United States. Several serological tests are used to identify horses infected with *B. caballi* and *T. equi*. However, they have various

Received 29 July 2013 Returned for modification 23 August 2013
Accepted 11 September 2013
Published ahead of print 18 September 2013
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doi:10.1128/CVI.00479-13

degrees of specificity and sensitivity (5, 6, 13). The complement fixation test (CFT) was the official regulatory test in the United States until it was determined to yield an unacceptable number of false-negative results due to the inability of equine IgG subclass 5 to fix complement (14, 15). The failure of the CFT to detect all persistently infected horses with *T. equi* may have resulted in the reintroduction of this pathogen, leading to the recent outbreaks in the United States (2, 8, 12). In 2005, the CFT was replaced with the competitive enzyme-linked immunosorbent assay (cELISA) as the official regulatory test for EP.

In response to the recent EP outbreaks in the United States, a national *B. caballi* and *T. equi* surveillance effort emerged through state- and industry-driven movement testing and widespread testing for epidemiologic and export purposes. Official testing is conducted by approved state and university laboratories as well at the USDA-APHIS-National Veterinary Services Laboratories, Ames, IA. Sera from ~220,000 U.S. horses were tested by (rhoptry-associated protein 1) RAP-1–cELISA, and 145 of these horses tested positive for antibodies against *B. caballi*. None of these seropositive horses had a history of clinical disease or epidemiological history of possible exposure. Moreover, none had been outside the United States or had a history that would suggest iatrogenic transmission. The current study was conducted in an effort to evaluate the suitability of RAP-1–cELISA as a single serological test to determine the infection status of *B. caballi* in native U.S. horses. A combination of immunoblot analysis, determination of antibody titers using cELISA, and blood transmission studies was performed. It was demonstrated that antibody reactivity to immunoblots by cELISA-positive U.S. horse sera was not consistent with *B. caballi* infection.

MATERIALS AND METHODS

Antigen preparation. Partially engorged adult *Dermacentor nitens* ticks were obtained from a horse that was naturally infected with *B. caballi* in Puerto Rico, where both the parasite and tick vector are endemic. *D. nitens* ticks infected with *B. caballi* were allowed to feed on a naive horse for transmission (16). Blood samples from the recipient horse were collected into EDTA tubes at peak parasitemia to establish parasitized erythrocyte cell cultures. Erythrocytes were prepared for culture by being washed in phosphate-buffered saline (PBS) with 0.05% EDTA to deplete leukocytes, and then 100 μl of washed packed cells containing approximately 3% parasitized erythrocytes was added to 100 μl of normal equine erythrocytes in 1 ml of HL-1 medium (Lonza Group) buffered with 20 mM HEPES, containing 20% normal horse serum (HyClone), 4 mM L-glutamine, 200 units of penicillin, and 58 mM streptomycin in 24-well tissue culture plates. Cultures were maintained in a microaerophilic stationary phase at 37°C in 93% nitrogen, 5% carbon dioxide, and 2% oxygen. To generate *B. caballi* antigen for immunoblot assays, cultures were expanded to a 20% parasitemia. Infected erythrocytes were lysed using RBC lysis solution (Qiagen), and the parasites were harvested by centrifugation at 2,800 × *g* for 15 min. Parasites were suspended in protease inhibitor buffer (50 mM Tris [pH 8], 5 mM EDTA, 5 mM iodoacetamide, 1% Nonidet P-40) with freshly added protease inhibitor mixture (ProteCEASETM; G-Biosciences), and the protein concentration was determined by bicinchoninic acid protein assay (Thermo Scientific). *B. caballi* antigen was stored at −20°C. Erythrocytes from an uninfected horse were processed in a similar manner and were used as a negative control. Finally, *B. caballi* RAP-1 protein was immunoaffinity purified using a monoclonal antibody as previously described (17). The purified RAP-1 protein was used for immunoblotting to determine horse antibody specificity.

Sarcocystis neurona (strain SN3) and *Neospora hughesi* (Oregon isolate) were propagated by serial passage in monolayers of bovine turbinate cells to generate antigens for immunoblots. After parasite growth disrupted the host cell monolayer, the culture medium containing cell debris and parasites was passed sequentially through 20-, 23-, and 25-gauge needles and filtered through a 3.0-μm-pore-size Nuclepore membrane (Whatman) to remove host cell debris. The harvested parasites were counted with a hemocytometer, washed with PBS, suspended in protease inhibitor buffer, and stored at −20°C until used.

Serum samples. *Babesia caballi* RAP-1–cELISA-positive and -negative equine sera representing geographically distinct regions were used in this study and included (i) 43 samples from the United States, (ii) 36 samples from Europa, (iii) 87 samples from Latin America, where EP is endemic, and (iv) 59 samples from a closed herd with no *B. caballi* exposure, located at Washington State University/USDA-ARS-Animal Disease Research Unit.

To determine if exposure to related apicomplexan parasites elicited antibodies that cross-react with *B. caballi*, sera from 10 horses naturally exposed to *N. hughesi*, 10 horses naturally exposed to *S. neurona*, one horse naturally exposed to *Besnoitia bennetti*, and three donkeys infected with *B. bennetti* were included in this study. Horses infected with *N. hughesi* and *S. neurona* were identified by neurological signs and by an indirect fluorescent antibody test (IFAT) (18, 19). The *N. hughesi*-positive horses with neurologic signs had antibody titers ranging from 1:320 to 1:40,960, and *S. neurona*-positive horses with neurologic signs had antibody titers ranging from 1:320 to 1:5,120. One horse exposed to *B. bennetti* and three donkeys with a history of clinical besnoitiosis had *Besnoitia*-specific IFAT antibody titers ranging from 1:200 to 1:3,200 (20).

Sera collected at multiple time points throughout the course of infection from five horses experimentally infected with *B. caballi* were used as positive controls.

Serology. The *B. caballi* RAP-1–cELISA was performed to detect equine antibodies against *B. caballi* using sera at dilutions of 1:2, 1:4, 1:8, and 1:16 (17). The cELISA uses a recombinant RAP-1 to detect specific antibodies against *B. caballi* (VMRD Inc.). To identify immunoreactivity against *B. caballi*, *N. hughesi*, or *S. neurona* proteins, immunoblotting was performed using these same sera. Approximately 100 ng of lysates from *B. caballi*, *N. hughesi*, or *S. neurona* was loaded into SDS-PAGE gels and separated by electrophoresis. The proteins were transferred to nitrocellulose membranes and were blocked with 10% nonfat dry milk in PBS with 0.2% Tween 20. The membrane was cut into strips and incubated in sera diluted 1:5 in 10% nonfat dry milk in PBS with 0.2% Tween 20. The membrane strips were washed and incubated with a 1:5,000 dilution of horseradish peroxidase-labeled goat anti-horse IgG (KPL) in 10% nonfat dry milk in PBS plus 0.2% Tween 20. After the washing step, immunoreactivity was detected by using ECL chemiluminescent substrate solution (GE Healthcare) and a ChemiDoc Image analyzer (Bio-Rad).

Whole-blood transmission studies. From a subset of five U.S. RAP-1–cELISA-positive horses, 120 ml of whole blood was collected in citrate phosphate dextrose anticoagulant (Fenwal Inc.) and shipped on ice overnight to the USDA-ARS-ADRU facility, Pullman, WA, for subsequent intravenous inoculation into five individual naive horses. All recipient horses were monitored for clinical signs of piroplasmosis daily for approximately 90 days post-blood transfer. Blood samples were collected weekly to determine seroconversion status by *B. caballi* RAP-1–cELISA and immunoblotting against *B. caballi* lysates. All experiments involving animals were approved by the respective University of Idaho and Washington State University Institutional Animal Care and Use Committees, in accordance with institutional guidelines based on the U.S. National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

RESULTS

Serum antibody reactivity by immunoblot assay. Sera from 81.8% (18/22) of the cELISA-positive U.S. horses reacted exclusively with an ~50-kDa protein using *B. caballi* lysate (Table 1). Interestingly, sera from U.S. horses negative by RAP-1–cELISA recognized a single band pattern at ~50 kDa by the immunoblot assay (Fig. 1). Overall, cELISA and immunoblot results for the

TABLE 1 Results of immunoblot analysis of sera from horses with either positive or no reactivity to *Babesia caballi* by RAP-1–cELISA

Horse	No. of sera						
	cELISA positive			cELISA negative			
	No bands	Single band	Multiple bands	No bands	Single band	Multiple bands	Total
WSU/ADRU[a]	0	1	0	55	3	0	59
United States	4	17	0	11	11	0	43
Europe	1	7	4	21	3	0	36
Latin America	1	3	55	27	1	0	87
Total	6	28	59	114	18	0	225

[a] A closed herd with no *B. caballi* exposure at Washington State University/USDA-ARS-Animal Disease Research Unit.

U.S. horses were discordant, with 18.2% (4/22) of the cELISA-positive horses being immunoblot assay negative, and 17.5% (14/80) of the cELISA-negative horses having a single banding pattern by immunoblot assay (Table 1). Importantly, no sera from U.S. horses, regardless of cELISA status, recognized a multiple banding pattern by immunoblot assay (Table 1).

In contrast, 100% (5/5) of sera taken at multiple time points following infection of horses experimentally infected with *B. caballi* (Fig. 2) and sera from 83% (59/71) of the cELISA-positive non-U.S. horses recognized three predominant bands ranging from ~30 to ~50 kDa on immunoblot assay (Table 1). The cELISA and immunoblot results were in agreement for the non-U.S. horses, with only 2.8% (2/71) of the cELISA-positive horses having no reactivity by immunoblot assay, and only 7.7% (4/52) of the cELISA-negative horses having a single band. Importantly, sera from the experimentally infected horses collected at several time points during early and late infection recognized the same multiple banding pattern (Fig. 2). Thus, the absence of a multiple banding pattern in the seropositive U.S. horses was not likely due to a narrow antibody response associated with early infection.

Overall, there were 14 U.S. and 4 non-U.S. horses that were RAP-1–cELISA negative and reacted with a single *B. caballi* protein by immunoblot assay (Table 1). Each of these samples (18/18), regardless of geographical region, recognized a single banding pattern by immunoblot assay. No sera from cELISA-negative horses, regardless of geographical region, recognized a multiple banding pattern on immunoblot assay (Table 1).

Serum antibody reactivity to native *Babesia caballi* RAP-1. Native RAP-1 protein was immunopurified from *B. caballi* lysates and used for immunoblot assays to confirm that antibodies from U.S. horse sera that reacted against the single ~50-kDa protein were, in fact, directed against *B. caballi* RAP-1. Regardless of cELISA status, all U.S. horse samples recognizing a single band exhibited specific reactivity to the native *B. caballi* RAP-1 protein, as did positive-control serum from horses experimentally infected with *B. caballi* (Fig. 3). Interestingly, samples from some cELISA-negative U.S. horses also reacted with *B. caballi* immunopurified native protein (Fig. 3).

Effect of serum dilution on cELISA reactivity. Sera from cELISA-positive U.S. horses, experimentally infected horses, and cELISA-positive non-U.S. horses were serially diluted and tested for reactivity by *B. caballi* cELISA. At a dilution of 1:4, only one of 22 U.S. horse samples tested remained positive by cELISA (i.e., demonstrated a percent inhibition above 40%). In contrast, sera yielding multiple banding patterns from horses experimentally infected with *B. caballi* and from non-U.S. horses remained cELISA positive even when diluted to 1:16 (Fig. 4).

Serum antibody reactivity against other apicomplexan parasites. In an attempt to determine if cross-reactivity could explain the positive cELISA and single-banding-pattern immunoblot results, sera from equids seropositive for *N. hughesi*, *S. neurona*, or *B. bennetti* were tested in the cELISA but failed to react against *B. caballi* RAP-1 (data not shown). Sera from *B. caballi* cELISA-positive U.S. horses were also tested by immunoblot assays against *N. hughesi* and *S. neurona* lysates, and no specific reactivity was observed (Fig. 5). In addition, no specific reactivity at ~50 kDa was observed when *N. hughesi*- or *S. neurona*-positive sera were immunoblotted against *B. caballi* lysate (Fig. 5).

Blood transfusion. To determine if the U.S. RAP-1–cELISA-positive horses presented a risk for transmission of *B. caballi* to naive horses, blood transfer from a subset of U.S. horses into naive seronegative recipient horses was performed. None of the five naive recipient horses transfused with whole blood from cELISA-positive U.S. horses developed clinical signs of *B. caballi* infection. Moreover, no recipient seroconverted, as determined by *B. caballi* cELISA and immunoblot assay.

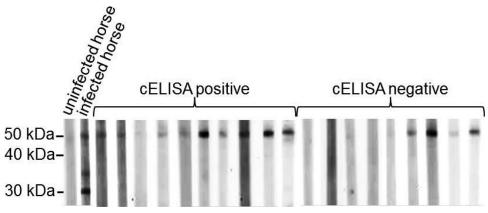

FIG 1 Immunoreactivity of sera from United States horses against *B. caballi* proteins in lysates from infected equine erythrocyte culture.

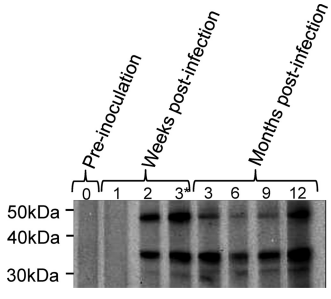

FIG 2 Detection of antibodies by immunoblot assay from a representative horse experimentally infected with *B. caballi* at multiple time points during infection. *, acute infection.

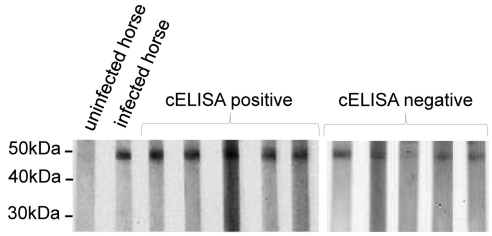

FIG 3 Immunoblot analysis of U.S. horse sera with antibody reactivity to immunopurified *B. caballi* RAP-1 native protein.

DISCUSSION

Restricting animal movement has been a critical strategy in preventing the spread of infectious diseases (9, 21, 22). The restriction of movement is based on the identification of infected animals by direct and indirect methods (5, 6). However, several diagnostic assays have failed to detect all infected animals due to a lack of sensitivity in the diagnostic tests, pathogen mutation or recombination, or deficiencies in host immunity (6, 23). A combination of these possibilities could explain the recent outbreaks of EP associated with *T. equi* in the United States. Importantly, the presence of competent ticks and infected horses in the United States has increased concerns regarding the prevalence of agents that cause EP by either *T. equi* or *B. caballi* (12).

With the intent of maintaining an infection/disease-free status, serological surveys in regions where *B. caballi* is nonendemic use a test with high specificity to identify serologically positive animals. However, as expected for any diagnostic assay that has high specificity, false-positive results will occur. The positive predictive value will decrease in areas where *B. caballi* is nonendemic or during low infection activity periods (http://www.cdc.gov/flu/pdf/professionals/diagnosis/clinician_guidance_ridt.pdf). A previous study demonstrated that 8.3% of tested sera from areas where *B. caballi* is endemic were positive by RAP-1–cELISA and negative by CFT and IFAT (17). For these results, the absence of test concordance was due in part to the lack of CFT sensitivity for persistent infections and the need to dilute sera for the IFAT to enhance specificity; therefore, a final conclusion was not provided concerning the true infection status of the 8.3% of results that were cELISA positive. Interestingly, a RAP-1–cELISA survey of ~220,000 U.S. horses detected 145 *B. caballi*-positive sera, although there has not been a report of *B. caballi* detection in the United States since its eradication from Florida in the late 1980s. The results of our two-tiered serological testing approach indicate that the U.S. horses identified as serologically positive for *B. caballi* by RAP-1–cELISA were not infected. The immunoblot assay demonstrated that sera from these U.S. horses recognized only a single band corresponding to *B. caballi* RAP-1 protein at ~50 kDa, while antibodies from horses either experimentally infected with *B. caballi* via tick transmission or blood transfer from areas where *B. caballi* is endemic consistently recognized a multiple banding pattern, including the protein at ~50 kDa. A previous report suggested that these proteins are immunodominant and consistently detected by serum antibodies from horses infected with *B. caballi* (24).

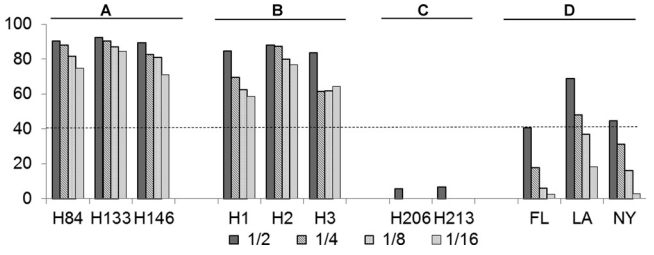

FIG 4 Effect of antibody serial dilution on *B. caballi* cELISA reactivity. Sera: horses experimentally infected with *B. caballi* (A); horses from regions where *B. caballi* is endemic (B); uninfected horses (C); horses from the United States (D). Percent inhibition of ≥40% is considered seropositive.

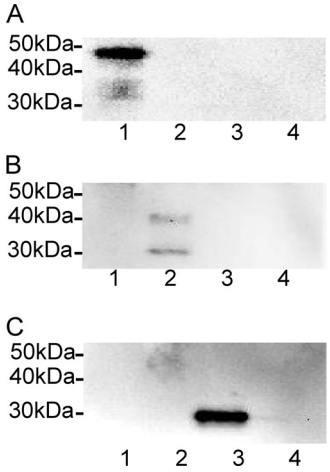

FIG 5 Immunoblot analysis of sera from horses infected with *Babesia caballi*, *Neospora hughesi*, or *Sarcocystis neurona*. Representative sera from a *B. caballi* cELISA-positive U.S. horse (A), an *N. hughesi*-infected horse (B), and an *S. neurona*-infected horse (C). Antigens: *B. caballi* lysate (1), *N. hughesi* lysate (2), *S. neurona* lysate (3), and control equine kidney cell lysate (4).

In the current study, attempts were made to determine if the sera from U.S. horses reacting against an epitope on RAP-1 by cELISA represented antibody cross-reactivity to closely related apicomplexan pathogens. Antibody cross-reactivity in serological tests can be a common occurrence linked with either vaccine or natural exposure to the test antigen or other antigens with similar binding properties (25, 26). In some cases, cross-reactivity can be associated with shared epitopes between closely related pathogens (27). There are several other apicomplexan pathogens that can infect equids (28, 29), and these are known to express protein orthologs hypothesized to mediate adhesion to host cells during pathogen invasion (30–32). Some of these proteins have been utilized to develop diagnostic assays to determine infection status (17). Because *S. neurona*, *N. hughesi*, and *B. bennetti* are apicomplexan pathogens that infect equids and have rhoptries as part of their apical complex (20, 28, 29), it was of interest to determine if cross-reactive antibodies against these organisms might explain the RAP-1–cELISA-positive and immunoblot single-banding-pattern results for U.S. horses (33, 34). Despite the failure to demonstrate cross-reactive antibodies against the other apicomplexans in this study, it is still possible that serum antibodies elicited against other organisms by U.S. horses may result in cross-reactivity against the *B. caballi* RAP-1 protein.

Reactivity directed exclusively against a single *B. caballi* protein in U.S. horses might also be explained by early infection prior to development of a more broad humoral response. In this study, sera from experimentally infected horses strongly indicated that

horses infected with *B. caballi* rapidly produce antibodies that recognize multiple *B. caballi* proteins as early as the second week of infection. A previous study also demonstrated that antibodies from horses experimentally infected with *B. caballi* reacted with multiple bands on an immunoblot assay. However, sera from the first week of infection recognized only a single band on the immunoblot (24), while, in our study, sera from the first week of infection showed no immunoblot reactivity to *B. caballi* proteins. Potential reasons for the inconsistency in the early infection between our results and the previous report include differences in the concentration of the proteins loaded and/or the dilution of serum antibodies. Interestingly, cELISA of diluted sera demonstrated a consistently high antibody titer from horses experimentally infected with *B. caballi* or naturally infected from regions where *B. caballi* is endemic, likely due to the persistent replication of parasites and carrier state that is common with piroplasmosis. In contrast, serum dilution indicated that the U.S. horses had lower levels of antibodies, as measured by the *B. caballi* cELISA, suggesting that these U.S. horses are most likely not infected with *B. caballi*. The results obtained in this study suggest that a modification in the serum dilution for the cELISA should be considered to allow for improved accuracy of the diagnostic test. Consequently, this would prevent unnecessary economic losses associated with quarantine, treatment, euthanasia, or export of false-positive cELISA horses.

Divergent *B. caballi* isolates infecting U.S. horses could be another possibility for a single banding pattern by immunoblot assay. Horses infected with a divergent *B. caballi* isolate have been identified in South Africa based on deletion and insertion of amino acids within the N-terminal repeat region of RAP-1 (23). Because this region contains the epitope recognized by the monoclonal antibody used in the cELISA, false-negative results can be obtained with the RAP-1–cELISA. Therefore, introduction of horses infected with divergent *B. caballi* into the United States could be another potential explanation for the results obtained by cELISA and immunoblot assay. In an attempt to determine if U.S. horses were infected with divergent *B. caballi*, a subset of the cELISA-positive U.S. horses was randomly chosen for blood transmission experiments. The observation that none of the recipient horses became infected with *B. caballi* suggests that the U.S. horses were not infected with a *B. caballi*-divergent isolate. Importantly, our previous study demonstrated that blood from carrier horses with *B. caballi* successfully infected naive seronegative recipient horses via blood transfusion (6).

Regardless of endemicity, the absence of a multiple banding pattern on the immunoblot directly correlated with RAP-1–cELISA negativity in every case and, presumably, the absence of *B. caballi* infection. In contrast, serum antibodies from the overwhelming majority of RAP-1–cELISA-positive horses that were either experimentally infected or from regions where *B. caballi* is endemic recognized a multiple banding pattern, suggesting that a multiple banding pattern on the immunoblot correlates with *B. caballi* infection. All of the RAP-1–cELISA-positive U.S. horses had only a single band. In contrast, only a small percentage of RAP-1–cELISA-positive non-U.S. horses had a single banding pattern. Moreover, some the cELISA-negative U.S. and non-U.S. horses had a single banding pattern. The lack of RAP-1–cELISA reactivity, but immunoblot detection of a single band at ~50 kDa, represents equine antibodies recognizing epitopes on native RAP-1 that do not compete with monoclonal antibody binding in the cELISA. These results suggest that a single banding pattern by immunoblot assay does not indicate *B. caballi* infection. Overall, these data strongly suggest that *B. caballi* infection results in both cELISA-positive and an immunoblot multiple banding pattern at ~30 to 50 kDa. In contrast, cELISA-positive results with either no immunoblot reactivity or a single banding pattern at ~ 50 kDa occur in the absence of *B. caballi* infection. In this study, we demonstrated that a combination of cELISA and immunoblot assay can enhance the accuracy of *B. caballi* serodiagnosis, thus preventing unnecessary economic losses associated with quarantine, treatment, euthanasia, or export. Although the RAP-1–cELISA recognized a limited number of false positives, the overall specificity and sensitivity of this diagnostic test indicate that it should be retained for primary import screening. Importantly, due to the increase of international movement of horses, all horses identified as RAP-1–cELISA positive should be retested by immunoblot assay for diagnostic confirmation. This combination of tests would be consistent with diagnostic confirmation of other infectious diseases of animals and humans, including equine infectious anemia, Lyme disease, and human immunodeficiency virus (35–37).

ACKNOWLEDGMENTS

This work was supported by USDA-ARS 5348-32000-034-00D.

We appreciate the excellent assistance of Nancy Kumpula-McWhirter, Ryan Evanoff, Shelby Beckner, James Allison, Ralph Horn, and Melissa Schmidt. We also thank Amber Gorsch of the USDA-APHIS, Veterinary Services, National Veterinary Services Laboratories, Ames, IA, for her critical support assistance.

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