Case Report: Field-Acquired Subclinical *Babesia equi* Infection Confirmed by In Vitro Culture

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A horse with no prior clinical history of equine piroplasmosis tested negative for *Babesia caballi* and *Babesia equi* in the complement fixation test before importation into the United States from France. After 5 years in residence in the United States, the animal tested serologically positive for *B. equi* by the complement fixation test, the immunofluorescent antibody test, and Western blot analysis. The carrier status of the horse was confirmed by culture of *B. equi* parasites. In vitro culture offers an efficient and comparatively inexpensive method to determine the carrier status of horses suspected of harboring *B. equi*.

Babesia equi and *Babesia caballi* are hemoprotozoan parasites that cause equine piroplasmosis, a disease of worldwide economic importance to the horse industry. During the 1960s, both *B. equi* and *B. caballi* entered the United States via horses imported from Cuba and South America into Florida (11, 13, 14). Since *Dermacentor nitens*, a vector tick for *B. caballi*, was widespread and the horse population throughout the country was considered highly susceptible to infection, in 1969 the U.S. Department of Agriculture (USDA) implemented the complement fixation (CF) test as the official regulatory test to prevent disease outbreaks (3, 9). Horses being imported into the United States must be CF test negative for both disease agents, and those that are CF test positive for *B. equi* are denied entry. If the horses are in an approved quarantine facility at the time of testing, they are either returned to the country of origin or euthanized. Similarly, domestic horses that are CF test positive for *B. equi* are also quarantined, exported, or euthanized. Horses that test positive for *B. caballi* may be allowed entry after appropriate drug treatment. Similar test regulations have been adopted by a number of other countries considered equine piroplasmosis free (4).

Case history. A 10-year-old Selle Français warmblood gelding residing in California was found CF test positive for *B. equi* in March 1993 (Table 1), before export to compete in an international show jumping event. At the time, serology was negative for *B. caballi*, equine infectious anemia, vesicular stomatitis (Indiana and New Jersey), equine encephalitis (Eastern, Western, and Venezuelan), dourine, and glanders.

The animal had been imported as a 5 year old into the United States from France in 1988, at which time its CF tests for *B. equi* and *B. caballi* were negative (Table 1). After residing for 6 months in Florida, the animal was shipped to a permanent residence in California and for the next 4 years was actively trained and shown in the competitive show jumping circuit throughout the country. The horse exhibited good health from 1988 through 1993 with the exception of a single clinical respiratory infection in the spring of 1990 that responded to supportive therapy.

Diagnosis. After the first CF test positive result in March 1993, five serum samples were submitted for CF tests during the following 4-month period. All samples were positive for *B. equi* antibody activity (Table 1), except the April submission which was questionable (a reaction of 1+ at a 1:5 dilution). A sample evaluated by the immunofluorescent antibody (IFA) test was also positive (Table 1). Serum and blood in EDTA were shipped to Texas A&M University, College Station, Texas, in June 1993 for further serologic evaluation by the IFA test and Western blot analysis and for cell culture.

The *B. equi* strain used for preparation of the IFA antigen and the positive control serum has been described previously (8). For the IFA test (5), cultured *B. equi*-infected erythrocytes (8) and fluorescein isothiocyanate-protein G (Sigma Chemical Co., St. Louis, Mo.) were used as the antigen and conjugate, respectively. Control sera included positive serum from horse 4 (experimentally infected with *B. equi* and housed at the National Veterinary Services Laboratory, USDA, Ames, Iowa) and a pool of negative normal adult horse sera (Hyclone Laboratories, Inc., Logan, Utah).

B. equi erythrocyte cultures at 14.7% parasitemia and uninfected erythrocytes from a donor horse (8) were used to prepare antigens for Western blot analysis (6). Replicate antigen samples were electrophoresed in a continuous sodium dodecyl sulfate-polyacrylamide gradient gel, and the separated proteins were transferred to a polyvinylidene difluoride membrane (Westran; Schleicher & Schuell, Inc., Keene, N.H.) by a modified Towbin procedure (16). Replicate membrane lanes were incubated in either serum from the suspected infected horse, positive serum from horse 4, or negative horse serum. After incubation, the membranes were washed, incubated with horseradish peroxidase-protein G conjugate (Sigma Chemical Co.), and then developed in substrate.

Erythrocytes from the suspected infected horse were prepared for culture as previously described (7), but with extra care for complete removal of the leukocyte buffy layer by centrifugation at each of three washes. Culture conditions were as described for *B. caballi*, but with medium devised for *B. equi* cultures (7, 8).

The horse was serologically positive for *B. equi* as shown by the CF and IFA tests (Table 1). In addition, Western blot analysis showed serum antibody from the suspected infected

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TABLE 1. CF and IFA test results for serum samples from the suspected *B. equi*-infected horse^a

Date	<i>B. equi</i> CF ^b	<i>B. equi</i> IFA	<i>B. caballi</i> CF
September 1988	Neg	ND	Neg
March 1993	2+ Pos, 1:5	ND	Neg
April 1993	1+ Pos, 1:5	ND	Neg
May 1993	4+ Pos, 1:5	Pos	Neg
May 1993	ND	Pos ^c	ND
June 1993	2+ Pos, 1:10	ND	Neg

^a Abbreviations: Neg, negative result; Pos, positive result; ND, not done.

^b Test is scored as 1 to 4+ at the last dilution showing positive; the result considered the minimum for a serological positive for regulatory purposes is 2+ Pos, 1:5.

^c Test performed at Texas A&M University; all other tests performed at the National Veterinary Services Laboratory, USDA.

horse reacting with specific *B. equi* antigens (major bands at 170, 57, 45.5, 43, and 18 kDa; minor bands at 137, 54, 39, and 26 kDa) (Fig. 1). Specific *B. equi* antigens of similar molecular weights have been reported by others (1, 10).

After 2 days in culture, *B. equi*-parasitized erythrocytes were observed in Giemsa-stained smears. The carrier status of the horse was positively confirmed by the observation of a typical *B. equi* tetrad formation (Fig. 2).

Discussion. An in vitro culture method was used to determine the positive carrier status of a horse suspected of harboring *B. equi*. The horse had no known exposure to *B. equi* and

FIG. 1. Western blot. *B. equi* antigen prepared from cultured parasitized erythrocytes (RBC) was run in lanes 1 to 6 and normal horse erythrocyte antigen was run in lanes 8 to 13. Lanes 1 to 3 and 8 to 10 were immunoblotted with serum from the suspect horse. Lanes 4 and 11 were immunoblotted with positive serum from a known *B. equi*-infected horse. Lanes 5 and 12 were immunoblotted with known negative horse serum. Lanes 6 and 13 were conjugate controls. Lanes 7 and 14 contain molecular mass markers. Specific *B. equi* major antigens of 170, 57, 45.5, 43, and 18 kDa detected with antibody from the suspect horse are indicated by arrowheads; arrows show specific *B. equi* minor antigen bands of 137, 54, 39, and 26 kDa.

FIG. 2. Giemsa-stained thin smear showing an erythrocyte parasitized with *B. equi* in the typical tetrad formation. The smear was observed on day 2 of culture. Bar, 10 μ m.

no clinical history of equine piroplasmosis, but sometime between importation in 1988 and subsequent testing in 1993, the animal became serologically positive by the CF test.

The positive serology by CF was confirmed by both IFA and Western blot analyses. Western blot analysis demonstrated the presence of specific antibody activity to a number of *B. equi* antigens. The observed antibody reaction to some specific antigens varied in intensity compared to the positive control serum. This variation may be due to differences in individual host responses to infection or is possibly due to variation in antibody produced because of differential antigenic expression in the two parasite strains.

The source of *B. equi* exposure in this case remains unclear. Eight other horses on the same California premises were found CF test negative for *B. equi* and *B. caballi*. It is possible that the horse was a carrier when imported into the United States in 1988, but perhaps insufficient time had elapsed to induce detectable antibody activity. Experimentally, positive CF serology does not result until at least 4 weeks after inoculation (12). A major problem with the CF test for piroplasmosis is the failure to detect antibody activity before the establishment of parasitemia (2, 12), which generally is not observed until approximately 1 month after exposure.

It is possible that this horse acquired the infection during the brief time in Florida. Although there are no official reports of the disease during that period, occasional focal outbreaks have occurred in that state (13–15). Another possibility is that the animal became infected while on the competitive circuit. This seems unlikely since other cases have not been reported.

Severe regulatory and economic repercussions result when an animal is found positive for *B. equi* by the CF test. Occasionally in such circumstances, horse owners request subinoculation of blood from the positive horse into a *B. equi*-susceptible animal to demonstrate the presence of the hemoparasite. This procedure is expensive, laborious, time-consuming, and very difficult to ethically justify. Successful methods for erythrocyte cultures of *B. equi* (8, 17) suggested the use of culture to circumvent subinoculation procedures.

The predictive accuracy of the CF test was confirmed in this case. Additional serologic tests supported the initial test results, but detection of *B. equi* parasites in cultured erythrocytes proved unequivocally that the horse was infected at the time. This report demonstrates the utility of the in vitro culture

method in determining the carrier status of animals suspected of harboring *B. equi*. No experimental horses are required. The culture method is less expensive and time-consuming and is an attractive alternative to the subinoculation of live animals to detect hemoparasites. A culture assay might prove to be a valuable supplemental test for importation screening of horses from areas where piroplasmosis is endemic.

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