Seroepidemiologic Studies on Babesia caballi and Babesia equi Infections in Japan

Hiromi IKADA¹,², Akiko NAGAI¹, Xuenan XUAN³, Ikuo IGARASHI¹, Tsugihiko KAMIO¹,², Naotoshi TSUJI³, Takashi OYAMADA², Naoyoshi SUZUKI¹,² and Kozo FUJISAKI¹,*

¹National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080–8555, ²Department of Veterinary Parasitology, School of Veterinary Medicine and Animal Sciences, Kitasato University, Towada, Aomori 034–8628 and ³National Institute of Animal Health, Tsukuba, Ibaraki 305–0856, Japan

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ABSTRACT. Antibodies to Babesia caballi and Babesia equi were examined on a total of 2,019 horse serum samples that had been collected in 1971–1973 by the National Institute of Animal Health by enzyme-linked immunosorbent assay (ELISA) using recombinant proteins and by Western-blot analysis. Based on the criterion for positivity by ELISA, 5.4% (109/2,019) and 2.2% (44/2,019) had antibodies against B. caballi and B. equi, respectively. The ELISA-positive sera were further examined by Western blot; 30/109 for B. caballi and 2/44 for B. equi were positive for native B. caballi or B. equi, but none of them was seropositive for both infections. Based on the results of this study, further investigations should be required to survey horses that have arrived in Japan recently and tick vectors of equine Babesia using ELISA with some recombinant protein, a parasite detection method in an in vitro culture of equine Babesia, and PCR testing.

KEY WORDS: Babesia caballi, Babesia equi, ELISA, Japan, Western blot.

Equine babesiosis, also known as biliary fever, is an acute, subacute, or chronic tick-borne disease of Equidae caused by the hemoprotozoan parasites, Babesia caballi and Babesia equi. The disease, which is widely distributed in tropical and subtropical areas worldwide, causes significant economic loss to the horse industry. Babesiosis is generally characterized by fever, anemia, jaundice, and edema. In some cases, it causes the death of infected horses [4, 6, 18, 19]. Up to the present, no clinical equine babesiosis has been reported in Japan, and therefore Japan is still considered free from the disease. However, there has been a long-standing increase in the number of imported horses from foreign countries including endemic areas, and the existence of tick vectors, Dermacentor reticulatus and Rhipicephalus sanguineus, has also been reported in Japan [24]. In this recent survey, Dermacentor sp. seemed to have been exterminated in Japan; furthermore, Haemaphysalis longicornis has raised questions about the real vector of equine Babesia which was widely distributed in Japanese pastures [13]. These situations indicate that Japan is facing the risk of introducing infected or carrier horses. Although the complement fixation test (CFT) has been used as the official test to detect antibodies against equine Babesia parasites by the United States Department of Agriculture (USDA) and Japan, it has been shown to yield both false-positive and false-negative results for B. caballi [5, 6, 21, 22]. In this study, a serological survey of equine babesiosis in Japan was conducted on sera collected during 1971–1973 by enzyme-linked immunosorbent assay (ELISA) and Western blot using recombinant proteins and native proteins, respectively.

MATERIALS AND METHODS

Serum samples: Serum samples of 2,019 horses were collected at random from every prefecture in Japan from 1971 to 1973 by the National Institute of Animal Health for a survey of equine infectious anemia and were kept at –20°C until use.

ELISA: The ELISA was performed as described [10, 12, 23]. Briefly, B. caballi 48-kDa merozoite rhpoly protein (GST-BC48) expressed by a pGEX4T expression vector (Amersham Pharmacia Biotech, England) in Escherichia coli as glutathione S-transferase fusion protein and a recombinant baculovirus expressing B. equi merozoite antigen-1 (EMA-1), which were secreted into the supernatant of insect cell cultures, were used for the ELISA for B. caballi- and B. equi-infection, respectively [10, 12, 23]. Ninety-six-well microtitration plates (Nunc-Immuno Plate; Nunc, Denmark) were coated with 50 µl of purified GST-BC48 (0.1 µg/µl) or the secreted EMA-1 diluted in a 0.05 M carbonate-bicarbonate buffer (pH 9.6) overnight at 4°C. To reduce non-specific binding, the plates were blocked with PBS containing 3% skim milk (PBS-SM) for 1 hr at 37°C. The blocking agent was removed, and individual horse serum diluted at 1:80 in PBS-SM was added to each well and then incubated for 1 hr at 37°C. After washing six times with PBS containing 0.05% Tween-20, 50 µl of peroxidase-conjugated goat anti-horse IgG antibody (Cappel, N.C.) diluted at 1:4,000 in PBS-SM was added to each well and incubated for 1 hr at 37°C. The plates were washed as described above, and then 100 µl of substrate solution (0.1 M citric acid, 0.2 M sodium phosphate, 0.003% H₂O₂, and 0.3 mg/ml 2,2'-Azide-bis[3-ethylbenzthiazoline-6-sulfonic acid]) was added to each well. Absorbance at 415 nm was read after 1 hr incubation at room temperature using an ELISA reader (Corona micro-
plate reader MTP-120; Corona, Japan). The optical densities (OD) over 0.2 were interpreted as positive [10, 12, 23].

Western blot: The positive sera detected by ELISA were further analyzed by Western blot. Western blot was performed on an antigen prepared from *B. caballi*- and *B. equi*-infected erythrocytes obtained from microaerophilous stationary-phase cultures as described [11]. Horse serum samples diluted at 1:80 in PBS-SM and the peroxidase-conjugated goat anti-horse IgG (Cappel) diluted at 1:1,000 were used as first and second antibodies in Western blot, respectively. After being washed three times with PBS for 5 min, the samples were incubated with enhanced chemiluminescence (ECL) detection reagents (Amersham Pharmacia Biotech) for 1 min. In the case of ECL detection, the membrane was exposed to a film.

RESULTS AND DISCUSSION

When GST-BC48 and EMA-1 recombinant proteins were used in the ELISA, we detected *B. caballi*- and *B. equi*-specific in sera from 2,019 horses in Japan. Based on the criterion for positivity described above, 5.4% (109/2,019) and 2.2% (44/2,019) had antibodies against *B. caballi* and *B. equi*, respectively (Fig. 1a, b). These positive sera were further examined by Western blot using native antigens; 30/109 for *B. caballi* and 2/44 for *B. equi* were positive for native *B. caballi* or *B. equi* proteins, respectively, but none of them was seropositive for both infections. Western-blot analysis showed that ELISA-positive reacted with specific *B. caballi* proteins (major bands at 27, 29, 48, and 50 kDa; minor bands at 70 and 112 kDa) and with *B. equi* proteins (bands at 34 and 43 kDa) (Fig. 2).

Currently, regulatory control of equine babesiosis in the U.S.A., Brazil, Canada, Japan, and Australia, relies on serological tests [7]. Although CFT is the official test used by the USDA and Japan, it has been shown to yield both false-positive and false-negative results for *B. caballi* [5, 21, 22]. Horse sera in Japan were tested by IFAT, and some pseudo (suspicious)-positive sera of *B. caballi* and *B. equi* were detected [21]. However, some pseudo (suspicious)-positive sera of *B. caballi* and *B. equi* were not precisely ascertained to be equine *Babesia* infections. At present, ELISA using recombinant protein has the highest sensitivity for equine *Babesia* infections [10, 12, 14, 15, 20, 23]. In the present study, ELISA using recombinant antigens detected serologically positive cases, and specific bands were also observed in some ELISA positive ones by Western-blot analyses. Western-blot analysis has been suggested as a confirmatory test that provides a species-specific diagnosis [3]. Moreover, *B. equi* parasites were detected by an in vitro culture system from a horse with a Western-blot positive result [8].

The ELISA using GST-BC48 of *B. caballi* and EMA-1 of *B. equi* were considered that might enable detection of antibody to the protein up to a year or more after infection [11, 15]. Moreover, specific *B. caballi* or *B. equi* proteins with similar molecular weights as those reported by others [1, 2, 4, 9, 16] were detected in the present study. For *B. caballi*, Böse et al. [2, 3] analyzed proteins of *B. caballi* (USDA

![Fig. 1. a, b. Distribution of ELISA results using recombinant protein by OD value. A total of 2,019 horse serum samples were tested in ELISA with GST-BC48 (a) and EMA-1 (b).](image-url)
strain)-infected erythrocytes by Western blot with sera from horses that had been experimentally and naturally infected with *B. caballi*. Dominant proteins of 48, 50, 70, 112, and 141 kDa were recognized by European and Brazilian horse sera tested. In the present study, not only similar proteins of 141 kDa were recognized by European and Brazilian horse sera tested. 

In conclusion, the present study suggests the existence of equine babesiosis in Japan by the detection of antibodies against *B. caballi* and *B. equi* in 1971–1973. The vectors for *B. caballi* and *B. equi*, such as *D. reticulatus* and *R. sanguineus*, have been reported in Japan [24]. *D. reticulatus* was confirmed in Japan in 1974, but the actual distribution has not been accurately determined [13]. Autochthonous infections have not been observed in Japan, although the potential vector, *H. longicornis*, also occurs in this country [13]. Thus, it is necessary that the actual conditions of the equine Babesia will be investigated in Japan. Concretely, further studies should be required to examine the horses that are definitely infected with equine *Babesia* and survey the tick vector of equine *Babesia* in Japan using ELISA with some recombinant protein of equine *Babesia*, a detection method in an *in vitro* culture of equine *Babesia*, and PCR testing.

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REFERENCES


