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Preliminary Survey on Horse Serum Indirect Fluorescence Antibody Titers in Japan against *Babesia equi* and *Babesia caballi* (Onderstepoort strain) Antigen

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ABSTRACT

At present no clinical surveys concerning equine babesiosis in Japan have been reported. A total of 494 horse serum samples (439 domestic horses and 55 imported) were collected from Kyushu and Hokkaido, Japan. Indirect fluorescent antibody test (IFAT) of the serum samples did not show any positive reactions in 1:80 serum dilution. However, 3 of the 19 trace or false-positive (±) cases were observed to be pseudo-positives (±~+). These had a weak and sharp fluorescence around the parasite of *B. equi* and/or *B. caballi*. Based from the results of this study, further investigations should be undertaken until the accurate diagnostic method in in vitro culture system can be established in Japan.

INTRODUCTION

Babesia equi and *Babesia caballi* are horse intraerythrocytic protozoan parasites transmitted by ticks almost worldwide (Tenter and Freidhoff 1986). Up to now, there have been no clinical reports concerning equine babesiosis in Japan, and Japan is still considered free from the disease. However, the possible vectors of horse piroplasma, *Dermacentor reticulatus* and *Rhipicephalus sanguineus* exist in Japan. Complement fixation test (CFT) is used as a standard test for horse piroplasmosis in Japan for disease control and safe importation of horses from foreign countries. In this study, indirect fluorescent antibody test (IFAT) was used in the preliminary survey of horse babesiosis in Japan.

MATERIALS AND METHODS

Strains of Babesia equi and Babesia caballi

Slide smears of *B. equi* and *B. caballi* for IFAT and standard positive and negative sera were transported at -20°C from Onderstepoort Veterinary Institute, South Africa. The antigen spots on the slides were encircled with a pap pen and fixed in cold acetone. Slides were wrapped in tissue papers covered with aluminum foil, and stored at -20°C until use.

Serum samples collected

Serum samples collected from Kyushu and Hokkaido were kindly supplied by various veterinary practitioners in 1992-1993. The total of 494 was from 439 domestic horses bred in Japan and from 55 horses imported from foreign countries. Ten samples of equine babesia CFT positive sera were kindly provided by Dr. N. Komatsu, Yokohama Animal Quarantine Service, the Ministry of Agriculture and Fishery. They were kept at -20°C until use.

IFAT technique

Eighty fold serum dilutions in PBS were prepared for each sample. Every test included *B. equi* and *B. caballi* standard positive and negative sera provided by Onderstepoort Veterinary Institute as well as PBS control. Ten microliters of each serum dilution was then placed on the antigen spots and slides were incubated in a humid chamber at 37°C for 30 min. The slides were then rinsed in fresh PBS and washed once for 10 min in PBS followed by a further washing with deionized-distilled water for 5 min. Goat anti-horse IgG FITC conjugate (Bethyl Lab., Inc., USA) was diluted 1:80 in 0.01% Evans blue-PBS to reduce nonspecific fluorescence and 10 ml of diluted conjugate was pipetted onto each spot. The slides were incubated, rinsed and washed as described above. After the last wash slides were slightly air dried before being overlaid with glycerin-PBS (1:1) and covered with a 24 x 60 mm cover slip. The slides were examined under a fluorescence microscope using a 40x objective lens (Microphot EPI-FL, Nikon Co., Japan). Fluorescence was interpreted as positive (++), pseudo-positive (+~±), trace (false-positive)(±), or negative (-).

RESULTS

Determination of equine sera by IFAT

With a serum dilution of 1:80, 494 samples were tested by IFAT either using *B. equi* or *B. caballi* spotted blood smears, together with the positive and the negative standard sera. As shown in Table 1, 19 out of 494 serum samples showed trace (false-positive)(±) results for *B. equi*. Three of the 19 cases gave a strong

and sharp trace, that is, a weak and clear fluorescence around the parasites. These were marked as pseudo-positives (+~±), in both *B. equi* and *B. caballi*. The other 475 samples were all negative. Of the three IFAT pseudo-positive sera of *B. equi* and *B. caballi*, two cases were positive in 1:5 dilution for *B. caballi* in the CFT as tested by Dr. T. Kamio, NIAH, Tsukuba (personal communication).

Table 1. IFA test of equine sera against *Babesia equi* (Onderstepoort strain) antigen.

Serum tested	Negative (-)	Trace (±)	Pseudo-positive (±~-)	Positive (+~++)	Total
1. Onderstepoort					
Positive serum				1	1
Negative serum	1				1
No. of control samples	1			1	2
2. Japan					
A. Hokkaido					
A stock farm	327	7	1	0	335
B stock farm	100	1	0	0	101
B. Kyushu					
C stock farm	48	8	2	0	58
No. of samples	475	16	3	0	494

Note: All samples at serum dilution of 1:80.

Comparison of the CFT positive sera with IFAT

Ten equine babesia CFT positive sera wherein the National Animal Quarantine Service, Yokohama, were able to detect babesia parasites inside erythrocytes in blood smears, were tested by IFAT. As shown in Table 2, in 7/10 cases (70%) identical results were obtained with IFAT. Test samples 5, 6 and 7 in *B. equi* were lower than 1:5 to 1:5 by the CFT. In the IFAT, they had a titer of 1:80 to 1:320. Sample No. 7 which had a titer lower than 1:5 in CFT was positive in IFAT showing a titer of 1:80.

Cross reactions between B. equi and B. caballi

In the IFAT as shown in Table 3, *B. equi* antigen reacted to 1:320 of anti-*B. equi* serum, and to 1:40 of anti-*B. caballi* serum as the positive titre, respectively. *Babesia caballi* antigen reacted also to 1:320 of anti-*B. caballi* serum and to 1:20 of anti-*B. equi* serum (trace(±) in 1:40) as the positive titre, respectively.

Table 2. Validation of the CFT for *Babesia equi* and/or *Babesia caballi* given by the Yokohama Animal Quarantine Service , as compared with IFAT.

Case No.	Sera from horses infected with *B. equi* and/or *B. caballi**											
	CFT						IFAT					
	B. equi			*B. caballi*			*B. equi*			*B. caballi*		
No. of Exam.	1	2	(S**)	1	2	(S**)	1	2	(S**)	1	2	(S**)
1 (EI-2)	5-10	5	(+)	<5	<5	(-)	80	80	(+)	<80	<80	(-)
2 (EI-5)	<5	<5	(-)	<5	<5	(-)	<80	<80	(-)	<80	<80	(-)
3 (EI-13)	5	10	(+)	20	20	(+)	80	80	(+)	160	80	(+)
4 (EI-17)	<5-5	<5-5	(±)	40	40	(+)	<80	<80	(-)	320	80	(+)
5 (EI-24)	<5	<5	(-)	20	20	(+)	320	320	(+)	80	80	(+)
6 (EI-25)	<5-5	<5-5	(±)	5	5	(+)	160	160	(+)	80	80	(+)
7 (EI-32)	<5-5	<5-5	(±)	<5	<5	(-)	80	80	(+)	80	<80	(±)
8 (EI-39)	40	40	(+)	10	5-10	(+)	320	160	(+)	160	160	(+)
9 (EI-46)	<5	<5	(-)	<5	<5	(-)	<80	<80	(-)	<80	<80	(-)
10(EI-49)	5-10	5-10	(+)	5-10	10	(+)	80	160	(+)	80	160	(+)

*Nos. 1-10 horses examined contained intraerythrocytic parasites in each blood smear slide. Results of the CFT for *B. equi* and *B. caballi* were tested by Dr. N. Komatsu at the Yokohama Animal Quarantine Service.

All sera tested by the IFAT were diluted more than 1:80 as a standard positive result.

S** means the score mark, serum positive (+), trace or false positive (±), or negative (-) result.

Table 3. Cross reactions between *B. equi* and *B. caballi* in equine piroplasma.

Serum dilutions	Antigens of *Babesia equi* and *Babesia caballi**									
	B. equi					*B. caballi*				
	1:20	1:40	1:80	1:160	1:320	1:10	1:20	1:40	1:80	1:320
Anti-*B. equi* serum** (SA. H-460-9)	+	+	+	+	+	+	+	±	-	-
Anti-*B. caballi* serum** (SA. H-442-9)	+	+	-	-	-	+	+	+	+	+

* Strains of *B. equi* and *B. caballi* were from Onderstepoort Veterinary Institute, South Africa.

**Standard sera used in this test were from experimentally infected horses, Onderstepoort Veterinary Institute, South Africa (SA).

DISCUSSION

Currently, regulatory control of equine babesiosis in the United States and other countries (Brazil, Canada, Japan, and Australia), relies on serological testing (Holman et al. 1993). Although CFT is the official test used by the U.S.

Department of Agriculture, it has been shown to yield both false-positive and false-negative results for *B. caballi* (Enigk 1950; Tenter & Friedhoff 1986). At present, ELISA has the highest sensitivity for *Babesia* infections. Western blot and IFAT provide comparable results, while CFT may be regarded as obsolete due to it's low sensitivity which does not meet the requirements for export testing or epidemiological studies (Boese and Peymann 1994). For the final confirmation of *Babesia* infection in horses, the parasites should be identified from the horse, and consequently, the ability to expand the parasite population to detectable levels through in vitro culturing, represents a final diagnostic procedure that might be used to confirm current methods (Holbrook et al. 1968). IFAT is laborious and provides a somewhat subjective method. Since, *Babesia* can be observed directly on every slide, a survey on the horses in some regions of Japan was carried out to determine whether they are completely free from infection through the IFAT Babesia tests. Three out of the 494 horse sera were not completely negative from the test at 1:80 serum dilutions, showing a weak and clear fluorescence around the parasites. Two of the three cases were positive at 1:5 serum dilution by CFT as examined by Dr. T. Kamio, National Institute of Animal Health (personal communication). The serum samples were collected at random from 2 different regions in Japan. Unfortunately, we could not do a retest of these three cases. Thus, it is suggested that further studies on equine babesiosis in Japan should be undertaken until such time that the accurate diagnostic methods and the in vitro culture system for the improved tests can be established.

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