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Survey on Tick-Borne Pathogens in Thoroughbred Horses in the Hidaka District, Hokkaido, Japan

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ABSTRACT. A total of 87 Thoroughbred horses and 10 ixodid ticks from a ranch in Hidaka district, Hokkaido were tested for tick-borne diseases. Using the indirect fluorescent antibody (IFA) method, 3.4, 92.0 and 97.7% of the horses showed antibody titers of ≥80 against *Anaplasma phagocytophilum, Rickettsia helvetica*, and *Borrelia garinii*, respectively. This is the first report of infection with the 3 pathogens in horses in Japan. Using PCR, DNAs from the peripheral blood of all horses were found negative with any *Anaplasma*, *Rickettsia* and *Borrelia* spp., while those from *Haemaphysalis megaspinosa* ticks were found positive for *Anaplasma* sp. closely related to *A. phagocytophilum* in Japan, and *A. bovis*. *B. japonica* was also detected in an *H. flava* tick for the first time.
KEY WORDS: *Anaplasma* sp., *Borrelia* sp., *Rickettsia* sp., Thoroughbred horse, tick-borne diseases.

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The epidemiology of tick-borne diseases in horses has been well studied in several countries. In the U.S.A., these diseases include babesiosis [21], borreliosis [22] and anaplasmosis [6]. In Brazil, Rickettsia infection in horses was also observed [39]. These diseases appear to be distributed in most part of the world [4, 7, 8, 34, 38, 41]. However, although these pathogens are known to be potentially zoonotic, their distribution in horses in the Asian area has not been well reported.

Reports on equine tick-borne diseases in Japan have been limited. Most reports deal with *Babesia* spp. [9, 12]. Other pathogens like *Anaplasma* spp. [19, 26, 35, 36, 44], *Rickettsia* spp. [2, 13, 22, 42] and *Borrelia* spp. [18, 25] are less studied in horses, although most of them have been detected in humans and several other animals in Japan. To date, the spotted fever group (SFG) *R. japonica* is considered to be the most important *Rickettsia* sp. in Japan because of its potential zoonoses, higher occurrence and potential severity, which can be fatal in humans [23, 24, 28].

In the present study, blood and ixodid tick samples from Thoroughbred horses kept in a ranch in Hidaka district, Hokkaido, Japan, were tested for tick-borne diseases. The present study aimed to clarify the exposure or infection of the horses with *Anaplasma*, *Rickettsia* and *Borrelia* spp. using serological and molecular methods. It also aimed to detect the pathogens found in the ticks from the horses.

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MATERIALS AND METHODS

Samples: Peripheral blood and serum samples were collected from 87 Thoroughbred horses (13 broodmares, 9 foals and 65 yearlings) that were kept in a ranch in the Hidaka District, Hokkaido, Japan, in 2010. All these horses were clinically normal. In addition, 10 ticks (4 *Haemaphysalis flava* and 6 *H. megaspinosa*) obtained from 3 horses were also collected. DNAs from EDTA-treated peripheral blood and ticks were extracted using a QIAamp DNA Mini Kit (QIAGEN, Valencia, CA, U.S.A.), eluted with 200 μl of TE buffer, and stored at −30°C until further use. The separated sera were kept at −20°C until use for indirect fluorescence analyses (IFA).

IFA: IFA was conducted using similar procedures previously performed [16]. In brief, the antigens used were prepared from cultured cells for *A. phagocytophilum* and *R. helvetica*, and from bacterial culture of *B. garinii*. Sera were screened at a 1:20 dilution in phosphate-buffered saline (pH 7.4) Tween 0.5% (PBST), and an optimized dilution (1:200) of fluorescein isothiocyanate-labelled IgG conjugate in PBST was used as the second antibody. Reactive antibodies were then detected using a fluorescence light microscope. Samples which reacted with any one of the antigens at the screening dilution were then titrated using serial two-fold dilutions to determine end titers. The antibody levels in the test samples were determined by comparing with the control samples. The positive controls used were *R. helvetica*-posi-

Table 1. Serological test results using indirect fluorescent antibody (IFA) technique

Pathogen	Animal	Antibody Titer					≥×80 (%)
		<×20	×40	×80	×160	×320	
A. phagocytophilum	Foal (<1 year)	9	-	-	-	-	-
	Yearling (1 year)	55	8	1	1	-	3.1
	Broodmare (<10 years)	8	-	-	1	-	11.1
	Broodmare (≥10 years)	4	-	-	-	-	-
	Total	76	8	1	2	-	3.4
R. helvetica	Foal (<1 year)	-	1	5	2	1	88.9
	Yearling (1 year)	1	5	18	15	26	90.8
	Broodmare (<10 years)	-	-	1	5	3	100.0
	Broodmare (≥10 years)	-	-	-	-	4	100.0
	Total	1	6	24	22	34	92.0
B. garinii	Foal (<1 year)	-	-	-	-	9	100.0
	Yearling (1 year)	1	-	3	2	59	98.5
	Broodmare (<10 years)	-	-	-	-	9	100.0
	Broodmare (≥10 years)	1	-	-	-	3	75.0
	Total	2	-	3	2	80	97.7

tive sera from a sika deer [16], *A. phagocytophilum*-positive sera, and *B. garinii*-positive sera. The negative control used was PBST.

PCR Assay: For the detection of *A. phagocytophilum* and the closely related *Anaplasma* sp. of Japan, a 16S rRNA-based nested PCR (nPCR) method was used to amplify a final 770-bp DNA fragment of either the 2 pathogens. The primers EC9/EC12A were used for the first-round PCR [19], followed by the primers AP-f1/AP-r1 for the second-round PCR [36]. For *Borrelia* spp., a nPCR based on 5S-23S rRNA intergenic spacer was used to amplify a final 250-bp fragment. The primer sets RIS1/RIS2 [30] and rrf2/rrl2 [11] were used for the first- and second-round PCR, respectively. For *Rickettsia* spp., a citrate synthase gene (*gltA*)-based nPCR was used to amplify a final 322 bp-DNA fragment. The primer sets RpCS.877p/RpCS1273r [33] and RpCS.896f/RpCS.1258n [10] were used for the first- and second-round PCR, respectively. Respective PCR cycling conditions were performed according to the primer source references. Amplification products were visualized using 1.5% agarose gel after electrophoresis, and were purified using a QIAquick PCR purification kit (QIAGEN). DNAs from the following species were used as positive controls: *A. phagocytophilum* from human (supplied by Dr. P. Brouqui, France), *Anaplasma* sp. closely related to *A. phagocytophilum* [43], *A. bovis* [36], *R. tarasevichiae* [13] and *Borrelia* sp. (EF160138). The negative control used was double distilled water (DDW).

Sequencing and analyses: Direct sequencing method was performed using the same PCR inner primers. Nucleotide sequence results were checked using the BLAST program hosted by the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for comparison with other known sequences. Gaps were not considered in the final computation of identities.

RESULTS

IFA testing showed that 3.4, 92.0 and 97.7% of the horses had antibodies ≥80 against *A. phagocytophilum, R. helvetica* and *B. garinii* antibodies, respectively (Table 1). In contrast with the PCR results, DNAs from horses were found negative with any *Anaplasma*, *Rickettsia* and *Borrelia* spp. For the ticks, 3 *H. megaspinosa* (H-7, H-8, H-9) were found to be positive for *A. bovis*, while 1 *H. megaspinosa* (H-10) was found to be positive for both *A. bovis* and *Anaplasma* sp. closely related to *A. phagocytophilum* in Japan. All ticks were found negative with any *Rickettsia* sp. Only 1 *H. flava* tick was found positive with *B. japonica*. Other relevant information about the obtained ticks is summarized in Table 2.

Sequencing results of selected positive amplicons revealed identities of 99.7% (723 bp), 100% (516 bp), and 100% (230 bp) with *Anaplasma* sp. closely related to *A. phagocytophilum* of Japan, *A. bovis*, and *B. japonica* (strain H014), respectively. All nucleotide sequences obtained in the present study were registered in GenBank with the following accession numbers: JX082004 (*B. japonica* from *H. flava*), JX082005 (*Anaplasma* sp. closely related to *A. phagocytophilum* from *H. megaspinosa*) and JX082006 (*A. bovis* from *H. megaspinosa*).

DISCUSSION

The Hidaka district of Hokkaido, where the horse ranch is located, is famous for the production of Thoroughbred horses. It has an endemic occurrence of Rickettsial infection in cattle and sika deer. Seropositive cattle were found PCR negative, while seropositive sika deer were found PCR positive with *R. asiatica* despite using *R. helvetica* antigens [16]. DNA fragments of *R. helvetica* were also detected in sika deer in Nakanoshima Island, Japan [14]. On the other hand, *A. phagocytophilum* has only been detected in *H. megaspinosa* ticks in the district [44], although the nucleotide se-

Table 2. PCR results of 10 ticks obtained from 3 horses

Tick no.	Horse source	Species	Stage	Engorged with blood	*A. phagocytophilum* or the closely-related *Anaplasma* sp. of Japan	*A. bovis*	*Rickettsia* spp.	*Borrelia* spp.
H-1	A	*H. flava*	Female	Yes	−	−	−	+
H-2	A	*H. flava*	Male	ND*	−	−	−	−
H-3	A	*H. flava*	Nymph	Yes	−	−	−	−
H-4	A	*H. flava*	Nymph	Yes	−	−	−	−
H-5	B	*H. megaspinosa*	Nymph	Yes	−	−	−	−
H-6	B	*H. megaspinosa*	Nymph	Yes	−	−	−	−
H-7	B	*H. megaspinosa*	Nymph	Yes	−	+	−	−
H-8	C	*H. megaspinosa*	Female	ND*	−	+	−	−
H-9	C	*H. megaspinosa*	Female	Yes	−	+	−	−
H-10	C	*H. megaspinosa*	Female	Yes	+	+	−	−

*ND=Not determined.

quence identities of the detected species appear to be closer to the potentially novel *Anaplasma* sp. of Japan than to *A. phagocytophilum* [43]. In different studies, DNA fragments of a pathogen closely resembling *B. garinii* were detected from an ixodid tick [11], while a feral raccoon was found to carry antibodies against *B. garinii* [15] in Hokkaido, Japan. Until the present study, infection in horses has not been reported in Japan.

The positive serological reaction (IgG antibody titer ≥×80) of the horses against the tick-borne pathogens coupled with the negative PCR results may indicate previous or repeated exposure with the pathogens, and that the seropositive animals were probably clearing these pathogens [5]. It is also possible that the levels of bacteremia were low and undetectable by PCR [37]. In the present study, all the yearlings were solely sourced from the Hidaka region. Thus, it can be presumed that the development of antibodies against the tick-borne pathogens is due to pathogen exposure that is probably confined in the endemic region (Hidaka).

The detection of antibodies against *R. helvetica* in horses in Japan presented in the current study is the first report elsewhere. This pathogen was previously reported only in European countries, but it has been detected in several areas of Japan [10]. In Hidaka district, *R. helvetica* infection was detected in cattle and sika deer using IFA. However, the infected cattle were found PCR negative, while sika deer found PCR positive revealed infection with *R. asiatica* instead of *R. helvetica* [16]. Thus, negative PCR results but with detectable antibodies in the animals may imply possible cross-reaction of *R.helvetica* antibodies with other *Rickettsia* spp., which may not be detected by the PCR assay used. Similarly, *A. phagocytophilum* infection in horses in Japan has not been reported until this study. *A. phagocytophilum* is also known to infect humans and horses [41]. However, it is also possible that instead of *A. phagocytophilum*, the closely related *Anaplasma* sp. of Japan cross-reacted with the *A. phagocytophilum* antigens in the examined horses, since the former was also detected in ticks in the area [44].

The horses were also found PCR negative with any *Borrelia* spp. However, the detection of antibodies against *B. garinii* in the horses and the detection of DNA fragments of *B. japonica* from an *H. flava* tick obtained from 1 of the horses connote possible exposure to the *Borrelia* pathogen. Moreover, PCR negative results do not rule out infection, since false-negative results can still occur even when the procedure is properly performed using DNA samples from blood [1]. Thus, serological tests can be also used as a good diagnostic aid. *B. japonica* appears to mainly occur only in Japan [31]. It has been detected in *I. ovatus* [17]. Although it is mainly considered to be non-pathogenic in humans [30], a human case was suspected in Shizuoka, Japan [27].

The *H. megaspinosa* tick (H-10) was found to be dually positive for *Anaplasma* sp. closely related to *A. phagocytophilum* in Japan and *A. bovis*. Another study also reported the detection of DNA fragments of the 2 pathogens in the same tick species [44]. The potentially novel *Anaplasma* sp. of Japan has also been previously detected in *I. persulcatus* and *I. ovatus* [29]. The different range of possible vectors is one of the evidences supporting its novelty [43], although the *I. persulcatus* tick is found to be a common vector of *A. phagocytophilum* [20] and the closely related *Anaplasma* sp. of Japan [26]. On the other hand, *A. bovis* has also been detected in *H. longicornis* [19] and other *Haemaphysalis* spp. [36] in Japan. Different species of ticks may be abundant in a particular season, as tick activities are affected by seasonal variations [3, 32]. It will be interesting to obtain more tick samples from the horses at different seasons to determine temporal patterns of the tick-borne pathogens in the area.

All horses used in the study showed no apparent clinical signs at the time the samples were collected. However, swelling on the tick bite sites and sudden flu-like symptoms (like fever) with unknown cause (most frequently in young horses) were observed. These signs may be caused by any of the tick-borne pathogens, which can also be asymptomatic or non-specific in nature [6, 40, 41]. Hence, infection with the pathogens, or the clinical signs associated with the infections may have been missed. Nonetheless, humans and horses in the Hidaka district are exposed to constant health threat due to the zoonotic potential of the pathogens present in the area. Infection should be suspected especially when there is a high risk of exposure to ticks, or when signs possibly related to tick-borne infections are seen.

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