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Problems in the Protection of Reintroduced Przewalski's Horses (*Equus ferus przewalskii*) Caused by Piroplasmosis

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ABSTRACT: The objectives of our research on equine piroplasmosis were to clarify the prevalence of the piroplasms *Babesia caballi* and *Theileria equi* among reintroduced Przewalski's horses (takhi, *Equus ferus przewalskii*) in comparison with horses (*Equus caballus*) of nomads from around Hustai National Park in Mongolia and to determine the mortality among takhi during 2012 to 2015. The prevalence of *T. equi* was high in 2014 at 80% among nomadic horses and 84% among takhi. We found no evidence of *B. caballi* infection among takhi. A homology comparison of the 18S rRNA base sequence of *T. equi* showed the same sequence in both domestic horses and takhi. According to the national park records, piroplasmosis is a cause of high mortality in young takhi (19%). The results suggested that takhi were infected with *T. equi* in this park, and the infection is an important issue when reintroducing the takhi into the wild and increasing the numbers of the animals in nature. Continuous monitoring and vector control were noted as important issues in future conservation programs.

Key words: Babesiosis, epidemiology, Mongolia, Przewalski's horse, takhi, theileriosis.

The takhi (Mongolian name) is a wild horse that was discovered on Mongolia's steppes during the 1870s by Russian colonel N. M. Przewalski and scientifically named Przewalski's horse (*Equus ferus przewalskii*). Unfortunately, takhi became extinct in the wild from the pressures of hunting and pasturing of livestock in the 1960s. In 1992, takhi were transported from European zoos back to southwest and central Mongolia (Boyd and Bandi 2002). Takhi are listed as endangered on the International Union for Conservation of Nature's Red List and the Mongolian Red List and Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora. Hustai National Park is

one of the reintroduction sites in a 50,600 ha protected area; geographically, it consists of steppe, mountain steppe, mountain zones, and forest (47°35'N to 47°52'N, 105°23'E to 105°37'E) in Tov Province, Mongolia, at an altitude of 1,100–1,840 m. The average temperature in the summer is 25 C; average temperature in the winter is –25 C; and the annual precipitation ranges from 130 to 220 mm. The national park is 95 km west of the Mongolian capital city, Ulaanbaatar, Mongolia, Central Asia. The park has been managed and protected by a nongovernmental organization and the Mongolian government. In five reintroductions from 1992 to 2000, a total of 84 takhi were transported to this park and have since increased in number to 336 takhi in the wild.

Theileria equi (formerly *Babesia equi*) and *Babesia caballi* are hemoprotozoan parasites in equines and are transmitted by ticks (Wise et al. 2013, 2014; Scoles and Ueti 2015). During reintroduction at sites in Hustai National Park and Gobi B Strictly Protected Area, deaths of adult takhi occurred during their first spring from April to May (Walzer et al. 2000). Pathologic findings in two takhi that died in 1999 strongly suggested that equine piroplasmosis (Mehlhorn and Schein 1998) was responsible for the deaths. No antibodies were detected in the introduced takhi prior to their translocation to Mongolia because they originated from captive breeding programs where piroplasmosis only occurs sporadically or is not endemic (Walzer et al. 2000). Our objectives were to determine the prevalence of *Babesia* and *Theileria* species among reintroduced takhi to compare morbidity and mortality of takhi to that of domestic horses

(*Equus caballus*) belonging to nomads around the park.

We examined domestic horses (*E. caballus*) belonging to five families of nomadic herders in the buffer zone around Hustai National Park in Tov Province, Mongolia. Approximately 70–120 nomadic families are currently living around the park, and each family has 15–200 horses. We referred to reports regarding the park's animal records (2012, 2014, and 2015) to investigate cases of mortality and disease occurrences in the wild animals. Sampling was carried out on 47 domestic horses in April 2014 and 45 domestic horses in August 2014 from the same five herding families (BA, BJ, BT, MU, and SA). The horses' ages ranged from 2 to 18 yr, as indicated by the dentition; sex information was provided by the herders. The nomad's domestic horses are normally free roaming in the wild and are used for riding; the mares are milked. The five herding families lived at distances between 2 km and 20 km away from each other. Blood samples from a total of 19 takhi (yearlings to adults) were collected from the jugular vein by the staff of Hustai National Park and the Institute of Veterinary Medicine at Mongolia.

The DNA was extracted from each blood sample (100 µL) by the magnetic bead method and analyzed with a MagExtractor - Genome- kit (Toyobo, Osaka, Japan). After measuring concentrations, the DNA samples were stored at –30 C. The extracted genomic DNA was used as a template and was subjected to PCR with *T. equi* and *B. caballi* primers that target specific 18S rRNA regions (Alhassan et al. 2005). The PCR reaction was performed using the following set of primers to amplify DNA fragments of 392 and 540 base pairs from *T. equi* and *B. caballi*, respectively: Bec-UF2 5'-TCGAAGACGAT-CAGATACCGTCG-3', Equi-R 5'-TGCCTTAAAC TTCCTTGCGAT-3', and Cab-R 5'-CTCGTT CATGATTTAGAATT-GCT-3'. The reaction conditions were: pre-heating at 95 C for 5 min, denaturation at 94 C for 15 s, annealing at 60 C for 30 s, and extension at 72 C for 30 s, and reactions were carried out using a Takara ExTaq polymerase

(Takara, Siga, Japan); 35 cycles were performed using the Mastercycler (Eppendorf, Tokyo, Japan). For the determination of the protozoan sequence, two of the PCR-positive samples from each nomad's horse from the five nomadic families and the takhi samples were ligated into a pTA2 cloning vector (Toyobo), and then the cloned plasmid was subjected to the cycle sequence using Big-Dye® Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems, Waltham, Massachusetts, USA) and analyzed with an ABI sequencer (Applied Biosystems). Gene sequences were analyzed using the BioEdit Sequence Alignment Editor and MEGA6 (Tamura et al. 2013), and the search was performed using BLAST (National Center for Biotechnology Information 2016). The phylogenetic analysis was performed using the neighbor joining method in MacVector 15.1 (MacVector, Inc., Apex, North Carolina, USA). The 18S rRNA gene nucleotide sequence data for the comparison piroplasms were obtained from GenBank (*Theileria parva*: L02366; *Theileria annulata*: M64243; *T. equi*: AB515309, AB515315, AY150062, AY150063, AY150064, AY534882, EU642507, EU642508, EU642509, EU642510, EU642511, EU888902, EU888903, EU888905, EU888906, HM229407, JQ657703, KF559357, Z15105; *B. caballi*: AY309955, AY534883, EU642512, EU642514, EU888900, EU888901, EU888904, JQ288735, Z15104). All work was conducted under the approval of the research ethics committee for DNA experiments at Rakuno Gakuen University (approval no. 140).

In total, 92 domestic horses from five nomadic herding families were examined for the detection of *Piroplasma* protozoa by PCR method. The prevalence of *T. equi* in nomad's horses was 80% (74/92) in total, and the prevalence varied from 64% to 100% at each farm. *Babesia caballi* was detected in only one domestic horse (1%, 1/92) from a nomadic herder's family (Table 1). The prevalence of *T. equi* in takhi was 84% (16/19); however, there was no detection of *B. caballi* infection among takhi.

TABLE 1. Prevalence of piroplasm protozoa determined by PCR testing of domestic horses (*Equus caballus*) from nomadic herders and of takhi (Przewalski's horse, *Equus ferus przewalskii*) around Hustai National Park, Mongolia, in 2014.

Horses ^a	Percent positive (no. positive/no. tested)	
	<i>Theileria equi</i>	<i>Babesia caballi</i>
BA	100 (15/15)	0 (0/15)
BJ	65 (11/17)	0 (0/17)
BT	75 (15/20)	5 (1/20)
MU	80 (16/20)	0 (0/20)
SA	85 (17/20)	0 (0/20)
Total	804 (74/92)	1 (1/92)
Takhi	84 (16/19)	0 (0/19)

^a The domestic horses from each nomadic family were identified by the initials of the family.

We performed an examination to identify the strain of *T. equi* in both takhi and nomad's horses. Eight clones of *T. equi* from takhi were analyzed for sequencing at the 18S rRNA regions. We also examined the sequences of 10 clones from five different nomadic family's horses (two clones from each nomadic family's horses). The resulting 391 base pair fragment was compared with the corresponding sequence of *T. equi* given earlier. The results showed *T. equi* sequences from nomad's horses were the same sequence as previously reported from isolated strains in Mongolia (GenBank accession no. JQ657703; Fig. 1). The *T. equi* sequence from all the takhi was the same sequence as was found in the nomadic horses (GenBank accession no. LC229307, Takhi 2).

In Hustai National Park, the population of takhi has increased to 336 free-roaming animals. There is high mortality among newborns: On average, 50% of newborns die each year. Among takhi of other ages (yearlings, juveniles, and adult horses), approximately 10 to 15 die each year. The mortality incidence from *Piroplasma* infection was 19% (24/127) over 3 yr (Table 2). All individuals that died of piroplasmosis were young takhi born in the park. The rates of mortality from piroplasmosis were 19% (10/54) in 2012, 14%

(7/49) in 2014, and 29% (7/24) in 2015 of total deaths.

Equine piroplasmosis is one of the issues being raised that influences morbidity and mortality in the population of introduced takhi in Hustai National Park. However, the nomad's horses did not show mortality due to piroplasm infection during the observation period. The prevalence was 80% among nomad's horses and 84% among takhi in the national park from 2012 to 2015. All yearlings that died showed the same symptoms of loss of appetite, depression, and hair loss due to louse (*Damalinia equi*) infection. Those yearlings were usually feverish (>40 C), with nasal discharge and signs of anemia such as whitened mucous membranes. Approximately 12–17 ticks infested each yearling, and the tick was identified as *Dermacentor nuttalli*, which is a vector for *T. equi* and *B. caballi* (Battsetseg et al. 2001; Wise et al. 2013).

Nomad's horses have freely ranged in and out of the protected area, according to the park rangers. The tick vector that infests domestic horses might transmit piroplasm to takhi in the park. Understanding this disease and monitoring of takhi are important to understanding the prevalence of piroplasmosis and managing the hemoprotozoan disease risk in the national park. Piroplasm infection can be an important factor for the conservation of vulnerable reintroduced takhi, as the infection has spread among the population (Rüegg et al. 2006). Piroplasm infection could result in a decrease in reproduction due to abortions and deaths of juveniles. The reintroduction of takhi as a key species that affects the conservation of the ecosystem has brought a new understanding to the community in the country. The buffer zone surrounding the park environment is fully occupied with cropland and families who own livestock, thus causing the overgrazing of pastureland, and entry to pastures in the park transmits the diseases and parasites of the livestock and nomad's horses. Of special concern, nomad's horses can hybridize with takhi. It is necessary to keep track of such behavior in the future.

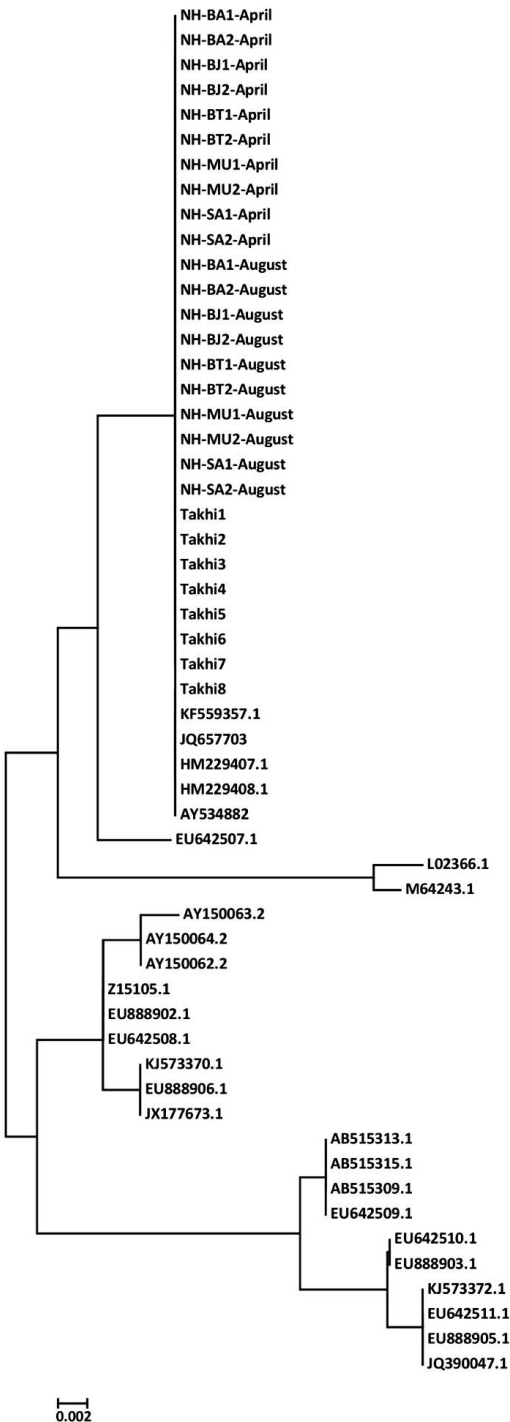


FIGURE 1. Phylogenetic analysis of *Theileria equi* in takhi (Przewalski's horse, *Equus ferus przewalskii*) and domestic horses (*Equus caballus*) in Hustai National Park, Mongolia, based on the 18S rRNA sequences of *T. equi* in two domestic horses (*E. caballus*) from each of

TABLE 2. The number of deaths of takhi (Przewalski's horse, *Equus ferus przewalskii*) due to piroplasmosis (*Theileria equi* and *Babesia caballi*) over 3 yr in Hustai National Park, Mongolia. All the takhi were born in Hustai National Park.

Mortality	2012	2014	2015 ^a	Total
Piroplasmosis	10	7	7	24
Total no. of death	54	49	24	127
Mortality rate (%)	18	14	29	19

^a The 2015 data were from January to June.

Although there is a limit to investigations of disease prevalence in wild horses, we focused on examining the prevalence of protozoan disease in both nomad's horses and takhi. We showed that *T. equi* infected both types of horses at high rates. Young takhi are a vulnerable population and are susceptible to death from *Theileria* infection. In conclusion, disease prevalence surveys of both nomad's horses and vectors in the habitat are important elements in the protection of takhi.

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