Molecular and serological surveillance of equine piroplasmosis in the Republic of Korea between 2016 and 2017

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Abstract

Equine piroplasmosis (EP), a tick-borne disease affecting horses, is caused by Babesia caballi and Theileria equi. We investigate antigen and antibody of EP in whole bloods and sera of horses in the Republic of Korea during 2016-2017. We collected 1,650 whole bloods and sera of horses in 16 regions and 222 farms to examine antigen and antibody of EP by polymerase chain reaction (PCR) of 18S rRNA gene and enzyme-linked immunosorbent assay (ELISA), respectively. T. equi antigen and antibody was detected in 1 of 1,650 samples (0.06%). Phylogenetic analysis of 18S rRNA revealed that T. equi was highly homologous with the strains from China, Mongolia, or Spain. At EP-positive ranch, 52 and 10 ticks were collected in 2016 and 2017, respectively. All of them were identified as Haemaphysalis longicornis, but EP pathogens were not detected from the collected ticks. From 1,650 whole bloods, 2 of Theileria spp. were also detected and highly homologous with T. buffeli, T. luwehshuni, and T. orientalis group. EP infectious status was very low in Korea, and routine surveillance should be needed to attain the free status of EP in Korea.

1. INTRODUCTION

Equine piroplasmosis (EP) is a tick-borne protozoal disease in which horses, mules, donkeys, and zebras are infected with the blood apicomplexans, *Babesia caballi* or *Theileria equi* from blood-sucking ticks (Onyiche et al., 2019; Ozubek & Aktas, 2018; Souza et al., 2019; Wise, Kappmeyer, Mealey, & Knowles, 2013). EP is not a fatal disease, but it causes serious economic damage and an important obstacle to international trade as major diseases designated by World Organization for Animal Health (Ozubek & Aktas, 2018; Seo et al., 2013; Seo et al., 2011; Short et al., 2012). EP is an endemic disease prevalent in the tropical and subtropical regions and in some temperate regions (Onyiche et al., 2019; Ozubek & Aktas, 2018; Wise et al., 2013). It has predominantly been reported in Asia, South and Central America, Africa, Southern Europe, and some parts of southern USA (Onyiche et al., 2019; Ozubek & Aktas, 2018; Wise et al., 2013). In Korea, EP by *T. equi* had been confirmed as 1.1% seroprevalence and 0.9% antigen prevalence from 224 horse samples from only three regions of whole country during 2007 - 2010 (Seo et al., 2013; Seo et al., 2011). After that, there was no surveillance of EP at national level, and it is needed to expand the monitoring the EP infectious status of all regions in Korea.

We examined antigens and antibodies from blood and serum of horses raised in the Republic of Korea (ROK) during 2016 - 2017, using polymerase chain reaction (PCR) for antigen detection and enzyme-linked immunosorbent assay (ELISA) for antibody detection. Subsequently, for antigen-positive samples, phylogenetic analysis was used to determine the genetic relationships with previous ones. In addition, the species of ticks distributed in the horse ranch and EP pathogens in the ticks were examined.

2.1 Sample collection

Whole blood and serum samples were collected by Korea Racing Authority as national horse seroprevalence of major infectious diseases according to the Animal Ethics regulations with the horse owners' informed consent. We surveyed the EP antigen and antibody in blood and serum samples collected from 1,650 horses at 222 horse stables in 6 metropolitan cities and 9 provinces. The collected blood and serum samples were stored at 4°C until use.

2.2 Molecular and serological detection of *T. equi* and *B. caballi*

Blood samples were tested for antigens of *B. caballi* and *T. equi* using polymerase chain reaction (PCR). DNA was extracted from the blood samples using a Maxwell RSC Whole Blood DNA kit (Promega, Madison, WI). DNA was eluted in 50 μL volumes of elution buffer and stored at -70°C until use. The PCR reactions included 2 μL of extracted DNA and 10 pmol of specific primer sets (Alhassan et al., 2005) (**Table 1**) and run on a C1000 Touch^{TM} Thermal Cycler (Bio-Rad, Pleasanton, CA) (95 for 10 min followed by 35 cycles at 95 for 1 min, 60 for 1 min, and 72 for 1 min; and finally 72 for 5 min). PCR-positive samples were sequenced using Macrogen Co. (Seoul, South Korea)

Nucleotide sequence homology searches were analyzed by the National Center for Biotechnology Information BLAST network service and aligned using the MegAlign software package (Windows version 7.1; DNA-STAR, Madison, WI, USA). Phylogenetic trees were generated using neighbor-joining algorithms and the Jukes and Cantor matrix. Support for topology was calculated using 1,000 bootstrap replications.

Serum samples were tested for anti-*B. caballi* and anti-*T. equi* antibodies by competitive enzyme-linked immunosorbent assay (cELISA) using *Babesia caballi* and *Theileria equi* antibody test kits (VMRD, Pullman, USA), respectively. cELISA tests were performed according to the manufacturer's recommendation. Briefly, 50 μl of serum samples and controls were loaded into the antigen-coated plate. After incubation, 50 μl of primary antibody was added to each well. And 50 μl of secondary antibody-peroxidase conjugate was added to each well. Finally, substrate solution and stop solution was consecutively added to each well and the ELISA plates were read on its optical density. Calculation of the percent inhibition (%I) was as follows according to the manufacturer's manual:

%I (Inhibition rate) = 100 [1 – (sample optical density [OD]/negative control OD)]

The one ELISA positive serum from Ulsan region was collected as a total of three times after 4 months and 1 year later.

2.3 Tick collection and tick PCR for detection of *T. equi* and *B. caballi*

Ticks were collected from the areas surrounding the farms that were confirmed to be positive for *T. equi.* BioQuip's tick drag was used to monitor ticks. Ticks were removed from traps, and transported to the laboratory where they were stored at -70°C until species identification by microscopic morphology on a cold table using standard keys (Yamaguti N, 1971). Up to 30 specimens were analyzed by species.

RESULTS AND DISCUSSION

During 2016-2017 in ROK, one of 1,650 horses (0.06%) was confirmed *T. equi* antigen and antibody (**Table 2**). In addition, *Theileria* spp. was found in 2 of the 1,650 horses (0.12%), which showed seronegative for *T. equi* antibody test. In the previous study in ROK, 83 samples in Jeju, 37 in Gyeongbuk and 104 in Gyeonggi were analyzed for EP pathogens (Seo et al., 2013), and the antigen positive rate of *T. equi* was 0.9%. *B. caballi* was not detected both studies. In this study, the samples were collected at national level in Korea, which were Jeju ($n = 661$), Gyeongbuk ($n = 114$), Gyeonggi ($n = 378$), and the other 12 regions ($n = 497$) (**Table 2**). In this study, *T. equi* was detected in Ulsan, while *T. equi* was found in Gyeonggi during 2007-2010 in the previous study (**Table 2**). The positive regions between two studies was not related epidemiologically each other. Ticks are an important vector to transmit EP. It has been known that there are 15 tick species that transmit *B. caballi* - 7 *Dermacentor*spp., 6 *Hyalomma* spp., and 2 *Rhipicephalus* spp. (Wise et al., 2013). And there are 14 *T. equi*- transmissible tick species, which are 4 *Dermacentor* spp., 4 *Hyalomma* spp., 5*Rhipicephalus* spp., and *Amblyomma cajennense* (Wise et al., 2013). In Korea, the predominant horse tick

species is *Haemaphysalis longicornis* (Seo et al., 2016). To date, EP-transmissible tick species have not been found in ROK (Jiang et al., 2019; Yun et al., 2014). We collected the 62 ticks in *T. equi* -positive horse ranch, which were 52 ticks (2 nymphs and 50 larvae) in 2016 and 10 (larvae only) in 2017. All of them were *H. longicornis* , in which EP pathogens were not detected. In 2010, the positive rate of *T. equi* was 0.9%, while in 2017, that was 0.06%. It might be implied that EP in the ROK has not spread from Gyeonggi after 2010. This result might be due to absence of the transmissible tick species of EP pathogens in ROK and no transport of *T. equi* -positive horses. However, the artificial infection experiments have reported that the propagation of *T. equi* by *H. longicornis* is possible (Ikadai et al., 2007). Therefore, it is needed consecutively to examine EP pathogens in horse ticks with identification of collected horse ticks.

The antibody titer of *T. equi* -positive horse at the first test was 54.63 %I using *T. equi* antibody test by cELISA kit. The second and third test of it were performed at 4 months and 1 year later, in which *T. equi* -antibody titers were showed 56.79 %I and 67.33 %I, respectively. At that time, we have also tested for 5 co-breeding horses with *T. equi* -positive horse in Ulsan at the same time to bleed *T. equi* -positive horse. No antibodies of *T. equi* or *B. caballi* were found in the other 5 co-breeding horses. In other countries, seroprevalence of *T. equi* in horses have been various from 78.8% to 0.0% depending on its infection and sanitation status. Sudan has the highest seroprevalence at 78.8%, followed by Mongolia (72.8%), Brazil (34.8%), and China (3.8%) (Boldbaatar et al., 2005; Elata et al., 2020; Li et al., 2019; Souza et al., 2019; Xuan et al., 2002). In Japan, no *T. equi* antibody-positive cases were found (Huang et al., 2006). Meanwhile, the seroprevalence (0.06%) of *T. equi* in ROK was close to "EP-free" status. *B. caballi* -positive horse was not found until now, but the seroprevalence of *B. caballi* was also different from each country. The seroprevalence of *B. caballi* was the highest in Mongolia at 40.1%, followed by Brazil (27.2%), China (20.1%), Japan (7%), and Sudan (5.1%) (Boldbaatar et al., 2005; Elata et al., 2020; Li et al., 2019; Souza et al., 2019; Xuan et al., 2002). To sustain "EP-free" status in ROK, a thorough border quarantine inspection should be conducted continuously and the monitoring of domesticated horses of EP should be performed consecutively. In addition, a preemptive monitoring system for horse ticks should be established, implementing the examination of EP pathogens.

Three of 1,650 horses (0.18%) were *Babesia* spp. and *Theileria* spp. dulex PCR-positive, which were collected from Ulsan, Gyeonggi-do, and Gyeongbuk-do (Table 1 and 2). All 3 positive samples were negative for *B. caballi* PCR. *T. equi* PCR revealed that one sample (0.06%) from Ulsan was positive (**Table 2**), in which *T. equi* antibody was also detected (**Table 2**). The 17H107 only identified as *T. equi* in this study was from a horse ranch located in Ulsan regions. This *T. equi* strain exhibited the highest genetic homology with two strains in ROK (HM229407, HM229408) through phylogenetic analysis of 18S rRNA gene (**Fig. 1A**). The *T. equi* found in this study was confirmed to be the same genotype as the previously detected Korean strain (**Fig. 1A**). Compared with those of other countries, 17H107 showed highly homologous with the strains found in Chinese (KF559357) and Spanish (AY534882) horse blood and the strain identified in Mongolian horse ticks (JQ657703) (**Fig. 1A**). It was genetically similar to strains prevalent in China, Mongolia, and Spain (**Fig. 1A**). In 2008, EP-positivity was confirmed in horses imported illegally from Mexico into USA (Short et al., 2012), indicating the importance of EP prevention through border quarantine. It is important to quarantine animals at international border for national-free infectious disease control. In addition, the phylogenetic analysis will give the molecular epidemiologic data between each country.

In this study, *Theileria* spp. was confirmed in 2 of 1,650 horses (**Fig. 1B**). The *Theileria* spp. identified in this study, 1st _427 and 2nd _128, was found to be similar to the *T. buffeli* derived from cattle and *T. luwenshuni* derived from deer in the ROK. In addition, the genetic homology was high with *T. luwenshuni* , *T. orientalis* , and *T. sinensis* from China and *T. annulata* from India (**Fig. 1B**). *T. luwenshuni* , *T. orientalis* , *T. sinensis* , and *T. annulata* have never been observed to infect horses in any previous report. Furthermore, there are no reports of *T. sinensis* and *T. annulata* among 4 *Theileria* spp. in Korea. Meanwhile, *T. luwenshuni* and *T. orientalis* have been reported in infected water deer and cattle in the ROK, respectively (Han et al., 2017; Park et al., 2019). Two horses confirmed with the infection of *Theileria* spp. showed no clinical signs. Thus, *Theileria* spp. identified in 2 of 1,650 horses might be non-to-mildly pathogenic *Theileria* spp., not *T. annulata* . It is needed to further identification of *Theileria* spp. at species level.

In conclusion, EP should be regularly monitored in horses reared within the ROK to maintain the EP-free status of the country. Furthermore, strengthening the surveillance system for this disease by examining the status of the distribution of horse ticks and pathogens in the ticks is important for proactively monitoring horse tick-borne infectious diseases, such as EP.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS APPROVAL

Field samples in this study were collected as part of the health management program by Horse racing authority in Korea. This article does not have any studies with human participants or animals done by any of the authors.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in this manuscript.

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Tables

Table 1. PCR primer sets for the detection of equine piroplasmosis

Primers	**Sequences (5'-3')**	**Products size (bp)**	**Target gene**	**Reference[a]**
Bec-UF1	GTTGATCCTGCCAGTAGTCA	*B. caballi*: 867 *T. equi* : 913	18S ribosomal RNA gene	1

Primers	**Sequences (5'-3')**	**Products size (bp)**	**Target gene**	**Reference[a]**
Bec-UR	CGGTATCTGATCGTCTTCGA			

[a] Reference: 1, Alhassan et al. (2005).

Table 2. Polymerase chain reaction and enzyme-linked immunosorbent assay findings for equine piroplasmosis in the Republic of Korea between 2016 and 2017

Regions	No. of farms	No. of horses	No. of Ag positive by PCR	No. of Ag positive by PCR	No. of Ag positive by PCR	No. of Ab positive by ELISA	No. of Ab positive by ELISA
			Babesia spp. or *Theileria* spp.	*B. caballi*	*T. equi*	*B. caballi*	*T. equi*
Seoul	2	28	-	-	-	-	-
Incheon	2	14	-	-	-	-	-
Daegu	2	10	-	-	-	-	-
Daejeon	2	12	-	-	-	-	-
Ulsan	4	16	1	-	1	-	1
Gwangju	5	24	-	-	-	-	-
Gyeonggi-do	24	378	1	-	-	-	-
Kangwon-do	7	96	-	-	-	-	-
Chungbuk-do	7	55	-	-	-	-	-
Chungnham-do	8	65	-	-	-	-	-
Gyeongbuk-do	21	114	1	-	-	-	-
Gyeongnham-do	12	57	-	-	-	-	-
Jeonbuk-do	13	62	-	-	-	-	-
Jeonnham-do	8	55	-	-	-	-	-
Jeju-do	102	661	-	-	-	-	-
Others	3	3	-	-	-	-	-
Total (%)	222	1,650 (100.00)	3 (0.18)	0 (0.00)	1 (0.06)	0 (0.00)	1 (0.06)

Ag: Antigen; Ab: Antibody; PCR: polymerase chain reaction; ELISA: enzyme-linked immunosorbent assay

Figure Legends

Fig. 1. Phylogenetic relationship of *Theileria equi* from an infected horse and *Theileria* spp. in the Republic of Korea

The 18S rRNA region sequences of *T. equi* and *Theileria*spp. in this study, 17H107 from the Ulsan horse,

1^{st}_427 from the Gyeonggi-do horse, and 2^{nd}_128 from the Gyeongbuk-do horse, were evaluated for their evolutionary relationships with the known sequences of *Theileria equi* (A) and *Theileria* spp. (B).

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