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Theileria buffeli infections in grazing cattle in the Republic of Korea

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Abstract. *Theileria* infections are encountered worldwide, occasionally resulting in serious economic losses for the livestock industry. This study is an epidemiological survey of *Theileria* infections in Korean indigenous cattle populations in the Republic of Korea (ROK). Blood samples were collected from 100 cattle in April (n=50) (prior to pastureland grazing), and again four months later, in August (n=50) (half of the cattle put out for grazing and the other half kept in housing). All samples were tested for the presence of *Theileria* infection based on PCR amplification of the small subunit of ribosomal RNA gene. Twenty-two samples across the whole study were verified as positive for *Theileria* infection by PCR methods. In August, *Theileria* infection was markedly increased in grazing cattle (16/25 animals, 64%) compared with indoor cattle (4/25 animals, 16%); affected animals exhibited no clinical signs of infection. The red blood cell, hematocrit, and hemoglobin values were significantly lower in *Theileria*-positive cattle than in *Theileria*-negative cattle. Phylogenetic analysis demonstrated that the isolates from this study belonged to the *T. buffeli* species, and were significantly related to Types A, B, C, and E, and were distinct from *T. buffeli* Type D, which is known to be more pathogenic. These findings indicate that *T. buffeli* identified in Korean indigenous cattle have a low-to-mild pathogenicity. These results suggest that the *T. buffeli* infection is relatively higher in the ROK, and the infection rate may increase following grazing. Taken together, *T. buffeli* infection may not only be seasonally correlated, but also may be affected by management practices such as pastureland grazing.

INTRODUCTION

Theileriosis is a tick-borne infectious disease caused by parasitic protozoa belonging to the species *Theileria*, and is transmitted by ixodid ticks of the genus *Haemaphysalis* (Perera *et al.*, 2013). *Theileria* parasites affect a range of domestic and wild animals, particularly ruminants, and cause economically significant diseases in the livestock industry worldwide. *Theileria* species are divided into two groups, host-cell transforming (*T. parva*, *T. annulata*, *T. lestoquardi*, and *T. taurotragi*) and non-transforming (*T. orientalis*, *T. mutans*, *T. velifera*, and *T. cervi*), based on the ability to transform host leukocytes (Sivakumar *et al.*, 2014). Whereas *T. parva* and *T. annulata* are considered the most pathogenic to cattle, *T. sergenti*, *T. buffeli*, and *T. orientalis*, are known to be the main causative agents of benign theileriosis. Accurate classification of these benign parasites is difficult, as it is complicated by their similar morphology, serology, and mechanism of vector transmission (Kakuda *et al.*, 1998). In fact, it is unclear whether these organisms represent the same species or even several different

species. Of the three species mentioned above, *T. sergenti* is not a valid one, since it is known to be a sheep parasite (Uilenberg, 2011). *T. orientalis* is widely distributed in many Asia-Pacific countries (Altangerel *et al*., 2011; Aparna *et al*., 2011; Inoue *et al*., 2001; Kamau *et al*., 2011; Yokoyama *et al*., 2011) and infects a variety of hosts, such as cattle, buffalo, and yaks (Altangerel *et al*., 2011; Fujisaki *et al*., 1994; Yin *et al*., 2004). *T. buffeli* refers to a cluster of benign species within the *T. buffeli/orientalis* group, which have been grouped together based on molecular data and their ability to infect buffalo (Chaisi *et al*., 2014; Gubbels *et al*., 2000). Clinical symptoms caused by infection with the *T. buffeli/orientalis* group may include fever, hemolytic anemia, anorexia, depression, jaundice, abortion, and recumbency; in severe cases, the infections can be associated with high morbidity rates and death (Ceci *et al*., 1997; Izzo *et al*., 2010; Kamau *et al*., 2011; Stockham *et al*., 2003). Research aimed at understanding the diversity within the *T. buffeli/orientalis* group is still in progress.

Although epidemiological studies of localized *T. buffeli* disease outbreaks have been previously conducted in several countries (Chaisi *et al*., 2014; Gubbels *et al*., 2000; Hornok *et al*., 2014; Ochirkhuu *et al*., 2015; Yu *et al*., 2010), comprehensive detailed analyses of these infections are still lacking. The objective of this study was to investigate the prevalence of *T. buffeli* infections before and after pasture grazing in the Republic of Korea (ROK). Therefore, we determined the genetic variation between Korean isolates identified in this study and known *T. buffeli* genotypes as well as their pathogenicity.

MATERIALS AND METHODS

Ethical statement

The collection of animal blood samples were carried out according to ethics guidelines for the use of animal samples as permitted by Chonbuk National University (institutional animal care and use committee [IACUC] decision No. CBU 2014-00026).

Blood sample collection

This study was conducted using Korean indigenous cattle from Hongeoeng, Kangwon province, ROK, in 2014. Historically, the herds from Heongseong graze on pastureland during warmer months (May-October) and are housed in stables during cooler months (November-April). Blood samples were collected from the cattle in April (prior to pastureland grazing), and again four months later, in August (half of the cattle put out for grazing and the other half kept in housing) (Table 1). All cattle studied displayed no outward clinical symptoms. Whole blood samples were frozen immediately and stored at -80°C until DNA extraction was performed.

Hematological examinations

Blood samples from each animal were collected in Vacutainer® tubes containing EDTA (Beckton Dickinson, Franklin Lakes, NJ, USA). Blood tests including red blood cell (RBC), hemoglobin (Hb), hematocrit (HCT), and white blood cell (WBC) counts were determined using the VetScan HM5 Hematology System (Abaxis, Union, CA, USA). Animals with RBC, HCT or Hb values lesser than 5.0 x 10^{12}/L, 24%, and 8 g/dL, respectively, were considered as anemic.

PCR, sequencing, and phylogenetic analysis

Genomic DNA was extracted from whole blood samples using the DNeasy Blood & Tissue Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. *Theileria* infection was diagnosed by PCR amplification of the small subunit of ribosomal RNA (SSU rRNA), using gene specific primers (yielding a 1800 bp PCR product) (Kamau *et al*., 2011), based on previously described conditions (Chae *et al*., 1998a). The PCR products were purified with the QIAquick PCR purification Kit (Qiagen) and cloned directly into the pGEM®-T Easy vector, according to the manufacturer's instructions (Promega, Madison, WI, USA). Clones containing an insert of the correct size were chosen and sequenced on both strands using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster

City, CA, USA). An ABI PRISM® DNA Analyzer (Applied Biosystems) was used to determine the nucleotide sequences, and the sequence data were initially aligned using Clustal X (version 1.8) (Thompson *et al.*, 1997). Additional sequences from representative isolates of *T. buffeli*, as well as other highly virulent species, were obtained from the GenBank database and included with each set of alignments. Based on the nucleotide alignments, a phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987). Bootstrap analysis was carried out using 1,000 replications, and the phylogeny was visualized using TreeView software (Page, 1996).

Statistical analysis

Statistical analyses were performed using a one-way ANOVA with IBM SPSS Statistics (version 19.0) and GraphPad Prism (version 6.0) software. In all statistical tests, P values <0.05 were considered significant. In addition, the seasonal changes in hematological parameters, especially due to grazing of animals were also analyzed. Data were expressed as mean ± standard deviation (SD). Normally distributed quantitative data were analyzed by a two-tailed, two-sample, Student's t test assuming unequal variance; variation was presented as SD.

RESULTS AND DISCUSSION

To determine the prevalence of *Theileria* infection in cattle, prior to and following pasture grazing, blood samples were collected and analyzed for the presence of *Theileria* genomic DNA by PCR. As summarized in Table 1, 22 of 100 blood samples (22%) were found positive for *Theileria* infection based on the presence of the *Theileria* SSU rRNA gene. The prevalence of infection in cattle housed indoors was lower at the beginning of the study (April) than when cattle were allowed to graze for four months until the end of the study (August) (Table 1). In the case of cattle that were housed indoors during the entire period of the study, the infection rate increased from 4% (at the beginning) to 16% (at the end of study). However, in animals that were housed indoors, and then allowed to graze on pasture for four months, the prevalence of *Theileria* infection considerably increased from 4% to 64% (Table 1). Despite the high percentage of *Theileria* infection in grazing cattle, none of them exhibited any clinical manifestations. As shown in Table 2, for cattle housed indoors for the duration of the study, RBC, Hb, and HCT values were decreased slightly at the end of study compared to the beginning of study. However, for pasture-grazed cattle, these parameters were significantly reduced in *Theileria*-positive cattle compared to negative animals (Table 2). The WBC profiles did not show any consistent changes across the different groups (Table 2).

To examine variations within the *Theileira* SSU rRNA gene isolated from different cattle, we randomly selected 22 PCR-positive samples, cloned the PCR products, and sequenced them. The samples were found to share 99.6%–100% sequence homology, and the two representative sequences identified were included in the

Table 1. Prevalence of *Theileria buffeli* infection in calves

Location	Number of samples	Season	*Theileria*-positive cattle
Hoengseong	100	April (Housing, n = 50) ‡	2/50
		August (Housing, n = 25)	4/25
		August (Grazing, n = 25)	16/25

This farm raised cattle both in housing and on pastureland.
‡Sampling occurred prior to release for pastureland grazing
Theileria infection in Korean indigenous cattle, before and after pastureland grazing, assessed by PCR

Table 2. Haematological changes in *Theileria buffelis*-infected calves

Season	Growth type	*T. buffelis* infection	RBC (M/mL) 5.0–10.0	Hb (g/dL) 8.0–15.0	HCT (%) 24.0–46.0	WBC (M/μL) 4.0–12.0
April	Housing (n=50)	Negative (n=48)	10.3±1.1	12.2±1.2	37.0±3.5	8.1±2.2
		Positive (n=2)	10.6±0.6	12.4±0.8	36.8±0.7	9.0±1.5
August	Housing (n=25)	Negative (n=21)	9.6±1.3	11.8±1.1	35.3±2.9	9.0±1.8
		Positive (n=4)	9.5±0.8	11.3±1.0	34.3±2.5	9.8±3.0
	Grazing (n=25)	Negative (n=9)	9.2±0.9	11.2±0.9	33.6±2.6	11.5±3.5
		Positive (n=16)	8.6±1.1*	10.2±1.2**	32.6±3.1**	11.3±1.9

* denotes P <0.05, compared to *Theileria*-negative cattle
** indicates P <0.01, compared to *Theileria*-negative cattle
Abbreviations: RBC = red blood cells; Hb = hemoglobin; HCT = hematocrit; WBC = white blood cells
Normal reference values were established with data obtained from Schalm's Veterinary Hematology (Feldman *et al.*, 2000).

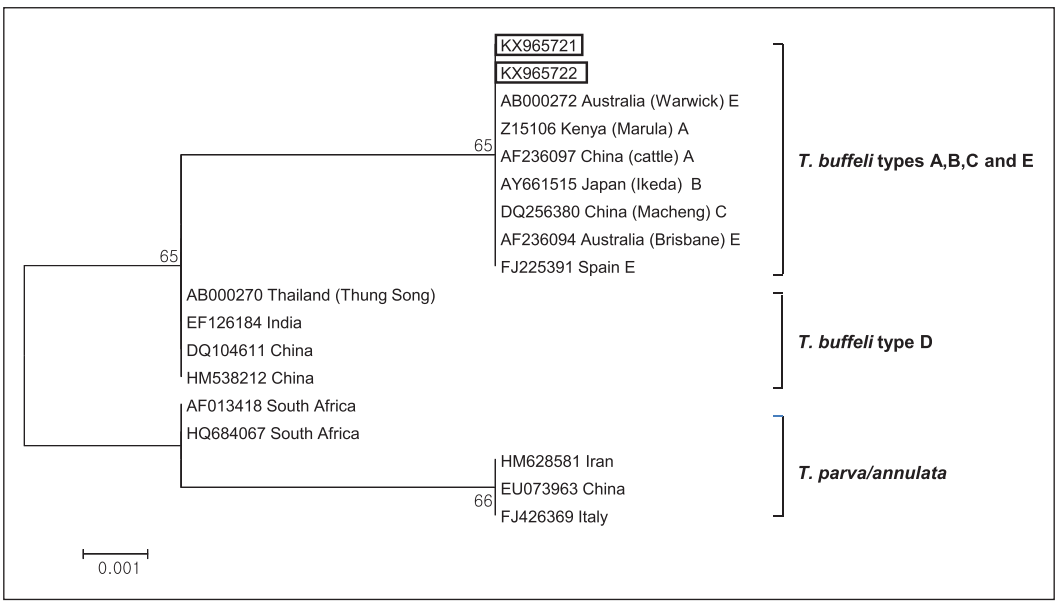

Figure 1. Phylogenetic analysis of known *T. buffeli* SSU rRNA sequences along with sequences of the 18S SSU rRNA gene derived from *Theileria*-positive cattle blood isolated in the present study. Also included are previously registered Genbank sequences for *T. annulata* and *T. parva*. The gene sequences identified in the present study are shown in the black-lined box. The unrooted phylogenetic tree was constructed using the neighbor-joining method. Bootstrapping was carried out using 1,000 replications.

phylogenetic tree. The sequences obtained from in this study were deposited in GenBank database under the accession numbers KX965721-KX965722. Phylogenetic analysis showed that Korean isolates were identified as *T. buffeli*, and these isolates were clearly distinguishable from those of *T. annulata* and *T. parva* (Fig. 1). The first clade comprised *T. buffeli* Types A, B, C, and E, and the second clade was closely related to *T. buffeli* Type D (Fig. 1). The isolates derived from grazing cattle showed very high similarity (98.6%–99.1%) to *T. buffeli* Types A, B, C, and E isolated from different geographical locations, and were obviously distinct from pathogenic *T. buffeli* Type D (Fig. 1).

The present study demonstrated that *T. buffeli* infections are prevalent in Korean indigenous cattle. Given the large number of grazing cattle found positive for *T. buffeli* infection, this indicates that the cattle became infected with *T. buffeli* after being put out for pasture grazing. Despite the fact that none of the *T. buffeli*-infected cattle exhibited any clinical signs of disease in this study, the RBC, HCT, and Hb values were significantly decreased in *T. buffeli*-positive grazing cattle compared to housed cattle (Table 2). Finally, the phylogenetic analyses reported here indicate that Korean isolates are closely related to *T. buffeli* identified in cattle from several different countries (Fig. 1) (Chae *et al*., 1998b; Chaisi *et al*., 2014; Gubbels *et al*., 2002).

Theileria buffeli is a species of *Theileria* that is present in some parts of Asia and belongs to the *T. orientalis/buffeli/sergenti* group (Chae *et al*., 1998a; Kamau *et al*., 2011). The taxonomy of this group has been debated for many years. Based on molecular and serological studies, these three species have actually been proposed to be one species, namely *T. orientalis* (Sivakumar *et al*., 2014; Kamau *et al*., 2011). A previous study, in the ROK, showed that *T. buffeli* could be identified by analysis of the major piroplasm surface protein (MPSP) gene (Chae *et al*., 1998a; Yu *et al*., 2010). In this study, we chose to determine *T. buffeli* infection using the sequence of the 18S SSU rRNA gene. The resulting phylogenetic analysis revealed that Korean isolates formed one group, together with *T. buffeli* Types A, B, C, and E, and were found to be distinct from *T. buffeli* Type D. The results show that there is genetic variation among *T. buffeli* types in agreement with other reports (Chae *et al*., 1998b). Types A, B, C, and E are mainly cattle parasites and are considered to have low-to-mild pathogenicity, whereas *T. buffeli* type D is considered more pathogenic than the other *T. buffeli* types. These results suggest that the *T. buffeli* identified in this study have low-to-mild pathogenicity.

The hematological analyses conducted during the month of August showed that RBC, Hb, and HCT values were significantly lower in *Theileria*-positive grazing cattle than in housed cattle. This result supports the possibility that cattle may be at higher risk for exposure to ecto-parasite infections through grazing. Based on our findings, the increase in *T. buffeli* infection during the grazing period could be closely related to the seasonal activity of tick vectors in the pasture. However, no ticks were collected from the cattle in this study, hence no firm conclusion about tick activity can be made. According to our results, the incidence and infection rates of *T. buffeli* were lower in housed cattle than in grazing cattle over the same period. This can be explained by the fact that the tick vector is less capable of transmitting the infectious organism to housed livestock compared to animals grazing on pastureland. The vectors for this organism might act as a source of infection in naïve cattle, and thus, have an effect on the epidemiology of theileriosis in the ROK. As a result, this infectious disease may become an increasingly serious problem for the livestock industry in the ROK.

In conclusion, our study demonstrated a high prevalence of *T. buffeli* infection in the small population studied, and that these infections are more common in grazing areas than in indoor housing areas. However, the small number of animals examined in the present study may not be sufficient to draw conclusions regarding the status of this disease in the broader cattle population in the ROK. Therefore, extensive epidemiological studies will be required to gain a better understanding of the geographic distribution of this parasite in the ROK and the relationship with tick vector populations.

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Conflict of interest

The authors declare that no conflicts of interest exist.

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