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Short communication

Occurrence and genetic diversity of *Blastocystis* in Korean cattle

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ABSTRACT

Blastocystis is one of the most commonly detected intestinal protozoan parasites worldwide and has been found in humans and other animals. Therefore, many countries have actively researched this parasite. However, to our knowledge, no study of *Blastocystis* has been conducted in Korea. Therefore, we conducted a study of the current status of *Blastocystis* infection in domestic cattle, the various genotypes involved, and its zoonotic potential through a phylogenetic comparison with subtypes found in other studies. The feces of cattle were randomly collected throughout Korea; basic information, including collection date, sex, and cattle type was recorded, and DNA extraction, PCR, and phylogenetic analyses were performed. A total of 1,512 fecal samples were tested. The 101 *Blastocystis*-positive samples were obtained, yielding an approximate infection rate of 6.7%. Differences in age, cattle type, fecal type, and season were statistically significant between *Blastocystis*-positive and -negative cattle. In this study, four subtypes of *Blastocystis* (ST1, ST5, ST10, and ST14) were confirmed by phylogenetic analysis. ST1 and ST5 are potential zoonotic subtypes, therefore the possibility of zoonotic transmission cannot be ignored. Further research and clarification of the infection and transmission patterns of *Blastocystis* are warranted.

1. Introduction

Blastocystis is one of the most common intestinal protozoan parasites detected in humans as well as many animals, including cattle. It is found worldwide in healthy and symptomatic humans and other animals (Lepczyńska et al., 2017). Transmission pathways and pathogenesis are thought to involve the fecal–oral route and the cyst form, but this is still under debate, because sufficient evidence is lacking (Ramírez et al., 2016; Song et al., 2017). Thus far, a total of 17 subtypes have been identified via small subunit rDNA (SSU rRNA) gene sequencing in mammals and birds (Alfellani et al., 2013).

The infection rates of animal handlers, such as those working in research institutions, zoos, and abattoirs, are higher than those of people who are not in frequent contact with animals. Thus, it is necessary to consider the zoonotic potential of *Blastocystis* (Abe et al., 2002). Many countries are studying the zoonotic potential and host range of *Blastocystis* and are reporting new subtypes. In the United Kingdom, genetic diversity was recently revealed in non-primate animal infection of *Blastocystis* (Betts et al., 2018). In China, a possible novel subtype was reported in goats, cattle, and sheep (Song et al., 2017; Zhu et al., 2017; Li et al., 2018). In France, a potential zoonotic risk in zoo animals was reported (Cian et al., 2017).

However, studies on *Blastocystis* in Korea are lacking. This study is the first attempt at *Blastocystis* research in Korean cattle. The aim is to identify the current state of infection in domestic breeding cattle, the associated genetic diversity, and the zoonotic potential through a phylogenetic comparison with subtypes found in other studies.

2. Materials and methods

2.1. Study area and collection of fecal samples

Korea is located between 34°200 and 37°110 North and 126°070 and 129°190 East, with an annual mean precipitation of 1,300 mm (Jung et al., 2014). Fecal samples were collected from a total of 1,512 cattle throughout Korea from November 2013 to October 2017. Samples were collected from more than 520 farms by practicing veterinarians, and only one sample was collected from each individual. Basic information, including collection date, sex, cattle type (dairy or beef), region, and fecal type, was recorded. Regions were classified into three

Abbreviations: SSU rRNA, small subunit rDNA

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groups according to provincial administrative boundaries: northern (Gangwon and Gyeonggi), central (Chungbuk, Chungnam, Gyeongbuk, and Jeonbuk), and southern (Gyeongnam and Jeonnam). Any information that was unclear or questionable was marked as unknown. The minimum number of samples required for the study was 246 according to the formula $n = \frac{1.96^2 p_{exp}(1-p_{exp})}{d^2}$ (Thrusfield, 2005), with an expected prevalence (p_{exp}) of 20% and a desired absolute precision (d) of 5%. An average 2.9 fecal samples were collected per farm, with a standard deviation of 8.8.

2.2. DNA extraction, PCR, and sequencing

DNA was extracted with the QIAamp® Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions, and stored at −20 °C until use. PCR amplification was done using the AccuPower® HotStart PCR Premix (Bioneer, Daejon, Korea). *Blastocystis* was detected based on the SSU rRNA gene using the RD5 (5′ ATCTGG TTGATCCTGCCAGT 3′) and BhRDr (5′ GAGCTTTTTAACTGCAACAACG 3′) primers (Scicluna et al., 2006; Ramírez et al., 2014). In each tube, 17 μL of solution containing the pair of PCR primers (1 μL RD5, 1 μL BhRDr), 15 μL of distilled water, and 3 μL of template DNA were mixed.

All PCR amplifications were performed using the Mastercycler Pro (Eppendorf, Hamburg, Germany). The conditions used were initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min. The PCR products were analyzed by 1% agarose gel electrophoresis with ethidium bromide staining. Finally, positive products (unseparated tubes of PCR mix) were sent to Macrogen (Seoul, Korea) for direct DNA sequencing.

2.3. Phylogenetic and statistical analysis

Thirty samples from the positive PCR products were sequenced and their characteristics, including region, season, and fecal type, were examined. The characteristics of the selected samples were as follows: region [northern (n = 6), central (n = 12), and southern (n = 12)]; season [spring (n = 7), summer (n = 7), autumn (n = 10), and winter (n = 6)]; and fecal type [hemorrhagic (n = 5), watery (n = 7), pasty (n = 9), normal (n = 6), and unknown (n = 3)]. Of these 30 sequenced samples, 10 sequences (see below) were used for phylogenetic analysis after eliminating overlapping data.

Phylogenetic analysis was performed using the *Blastocystis* SSU rRNA gene, and a phylogenetic tree was constructed using MEGA7 software (Pennsylvania State University, State College, PA, USA). Phylogenetic inference was conducted by the maximum likelihood method with 1,000 bootstrap replications. Statistical analysis was performed with the χ^2 test using SPSS V. 23.0 (SPSS Inc., Chicago, IL, USA); p-values < 0.05 were considered statistically significant. "Unknown" data were excluded from statistical analysis.

The 10 sequences used in phylogenetic analysis were submitted to GenBank as accession nos. MH201330, MH201331, MH201332, MH201334, MH201335, MH201336, MH201337, MH201338, MH201339, and MH201341.

3. Results and discussion

A total of 1,512 fecal samples were tested. The 101 *Blastocystis*-positive samples were obtained, indicating a 6.7% infection rate. The following factors were considered in the analysis: cattle type, age, sex, fecal type, region, and season (Table 1). The overall infection rate in this study was lower than that reported for other countries in previous studies. Recent results for Chinese cattle revealed a 10.3% infection rate (Zhu et al., 2017), which was similar to the 9.6% infection rate in Iran (Badparva et al., 2015). However, these results are somewhat different from those reported in the United States (19.2%) (Fayer et al., 2012) and the United Kingdom (22.6%) (Alfellani et al., 2013). Even in the same species or breed, the incidence may vary depending on differences in environment, such as geographical factors. Since no positive control was tested during amplification, inhibitors present in the animal feces may account for the low prevalence observed.

Table 1
Infection of *Blastocystis* spp. in Korean cattle.

Group		No. tested	No. positive (%)	χ^2	*p*-value
Cattle type	Beef	1,285	90 (7)	4.151	0.040*
	Dairy	106	2 (1.9)		
	Unknown	121	9 (7.4)		
Age	< 3 m	1,089	44 (4)	75.576	< 0.001*
	3 m–1 y	197	41 (20.8)		
	> 1 y	111	8 (7.2)		
	Unknown	115	8 (7)		
Sex	Male	517	25 (4.8)	1.403	0.276
	Female	291	9 (3.1)		
	Unknown	704	67 (9.5)		
Fecal type	Hemorrhagic	133	10 (7.5)	51.027	< 0.001*
	Pasty	627	35 (5.6)		
	Watery	524	24 (4.6)		
	Normal	90	22 (24.4)		
	Unknown	138	10 (7.2)		
Region	Northern	389	26 (6.7)	0.114	0.960
	Central	309	19 (6.1)		
	Southern	763	51 (6.7)		
	Unknown	51	5 (9.8)		
Season	Spring	404	17 (4.2)	33.047	< 0.001*
	Summer	422	53 (12.6)		
	Fall	348	15 (4.3)		
	Winter	279	12 (4.3)		
	Unknown	59	4 (6.8)		
Total		1,512	101 (6.7)		

m, months; y, year.
* Statistically significant ($p < 0.05$) correlation with infection.

In this study, the correlation between *Blastocystis* infection and cattle type was statistically significant (p = 0.040), and the infection rate in beef cattle (7.0%) appeared higher than that in dairy cattle (1.9%). This discrepancy was assumed to be due to the difference in the number of tested samples; since too few dairy cattle fecal samples were collected for adequate statistical analysis, the observed infection rate may be lower than the actual rate. Further studies including more dairy cattle samples are warranted.

According to age data, the highest infection rate was observed in cattle aged 3 months to 1 year (20.8%), followed by adults aged over 1 year (7.2%), with the lowest infection rate observed in calves aged less than 3 months (4.0%). These results are similar to those in a report on Chinese cattle (Zhu et al., 2017), and to reports of *Blastocystis* infection in other animals and in humans. In humans, the infection rate is lowest in young children and increases with age, but the rate tends to decrease in adults (Beyhan et al., 2015). In pigs, the rate is higher in weaned and adult pigs than in piglets, indicating that the age of animals is an important factor (Navarro et al., 2008).

Gender data revealed that the infection rate in males was slightly higher than that in females (p = 0.276). Specifically, 4.8% of males and 3.1% of females were positive for *Blastocystis* infection. This slight difference was also observed in previous reports (Beyhan et al., 2015; El Safadi et al., 2016). However, since the highest infection rate was found in the unknown sex group in the present study, further study will be necessary to clearly confirm sex as a risk factor.

When considering fecal type, the infection rate in cattle with normal feces (24.4%) was about 5-times higher than that in cattle with diarrhea (5.4%). Regarding the type of diarrhea, the infection rates of 7.5% in the hemorrhagic group, 5.6% in the pasty group, and 4.6% in the watery group were not significantly different. This suggests that