Research paper

Multilocus typing of *Cryptosporidium* spp. in young calves with diarrhea in Korea

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ABSTRACT

We assessed the prevalence and performed molecular analysis of *Cryptosporidium* spp. in diarrheal feces of calves in Korea. Diarrheal fecal samples were collected from 951 young calves (<3 months) on 425 farms. *Cryptosporidium* prevalence was assessed by PCR and ELISA, and molecular characterization was performed by targeting the 18S rRNA, heat-shock protein 70 (*hsp70*), and glycoprotein 60 (*gp60*) genes. Data were analyzed according to the sex, type of cattle, region, season, and type of diarrhea. PCR analysis revealed *Cryptosporidium* spp. in 9.9% (94/951) of diarrheal fecal samples. *C. parvum* and *C. bovis/ryanae* were present in 6.1% (58/951) and 4.1% (39/951) of diarrheal fecal samples, respectively. In addition, ELISA showed positive results for *C. parvum* in 9.7% (92/951) samples. Statistical analysis of the PCR and ELISA results revealed a lower prevalence of *C. parvum* in the hemorrhagic diarrheal samples ($P < 0.05$). For *C. bovis/ryanae*, seasonality and high prevalence in hemorrhagic diarrhea were observed ($P < 0.05$). Of the 951 samples tested for *C. parvum*, 903 samples showed agreement with a κ value of 0.65, indicating good agreement between the two tests. Although *C. bovis* and *C. ryanae* share highly similar 18S rRNA sequences, PCR based on *hsp70* successfully distinguished *C. bovis* from *C. ryanae*. Sequence analysis of *gp60* revealed that *C. parvum* belonged to the IIa families and was further subtyped as IIaA18G3R1 and IIaA16G3R1, which have not been previously reported in Asia. These findings indicate that *Cryptosporidium* spp. play an important role in diarrhea in young calves in Korea. Considering the zoonotic significance of *C. parvum* IIa subtype and dense rearing system of cattle in Korea, prevention and continuous monitoring of *Cryptosporidium* are required.

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1. Introduction

Cryptosporidium is an apicomplexan protozoan parasite that is distributed worldwide (Ryan et al., 2014; Wang et al., 2014). The genus *Cryptosporidium* includes more than 30 species that affect different hosts (Ryan et al., 2014; Shrestha et al., 2014; Mirhashemi et al., 2015). For example, *Cryptosporidium parvum*, *C. andersoni*, *C. bovis*, *C. hominis*, and *C. ryanae* infect cattle, while *C. parvum* and *C. hominis* are responsible for human cryptosporidiosis (Smith et al., 2005; Ryan et al., 2014).

Cryptosporidium is transmitted via the fecal-oral route, and water plays an important role in the transmission (Karanis et al., 2007; Baldursson and Karanis, 2011; Moon et al., 2013). *Cryptosporidium* causes cryptosporidiosis, leading to diarrhea in various vertebrates. The clinical signs of cryptosporidiosis range from self-limiting to severe diarrhea in cattle and can cause death in small children or immunocompromised humans (Del Chierico et al., 2011; Mirhashemi et al., 2016). This disease affects 57,000 people annually in the United States (Scallan et al., 2011) and approximately 20% of children with diarrhea in developing countries (Mosier and Oberst, 2000).

In Korea, cryptosporidiosis was first identified in 1986 in chickens (Mo et al., 1988); subsequent studies identified *Cryptosporidium* infection in humans and animals (Rhee et al., 1991; Cho et al., 1993; Wee et al., 1996; Chai et al., 2001; Moon et al., 2013). A recent waterborne outbreak of cryptosporidiosis in humans prompted several
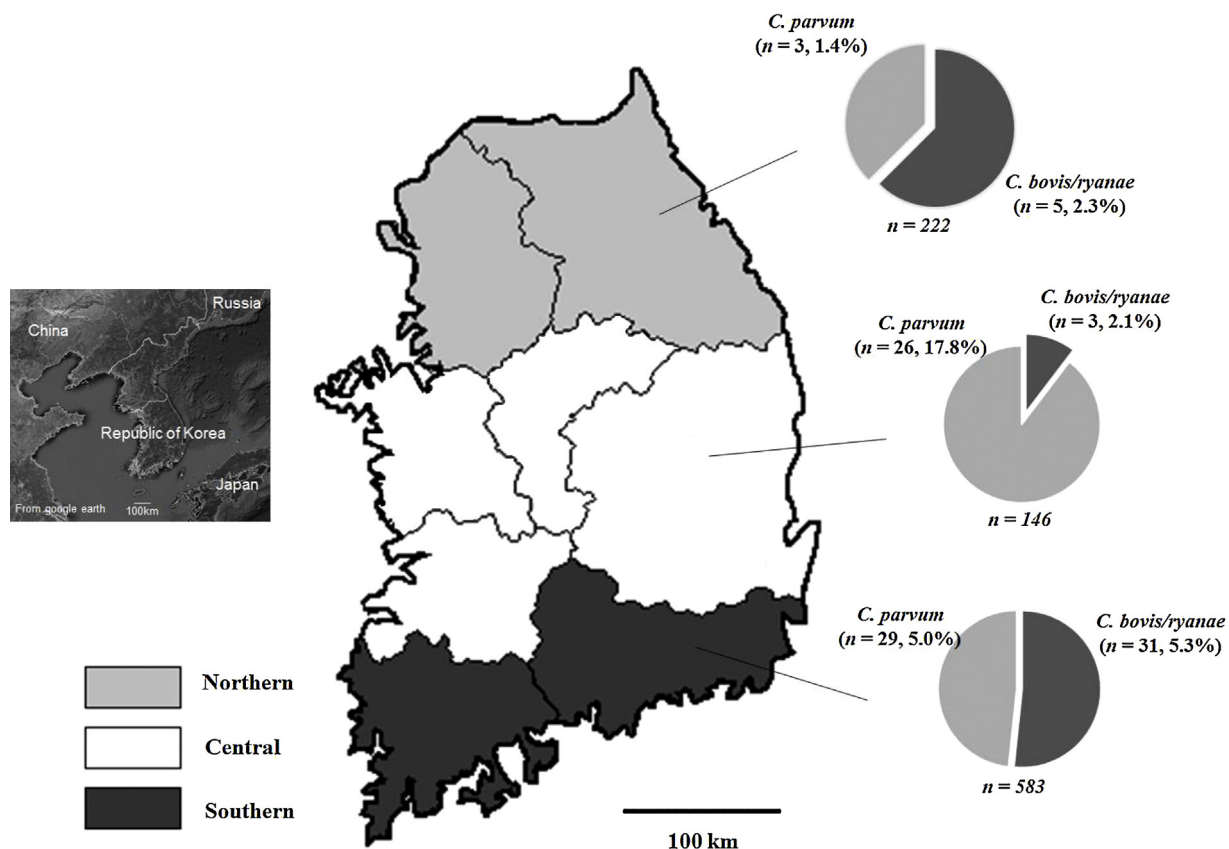
Fig. 1. Map of Korea showing the sampling points at which diarrheal feces from calves were collected and the prevalence of *Cryptosporidium* spp. determined by PCR analysis. For statistical analysis, the samples were assigned to the northern, central, and southern groups according to administrative boundaries.

studies on *Cryptosporidium* spp. in Korea (Moon et al., 2013). In calves, *Cryptosporidium* spp. were identified in the diarrheal feces of calves by using dimethyl sulfoxide-modified acid-fast staining, enzyme-linked immunosorbent assay (ELISA), and indirect immunofluorescence antibody assay (Wee et al., 1996). Previous studies on animals and humans in Korea focused on the regional prevalence, and only a few studies have evaluated the molecular characteristics of *Cryptosporidium*.

Cattle are one of the most important animals in the livestock industry in Korea. Approximately three million cattle, including dairy and beef cattle, are reared in Korea annually (Oh et al., 2016). According to the National Animal Health Monitoring System for U.S. dairy industries, diarrhea is responsible for 57% of deaths of weaning calves (US Department of Agriculture, 2008). Therefore, it is important to determine the cause of diarrhea in calves in order to prevent economic losses in the livestock industry.

The purposes of the present study were two-fold. First, we evaluated the prevalence of *Cryptosporidium* spp. in the diarrheal feces of calves reared in Korea, using PCR and ELISA. Second, genetic typing of *Cryptosporidium* spp. was conducted based on the gene sequences of 18S rRNA, heat-shock protein 70 (*hsp70*), and glycoprotein 60 (*gp60*).

2. Materials and methods

2.1. Study area and collection of diarrheal feces

Korea is located between 34°20′–37°11′ northern latitude and 126°07′–129°19′ eastern longitude and receives 1300 mm of precipitation annually (Jung et al., 2014). The annual mean temperature in Korea is 12.9 °C.

Between November 2013 and March 2016, 951 diarrheal fecal samples were collected from young calves (<3 months) reared in Korea (Fig. 1). The number of collected samples was determined using the following formula (Thrusfield, 2005b): $n = \frac{1.96^2 p_{exp}(1-p_{exp})}{d^2}$, where n = required sample size, p_{exp} = expected prevalence, and d = desired absolute precision. Expected prevalence and desired absolute precision were considered 15% and 5%, respectively, based on previously reported prevalence values in Korea (Wee et al., 1996). According to the formula, at least 196 samples were required; 951 samples were assessed in this study. The fecal samples were collected from 425 farms, and the mean number ± standard deviation of samples per farm was 2.2 ± 4.6.

For this study, practicing veterinarians collected diarrheal fecal samples in sterilized containers stored in a cold box (0–4 °C) during treatment or regular medical checkup after receiving consent from the cattle owners. For the molecular identification of *Cryptosporidium* species, samples in the cold box were sent to the Laboratory of Veterinary Parasitology at Kyungpook National University, Daegu, Korea. Sample collection was neither harmful nor against animal welfare, and thus required no ethical approval from any authority.

For statistical analysis, data were collected for sex (male, female, or unknown), type of cattle (beef, dairy, or unknown), region [northern (Gyeonggi and Gangwon Provinces), central (Chungnam, Chungbuk, Gyeongbuk, and Jeonbuk Provinces), or southern (Jeonnam and Gyeongnam Provinces)], season [spring (March–May), summer (June–August), fall (September–November), or winter (December–February)], and type of diarrhea (hemorrhagic, watery, or pasty). When data were insufficient, the result was indicated as "unknown."