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## 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  $\kappa$  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

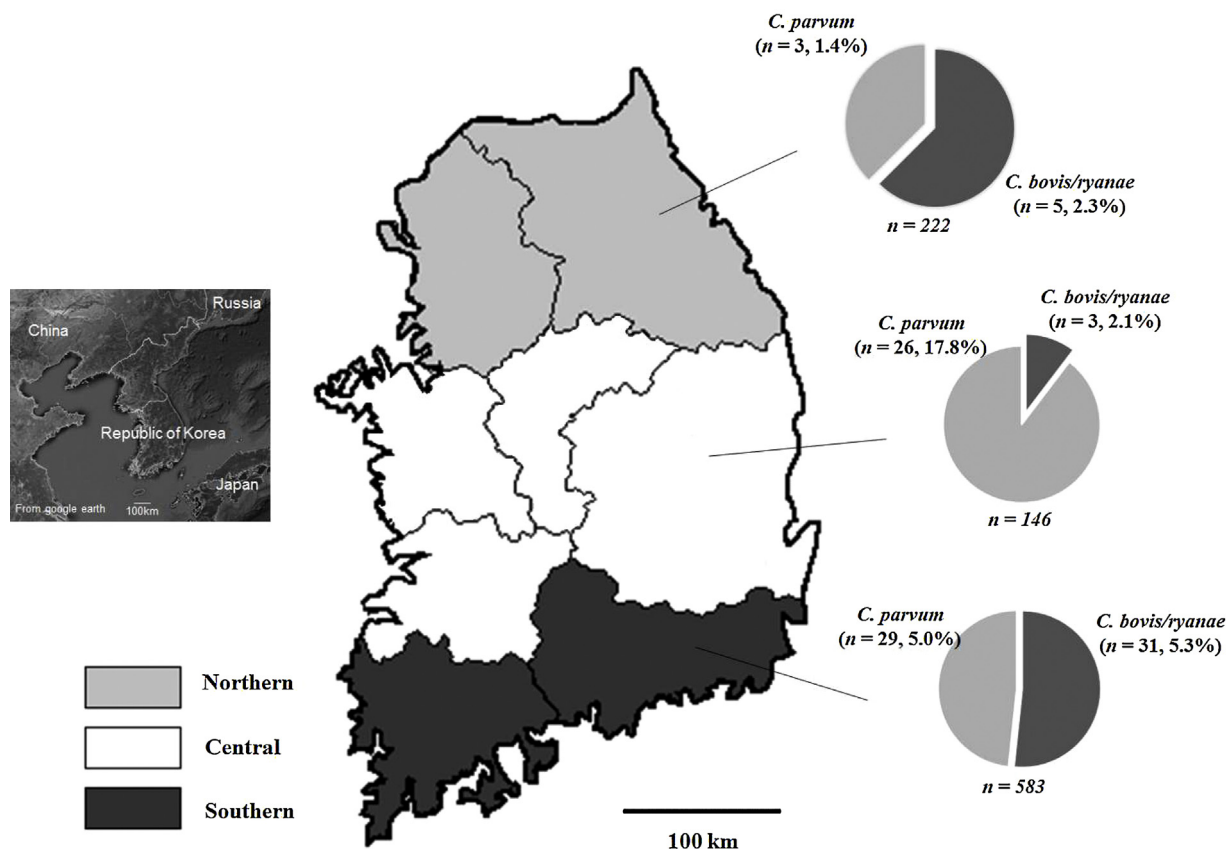
*Cryptosporidium* is an apicomplexan protozoan parasite that is distributed worldwide (Ryan et al., 2014; Wang et al., 2014). The genus *Cryptosporidium* includes more than 30 species that affect different hosts (Ryan et al., 2014; Shrestha et al., 2014; Mirhashemi et al., 2015). For example, *Cryptosporidium 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

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**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  $\pm$  standard deviation of samples per farm was  $2.2 \pm 4.6$ .

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.”

**Table 1**Primers used to amplify *Cryptosporidium* spp. 18S rRNA, *gp60*, and *hsp70* gene fragments in the diarrheal feces of young calves.

Target species	Target gene	Primer name	Primer sequence (5' to 3')	Tm (°C)	Reference
<i>Cryptosporidium</i> spp.	18S rRNA	18SiF	AGT GAC AAG AAA TAA CAA TAC AGG	55.8	Cheun et al. (2007)
		18SiR	CCT GCT TTA AGC ACT CTA ATT TTC	57.5	
	<i>hsp70</i>	CPHSPF	AGT GAT ATG ACT CAC TGG CCA TT	58.8	Satoh et al. (2005)
		CPHSPR	ACA ACA TCA TGT ACA GAT CTC TT	55.3	
<i>C. parvum</i>	18S rRNA <i>gp60</i>	CpaFc <sup>a</sup>	GAA ATA ACA ATA CAG GAC TTT TT	51.7	In this study
		AL3531	ATA GTC TCC GCT GTA TTC	51.4	Peng et al. (2001)
		AL3534	GCA GAG GAA CCA GCA TC	55.1	
		AL3532	TCC GCT GTA TTC TCA GCC	55.6	Peng et al. (2001)
		AL3533	GAG ATA TAT CTT GGT GCG	44.4	
<i>C. andersoni</i>	18S rRNA	Can18F <sup>a</sup>	ACG GAT CGC ATC TCT GAT GC	59.3	In this study
<i>C. bovis/ryanae</i>	18S rRNA	Cbrx18F <sup>a</sup>	CAA TAC AGA RCC TTA CGG TT	56.4	In this study

<sup>a</sup> 18SiR was used as the reverse primer.

## 2.2. DNA extraction, PCR, and sequencing

DNA was extracted from the diarrheal feces (200 mg) by using the QIAamp® Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and stored at -20 °C until use. The quality and quantity of the extracted DNA were evaluated using the Infinite® 200 PRO NanoQuant (Tecan, Mannedorf, Switzerland). PCR was performed with 100 ng fecal DNA and 10 pmole of each primer by adjusting 20 µl reaction volume with distilled water in the AccuPower® HotStart PCR PreMix Kit (Bioneer, Daejeon, Korea).

To detect *Cryptosporidium* spp. in feces, 18S rRNA of *Cryptosporidium* spp. was amplified using the primer set 18SiF/18SiR as described previously (Cheun et al., 2007). For samples that yielded positive results for *Cryptosporidium* spp., species identification was performed using species-specific primers (Table 1). For molecular characterization, *hsp70* and *gp60* genes were amplified for *Cryptosporidium* spp. and *C. parvum*, respectively (Peng et al., 2001; Satoh et al., 2005).

Because *C. bovis* and *C. ryanae* share highly similar 18S rRNA sequences, it is difficult to use PCR results to distinguish between *C. bovis* and *C. ryanae* (Mirhashemi et al., 2016). Therefore, primers were designed to amplify the 18S rRNA of both *C. bovis* and *C. ryanae*, and the positive results were indicated as *C. bovis/ryanae*.

For sequencing, we selected 9 *C. parvum* samples and 4 *C. bovis/ryanae* samples, all of which were PCR-positive. The factors of sex, type of cattle, region, season, type of diarrhea, and ELISA results

were considered. The *C. parvum* 18S rRNA gene was sequenced using the primer set 18SiF/18SiR or CpaFc/18SiR. Sequencing was performed using the BigDye Terminator V.3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and analyzed with the ABI 3730XL DNA Analyzer (Applied Biosystems).

## 2.3. ELISA

A commercial ELISA kit (*Cryptosporidium parvum* Antigen Test Kit, IDEXX Laboratories, Inc., Westbrook, ME, USA) was used to detect *C. parvum* in the diarrheal feces. The diagnostic test was performed according to the manufacturer's instructions, and in each experiment, positive and negative controls provided by the manufacturer were included.

To determine positivity, optical density (OD) was evaluated at 450 nm using the Infinite® 200 PRO NanoQuant plate reader (Tecan), and the sample-to-positive ratio (S/P) was calculated as manufacturer's instruction:  $\frac{S}{P} = \frac{OD_{\text{sample}} - OD_{\text{negativecontrol}}}{OD_{\text{positivecontrol}} - OD_{\text{negativecontrol}}}$ . The S/P greater than 20 was considered to be a positive result.

## 2.4. Phylogenetic analysis

For molecular characterization, phylogenetic analysis was performed using the 18S rRNA, *hsp70*, and *gp60* of *Cryptosporidium* spp. Phylogenetic trees were constructed using MEGA 6.0 with the max-

**Table 2**Detection of *Cryptosporidium* spp. at the genus level, using PCR analysis.

Group		No. tested	<i>Cryptosporidium</i> spp.		
			No. positive (%)	95% CI <sup>a</sup>	P-value
Sex	Male	433	34 (7.9)	5.3–10.4	0.634
	Female	248	17 (6.9)	3.7–10.0	
	Unknown	270	43 (15.9)	11.6–22.3	
Type of cattle	Beef	909	91 (10.0)	8.1–12.0	0.521
	Dairy	21	3 (14.3)	0–29.3	
	Unknown	21	–	–	
Region	Northern	222	8 (3.6)	1.2–6.1	<0.001
	Central	146	29 (19.9)	13.4–26.3	
	Southern	583	57 (9.8)	7.4–12.2	
Season	Spring (Mar–May)	252	17 (6.8)	3.7–9.8	0.105
	Summer (Jun–Aug)	240	31 (12.9)	8.7–17.2	
	Fall (Sep–Nov)	250	28 (11.2)	7.3–15.1	
	Winter (Dec–Feb)	209	18 (8.6)	4.8–12.4	
Type of diarrhea	Hemorrhagic	105	10 (9.5)	3.9–15.1	0.987
	Watery	419	42 (10.0)	7.2–12.9	
	Pasty	427	42 (9.8)	7.0–12.7	
Total		951	94 (9.9)	8.0–11.8	

<sup>a</sup> CI: Confidence interval.

imum likelihood method (Tamura et al., 2013). To support topology, 100 resamplings were performed.

### 2.5. Statistical analysis

To evaluate the association between prevalence and variables (sex, type of cattle, region, season, and type of diarrhea), the  $\chi^2$  test of SPSS V.21.0 (IBM Corporation, Armonk, NY, USA) was used. “Unknown” data in the categories of sex and type of cattle were excluded from the statistical analysis. A *P*-value less than 0.05 was considered statistically significant, and 95% confidence intervals were calculated.

The  $\kappa$  statistic was used to evaluate the agreement between the PCR and ELISA results to detect *C. parvum*. The  $\kappa$  value was interpreted as follows (Thrusfield, 2005a):  $\geq 0.81$ , very good agreement; 0.61–0.80, good agreement; 0.41–0.60, moderate agreement; 0.21–0.40, fair agreement; and  $\leq 0.20$ , poor agreement.

## 3. Results

### 3.1. Prevalence based on PCR and ELISA

The overall prevalence of *Cryptosporidium* spp., based on the PCR results, was 9.9% (94/951; Table 2). At the species level, 6.1% (58) and 4.1% (39) of samples were positive for *C. parvum* and *C. bovis/ryanae*, respectively, whereas *C. andersoni* was not identified in any samples (Table 3). Both *C. parvum* and *C. bovis/ryanae* were present in 3 samples. At the genus level, a statistically significant difference in different regions was observed ( $P < 0.001$ ). The *C. parvum* data revealed statistically significant differences in region ( $P < 0.001$ ) and type of diarrhea ( $P = 0.025$ ), while in *C. bovis/ryanae*, there were statistically significant differences in season ( $P < 0.001$ ) and type of diarrhea ( $P = 0.008$ ). At the farm level, PCR revealed that at least one *Cryptosporidium* spp. was present in 11.8% (50/425) of farms.

ELISA revealed that 9.7% (92/951) of samples were positive for *C. parvum*. Statistically significant differences were observed according to region ( $P < 0.05$ ) and type of diarrhea ( $P = 0.004$ ), supporting the PCR results. At the farm level, ELISA revealed that *C. parvum* was present in 11.1% (47/425) of farms.

### 3.2. Comparison of PCR and ELISA results for *C. parvum*

Of the 951 tested samples, 95.0% (903) samples showed consistent results between PCR and ELISA in the detection of *C. parvum* (Table 4), including 5.4% (51) positive and 89.6% (852) negative samples. The samples also included 0.7% (7) samples that were PCR+/ELISA- and 4.3% (41) samples that were PCR-/ELISA+. The  $\kappa$  value was calculated to be 0.65, indicating good agreement. In addition, no samples positive for *C. bovis/ryanae*, based on the PCR results, showed positive results using ELISA.

### 3.3. Sequencing and phylogenetic analysis

Of the 58 *C. parvum* PCR-positive samples, 9 samples were sequenced. After sequencing, 295, 758, and 467 bp of the 18S rRNA, *hsp70*, and *gp60* sequences, respectively, were obtained. Of the 39 *C. bovis/ryanae* PCR-positive samples, 4 samples were sequenced, and 270 and 758 bp of the 18S rRNA and *hsp70* sequences, respectively, were obtained. The obtained sequences were submitted to the GenBank database (Accession nos. KX342025–KX342059).

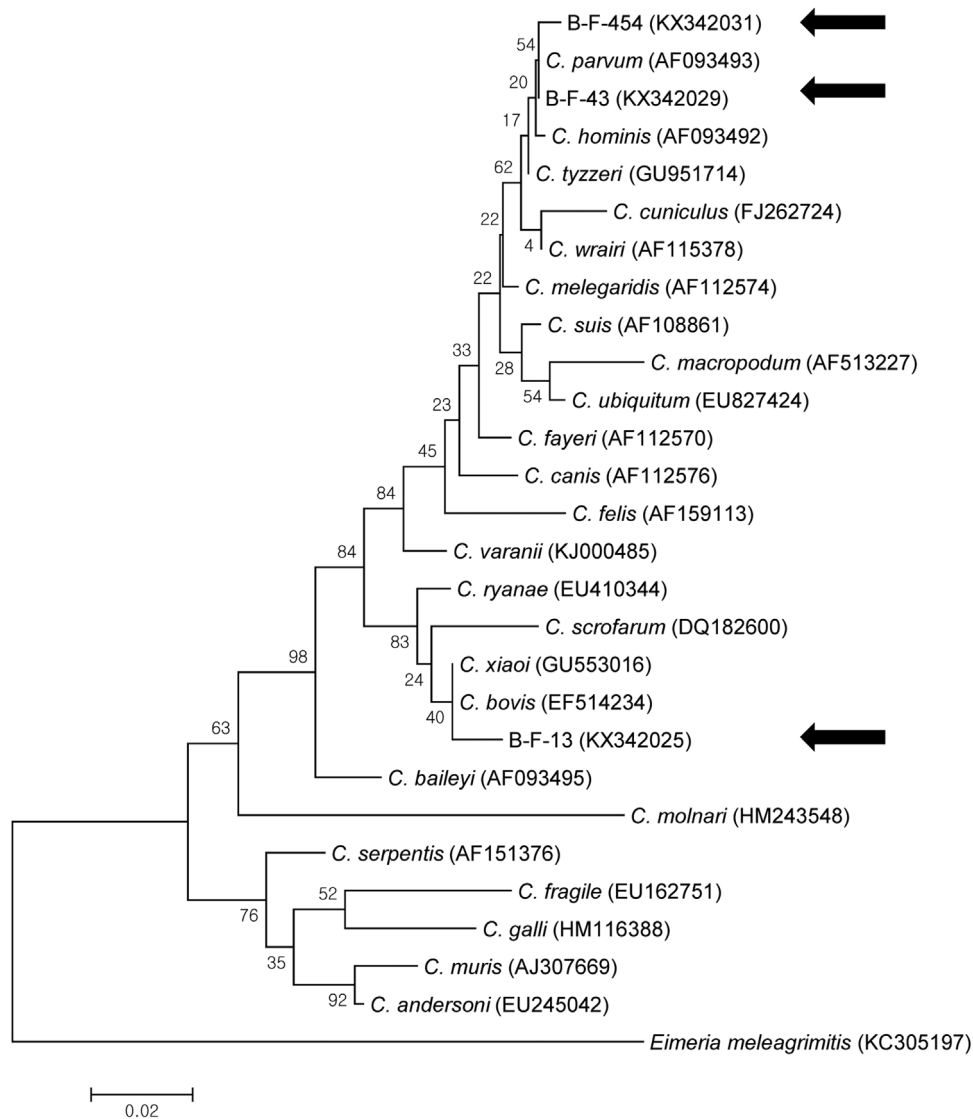
Phylogenetic analysis of 18S rRNA revealed that the sequences obtained in the present study belonged to *C. parvum* and *C. bovis* (Fig. 2). Although it was not possible to differentiate between *C. bovis* and *C. ryanae* using PCR based on 18S rRNA, phylogenetic analysis was used to successfully differentiate between the species. In

**Table 3**  
Detection of *Cryptosporidium parvum* and *C. bovis/ryanae* using PCR analysis and ELISA.

Group	No. tested	<i>C. parvum</i>				<i>C. bovis/ryanae</i>				
		No. positive (%)	95% CI <sup>a</sup>	<i>P</i> -value		No. positive (%)	95% CI <sup>a</sup>	<i>P</i> -value		
Sex	433	21 (4.8)	2.8–6.9	0.312	42 (9.7)	6.9–12.5	0.370	15 (3.5)	1.7–5.2	0.911
	248	8 (3.2)	1.0–5.4		19 (7.7)	4.4–11.0		9 (3.6)	1.3–6.0	
	270	29 (10.7)	7.1–14.4		31 (11.5)	7.7–15.3		15 (5.6)	2.8–8.3	
Type of cattle	909	56 (6.2)	4.6–7.7	0.529	90 (9.9)	8.0–11.8	0.954	38 (4.2)	2.9–5.5	0.895
	21	2 (9.5)	0–22.1		2 (9.5)	0–22.1		1 (4.8)	0–13.9	
	21	–	–		–	–		–	–	
Region	222	3 (1.4)	0–2.9	<0.001	10 (4.5)	1.8–7.2	<0.001	5 (2.3)	0.3–4.2	0.059
	146	26 (17.8)	11.6–24.0		29 (19.9)	13.4–26.3		3 (2.1)	0–4.4	
	583	29 (5.0)	3.2–6.7		53 (9.1)	6.8–11.4		31 (5.3)	3.5–7.1	
Season	252	15 (6.0)	3.0–8.9	0.854	18 (6.7)	3.7–9.8	0.158	2 (0.8)	0–1.9	<0.001
	240	13 (5.4)	2.6–8.3		22 (9.2)	5.5–12.8		18 (7.5)	3.5–9.8	
	250	18 (7.2)	4.0–10.4		25 (10.0)	6.3–13.7		13 (5.2)	2.5–8.0	
	209	12 (5.7)	2.6–8.9		28 (13.4)	8.8–18.0		6 (2.9)	1.2–6.4	
Type of diarrhea	105	0	–	0.025	1 (1.0)	0–2.8	0.004	10 (9.5)	2.5–12.7	0.008
	419	27 (6.4)	4.1–8.8		49 (11.7)	8.6–14.8		17 (4.1)	2.2–6.0	
	427	31 (7.3)	4.8–9.7		42 (9.8)	7.0–12.7		12 (2.8)	1.6–5.0	
Total	951	58 (6.1)	4.6–7.6		92 (9.7)	7.8–11.6		39 (4.1)	2.5–5.4	

<sup>a</sup> CI: Confidence interval.





**Fig. 2.** Phylogenetic relationships of 18S rRNA of *Cryptosporidium* spp. identified from the diarrheal feces of young calves. A phylogenetic tree was constructed using the maximum likelihood method with 100 replicates. The sequences identified in this study are indicated using black arrows. The sequences of KX342029, KX342030, and KX342032–KX342037 are identical and those of KX342025–KX342028 are identical.

**Table 4**  
Comparison of *Cryptosporidium parvum* detection using PCR and ELISA.

		PCR		Total
		No. positive	No. negative	
ELISA	No. positive	51	41	92
	No. negative	7	852	859
Total		58	893	951

$\kappa$  value = 0.65, indicating good agreement.

addition, phylogenetic analysis of *hsp70* yielded the same results as those obtained by analyzing 18S rRNA (Fig. 3).

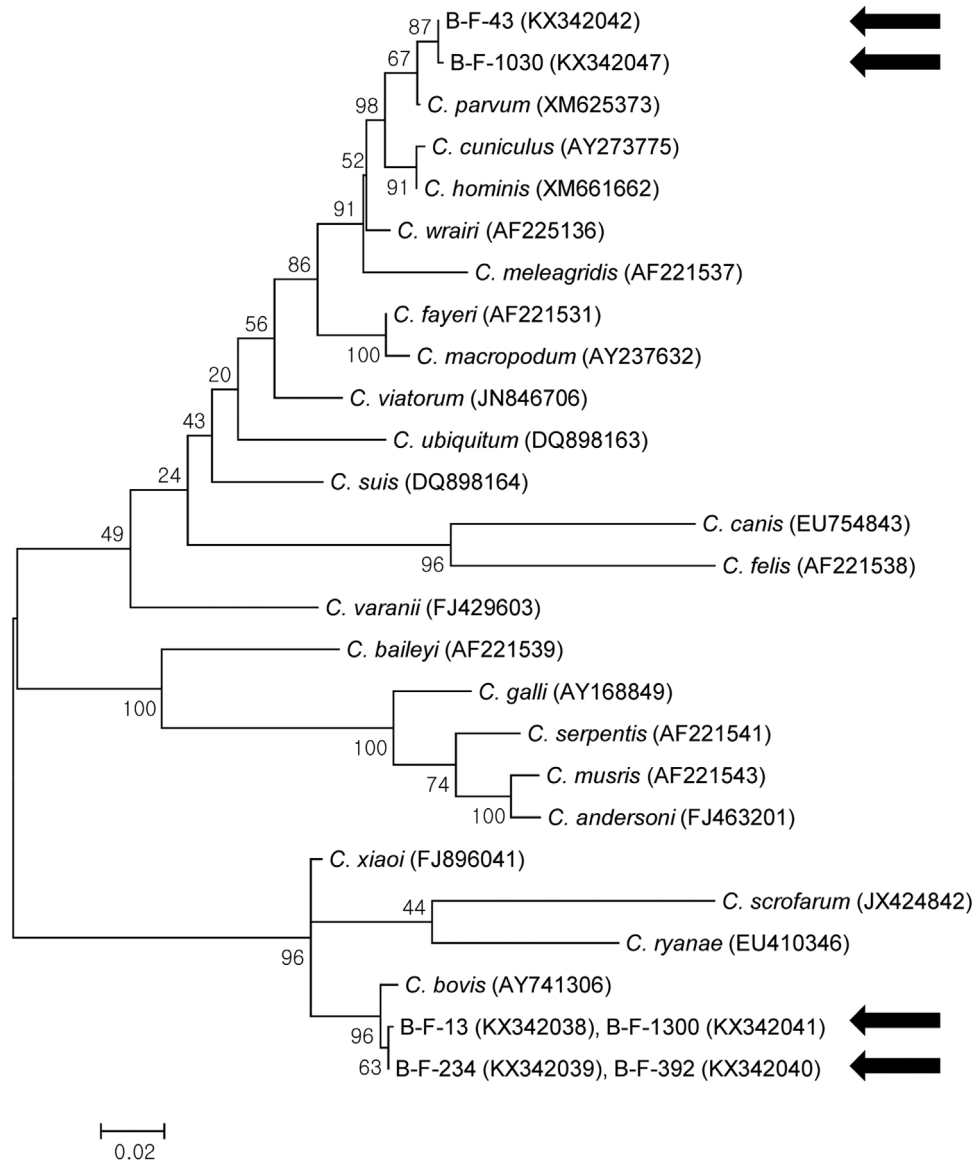
#### 3.4. Subtyping of *C. parvum* by using *gp60*

All sequences obtained from *C. parvum gp60* belonged to IIa according to phylogenetic analysis (Fig. 4). Furthermore, 8 sequences were identified as IIaA18G3R1, and one was identified as IIaA16G3R1 according to the nomenclature system suggested by Sulaiman et al. (2005).

#### 4. Discussion

*Cryptosporidium* spp. are distributed worldwide, and different species are responsible for diarrhea in pre-weaned or post-weaned calves (Santín and Zarlenga, 2009; Zhang et al., 2015; Ibrahim et al., 2016). Previous studies using different methods found variable prevalence rates of *Cryptosporidium* in cattle: 49.4% (39/79) in Hungary using IFA (Plutzer and Karanis, 2007), 11.9% (68/571) in the United States using PCR (Fayer et al., 2006), 75.0% (60/80) in Japan using PCR (Karanis et al., 2010), 20.0% (15/60) in New Zealand using IFA (Shrestha et al., 2014), 5.1% (150/2945) in China using PCR (Zhang et al., 2015), 21.5–22.5% in Ireland using direct fluorescence antibody testing (Mirhashemi et al., 2016), and 10.2% (49/480) in Egypt using microscopy with staining (Ibrahim et al., 2016).

Several studies in Korea have identified *Cryptosporidium* in cattle at rates of 41.2% (7/17) in a study using microscopy (Park et al., 2006), 14.4% (29/201) in a study using dimethyl sulfoxide modified acid-fast staining (Wee et al., 1996), and 22.2% (111/500) in a study using microscopy (Rhee et al., 1991). In the present study, the overall prevalence of *Cryptosporidium* detected by PCR was 9.9% (94/951), which was lower than that reported in previous stud-



**Fig. 3.** Phylogenetic relationships of *hsp70* of *Cryptosporidium* spp. identified from the diarrheal feces of young calves. A phylogenetic tree was constructed using the maximum likelihood method with 100 replicates. The sequences identified in the present study are indicated using black arrows. The sequences of KX342042–KX342050 are identical except for KX342047.

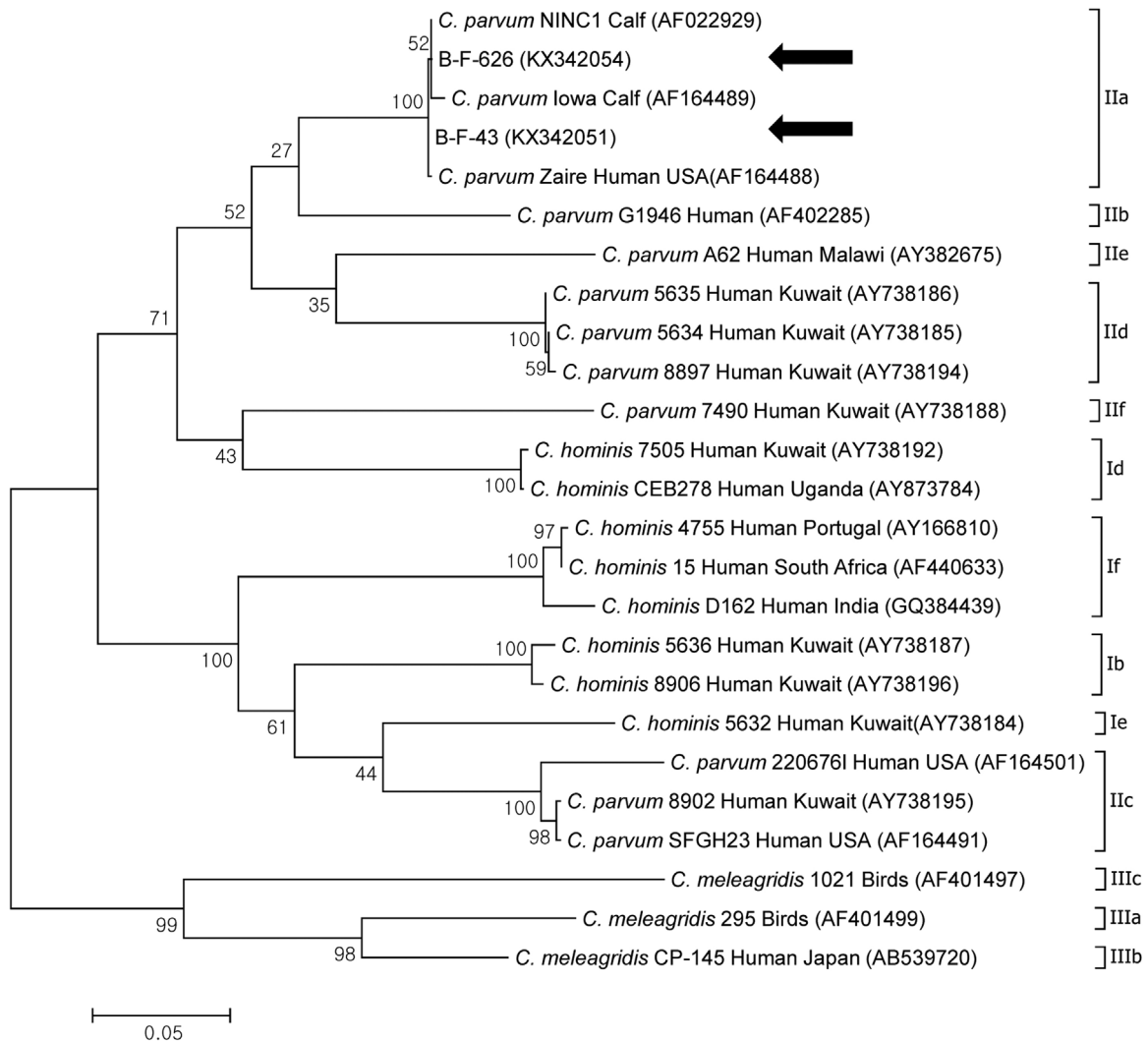
ies conducted in Korea. Our results may reflect improvements in hygiene, as the previous studies were performed more than 10 years ago (some were more than 20 years ago), when the hygiene of cattle breeding systems was inadequate. As poor hygiene may result in *Cryptosporidium* infection in humans (Ryan et al., 2014), improvements of hygiene in cattle rearing may have resulted in the lower prevalence observed in the present study.

Our data revealed that 6.1% (58) and 9.7% (92) of the 951 diarrheal fecal samples were positive for *C. parvum* according to the PCR and ELISA results, respectively. In addition, the PCR and ELISA results showed the same statistically significant differences with respect to the higher prevalence in the central region and lower prevalence in the case of hemorrhagic diarrhea. Analysis of the higher prevalence in the central region revealed that all positive samples in the central region were collected from a single farm. At this farm, 39 samples were collected between July 2014 and July 2015, and 66.7% (26) and 71.8% (28) samples were positive based on PCR and ELISA results, respectively. By excluding the data from this farm, the prevalence rates in the central region were 0% (0/107) and

0.9% (1/107) by PCR and ELISA, respectively, with *P*-values of 0.005 and 0.002, indicating a statistically lower prevalence in the central region. Therefore, the significant difference according to region was biased by the prevalence on one farm.

In addition, a higher prevalence at the farm level than the individual level was observed, regardless of the PCR or ELISA results, indicating a wide distribution of *Cryptosporidium* in calves in Korea. When the data were analyzed according to the type of diarrhea, there was low prevalence of *C. parvum* in calves with hemorrhagic diarrhea. Although the pathogenesis of *Cryptosporidium* remains unclear, *Cryptosporidium* is not considered to cause hemorrhagic diarrhea (Cho and Yoon, 2014; Di Genova and Tonelli, 2016) and appears to have a low association with this condition.

Several studies have shown that the oocysts of *Cryptosporidium* are resistant to adverse environmental conditions and survive well under temperate and moist conditions (Ryan et al., 2014). The higher prevalence of *Cryptosporidium* in the summer compared to in the winter may be related to the high temperature in the summer (Chai et al., 2001). In the case of *C. bovis/ryanae*, the prevalence



**Fig. 4.** Phylogenetic analysis of *gp60* of *Cryptosporidium* spp. identified from the diarrheal feces of young calves. A phylogenetic tree was constructed using the maximum likelihood method with 100 replicates. The sequences identified in the present study are indicated using black arrows. Species, isolation, host, region of identification, and GenBank accession number are included. According to *gp60* sequence variation, *C. hominis*, *C. parvum*, and *C. meleagridis* belong to subtypes I, II, and III, respectively. The sequences of KX342051–KX342053 are identical.

associated with season is consistent with the results from previous studies; however, *C. parvum* showed no association with seasonality according to PCR and ELISA results.

As discussed previously, *Cryptosporidium* is not considered to cause hemorrhagic diarrhea (Cho and Yoon, 2014). Therefore, the higher prevalence of *C. bovis/ryanae* in hemorrhagic diarrheal samples in the present study may have resulted from co-infection with other pathogens that cause hemorrhagic diarrhea in calves, such as *Clostridium perfringens*, *Salmonella* spp., bovine coronavirus, and *Eimeria* spp.

No statistically significant differences were observed for sex and type of cattle in the case of *C. parvum* and *C. bovis/ryanae* ( $P > 0.05$ ). A previous study showed a higher prevalence of *Cryptosporidium* in males than in females (Ibrahim et al., 2016); however, in the present study, although a slightly but insignificantly higher prevalence in males was noted.

Many studies have shown that the distribution of *Cryptosporidium* is related to age (Santín and Zarlenga, 2009; Zhang et al., 2015; Ibrahim et al., 2016). For example, *C. parvum* is mostly identified in pre-weaned calves; *C. bovis* and *C. ryanae* are found in post-weaned calves; and *C. andersoni* is present in adult cattle. Considering the target population in this study ( $\leq 3$  months), the results were con-

sistent with those of previous studies, with a higher prevalence of *C. parvum* than of *C. bovis/ryanae* and no detection of *C. andersoni*.

In previous studies, although diarrheic cattle showed a higher prevalence of *Cryptosporidium* spp., *Cryptosporidium* was also identified in non-diarrheic cattle (Azami, 2007; Ibrahim et al., 2016). These previous studies did not report that *Cryptosporidium* is an apathogenic organism, but suggested that other factors can affect *Cryptosporidium* infection. Age-related infection of *Cryptosporidium* in cattle and detection in many small children and immunocompromised individuals suggest that the immune response plays an important role in cryptosporidiosis (Ryan et al., 2014; Ibrahim et al., 2016).

Because there is no gold standard for the diagnosis of *Cryptosporidium* spp. (Mirhashemi et al., 2015), we used two tests, PCR and ELISA, to identify *C. parvum*. The  $\kappa$  value was used to evaluate agreement between the two different tests (Thrusfield, 2005a). In the present study, a  $\kappa$  value of 0.65, which indicates good agreement, was obtained. In addition, all *C. bovis/ryanae* PCR-positive samples yielded negative ELISA results. Our results indicated that the two tests could be applied compatibly to detect *C. parvum* in feces. While there was good agreement between the two tests, there were still 41 ELISA+/PCR- samples. This discrepancy may



be attributed to two factors: first, the presence of PCR inhibitors such as bilirubin, bile salts, and complex polysaccharides, which are included in feces, and second, limitations in the sensitivity and specificity in each assay (Morgan et al., 1998). Thus, additional studies are required to clarify the discrepancy in detecting *Cryptosporidium* spp. using the two assays.

To date, several genes have been used for the molecular characterization of *Cryptosporidium*, including 18S rRNA, *Cryptosporidium* wall protein, *hsp70*, and *gp60* (Xiao, 2010). In the present study, 18S rRNA was successfully used to identify *Cryptosporidium* spp.; however, because sequence identity is greater than 99% 18S rRNA among some *Cryptosporidium* spp. (*C. bovis*, *C. ryanae*, and *C. xiaoi*), it is not always possible to differentiate the species by PCR (Santín and Zarlenga, 2009; Mirhashemi et al., 2016). The sequences obtained in the present study also showed 99% 18S rRNA sequence identity for *C. bovis* and *C. ryanae*; however, phylogenetic analysis was used to differentiate these two species. In addition, we amplified *hsp70* in *C. bovis*, and this sequence clearly differed from those in *C. ryanae* (EU410346) and *C. xiaoi* (FJ896041), with 88.9% and 97.5% identity, respectively. Because there is only one *C. bovis hsp70* sequence (AY741306) in the GenBank database and the sequence contains only 384 bp, our sequence information will be useful for further investigations. We recommend targeting *hsp70*, rather than 18S rRNA, to identify *C. bovis*, *C. ryanae*, and *C. xiaoi*.

The gene *gp60* has been used for subtyping *C. parvum* and *C. hominis*; some other *Cryptosporidium gp60* genes have also been reported, such as *C. meleagridis*, *C. fayeri*, *C. cuniculus*, and *C. ubiquitum* (Ryan et al., 2014). The *C. parvum* and *C. hominis* subtype families can be distinguished based on variations in *gp60* sequences and according to repetition of the trinucleotide region (Sulaiman et al., 2005). Significantly different clinical presentations and virulence have been reported for the *C. parvum* and *C. hominis* subtype families (Del Chierico et al., 2011; Feng et al., 2012). Of the *C. parvum* subtype families (from Ila to Ilo), the Ila and Ild families are the most dominant families responsible for zoonotic cryptosporidiosis (Ryan et al., 2014; Wang et al., 2014). Ild families are considered to be dominant in Asian and African animals, while Ila families are dominant in American, European, and Oceanian animals (Wang et al., 2014).

In the present study, 8 *C. parvum* PCR-positive samples belonged to the Ila families according to phylogenetic analysis, and sequence analysis further identified the samples' subtypes as IlaA18G3R1 ( $n=8$ ) and IlaA16G3R1 ( $n=1$ ). This is the first report of these subtypes in the Asian region, although they have been previously identified in Canada, the United States, England, Northern Ireland, Spain, Australia, and New Zealand, which are geographically distant from Korea (Xiao et al., 2007; Xiao, 2010; Shrestha et al., 2014). Therefore, further studies on *C. parvum gp60* are required in other regions of Asia.

In Korea, the number of cattle produced annually is increasing, while the number of stock breeding farms is decreasing; therefore, the cattle population per farm is increasing (Oh et al., 2016). This makes the farm environment more vulnerable to *Cryptosporidium* spp., which are transmitted via the fecal-oral route with direct contact (Zhang et al., 2015). Because a very small number of oocysts can initiate *C. parvum* infection (Okhuysen et al., 1999), and there are a limited number of medicines available to treat cryptosporidiosis (Ryan et al., 2014), prevention is the most important method for reducing economic losses in the livestock industry.

This is the first nationwide epidemiological study on the prevalence of *Cryptosporidium* in the diarrheal feces of calves in Korea. Our study showed that *Cryptosporidium* spp. play an important role in diarrhea in young calves and that *C. parvum* is the dominant *Cryptosporidium* species. In addition, *gp60* analysis revealed that *C. parvum* in Korea belonged to the IlaA18G3R1 and IlaA16G3R1 families, which have not been previously reported in Asia. Considering

the zoonotic significance of *C. parvum* Ila subtype and the dense rearing system in Korea, prevention and continuous monitoring of *Cryptosporidium* are required.

## Conflicts of interest

The authors have no conflict of interest.

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