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Causative agents and epidemiology of diarrhea in Korean native calves

Sung-Hwan Lee [iD] [1], **Ha-Young Kim** [iD] [2], **Eun Wha Choi** [iD] [3,*], **Doo Kim** [iD] [1,*]

[1]Laboratory of Large Animal Medicine, College of Veterinary Medicine & Institute of Veterinary Science, Kangwon National University, Chuncheon 24341, Korea
[2]Animal Disease Diagnostic Division, Animal and Plant Quarantine Agency, Gimcheon 39660, Korea
[3]Department of Veterinary Clinical Pathology, College of Veterinary Medicine & Institute of Veterinary Science, Kangwon National University, Chuncheon 24341, Korea

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*Corresponding authors:
Eun Wha Choi
Department of Veterinary Clinical Pathology, College of Veterinary Medicine & Institute of Veterinary Science, Kangwon National University, 1 Kangwondaehak-gil, Chuncheon 24341, Korea.
E-mail: ewchoi@kangwon.ac.kr

Doo Kim
Laboratory of Large Animal Medicine, College of Veterinary Medicine & Institute of Veterinary Science, Kangwon National University, 1 Kangwondaehak-gil, Chuncheon 24341, Korea.
E-mail: kimdoo@kangwon.ac.kr

ORCID iDs
Sung-Hwan Lee [iD]
https://orcid.org/0000-0002-5135-068X
Ha-Young Kim [iD]
https://orcid.org/0000-0002-1332-3832
Eun Wha Choi [iD]
https://orcid.org/0000-0001-7213-695X
Doo Kim [iD]
https://orcid.org/0000-0003-4741-1352

ABSTRACT

Calf diarrhea caused by infectious agents is associated with economic losses in the cattle industry. The purpose of this study was to identify the causative agents and epidemiological characteristics of diarrhea in Korean native calves (KNC). In total, 207 diarrheal KNC aged less than 7 months were investigated. Fecal samples collected from the rectum were examined for causative agents using polymerase chain reaction (PCR) or real-time PCR and the number of oocysts were counted. Fourteen causative agents were detected from 164 of the 207 diarrheal KNC. Rotavirus was the most common agent (34.8%), followed by *Eimeria* spp. (31.7%), *Escherichia coli* (22.0%), *Giardia* spp. (14.0%), *Clostridium difficile* (9.8%), bovine viral diarrhea virus (8.5%), coronavirus (7.9%), *Cryptosporidium* spp. (7.3%), torovirus (6.7%), parvovirus (5.5%), norovirus (4.9%), kobuvirus (1.8%), adenovirus (1.2%), and *Salmonella* spp. (0.6%). About 95 (57.9%) of 164 calves were infected with a single causative agent and 42.1% were infected by multiple agents. No significant difference was observed in mortality between calves infected with a single agent and multiple agents. The occurrence of diarrhea caused by rotavirus, *Eimeria* spp., kobuvirus, and *Giardia* spp. was significantly different based on onset age, and the prevalence of diarrhea caused by rotavirus or *C. difficile* was significantly different between seasons. This study help the understanding of KNC diarrhea for the development of an effective strategy for disease prevention and control, especially in Eastern provinces of South Korea.

Keywords: Diarrhea; calf; Korean native calves; causative agents; prevalence

INTRODUCTION

Calf diarrhea caused by infectious agents is associated with enormous economic losses in the cattle industry, owing to calf death, growth retardation, and treatment costs [1]. Calf diarrhea is related to diverse and complex factors such as individual health conditions, management practice, rearing environment, as well as infectious causative agents. More than 50% of mortality in preweaned calves has been related to diarrhea, and most cases have occurred in calves less than 1 month of age [2].

Conflict of Interest
The authors declare no conflicts of interest.

Author Contributions
Conceptualization: Kim D; Data curation: Lee SH, Kim HY; Formal analysis: Choi EW, Kim D; Investigation: Lee SH, Kim HY; Writing - original draft: Lee SH; Writing - review & editing: Lee SH, Kim HY, Choi EW, Kim D.

The biggest cause for diarrhea in calves is the rapid reduction in the antibodies in the colostrum after birth. Korean native calves (KNC) are also susceptible to diarrhea, owing to the low colostrum yield as well as the low concentration of immunoglobulins in the colostrum [3-5].

Viruses, bacteria, and protozoans are known to cause infectious diarrhea, which may be associated with a single infection or mixed infections and increased damage. Rotavirus is the main viral causative agent of diarrhea in calves worldwide [6]. However, several other viruses, including coronavirus, bovine viral diarrhea virus (BVDV), adenovirus, kobuvirus, norovirus, parvovirus, and torovirus, have been recognized as the causative agents of diarrhea [3,7-13]. The major causative agents of bacterial diarrhea are *Escherichia coli*, *Clostridium* species (spp.), and *Salmonella* spp. [3,14-16]. *Eimeria* spp., *Giardia* spp., and *Cryptosporidium* spp. are recognized as the protozoans responsible for diarrhea [3,17-21].

The rearing environment may affect the prevalence of diarrhea. In seasonal breeding, as the calves are born within a short period of time, diarrhea often occurs owing to environmental contamination with infectious agents as well as fecal-oral contamination. Neonatal diarrheal calves born in cold winters are vulnerable to disease owing to poor adaptability to the environment and poor resilience due to low body temperature [22].

The clinical symptoms of diarrhea are very similar in spite of the differences in the causative agents. Therefore, the identification of the causative agents of diarrhea is essential for appropriate treatment and to adopt accurate preventive measures. Several studies have revealed the association between causative agents and diarrhea in calves [1]. However, in Korea, only a few small-scale studies have been performed on the causative agents of diarrhea in KNC [2,17]. Therefore, it is necessary to identify the causative agents and epidemiological characteristics of diarrhea in KNC. Here, the causative agents and epidemiology of diarrhea in KNC were investigated by assessing the prevalence of 14 infectious agents in fecal samples from diarrheic calves in South Korea.

MATERIALS AND METHODS

Animals
To investigate the causative agents and epidemiology of calf diarrhea, a total of 207 diarrheal KNC below 210 days of age from 96 farms were studied from July 2014 to June 2016 in Eastern provinces (Gangwon and Gyeongbuk provinces) of South Korea. The mean and median ages of 207 calves were 33.73 ± 42 and 20 ± 42 days, respectively. In general, the breeding environment of Korean native cattle is different from the isolation during artificial feeding of cow calves. All breeding cattle farms did not include grazing, but were housed in a roofed cowshed while sawdust was spread on the cowshed floors. The cleanliness of cowshed floors in various farming environments differed and no specific records were kept. This retrospective study was reviewed and approved by the Institutional Animal Care and Use Committee of Kangwon National University (KW-190207-1).

Clinical examination
To collect the clinical and epidemiological data, patient's history, including farm, breed, sex, age, season, recovery period, and prognosis, was recorded. Physical examination of each patient was conducted. The fecal samples were classified as pasty, watery, mucous, and hemorrhagic.

Detection of diarrheal causative agents

To identify the causative agents of calf diarrhea, 207 diarrheal fecal samples were collected from the recta and transferred to the laboratory under refrigeration. All experiments for detection of diarrheal pathogens were conducted at the Animal Disease Diagnostic Division (ADDD), Animal and Plant Quarantine Agency (APQA, Korea).

DNA and RNA were extracted from fecal samples using Patho Gene-spin DNA/RNA Extraction kit (iNtRON Biotechnology, Inc., Korea) according to the manufacturer's instructions. Real-time polymerase chain reaction (RT-PCR) was performed on BVDV, coronavirus, and rotavirus using an i-BD Multi Detection Kit (iNtRON Biotechnology, Inc.). In addition, RT-PCR for adenovirus, norovirus, kobuvirus, parvovirus, and torovirus was performed as previously described [9,23-25].

Pathogenic *E. coli* and *Salmonella* spp. were cultured as per the protocols provided in previous studies [26,27]. Briefly, the fecal samples were inoculated on MacConkey (BBL, USA) and blood agar (Asan Pharmaceutical Co., Ltd., Korea). After overnight incubation at 37°C, only pure cultured colonies were identified as *E. coli* using the VITEK II system (bioMérieux, France). Pathogenic *E. coli* genes, including *F5*, *F41*, *F17*, *eae*, *stx1*, *stx2*, *sta*, *hly*, *saa*, and *invA* were detected as previously described [26-30]. Briefly, colonies were suspended in 200 μL of water and boiled for 10 min. After centrifugation, the supernatant was used as a template for PCR reactions. The amplified products were visualized using electrophoresis on 2% agarose gels, which were stained with ethidium bromide. For isolation of *Salmonella* spp., fecal samples were inoculated in 9 mL of Rappaport-Vassiliadis (RV) R10 broth (BD, USA) and incubated at 42°C for 24 h. A loopful of RV culture was streaked onto CHROMagar Salmonella Plus (CHROMagar, France). PCR for *invA* gene of *Salmonella* spp. was carried out.

Isolation of *C. difficile* was carried out according to a previous study [30]. The samples were mixed with the same amount of 100% alcohol, allowed to stand for 1 h at room temperature, subjected to alcohol shock, and centrifuged at 3,800 ×*g* for 10 min. The pellet was inoculated on *C. difficile* selective medium (CDSA, BD) and anaerobically cultured at 37°C for 48 h or more. Samples without pretreatment of alcohol shock were inoculated in approximately 1 mL of TCCFB (cycloserine-cefoxitin fructose broth containing 0.1% taurocholate sodium) and incubated at 37°C for 7 days. The suspected unique colonies on the selective medium were subcultured on a sheep blood agar, followed by purification of DNA. PCR was performed to confirm the presence of *tcdB* toxin gene in the purely isolated *C. difficile*.

To detect and count *Eimeria* oocysts, oocysts were concentrated by fecal flotation technique at a specific gravity of 1.18 using sodium nitrate [31]. To detect *Cryptosporidium* spp. in feces, DNA was extracted from the fecal samples using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Germany) according to the manufacturer's instructions and stored at −20°C until use. The 18S rRNA of *Cryptosporidium* spp. was amplified using primer set 18SiF/18SiR as previously described [32]. To amplify β-giardin gene fragments from *Giardia duodenalis*, nested PCR was performed with two primer sets G7/G759 and G7n/G759n, as previously described [33].

Statistical methods

Statistical evaluations were performed with the SPSS (IBM SPSS Statistics, Desktop Base, v22.0; IBM Corporation, USA) software. Prevalence of causative agents was detected in diarrheal calves divided into eight age groups as follows: < 1 week old, 1–2 weeks old, 3–4

weeks old, 5–6 weeks old, 7–8 weeks old, 9–10 weeks old, 11–12 weeks old, and ≥ 13 weeks old. Seasons were grouped as spring (March–May), summer (June–August), fall (September–November), and winter (December–February) [34]. Diarrhea was classified as pasty, watery, mucous, or hemorrhagic. The degree of *Eimeria* spp. infection was determined on the basis of oocyst per gram of feces (OPG) as follows: light infection (OPG 1), OPG < 1,000; moderate infection (OPG 2), 1,000 ≤ OPG < 10,000; and heavy infection (OPG 3), OPG ≥10,000 [35]. A chi-square test was used to analyse the differences in the prevalence of diarrhea caused by each causative agent and the onset ages as well as seasons. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Prevalence of causative agents in diarrheal calves

Causative agents of diarrhea were detected in 164 of 207 diarrheal calves (79.2%). The remaining diarrheal calves were placed in the following diagnostic categories: abomasal impaction (n = 6), abomasal ulcer (n = 3), and others, which included diarrhea with unknown causes and undetected causative agents owing to insufficient fecal samples.

Fourteen species of causative agents were detected in 164 of 207 diarrheal calves. Rotavirus was the most common causative agent (57/164 calves, 34.8%), followed by *Eimeria* spp. (52/164, 31.7%), *E. coli* (36/164, 22.0%), *Giardia* spp. (23/164, 14.0%), *Clostridium difficle* (*C. difficile*, 16/164, 9.8%), BVDV (14/164, 8.5%), coronavirus (13/164, 7.9%), *Cryptosporidium* spp. (12/164, 7.3%), torovirus (11/164, 6.7%), parvovirus (9/164, 5.5%), norovirus (8/164, 4.9%), kobuvirus (3/164, 1.8%), adenovirus (2/164, 1.2%), and *Salmonella* spp. (1/164, 0.6%).

Viruses (117/164, 71.3%) were the most common causative agent, followed by protozoans (87/164, 53.1%) and bacteria (53/164, 32.3%). Although several causative agents were detected, rotavirus, *Eimeria* spp., *Giardia* spp., *E. coli*, *Cryptosporidium* spp., *C. difficile*, BVDV, and coronavirus were the main causative agents in KNC.

A total of 95 from 164 calves (57.9%) were infected with a single causative agent, while 69 calves (42.1%) were infected with multiple causative agents. A total of 48 of 164 calves (29.3%) were infected with viruses alone, while 18 (10.9%) and 29 (17.7%) were infected with bacteria and protozoans alone, respectively (**Table 1**).

In cases of mixed infection (69 out of 164 calves, 42.1%), 53 calves (32.3%) were infected with double causative agents and 9 (5.5%), 6 (3.7%), and 1 (0.6%) were infected with triple agents, quadruple agents, and quintuple agents, respectively (**Table 2**). *Eimeria* spp. (32/164, 19.5%), rotavirus (29/164, 17.7%), and *E. coli* (23/164, 14.0%) were the most commonly detected agents in calves with mixed infections (**Table 3**). Most causative agents were related to mixed infection rather than single infection.

Distribution of calves according to onset age of diarrhea based on causative agents

To identify the onset age of diarrhea according to the causative agents, the causative agents and onset age of diarrhea in 164 calves were examined. The mean onset age of diarrhea was 33.7 ± 42.0 days, with a minimum age of 2 days and maximum age of 210 days.

Table 1. Prevalence of calves infected with a single causative agent (n = 95) among diarrheal Korean native calves (n = 164)

Causative agents	No. of calves	Prevalence (%)
Virus		
Rotavirus	28	17.1
Coronavirus	5	3.0
Parvovirus	5	3.0
BVDV	3	1.8
Norovirus	3	1.8
Torovirus	3	1.8
Kobuvirus	1	0.6
Subtotal	48	29.1
Bacteria		
E. coli	13	7.9
C. difficile	4	2.4
Salmonella spp.	1	0.6
Subtotal	18	10.9
Protozoa		
Eimeria spp.	20	12.2
Giardia spp.	8	4.9
Cryptosporidium spp.	1	0.6
Subtotal	29	17.7
Total	95	57.9

BVDV, bovine viral diarrhea virus.

Distribution of major causative agents according to onset age of diarrhea is presented as **Table 4**. The occurrence of diarrhea caused by rotavirus gradually reduced with an increase in calf age. Rotavirus was undetected at 9 weeks of age and over. The occurrence of diarrhea caused by rotavirus differed significantly with onset age ($p < 0.001$).

No diarrhea was caused by *Eimeria* spp. in KNC within 20 days of age. The diarrhea caused by *Eimeria* spp. (31/52 calves, 59.6%) was most prevalent in KNC between 3 weeks and 6 weeks of age. The occurrence of diarrhea caused by *Eimeria* spp. differed significantly with onset age ($p < 0.001$).

Diarrhea caused by *Giardia* spp. occurred in KNC between 2 and 10 weeks of age.

The occurrence of diarrhea by *Giardia* spp. differed significantly with onset age ($p = 0.002$).

Viral and bacterial causative agents were mainly detected in KNC within 3 weeks of age, but these causative agents were usually distributed up to 8 weeks of age. Protozoal causative agents were mainly detected in KNC between 3 and 10 weeks.

Distribution of causative agents according to seasons

Distribution of 257 causative agents according to seasons in 164 diarrheal calves is presented in **Table 5**. The prevalence of diarrhea caused by rotavirus was significantly different between seasons ($p = 0.017$); spring was the most common season of occurrence of diarrhea. The prevalence of diarrhea caused by *C. difficile* was similarly significantly different between seasons ($p = 0.001$); spring was the most common occurrence season (**Table 5**).

Seasonal prevalence of diarrhea in diarrheal calves was 46.3% (76 out of 164 calves) in spring, 32.3% (53 out of 164 calves) in winter, 13.4% (22 out of 164 calves) in summer, and 7.9% (13 out of 164 calves) in fall. The prevalence rate of diarrhea in diarrheal calves was the highest in spring. However, considering calf birth, the prevalence rate of diarrhea caused by causative

Table 2. Prevalence of calves infected with mixed causative agents (n = 69) among diarrheal Korean native calves (n = 164)

Mixed form	No.	Causative agents	Prevalence (%)
Double	7	*E. coli*, *Eimeria* spp.	
	6	Rotavirus, coronavirus	
	5	Rotavirus, *E. coli*	
	5	Rotavirus, *C. difficile*	
	3	Rotavirus, *Eimeria* spp.	
	3	Rotavirus, *Giardia* spp.	
	3	*Eimeria* spp., *Giardia* spp.	
	2	Rotavirus, BVDV	
	2	Coronavirus, *Eimeria* spp.	
	2	Norovirus, *E. coli*	
	2	Torovirus, *E. coli*	
	2	*Eimeria* spp., *Cryptosporidium* spp.	
	1	Rotavirus, parvovirus	
	1	Rotavirus, norovirus	
	1	BVDV, *C. difficile*	
	1	BVDV, *Eimeria* spp.	
	1	Parvovirus, norovirus	
	1	Parvovirus, torovirus	
	1	Parvovirus, *Eimeria* spp.	
	1	Kobuvirus, *Eimeria* spp.	
	1	Kobuvirus, *Giardia* spp.	
	1	Torovirus, *Eimeria* spp.	
	1	*C. difficile*, *Giardia* spp.	
Subtotal	53		32.3
Triple	2	BVDV, *Eimeria* spp., *Cryptosporidium* spp.	
	1	BVDV, adenovirus, *Eimeria* spp.	
	1	BVDV, *E. coli*, *C. difficile*	
	1	Rotavirus, torovirus, *E. coli*	
	1	Rotavirus, *E. coli*, *C. difficile*	
	1	Adenovirus, torovirus, *Eimeria* spp.	
	1	Torovirus, *E. coli*, *C. difficile*	
	1	*Eimeria* spp., *Cryptosporidium* spp., *Giardia* spp.	
Subtotal	9		5.5
Quadruple	3	BVDV, *Eimeria* spp, *Cryptosporidium* spp., *Giardia* spp.	
	1	Rotavirus, torovirus, *E coli*, *Cryptosporidium* spp.	
	1	*E. coli*, *C. difficile*, *Eimeria* spp., *Giardia* spp.	
	1	*C. difficile*, *Eimeria* spp., *Cryptosporidium* spp., *Giardia* spp.	
Subtotal	6		3.7
Quintuple	1	Norovirus, *E. coli*, *Eimeria* spp., *Cryptosporidium* spp., *Giardia* spp.	
Subtotal	1		0.6
Total	69		42.1

BVDV, bovine viral diarrhea virus.

agents (spring, 0.9% [76/8,037]; summer, 0.4% [22/5,176]; fall, 0.4% [13/3,562]; and winter, 1.7% [53/3,065]) was the highest in winter ($p < 0.001$).

The seasonal mortality rate caused by diarrhea in diarrheal calves was 7.9% (13 out of 164 calves) in spring, 6.7% (11 calves) in winter, 3% (5 calves) in summer, and 0.6% (1 calves) in fall. The number of dead calves was the highest in spring. However, considering seasonal calf birth and the prevalence rate of diarrhea, the mortality rate caused by diarrhea was the highest in winter ($p = 0.003$).

Mortality rate of calves with single and mixed infection was 17.9% (17 out of 95 calves) and 18.8% (13 out of 69 calves), respectively. No significant difference was observed in mortality between calves with single and mixed infection.

Table 3. Prevalence of calves infected with a single or multiple causative agents (n = 257) among diarrheal Korean native calves (n = 164)

Causative agents	No. of tested causative agents	No. of calves with single agent	No. of calves with mixed agents
Virus			
Rotavirus	57	28	29
BVDV	14	3	11
Coronavirus	13	5	8
Torovirus	11	3	8
Parvovirus	9	5	4
Norovirus	8	3	5
Kobuvirus	3	1	2
Adenovirus	2	0	2
Subtotal (%)	117 (45.5)	48 (18.7)	69 (26.8)
Bacteria			
E. coli	36	13	23
C. difficile	16	4	12
Salmonella spp.	1	1	0
Subtotal (%)	53 (20.6)	18 (7.0)	35 (13.6)
Protozoa			
Eimeria spp.	52	20	32
Giardia spp.	23	8	15
Cryptosporidium spp.	12	1	11
Subtotal (%)	87 (33.9)	29 (11.3)	58 (22.6)
Total (%)	257 (100.0)	95 (37.0)	162 (63.0)

BVDV, bovine viral diarrhea virus.

Table 4. Distribution of major causative agents according to onset age of diarrhea

Onset age	Rota	Corona	BVDV	Adeno	Parvo	Kobu	Noro	Toro	*E. coli*	*Salmonella*	*C. difficile*	*Eimeria*	*Cryptosporidium*	*Giardia*
< 1 weeks	22	4	3	0	1	0	4	4	10	1	4	0	1	0
(n = 38)	38.6%	30.8%	21.4%	0.0%	11.1%	0.0%	50.0%	36.4%	27.8%	100.0%	25.0%	0.0%	8.3%	0.0%
1–2 weeks	17	3	1	0	1	1	2	2	7	0	3	0	1	2
(n = 26)	29.8%	23.1%	7.1%	0.0%	11.1%	33.3%	25.0%	18.2%	19.4%	0.0%	18.8%	0.0%	8.3%	8.7%
3–4 weeks	8	1	5	1	4	0	0	0	6	0	5	12	4	5
(n = 30)	14.0%	7.7%	35.7%	50.0%	44.4%	0.0%	0.0%	0.0%	16.7%	0.0%	31.3%	23.1%	33.3%	21.7%
5–6 weeks	7	1	2	1	1	0	2	2	5	0	2	19	4	7
(n = 30)	12.3%	7.7%	14.3%	50.0%	11.1%	0.0%	25.0%	18.2%	13.9%	0.0%	12.5%	36.5%	33.3%	30.4%
7–8 weeks	3	1	2	0	0	0	0	0	1	0	2	5	2	6
(n = 14)	5.3%	7.7%	14.3%	0.0%	0.0%	0.0%	0.0%	0.0%	2.8%	0.0%	12.5%	9.6%	16.7%	26.1%
9–10 weeks	0	1	0	0	0	2	0	1	4	0	0	7	0	3
(n = 12)	0.0%	7.7%	0.0%	0.0%	0.0%	66.7%	0.0%	9.1%	11.1%	0.0%	0.0%	13.5%	0.0%	13.0%
11–12 weeks	0	0	0	0	0	0	0	0	1	0	0	3	0	0
(n = 3)	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	2.8%	0.0%	0.0%	5.8%	0.0%	0.0%
≥ 13 weeks	0	2	1	0	2	0	0	2	2	0	0	6	0	0
(n = 11)	0.0%	15.4%	7.1%	0.0%	22.2%	0.0%	0.0%	18.2%	5.6%	0.0%	0.0%	11.5%	0.0%	0.0%
Total	57	13	14	2	9	3	8	11	36	1	16	52	12	23
(n = 164)	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%
Pearson's χ^2, p	< 0.001	0.732	0.608	0.834	0.208	0.014	0.444	0.464	0.736	0.852	0.634	< 0.001	0.337	0.002
Linear by linear association	< 0.001	0.963	0.887	0.922	0.422	0.261	0.052	0.873	0.519	0.244	0.164	< 0.001	0.920	0.050
Onset age (mean ± standard deviation, days)		44.0 ± 62.7	30.1 ± 31.6		58.4 ± 78.2		13.4 ± 11.9	48.9 ± 67.8	33.1 ± 45.3		20.1 ± 15.0	57.0 ± 49.7	28.3 ± 13.1	38.1 ± 15.0
Onset age (range, days)		2–180	3–129		7–210		3–34	3–180	3–210		3–55	20–210	7–52	10–70
Single infection, onset age (range, days)	3–45	2–180	3–129		16–210		3–12	7–180	5–60		15–55	22–210	7	13–64

BVDV, bovine viral diarrhea virus.

Table 5. Seasonal distribution of causative agents (n = 257) detected in diarrheal Korean native calves (n = 164)

Causative agents	Spring (Mar–May)	Summer (Jun–Aug)	Fall (Sep–Nov)	Winter (Dec–Feb)	*p* value
Rotavirus	33 (20.1)	2 (1.2)	6 (3.7)	16 (9.8)	0.017
Coronavirus	5 (3.0)	0 (0.0)	1 (0.6)	7 (4.3)	0.250
BVDV	3 (1.8)	4 (2.4)	0 (0.0)	7 (4.3)	0.061
Adenovirus	1 (0.6)	1 (0.6)	0 (0.0)	0 (0.0)	0.417
Parvovirus	4 (2.4)	1 (0.6)	0 (0.0)	4 (2.4)	0.745
Kobuvirus	0 (0.0)	0 (0.0)	1 (0.6)	2 (1.2)	0.143
Norovirus	1 (0.6)	1 (0.6)	0 (0.0)	6 (3.7)	0.058
Torovirus	5 (3.0)	0 (0.0)	2 (1.2)	4 (2.4)	0.361
E. coli	20 (12.2)	6 (3.7)	3 (1.8)	7 (4.3)	0.310
Salmonella spp.	0 (0.0)	1 (0.6)	0 (0.0)	0 (0.0)	0.090
C. difficile	7 (4.3)	3 (1.8)	5 (3.0)	1 (0.6)	0.001
Eimeria spp.	19 (11.6)	10 (6.1)	4 (2.4)	19 (11.6)	0.270
Cryptosporidium spp.	2 (1.2)	1 (0.6)	1 (0.6)	8 (4.9)	0.059
Giardia spp.	9 (5.5)	2 (1.2)	3 (1.8)	9 (5.5)	0.570
Total	109 (66.5)	32 (16.5)	26 (8.5)	90 (54.9)	

Values are presented as number of patients (%). The χ^2 test was used to analyse the differences in the prevalence of diarrhea caused by each causative agent across seasons.
BVDV, bovine viral diarrhea virus.

Comparison of fecal forms according to the causative agents

To examine fecal properties according to causative agents, fecal samples were classified by gross evaluation into pasty, watery, mucous, and hemorrhagic diarrhea. The fecal forms evaluated from 95 single infections and 162 mixed infections are presented in **Supplementary Table 1**. In all diarrheal KNC, rotavirus mainly caused watery diarrhea (71.9%, *p* < 0.001), while *Eimeria* spp. mainly caused hemorrhagic diarrhea (65.4%, *p* < 0.001). *Giardia* spp. mainly caused watery (39.1%) and mucous diarrhea (30.4%) (*p* = 0.001). Calves with a single infection of rotavirus often excreted a large amount of yellowish, greenish-yellow, or milky white watery diarrhea, which sometimes contained blood. Calves with mixed infections of rotavirus and other causative agents showed fecal forms very similar to those of calves with only rotavirus infection. In most cases, rotavirus and *E. coli* induced severe watery diarrhea.

Hemorrhagic diarrhea was observed in calves infected with rotavirus, coronavirus, parvovirus, torovirus, *E. coli*, *C. difficile*, and *Eimeria* spp. Of these agents, *Eimeria* spp. caused the most severe hemorrhagic diarrhea. *Giardia* spp. caused a characteristic mud-like feces, which occasionally contained mucous.

Comparison of fecal forms in 52 diarrheal calves detected with *Eimeria* spp. was conducted based on OPG. Of the 22 calves with OPG 1, pasty diarrhea was observed in 4 calves, while 6 and 12 (23.1%) calves presented watery diarrhea and hemorrhagic diarrhea, respectively. Of the 10 calves with OPG 2, one, three, one, and five (9.6%) calves presented pasty, watery, mucous, and hemorrhagic diarrhea, respectively. In 20 calves with OPG 3, watery diarrhea was observed in one calf, and mucous and hemorrhagic diarrhea was reported in two and 17 calves (32.7%), respectively. Calves with OPG 3 showed more mucoid and severe hemorrhagic diarrhea than calves with OPG 1 and 2. As the number of oocysts of *Eimeria* spp. in feces increased, the diarrheal symptoms of calves became more serious. However, no statistical significance was observed (*p* = 0.083). The number of OPG had no significant effect on the incidence of specific fecal forms of diarrhea (*p* = 0.083).

DISCUSSION

Fourteen species of causative agents were not detected in 43 of 207 diarrhoeal calves involved in this study. We found six calves with abomasal impaction and three with abomasal ulcers. It was difficult to identify the cause of diarrhea for the other calves because of the following reasons: 1) Insufficient fecal volume of the sample; 2) The fecal volume was sufficient, but the causative agent may be other than the 14 causal agents selected in the experimental design of this study. All individuals were not subjected to an autopsy, and it was difficult to clarify the cause. As we focused on the analysis of 14 causative agents, the other causative agents were not analyzed.

Sometimes there was a mass outbreak of diarrhea on a farm by viruses. The principle underlying the sampling was to have one sample for a one-day medical visit and the other sample for another treatment visit on the same farm on a different day. Thus, the cause of diarrhea outbreaks on the farms would not influence the results of the experiment.

Rotavirus was the most common causative agent detected in this study. The incidence of rotavirus varies from 19.4% to 69.2%, and this virus is known to have a very high prevalence in South Korea and other countries [1-3,6,36].

Although a previous study reported the detection of BVDV (at a very high rate of 66%) in the Gyeongnam province [22], BVDV was detected at a lower percentage in the present study. The difference in the incidence is thought to be due to the regional difference.

Coronavirus, a major cause of calf diarrhea, was detected in 5.3% to 16.5% of diarrheal calves [6,8,22]. Coronavirus induces winter dysentery in adult cattle but is a more important causative agent when mixed with rotavirus in calves [37,38]. In this study, detection rate of coronavirus was similar to that previously reported [22].

E. coli was reported in 14.7% to 20.3% of calves [3,6] and *C. difficile* was isolated from 7.6% of diarrheal calves [14]. In this study, the detection rate of *E. coli* and *C. difficile* was slightly higher than that reported in other studies. Heo et al. [19] reported an isolation rate of 7.2% for *Salmonella* spp. in calves from the south area of Gyeongnam. However, another report showed the absence of *Salmonella* spp. [3]. In the present study, only one case of *Salmonella* spp. was identified as a very rare causative agent in single suckle beef calves.

Jeong et al. reported that bacteria (45.4%) were the most common infectious agents, followed by viruses (39.0%), *Eimeria* spp. (0.4%), and mixed infection (10%) in diarrheal calves in Gyeongnam province [22]. In the present study, protozoan and viral causative agents were found to be the predominant causative agents in Gangwon and Gyeongbuk provinces of South Korea.

The prevalence of *Eimeria* spp. was reported to range from 21.2% to 83.67% [3,13,18,20,39], while that of *Cryptosporidium* spp. and *Giardia* spp. ranged from 11% to 15.6% [6,13,18,31] and 9.34% to 29%, respectively [18,31,33]. In the present study, protozoans were the most common causative agents. However, the prevalence of *Cryptosporidium* spp. was lower than that previously reported [6,13,18,31]. As mentioned above, *Eimeria* spp. is the most important of the protozoal causative agents. However, contrary to the result of our study, Gillhuber et al. [21] reported that the prevalence of *Cryptosporidium* spp., *Eimeria* spp., and *Giardia* spp.

was 41.3%, 13.3%, and 7.2%, respectively. In a previous study in South Korea, *Giardia* spp. was detected in 13.1% of diarrheal calves using PCR [33], consistent with the results of the present study.

The occurrence of diarrhea caused by rotavirus was significantly different according to onset age. It was reported that the peak time for the identification of rotavirus in diarrheal calves is from 0 to 2 weeks of age [40]. In a previous study, rotavirus was the most common pathogen identified in each of the four age groups of Australian dairy calves (0–4, 5–14, 15–21, and > 21 days), and the highest occurrence was reported in the age group of 5–14 days [1]. Coccidiosis, a disease commonly observed in young cattle (1–2 months to 1 year), is usually sporadic during the wet seasons of the year. Diarrhea due to *Eimeria* spp. was the most prevalent between 5 and 6 weeks of age in KNC (the mean age: 57 days old). Diarrhea due to *Giardia* was also most prevalent between 5 and 6 weeks of age (the mean age: 38.1 days old), and all diarrhea caused by *Giardia* spp. occurred between 2 and 10 weeks of age. Other studies have reported that the most prevalent age for diarrhea due to *Eimeria* spp. was 4.7 to 12 weeks [3], with a mean age of 55 days (median: 49 days old), in southern Germany [21]. The mean age of diarrhea caused by *Giardia* spp. was 46.5 days (median: 42 days old) in southern Germany [21], and the mean ages of diarrhea caused by *Eimeria* spp. and *Giardia* spp. in southern Germany were very similar to those reported in the present study.

A previous study conducted in the Gyeongnam province of Korea reported that the prevalence of diarrhea was 31.3% in spring, 28.5% in summer, 24.9% in fall, and 15.3% in winter [22]. In the present study, the prevalence of diarrhea was 46.3% in spring, 13.4% in summer, 7.9% in fall, and 32.3% in winter. Spring was the most dangerous season because the morbidity and mortality rates associated with diarrhea in diarrheal calves were the highest in this season. A previous study in south-west France reported that the month of birth was significantly associated with morbidity: The highest morbidity rates were observed in December and March, and the mortality rate was two times higher in December than in other months [6]. In the present study, the incidence of calf diarrhea increased in proportion to the number of calf births in spring. In summer and fall, the incidence of diarrhea decreased in proportion to the number of calf births. In winter, however, the ratio of diarrheal calves to calf births was doubled. As the number of calf births in spring is the highest, diarrhea occurs more frequently owing to changes in temperature and the increased chance of contact with causative agents of calf diarrhea. In winter, few calf births were reported, but the calves appeared to be more exposed to causative agents and diarrhea. These infections became more severe, owing to the decreased resistance of calves upon exposure to the cold weather. The reason underlying the increase in mortality and morbidity was associated with the rotaviral infection of calves. Rotavirus-associated mortality was the highest component (46.7%) of total mortality. This observation is consistent with the most frequent rotavirus infection within 1 week of age, especially in spring and winter. In general, viruses tend to be more stable during the cold season, presumably owing to low temperatures. The prevalence of diarrhea caused by rotavirus was significantly different between seasons.

No seasonal influence was observed on the occurrence of diarrhea caused by *Giardia* spp. and *Cryptosporidium* spp. [21,31]. Unlike the most outbreaks that occurred in spring and winter in the present study, infections with *Eimeria* spp. and *Giardia* spp. mainly occurred in summer (warm and humid season) [5,33]. The most likely reasons for the high detection rate of protozoans in the winter are the wet and dirty floors of crowded breeding spaces as well as the warm and crowded calf room heated by electronic blanket.

It was difficult to distinguish between different causes of diarrhea in calves by gross observation. However, a specific difference in diarrheal feces was observed based on the causative agents. These results are similar to those reported in other studies [33].

The number of OPG in *Eimeria* spp. had no significant effect on the incidence of hemorrhagic diarrhea. Hemorrhagic diarrhea caused by *Giardia* spp. did not occur as an infection caused by a single agent and was considered as a result of a mixed infection with *Eimeria* spp.

As it is difficult to predict the diarrheal causative agent based on the clinical symptoms of KNC, it is necessary to accurately diagnose calf diarrhea using tests that may be performed in the laboratory or at farm site.

This study elucidated the causative agents and epidemiological aspects of diarrhea in KNC and may help broaden our understanding of calf diarrhea for the development of an effective strategy for disease prevention and control, especially in the east area of South Korea.

SUPPLEMENTARY MATERIAL

Supplementary Table 1
Fecal forms of calves with single and mixed infections (n = 257) according to the causative agents (n = 164)

Click here to view

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