

Detection and Isolation of Winter Dysentery Bovine Coronavirus Circulated in Korea during 2002–2004

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ABSTRACT. Although winter dysentery (WD) has been suspected to occur frequently in Korea, to date the exact epidemiology of WD has remained unknown. Therefore, we investigated the causative agents of WD by using electron microscopy, ELISA, RT-PCR, and nested PCR from 97 fecal samples of 32 WD-affected herds collected from 8 provinces during 2002–2004. The bovine coronavirus (BoCV) was consistently detected in all herds with WD. Of other pathogens, only coccidian oocysts were inconsistently but concurrently detected with BoCV. Ten isolates were identified as BoCV by immune electron microscope, immunofluorescent test and ELISA with antiserum to BoCV, and RT-PCR. From these results, it is concluded that WD caused by BoCV occurred in relatively high frequency and was widespread in Korea. The results provide important epidemiological data for the control and establishment of a surveillance system for WD in Korea.

KEY WORDS: bovine coronavirus, Korea, winter dysentery prevalence.

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Bovine coronavirus (BoCV), a member of the Coronaviridae family, causes severe newborn calves diarrhea (CD) and is associated with winter dysentery (WD) in adult cattle [5, 10]. WD is characterized by a sudden onset of diarrhea that rapidly affects many adult cattle in a herd and has been reported in many parts of the world. WD-affected cattle lose body condition and result in a dramatic decrease in milk production [12]. It has been suspected that WD caused by BoCV does occur and causes enormous economic losses in both dairy and beef industries of South Korea. However, the exact epidemiology and the causative agent of WD have never been documented. We report here the prevalence of WD in South Korea.

Ninety-seven bovine fecal samples from 29 dairy (Holstein) and 3 Korean native beef (Hanwoo) cattle herds with WD have been collected from 8 provinces in the Laboratory of Animal Diseases, College of Veterinary Medicine, Chonnam National University (Table 1). These diarrheic fecal samples were collected during the winter from late November 2002 to late February 2004. The ages of tested cattle from all provinces ranged from 10 months to 8 years old. Fecal samples were subjected to routine tests and standard diagnostic procedures to detect the presence of common enteric pathogenic bacteria which included *Salmonella* spp., *Clostridium* spp., *Campylobacter* spp., and *Mycobacterium paratuberculosis*, and intestinal parasites including *Eimeria* spp., *Giardia* spp., and *Cryptosporidium* spp. Electron microscopic examination and an indirect antigen-capture

ELISA employing monoclonal antibodies to BoCV as capture antibodies were used to detect BoCV in the fecal samples as previously described [2, 9–11, 13]. To detect the BoCV-RNA, RT-PCR and nested PCR with specific primer pairs were performed with the extracted RNA from fecal samples as described by Cho *et al.* [4]. As a negative control, RNA was extracted from mock-infected human rectal tumor (HRT-18G) cells or mock-infected bovine fecal samples. For clarifying the involvement of bovine diarrhea virus (BVDV) and group A rotavirus to WD, we performed RT-PCR with the specific primer pairs reported previously [3, 7].

By electron microscopy (EM), 63 fecal samples from 21 WD-affected herds were examined (Table 1). Typical coronavirus particles, which were 80 to 150 nm in diameter and had short and long surface spikes or projection, were observed in 9 of 63 (14.3%) fecal samples from cows with WD (Table 1 and Fig. 1). However, coronavirus-like particles, which did not possess long surface projections, were detected in 23 of 63 (36.5%) fecal samples of WD-affected cattle (Table 1). The number of herds in which at least one fecal sample had either typical coronavirus or altered coronavirus particles was 12 of 21 (57.1%) (Table 1). No other virus particles including rotavirus and BVDV were detected in the fecal samples tested.

By use of a BoCV antigen-capture ELISA, 34 of 97 (35.1%) fecal samples were positive (Table 1). Of the 32 herds, 17 herds had at least one BoCV-positive fecal sample (Table 1). A 1-step RT-PCR assay, targeting a 730 bp fragment of the nucleocapsid (N) gene of BoCV detected 32 positive fecal samples (33%) of 97 fecal samples tested (Table 1 and Fig. 2). In the herd level, 15 (46.9%) of 32

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Table 1. Detection of winter dysentery (WD) bovine coronavirus (BoCV) in the fecal samples from herds with WD by EM, ELISA, RT-PCR, and nested PCR

Methods Provinces	EM (Typical coronavirus)		EM (Coronavirus-like)		ELISA		RT-PCR		Nested PCR	
	Gyeonggi	4/18 ^{a)}	2/7 ^{b)}	1/18	1/7	6/23	5/9	8/23	4/9	21/23
Jeonnam	0/16	0/3	11/16	2/3	16/32	5/6	13/32	4/6	24/32	6/6
Chungnam	3/17	1/6	5/17	2/6	9/24	5/9	7/24	5/9	21/24	9/9
Gyeongbuk	0/4	0/1	4/4	1/1	0/10	0/4	1/10	1/4	4/10	4/4
Gyeongnam	0/3	0/1	2/3	1/1	2/3	1/1	0/3	0/1	2/3	1/1
Chungbuk	0/3	0/1	0/3	0/1	0/3	0/1	2/3	1/1	3/3	1/1
Gangwon	1/1	1/1	0/1	0/1	1/1	1/1	0/1	0/1	1/1	1/1
Jeonbuk	1/1	1/1	0/1	0/1	0/1	0/1	1/1	1/1	1/1	1/1
Total	9/63	5/21	23/63	7/21	34/97	17/32	32/97	15/32	77/97	32/32

a) No. of positive fecal samples for BoCV/No. of tested fecal samples.

b) No. of positive herds for BoCV/No. of tested herds.

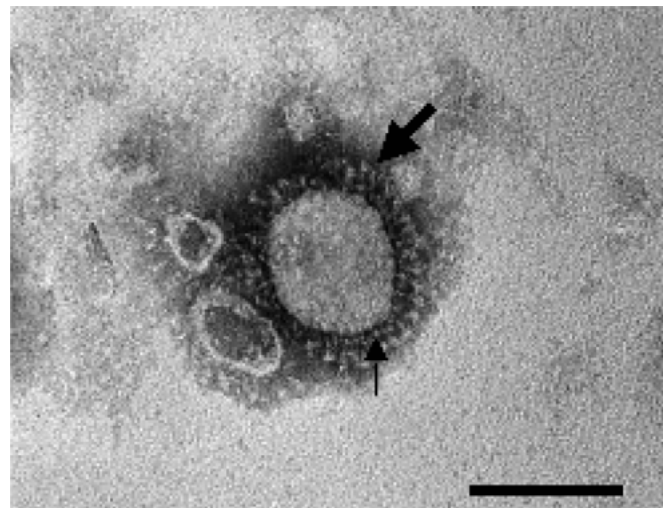


Fig. 1. Electron micrograph of characteristic bovine coronavirus particle detected from an adult cow with winter dysentery. Note outer long projections (thick arrow) and inner short hemagglutinin-esterase spike (thin arrow). Phosphotungstic acid negative staining. Bar=100 nm.

herds contained at least one BoCV positive fecal sample (Table 1). By nested PCR assay, targeting a 407 bp fragment of the N gene, 77 of 97 (79.4%) were positive (Table 1 and Fig. 2) and all 32 herds had at least one cow with a positive fecal sample (Table 1). As the cows were considered positive if a positive reaction was observed by at least one of EM, ELISA, RT-PCR and nested PCR, 90 (92.8%) of 97 diarrheic fecal samples and 32 (100%) of 32 herds with WD were positive for BoCV.

Bacteriological examination of fecal samples from cows in herds with WD was negative for *Salmonella* spp., *Campylobacter* spp., *Clostridium* spp., *Yersinia* spp. and enterotoxigenic *Escherichia coli*. Using standard flotation technique, a low number of *Eimeria* spp. was detected. In general, oocysts in feces of cows with WD were consistent in size and morphologic characteristics to that of *Eimeria bovis*.

Monolayers of HRT-18G cell cultures grown in 6-well plates were used for virus isolation, as described previously

[2, 13]. Of the 20 BoCV-positive fecal samples detected strongly by ELISA and RT-PCR, BoCV was isolated from 10 samples. After the second passage, the cytopathic effect (CPE) was observed in the cultures inoculated with each fecal sample from cattle with WD. CPE was characterized by enlarged, rounded, and densely granular cells in clusters at postinoculation days 2 to 3. These clustered cells resembled syncytia. No differences in CPEs were observed among the isolates. CPE was not observed in mock-infected HRT-18G cells. The direct immunofluorescence (IF) test detected BoCV-specific cytoplasmic fluorescence in HRT-18G cells inoculated with each of these samples at the third passage. Viruses were cloned by liquid-limiting dilution, and the highest dilution of the virus that caused CPE was passaged an additional 3 times in HRT-18G cells.

Coronavirus particles were seen in the culture supernatant of HRT-18G cells infected with each isolate; the particles were similar in morphology to those observed in the original

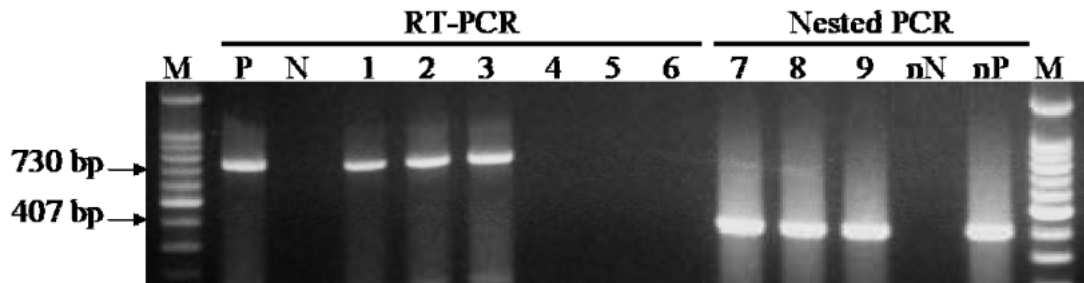


Fig. 2. Detection of bovine coronavirus (BoCV) by RT-PCR and nested PCR in the fecal samples from adult cattle with winter dysentery (WD). M; 100 bp marker. P; BoCV-inoculated culture medium. N; mock-infected fecal sample. Lanes 1–6; diarrheic fecal samples from adult cattle with WD. Lanes 7–9; diarrheic fecal samples negative for BoCV by RT-PCR. nN; mock-infected fecal sample. nP; 5,000-times diluted RT-PCR positive product.

feces and were agglutinated by hyperimmune anti-BoCV serum in immune electron microscopy (IEM). The ELISA detected BoCV antigen in these 10 isolates. By RT-PCR assay targeting a 730 bp fragment of the nucleocapsid gene of BoCV, a specific band was detected after amplification with all 10 isolates. We designated these 10 isolates as KWD1 to KWD10 strains, respectively.

To our knowledge, this study is the first to survey the prevalence of WD and isolate WD BoCV in South Korea. The clinical manifestation in the cattle of the present herds was identical to that of WD reported elsewhere [5, 6, 8, 10, 12]. The consensus from those studies is that coronaviruses are commonly isolated from clinically typical cases of WD. In the present study, the BoCV was the only enteric pathogen consistently identified in the adult cattle in 32 herds with WD. Although other diseases similar to WD can appear, including acute BVDV infection and salmonellosis [8], these pathogens were inconsistently detected and concurrently observed with BoCV in the fecal samples of cattle with WD [10]. In the present study, coccidian oocysts were also inconsistently detected and concurrently observed with BoCV in the fecal samples of cattle with WD. Therefore, it is suggested that WD is infectious in nature and, because of the similarities in clinical signs among outbreaks, that a single etiologic agent may be involved. Other pathogens including BVDV, rotavirus, salmonella, and coccidian may be incidentally associated with WD [10]. This suggestion is supported by the fact that experimental reproduction of WD is successfully induced in adult cows after inoculation only with BoCV isolated from the feces of WD-affected cattle [12].

The antigen-capture ELISA data in the present study demonstrated that the coronaviruses detected in the WD fecal samples were antigenically related to the Mebus strain of BoCV. Our report [10] and others [1, 2] demonstrated that WD BoCV isolates or strains were reacted with antibodies against the prototype Mebus BoCV. Furthermore, based on IEM, IF and ELISA, the coronaviruses isolated in the HRT-18G cells from the WD fecal samples in the present study reacted with antibodies against the prototype Mebus BoCV. These data indicate that our Korean WD BoCV isolates were also antigenically related to the Mebus

BoCV. In addition, Korean WD isolates induced severe diarrhea in colostrums-deprived calves (unpublished data). Traven *et al.* [12] reproduced severe diarrhea in the calves by the inoculation of BoCV isolates from the adult cattle with WD. Taken all together, WD and CD are strongly correlated in the etiological aspect. More detailed studies of the experimental transmission of coronaviruses isolated from cases of WD occurring in Korea, in susceptible bovine hosts including calves and adult cattle, may help to define more clearly the etiology of the disease in calves and adult cattle, and assign a causative role to WD BoCV.

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