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FULL PAPER Virology

Detection of Bovine Torovirus in Fecal Specimens of Calves with Diarrhea in Japan

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ABSTRACT. The aim of this study was to determine the prevalence of bovine torovirus (BoTV) in bovine fecal samples and to determine whether a relationship exists between BoTV and diarrhea in Japan. Ninety-nine diarrheic and 114 normal fecal samples from calves in Hokkaido Prefecture and 38 diarrheic fecal samples from calves in 10 other prefectures were examined by reverse transcription (RT)-PCR with primers designed in the spike (S) gene for the presence of BoTV. The specimens were also examined for the presence of other enteric pathogens, bovine rotavirus, coronavirus and *Cryptosporidium* spp. BoTV RNA was detected in 15 (15.2%) of the 99 diarrheic samples from Hokkaido and in 9 (23.7%) of the 38 diarrheic samples from the other prefectures. The incidence of BoTV in control specimens was 7.0%. In 11 of the 15 BoTV-positive specimens from Hokkaido, BoTV was the only pathogen detected among those examined, and 11 BoTV-positive specimens were obtained from calves less than 2 weeks of age. Rotavirus was confirmed to be associated with calf diarrhea, but coronavirus and *Cryptosporidium* spp. were not. Nucleotide sequences of 17 different BoTV RT-PCR products were determined. Phylogenetic analysis based on the sequences revealed that Japanese BoTVs could be classified into at least two groups. This study showed that BoTV is a common virus in fecal specimens of calves with diarrhea in Japan and may be an important pathogen of cattle, principally in young calves less than 2 weeks of age.

KEY WORDS: bovine, coronavirus, *Cryptosporidium*, rotavirus, torovirus.

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The etiology of infectious diarrhea in calves has been attributed to enteropathogens, such as rotavirus, coronavirus, parvovirus, calicivirus, astrovirus, and *Cryptosporidium* spp. [19]. Bovine torovirus (BoTV), a member of the genus torovirus of the family *Coronaviridae*, was first associated with enteritis in calves in 1982 [33]. BoTV produces mild to moderate diarrhea in calves under both field and experimental conditions [13]. After oral or intranasal inoculation of calves with BoTV, the virus infects epithelial cells in the middle and lower parts of villi extending into the crypt epithelium, inducing cytopathic effects and epithelial desquamation in the small intestine, in addition to areas of necrosis in the large intestine [6, 18, 33]. The BoTV has not been frequently reported and, unlike the prototype equine torovirus Berne strain [32], it cannot as yet be grown in cell culture, which has precluded the development of routine immunological diagnostic tests. However, the entire 3' end of the BoTV genome, which encodes viral structural proteins, has allowed for the application of reverse transcription-PCR (RT-PCR) for the detection of BoTV genome [5]. Recently, the complete sequence of the BoTV genome has been determined [3].

Epidemiologic studies have shown that BoTV is widespread in the Netherlands [15], Germany and Switzerland [31], the United Kingdom [1], and the United States [29, 34], with 55 to 90% of cattle being seropositive. Prospective studies in The Netherlands using an enzyme-linked immunosorbent assay (ELISA) and in Canada using RT-PCR have demonstrated that torovirus was present in 6.4% and 36.4% of calves with diarrhea and in 1.7% and 11.6% of asymptomatic controls, respectively [4, 16].

There has been no report on an epidemiologic study of BoTV in Japan. The aim of the present study was to determine the incidence of BoTV excretion in calves with diarrhea by RT-PCR and compare this with the excretion of other enteric pathogens, bovine rotavirus (BoRV), coronavirus (BoCV) and *Cryptosporidium* spp. Phylogenetic analysis was also conducted to reveal the genetic relationship among BoTVs detected in Japan.

MATERIALS AND METHODS

Clinical specimens: A total of 99 diarrheic calf stool specimens (H-D1 to H-D99) and 114 specimens from healthy calves (H-N1 to H-N114) were obtained from October 2003 to March 2004 from Hokkaido Prefecture, Japan. Thirty-eight stool specimens from calves with diarrhea were also obtained in March 2003 from 10 prefectures covering most of Japan: Aomori (3 samples, AM-1 to AM-3), Akita (1 sample, AK-1), Fukushima (4 samples, FK-1 to FK-4), Nagano (7 samples, NA-1 to NA-7), Kyoto (8 samples, KYO-1 to KYO-8) and Okayama (1 sample, OKA-1) in Honshu, Ehime (1 sample, EH-1) in Shikoku, Miyazaki (2 samples, MI-1 and MI-2) and Oita (5 samples, OT-1 through OT-5) in Kyushu, and Okinawa (6 samples, OK-1 to OK-6). All specimens were tested for oocysts of *Cryptosporidium* spp. by a flotation method [27] before viral examination.

RNA extraction: Fecal specimens were diluted with nine volumes of phosphate-buffered saline (pH 7.4) and clarified by centrifugation at 1,000 × g for 10 min at room temperature. Viral RNA was extracted from the supernatant using a QIAamp viral RNA mini kit (QIAGEN, Maryland, U.S.A.). Reverse transcription of the RNA was performed using a

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Table 1. Oligonucleotide primers used for RT-PCR

Target	Primer	Sequence (5'→3')	Position in genome	PCR product
BoTV S gene[a)]	S5[d)]	GTGTTAAGTTTGTGCAAAAAT	36–56	741 bp
	S3[d)]	TGCATGAACTCTATATGGTGT	758–777	
	S-IF	TGG ATT AATTCAGGAGGTGCC	94–114	653 bp
	S-IR	CACTCTACATAGAGCGGTGTC	726–746	
BoRV VP6 gene[b)]	VP6-F	ATGGATGTCCTGTACTCCTTG	24–44	1194 bp
	VP6-R	TCATTTGACAAGCATGCTTCTAATGG	1192–1217	
	VP6-IF	ATGGAATTGCACCACAATCAGA	343–364	327 bp
	VP6-IR	TTCGGAGCTGTACAATATGCTC	648–669	
BoCV N gene[c)]	N-F1[e)]	GCAATCCAGTAGTAGAGCGT	21–40	730 bp
	N-R1[e)]	CTTAGTGGCTACCTTGCCAA	731–750	
	N-IF[e)]	GCCGATCAGTCCGACCAATC	79–98	407 bp
	N-IR[e)]	AGAATGTCAGCCGGGGTAT	467–485	

a) Breda strain (GenBank accession number AF076621).
b) UK strain (GenBank accession number X53667).
c) Mebus strain (GenBank accession number M16620).
d) Reported by Hoet *et al.* [9].
e) Reported by Cho *et al.* [2].

First Strand cDNA Synthesis kit, ReverTra Ace-α (TOYOBO, Tokyo).

RT-PCR: All specimens were tested for BoTV, BoRV and BoCV RNAs by nested RT-PCR. Viral cDNA was used to amplify coding regions of BoTV spike (S) protein, BoRV VP6, and BoCV N protein using the PCR primers listed in Table 1. PCR amplification was carried out in a GeneAmp PCR System 2400 (Perkin Elmer, Norwalk, U.S.A.) as follows. For BoTV and BoRV, 1st and nested PCR amplifications using KOD-PLUS DNA polymerase (TOYOBO) were performed with 30 cycles of denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s for BoTV and at 58°C for 30 s for BoRV, and extension at 68°C for 30 s with an additional final 7-min incubation at 68°C to complete all extensions. For BoCV, 1st and nested PCR amplifications using an Expand High Fidelity PCR System (Roche Diagnostics, Mannheim, Germany) were performed with 30 cycles as for BoTV except for extension at 72°C. Amplified cDNA was analyzed by electrophoresis on 2% agarose gel.

Cloning and DNA sequencing: PCR products were purified by GenElute agarose spin columns (Sigma, Saint Louis, U.S.A.) and cloned into the pGEM-T plasmid vector (Promega, Madison, U.S.A.). Several clones were selected, and both strands were sequenced by the dideoxy method using a DyenamicET terminator cycle sequencing kit (Amersham Biosciences UK Limited, Buckinghamshire, England) and ABI PRISM 310 Genetic Analyzer (Applied Biosystems Japan, Tokyo). Computer analysis of sequence data was carried out with DNASIS Pro (Hitachi Software Engineering, Tokyo). The sequences of the S genes of BoTV Breda, B145 [21, 28], equine torovirus (EqTV) Berne, and porcine torovirus (PoTV) Markelo strains [17, 21] were obtained from the GenBank database (accession numbers AF076621, AJ575373, X52506, and AJ575372).

Statistical analysis: Chi-square analysis was used to determine the association between BoTV shedding and diarrhea.

Nucleotide sequence accession numbers: The newly determined sequences have been deposited in the DDBJ database under accession numbers AB254063 through AB254079.

RESULTS

Virus detection by RT-PCR and the presence of Cryptosporidium spp.: Frequency and percentage for fecal shedding of BoTV, BoRV, BoCV, and *Cryptosporidium* spp. are shown in Table 2. BoTV, BoRV, BoCV and *Cryptosporidium* spp. were detected in 15 (15.2%), 13 (13.1%), 2 (2.0%) and 14 (14.1%) of the 99 specimens from diarrheic calves in Hokkaido and in 8 (7.0%), 7 (6.1%), 7 (6.1%) and 17 (14.9%) of the 114 specimens from healthy calves in Hokkaido, respectively. In the 38 specimens from diarrheic calves in prefectures other than Hokkaido, BoTV, BoRV and *Cryptosporidium* spp. were detected in 9 (23.7%), 7 (18.4%), and 21 (55.3%) specimens, respectively. BoCV was not detected. In 11 of the 15 BoTV-positive specimens (73.3%) from diarrheic calves in Hokkaido, BoTV was the only pathogen detected among those examined. There was a significantly greater number of BoTV-positive specimens in the diarrheic calves than in the healthy calves in Hokkaido ($P<0.01$). BoRV was also significantly associated with the diarrheic calves ($P<0.01$). There was no significant difference in the presence of *Cryptosporidium* spp. between diarrheic and normal specimens from Hokkaido, though a high shedding rate (55.3%) was observed in diarrheic specimens from prefectures other than Hokkaido. In diarrheic specimens from the 38 calves in pre-

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Table 2. Summary of viruses and *Cryptosporidium* spp. present in the stools of diarrheic and healthy calves in Japan

	No. (%) of calves		
	Hokkaido		Other parts of Japan
Pathogens	diarrheic n=99	normal n=114	diarrheic n= 38
BoTV alone	11 (11.1)	5 (4.4)	1 (2.6)
+ BoRV	0	0	1 (2.6)
+ BoRV +Cryp[a]	2 (2.0)	0	2 (5.3)
+ BoCV	0	2 (1.8)	0
+ Cryp	2 (2.0)	1 (0.9)	5 (13.2)
Total	15 (15.2)*	8 (7.0)	9 (23.7)
BoRV alone	10 (10.1)	6 (5.3)	1 (2.6)
+ BoTV	0	0	1 (2.6)
+ BoTV + Cryp	2 (2.0)	0	2 (5.3)
+ BoCV	0	0	0
+ Cryp	1 (1.0)	1 (0.9)	3 (7.9)
Total	13 (13.1)*	7 (6.1)	7 (18.4)
BoCV alone	2 (2.0)	5 (4.4)	0
+ BoTV	0	2 (1.8)	0
+ BoRV	0	0	0
Total	2 (2.0)	7 (6.1)	0
Cryp alone	9 (9.1)	15 (13.2)	11 (28.9)
+ BoTV	2 (2.0)	1 (0.9)	5 (13.2)
+ BoTV + BoRV	2 (2.0)	0	2 (5.3)
+ BoRV	1 (1.0)	1 (0.9)	3 (7.9)
Total	14 (14.1)	17 (14.9)	21 (55.3)
Mock	62 (62.6)	79 (69.3)	14 (36.8)

a) *Cryptosporidium* spp.
* P<0.01, in comparison with the normal specimens.

fectures other than Hokkaido, 24 contained one or more pathogens (63.2%). Among those 24 specimens, 13 (54.2%) contained only one pathogen and the other 11 (45.8%) contained two or more pathogens. On the other hand, 37 (37.3%) of 99 diarrheic specimens from Hokkaido contained one or more pathogens. Among those 37 specimens, 32 (86.5%) contained only one pathogen and the other 5 (13.5%) contained two or more pathogens.

When analyzed by age of calves in Hokkaido, the proportions of BoTV-positive specimens were almost the same in the age groups of diarrheic calves (Table 3). However, the BoTV-positive rate differed significantly between diarrheic and healthy groups less than 2 weeks old (P<0.01). In BoRV, there was a significant difference between diarrheic and healthy calves of 3 weeks and 4 weeks of age (P<0.01). BoCV was detected in calves less than 4 weeks of age. *Cryptosporidium* spp. were detected principally in calves less than 4 weeks of age, and there was no significant difference between the positive rates in diarrheic and healthy age groups.

Sequences of BoTV-positive RT-PCR products: To determine the degree of heterogeneity among BoTVs detected in fecal specimens, RT-PCR products of 653 bp within the 5' region of the S gene from 17 specimens were cloned and sequenced. 611-bp sequences without primer sequences were used (Table 4). The origins of the 17 samples were as follows: five (H-D7, H-D27, H-D52, H-D-53 and H-D62) from diarrheic calves and four (H-N1, H-N11, H-N17 and H-N-32) from healthy calves were from Hokkaido, NA-5 and NA-7 were from Nagano, KYO-1 and KYO-8 were from Kyoto, MI-1 and MI-2 were from Miyazaki, OKA-1 was from Okayama, OKI-6 was from Okinawa. Pairwise comparison of nucleotide sequences of the S gene revealed that all of the 17 Japanese BoTVs except for OKA-1 show identities of 99.0 to 100% to each other, 89.9 to 92.8% to BoTV prototype Breda strain from the United States and B145 strain from The Netherlands, 73.5 to 74.0% to EqTV Berne strain and 63.9 to 64.8% to PoTV Markelo strain. OKA-1 showed identities of 92.6 to 93.6% to the other Japanese BoTVs and 94.6, 93.5, 75.2 and 66.3% to BoTV Breda, B145, EqTV and PoTV, respectively. Phylogenetic trees were generated for each sequence of the S gene by the neighbor-joining method (Fig. 1). Three distinct torovirus genotypes with apparent preferences for cattle, horse and pig could be discerned. Japanese BoTVs clustered with BoTV Breda and B145 strains. BoTVs could be divided into four groups, designated tentatively group 1 to group 4. Breda and the B145 belonged to group 1 and 3, respectively. Japanese BoTVs belonged to group 2 and 4. All BoTVs from normal specimens were classified to group 4 with most of the BoTVs from diarrheic specimens.

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Table 3. Prevalence of BoTV and other enteric pathogens in fecal specimens from 1 to 8-week-old calves in Hokkaido

Pathogen	fecal condition	Age (weeks) of Calves 1 (n=20)	2 (n=87)	3 (n=56)	4 (n=22)	5–8 (n=19)	Total (n=185)
BoTV	diarrheic	2/6 (33.3)[b]*	7/47 (14.9)*	3/23 (13.0)	1/7 (14.3)	2/12 (16.7)	13/95 (13.7)
	normal	0/14	2/40 (5.0)	3/33 (9.1)	2/15 (13.3)	1/7 (14.3)	7/110 (6.4)
BoRV	diarrheic	0/6	3/47 (6.4)	3/23 (13.0)*	4/7 (57.1)**	3/12 (25.0)	13/95 (13.7)
	normal	0/14	3/40 (7.5)	1/33 (3.0)	1/15 (6.7)	1/7 (14.3)	6/110 (5.5)
BoCV	diarrheic	0/6	1/47 (2.1)	0/23	0/7	0/12	1/95 (1.1)
	normal	0/14	4/40 (10.0)	2/33 (6.1)	1/15 (6.7)	0/7	7/110 (6.4)
Cryp[a]	diarrheic	0/6	8/47 (17.0)	4/23 (17.4)	1/7 (14.3)	1/12 (8.3)	14/95 (14.7)
	normal	3/14 (21.4)	6/40 (15.0)	6/33 (18.2)	2/15 (13.3)	0/7	17/110 (15.5)

a) *Cryptosporidium* spp.
b) No. of positive/tested. Numbers in parenthesis indicate pathogen-positive percents.
* P<0.01 and ** P<0.001, in comparison with the age-matched normal group.

Table 4. Percent of nucleotide identities of 611 bp within the 5' region of toroviral S genes

Virus	H-N1[a]	H-N11	H-D27	H-D62[b]	KYO-1[c]	NA-5[d]	OKA-1	BoTV B145	BoTV Breda	EqTV Berne
H-N11	99.8									
H-D27	99.5	99.7								
H-D62	99.2	99.0	99.0							
KYO-1	99.3	99.2	99.2	99.5						
NA-5	99.5	99.5	99.0	99.3	99.8					
OKA-1	93.1	92.6	93.0	93.6	93.5	93.3				
BoTV, B145	92.5	92.3	92.6	92.6	92.8	92.6	94.6			
BoTV, Breda	90.1	89.9	89.9	90.3	90.4	90.3	93.5	91.9		
EqTV, Berne	73.7	73.8	73.5	73.9	74.0	74.0	75.2	75.2	77.0	
PoTV, Markelo	64.0	63.9	64.0	64.8	64.4	64.2	66.3	66.3	65.0	67.4

a) The sequences of H-N17 and H-N32 coincide with those of H-N1.
b) The sequences of H-D52 and H-D53 coincide with those of H-D62.
c) The sequences of H-D7, KYO-8, OKI-6, MI-1 and MI-2 coincide with those of KYO-1.
d) The sequences of NA-7 coincide with those of NA-5.

DISCUSSION

This is the first report on the prevalence of torovirus infection in cattle in Japan. We found a significant difference in the detection rates of BoTV between diarrheic and normal fecal specimens from Hokkaido. BoTV-positive rate in diarrheic specimens was higher in calves less than 2 weeks of age than in age-matched normal specimens. Detection of BoTV in young calves has also been reported, and BoTV has been suggested to be one of etiological agents causing diarrhea [4, 9, 10, 14, 16, 20, 33, 34]. Hoet *et al.* [9] reported that BoTV was detected in fecal samples from cattle with diarrhea, principally in young calves less than 3 weeks of age. However, in their study, the actual etiologic role of BoTV in calf diarrhea was not clear because fecal samples from age-matched normal young calves were not examined. Our study clearly showed that BoTV was associated with diarrhea in calves, especially those less than 2 weeks of age. This suggests that apparent BoTV infection is limited to an extremely short period after birth. After passing the susceptible period, BoTV might cause inapparent infection in calves since BoTV detection rates in each age group with and without diarrhea were almost the same in our study. The reason for the low BoTV infection rate in normal specimens from calves less than 2 weeks of age is not clear. One possible explanation is that maternal BoTV antibody derived from colostrum might work well to prevent infection in the intestine as do other enteropathogens [23]. Koopmans *et al.* [15] reported that ninety percent of newborn calves had high levels of maternal BoTV antibodies. Therefore, low BoTV antibody levels in colostrum or insufficient colostrum intake might affect the pathogenicity of BoTV infection in neonatal calves. In Japan, there has been no seroepidemiologic study of BoTV. Examination of the distribution of BoTV antibodies is needed to reveal the epidemiology and pathogenicity of BoTV infection in Japan.

The data on incidence of rotavirus obtained in this study confirmed that rotavirus is one of major causes of diarrhea. Though BoCV is also known to be one of etiological agents causing calf diarrhea in Japan [26], we could not find a significant difference between BoCV detection rates in diarrheic and normal fecal specimens, as reported by others [4,

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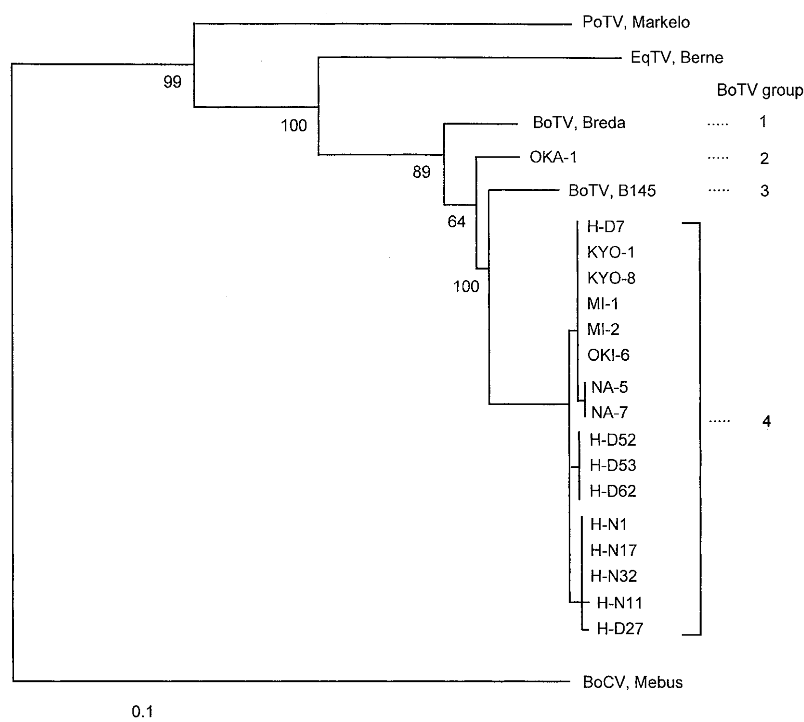

Fig. 1. Phylogenetic tree constructed from 611-bp nucleotide sequences within the S gene of bovine, equine and porcine torovirus. The corresponding sequence of the S gene of BoCV Mebus strain (GenBank accession number U00735) was used as an outgroup. The number on each branch shows the percent occurrence in 1,000 bootstrap replicates, and values over 50% are shown. Bar indicates 0.1 nucleotide substitutions per site.

16, 24]. This reason for this discrepancy is not clear. One possibility is that calves receive passive immunity to BoCV by colostrum, since most cattle in Japan possess serum antibody against BoCV [12, 25].

We did not find a significant difference between detection rates of *Cryptosporidium* spp. in diarrheic and normal fecal specimens from Hokkaido. In our study, *Cryptosporidium* spp. infection rate in diarrheic specimens from Hokkaido was about 14.1%. On the other hand, samples from symptomatic calves in regions other than Hokkaido showed a positive rate of 55.3%. Since we did not obtain age-matched normal fecal samples from those areas, we could not evaluate the value in association with the cause of diarrhea. Previous studies also showed no association between *Cryptosporidium* spp. infection and diarrhea in cattle [24, 30].

We found sequence diversities in 5' regions of the S gene and tentatively divided BoTVs into four groups. Japanese BoTVs were divided into two groups. Serogically, two types of BoTV have been recognized [34]. Serotype 1 represents Breda strain from Iowa, the United States and serotype 2 includes a second isolate from Iowa and an isolate from a diarrheic calf in Ohio, the United States. S protein is thought to be involved in viral infectivity and has been shown to be recognized by neutralizing antibodies [11, 22]. The molecular properties of the BoTV S gene resemble those of the S gene of coronaviruses [5]. In coronaviruses, the variation in host range and tissue tropism is largely attributed to variations in S protein [7, 8]. Therefore, it would be interesting to examine the relationship between antigenicity (or serotypes) and sequence differences in the S gene observed in our partial sequencing. It is necessary to determine the complete S gene sequences of our field BoTVs and isolates of serotype 2 to elucidate the relationships. However, owing to relatively high sequence variability in the BoTV S gene, we might have missed several BoTVs from fecal specimens by our primers targeting the S gene. It is necessary to detect BoTV by RT-PCR using genes coding for other proteins such as the membrane, hemagglutinin-esterase and nucleocapsid (N) proteins. We are now developing RT-PCR targeting the genetically most stable N gene to examine whether we can detect more BoTVs from the same specimens as those used in this study.

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