Epidemiological Analysis of Bovine Torovirus

（牛トロウイルスに関する疫学的研究）

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General Introduction

The family *Coronaviridae* consists of enveloped and single-stranded linear and positive-sense RNA viruses, specifically coronaviruses and toroviruses (Cavanagh., 1993 and 1997).

Coronaviruses are well known to have hosts in various mammalian and avian species. Although the most common target organs of coronaviruses are intestinal and respiratory tracts, they also possess a tropism to other various tissues; some coronaviruses can infect liver, kidney, nervous tissues, and so on (Brian and Baric., 2005; Cavanagh., 2005, Decaro and Buonavoglia., 2008; Foley and Leutenegger., 2001; Homberger., 1997).

The basic structures of toroviruses are similar to those of coronaviruses; both viruses are pleomorphic and enveloped viruses with 120 to 140 nm in the largest diameter. Similarly to coronaviruses, toroviruses have club-shaped spike proteins which are evenly dispread on the surface of viral particles. However, both viruses can be distinguished from each other by the morphology of nucleocapsid; toroviruses possess characteristic doughnut-shaped nucleocapsid with helical symmetry (Cornelissen et al., 1997; Horzinek et al., 1987; Lamouliatte et al., 1987;

Snijder and Horzinek., 1993; Weiss et al., 1983. Fig. A). Toroviruses, identified so far, have been further classified into human, bovine, porcine, and equine toroviruses on the basis of their own hosts (Beards et al., 1984; Kroneman et al., 1998; Weiss et al., 1983; Woode et al., 1982). Although several researches have established association of human, bovine and equine toroviruses with gastrointestinal symptoms in infected host animals (Beards et al., 1984; Jamieson et al., 1998; Pohlenz et al., 1984; Woode et al., 1984), the investigations on toroviruses including viral characterization and etiological significance have been limited, as compared to those of coronaviruses, primarily because toroviruses, with the exception of equine torovirus, are very difficult to propagate in cell cultures (Weiss et al., 1983).

Bovine torovirus (BToV) was first detected in the United States during an outbreak of diarrhea in cattle in 1982 (Woode et al et al., 1982). Subsequently, the experimental inoculation trial using cattle demonstrated that BToV infects crypt and villus epithelial cells of jejunum, ileum and colon, which results in gastroenteritis (Pohlenz et al., 1984; Woode et al., 1982). Electron microscopy (EM), enzyme-linked immunosorbent assay (ELISA), and reverse transcription-polymerase chain reaction (RT-PCR) on clinical materials have been often utilized for detection of BToV and an

epidemiological research on BToV infection because this virus can not grow in cell cultures (Koopmans et al., 1991; Liebler et al., 1992; Scott et al., 1996). The previous reports using these methods have shown that BToV is widespread worldwide including European countries and the United States and may act as one of risk factors for the gastrointestinal diseases in cattle (Canada: Duckmanton et al., 1998a; Netherlands: Koopmans et al., 1991; Austria: Haschek et al., 2006; United States: Hoet et al., 2002 and 2003; Germany: Liebler et al., 1992). However, there have been only a few reports dealing with spreading and circulation of BToV among Asian countries including Japan. Consequently, it is also unclear whether the BToV circulating in these areas have distinct characteristics with other countries.

Among the methods for BToV detection, RT-PCR is more sensitive than ELISA and nearly identical to EM in sensitivity (Duckmanton et al., 1998a; Hoet et al., 2002). Additionally, RT-PCR has the advantage that enables us to compare genetic diversity of BToVs from different sources by analyzing their RT-PCR products. Consequently, the genetic information of various BToVs has been accumulated to make its substantial database (Draker et al., 2006; Duckmanton et al., 1998b; Smits et al., 2003). Draker et al (2006) have determined the structure of BToV complete

genome by analyzing RT-PCR amplicons obtained from BToV infected stool specimen; the BToV genome is composed of 28,475 kb and consists of RNA polymerase, spike (S), membrane (M), hemagglutinin-estrase (HE), and nucleocapsid (N) genes. Several variety of genome size, encoding open reading frames and its sorting order has identified among the coronaviruses. On the other, such variety has not been identified so far among the reported toroviruses. The representative genome organization of the viruses included in the family *Coronaviridae* is indicated in Fig. B. The sequences of N and M coding regions have been suggested to be highly conserved among the BToVs compared with S coding region (Koopmans and Horzinek., 1994). On the contrary, the sequences of coding regions for S and HE genes have been shown to vary among BToVs investigated (Smits et al., 2003). On the other hand, the products of S gene of coronaviruses and equine torovirus are known to correlate closely with their antigenic properties (Gallagher and Buchmeier., 2001; Horzinek et al., 1987), and only one amino acid substitution in S gene product of bovine coronavirus (BCV) was shown to cause antigenic variation distinguishable by neutralization activity (Yoo and Deregt., 2001). These evidences appear to indicate that antigenic variations may exist among BToVs as reported in equine torovirus and coronavirus. However, antigenic properties of BToVs

using cultivatable viruses in cell cultures remain to be investigated because the virus is difficult to grow in cell cultures.

As mentioned first, the respiratory track is a major target of some coronaviruses besides the gastrointestinal tracts. For example, porcine respiratory coronavirus, which is antigenically closely related to another coronavirus, porcine transmissible gastroenteritis virus (TGEV) and believed to be a deletion mutant in S gene of TGEV, can easily infect respiratory tissues (Cox et al., 1990; Laude et al., 1993). Similarly to porcine coronavirus, BCV is also capable of infection in epithelial cells of both intestinal and respiratory tracts; consequently, both oral and nasal routes may be etiological risk factors for BCV infection among cattle (Clark., 1993; Cho et al., 2001; Park et al., 2007). On the contrary to porcine coronaviruses, however, enteric and respiratory BCVs have not been distinguished from each other on the basis of genetic analysis (Kanno et al., 2007).

On the other hand, there have been a few reports describing respiratory infection with BToV. Hoet et al. (2002) have reported that BToV was detected in both fecal and nasal swabs from feedlot cattle in the United States, suggesting that BToV infection may occur among cattle via not only oral but also nasal routes. However, no extensive surveys on the

relation between BToV infection and respiratory disorders in cattle have been reported. In addition, the genetic divergence between BToVs derived from fecal and nasal specimens remained to be investigated.

The investigation to solve the above issues, especially on prevalence of BToV infection among cattle, etiological involvement with bovine diseases, isolation of cultivatable BToV in cell cultures, virological, antigenic and genetic characterizations of BToV, and responses of infected cattle, are expected to produce fruitful data for understanding virology, epidemiology, etiological role, and immunological aspects of BToV.

In this thesis, the author performed epidemiological surveys using fecal and nasal swabs collected from cattle with intestinal and/or respiratory symptoms by RT-PCR, and RT-PCR products were genetically analyzed in comparison with BToVs previously reported. Concurrently, the samples positive for BToV gene were subjected to isolation of cultivatable BToV in cell cultures, and the author succeeded in isolation of cytopathogenic BToVs. Then, the isolated BToVs were subjected to virological, genetic, and antigenic characterization. Finally, the virological and immunological responses of cattle experimentally infected with the isolated virus were investigated, and in order to determine seroprevalence in Japan, serum samples collected from domestic farms were tested for

BToV antibodies by hemagglutination inhibition test using the isolated virus. The purpose of this thesis is to describe these investigations and to discuss about their results.

This thesis is composed of 5 chapters.

Chapter 1: 231 fecal samples obtained from Japanese cattle were analyzed by nested RT-PCR with primer sets designed specific for BToV N, M, and S genes, and the nucleotide sequences of RT-PCR positive specimens were compared to investigate genetic diversity among BToVs detected in fecal samples.

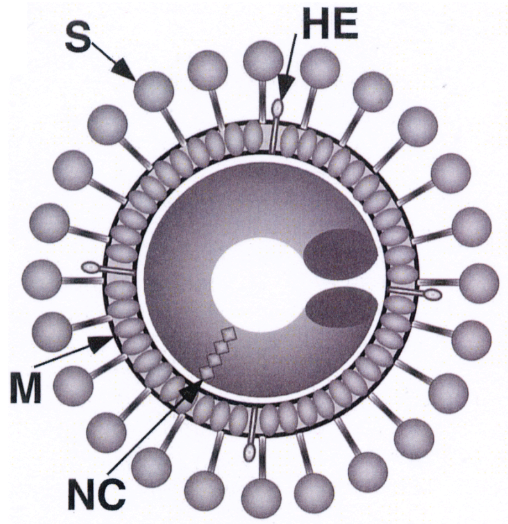
Chapter 2: The results of nested RT-PCR to amplify BToV N, S and HE gene on nasal samples collected from 311 cattle, and comparison of the nucleotide sequences of RT-PCR positive specimens with those of fecal samples previously reported are described.

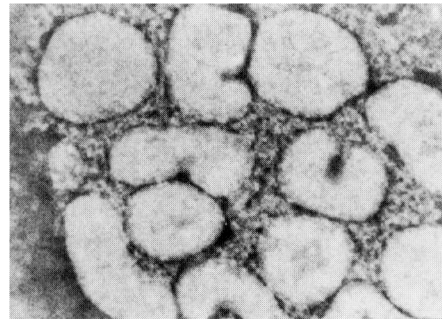
Chapter 3: The author made attempts to isolate BToVs from the RT-PCR positive specimens in HRT-18 cells derived from human rectal carcinoma, and succeeded in 4 cytopathogenic BToVs. In this chapter, the processes of their isolation and the results of their virological, genetic, and antigenic characterizations are described.

Chapter 4: Calves were experimentally inoculated with one of cytopathogenic BToVs described in chapter 3 and monitored for clinical manifestations of disease, excretion of the virus in feces and nasal secretions and antibody responses.

Chapter 5: 359 paired serum samples collected from 48 herds between December 2005 and March 2010 were tested for BToV antibodies to survey the relationship between clinical symptoms and the seroepidemiology of BToV.

(a)





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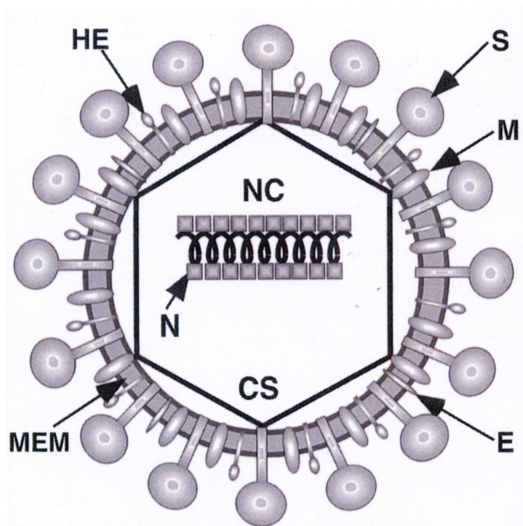


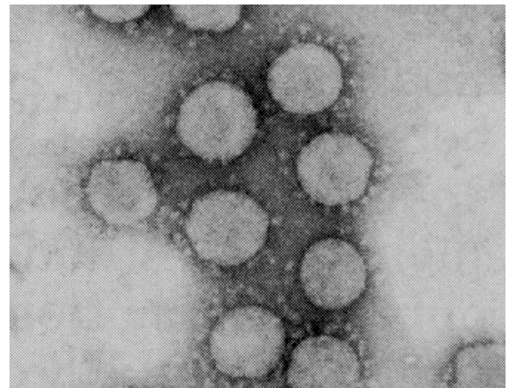
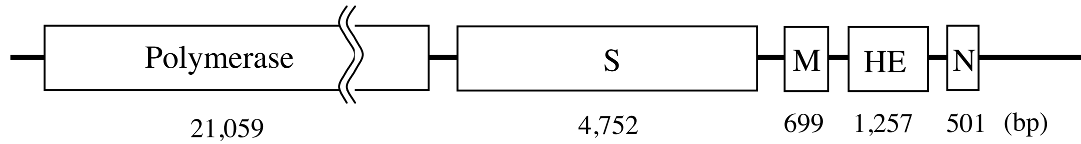


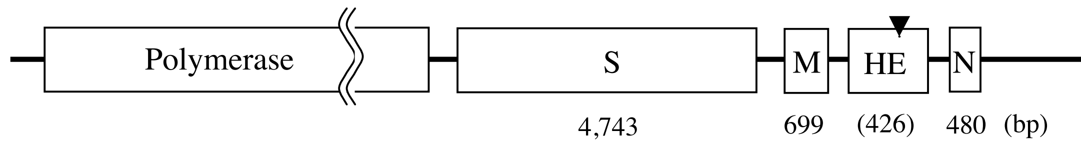
Fig. A.

Torovirus

Bovine torovirus - Bredavirus-1: 28.5 Kbp

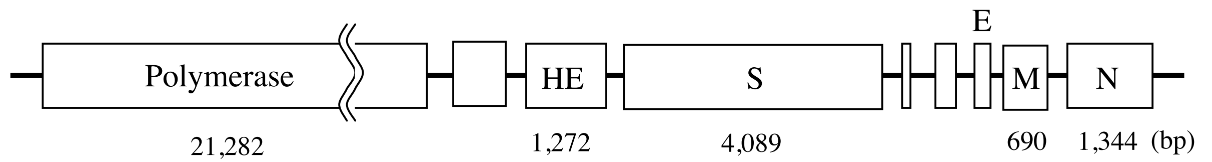


Equine torovirus - Bernevirus: 28 Kbp

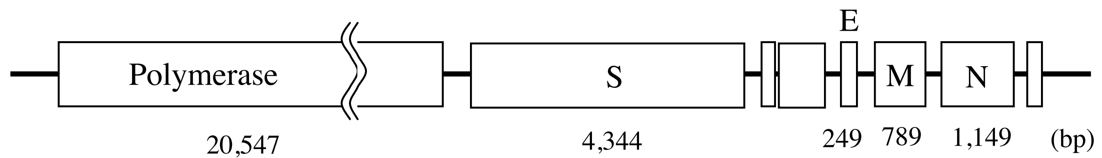


Coronavirus

Bovine coronavirus - Mebus strain: 31.0 Kbp



Transmissible gastroenteritis virus - Purdue 115 strain: 28.6 Kbp



Infectious bronchitis virus - Beaudette CK strain: 27.6 Kbp

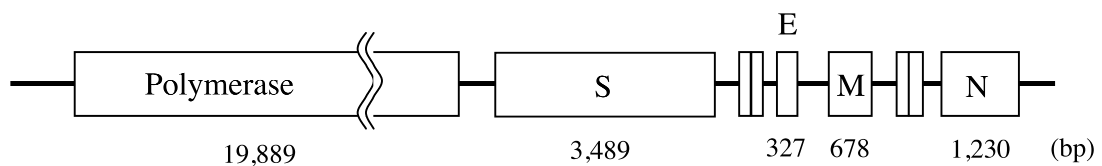


Fig. B.

Figure legends

Fig. A. Schematic interpretation and electron micrographic representation of (a) torovirus (Equine torovirus) and (b) coronavirus (Transmissible gastroenteritis virus). The localization of the structural protein and genome are indicated. S, spike protein; M, membrane protein; HE, hemagglutinin-estrase; N and NC, nucleocapsid; E, small-envelope protein; MEM, lipid membrane; CS, core-shell. (Reference: Virus Taxonomy VIII)

Fig. B. The representative genome organizations of viruses included in the family *Coronaviridae*. S, spike protein; M, membrane protein; HE, hemagglutinin-estrase; N, nucleocapsid; E, small-envelope protein.

CHAPTER 1

Molecular epidemiological analysis of bovine torovirus in Japan

Abstract

Bovine torovirus (BToV), a member of the *Coronaviridae*, is an established gastrointestinal infectious agent in cattle. No epidemiological research on BToV has been reported from Japan. In this study, the author performed a survey to detect BToV in Japan in 2004 and 2005 using 231 fecal samples (167 from diarrheic cattle and 64 from asymptomatic cattle) by nested reverse transcription (RT) PCR using primers located in the consensus sequences of the reported BToV nucleocapsid (N), membrane (M), and spike (S) genes. BToV N, M, and S genes were detected in 6.5% (15/231), 6.1% (14/231), and 5.6% (13/231) of samples by nested RT-PCR. During the research, detectability was improved compared to the results of the first round of RT-PCR. Among the 15 samples that were positive for the BToV N gene, BToV was detected at a significantly higher rate in diarrheic samples than in asymptomatic samples (14/167 diarrheic samples [8.4%] and 1/64 asymptomatic samples [1.6%]), suggesting that BToV may act as a risk factor for diarrhea in Japanese cattle. The nucleotide sequences of N and M fragments showed more than 97% identity, except for the N fragment of the Breda virus-1 and Breda virus-2, a member of the BToV reported by Duckmanton et al. Domestic samples were classified

into three clusters by phylogenetic analysis of the S gene fragment, which were considerably correlated with the geographic origin of the samples. These areas did not adjoin each other but were spread across a wide range, suggesting that BToV exists conventionally in Japan and is geographically differentiated. The author also developed an RFLP method to distinguish these clusters using two restriction enzymes, *Hae*III and *Acc*I. This method should be useful for comparing newly acquired BToV-positive samples with the reported BToVs.

Introduction

In 1982, a new virus was detected from diarrheic calves, which proved to be a bovine torovirus (BToV) (Woode et al., 1982). The detection of BToV in fecal samples has since been reported in various countries (Duckmanton et al., 1998a; Koopmans et al., 1991; Haschek et al., 2006; Hoet et al., 2002, 2003; Liebler et al., 1992). Furthermore, artificial inoculation trials have demonstrated the pathogenicity of BToV to cattle (Pohlenz et al., 1984; Woode et al., 1982). These data strongly suggest that BToV is associated with diarrhea in cattle. However, no epidemiological data from Japan has been reported. Since BToV cannot yet be grown in cell culture, identification of the N and M genes of the virus is thought be the most sensitive method for detecting BToV in fecal samples, and it can distinguish the virus from both coronaviruses and other toroviruses (equine isolate Berne virus, porcine torovirus, and human torovirus; Koopmans and Horzinek, 1994). Moreover, analysis of the S gene, which is known in other coronaviruses to influence antigenicity (Gallagher and Buchmeier, 2001), in BToV N and M gene-positive samples appears to be important in determining the pathogenicity of the virus.

In this chapter, the author reports the results of an analysis of 231

fecal samples from Japanese cattle, in which BToV was detected by nested RT-PCR using primers specific for the N, M, and S genes of BToV. Subsequently, the author compared these nucleotide sequences to assess viral genetic diversity.

Materials and methods

Specimens

A total of 231 individual fecal samples (167 from diarrheic cattle and 64 control samples from asymptomatic cattle) were collected from 32 farms in 12 prefectures in Japan (Fig. 1-1) between April 2004 and March 2005. All 231 fecal samples were used for BToV, bovine coronavirus (BCV), and rotavirus detection. The majority of cattle were between 1 week and 6 years old at the time of sample collection.

RNA extraction

The fecal samples were diluted in 10x volume of phosphate-buffered saline (PBS, pH 7.5) and centrifuged at 1,000 x *g* for 1 min at room temperature. The supernatant was transferred to a new tube and centrifuged again at 8,000 x *g* for 5 min at room temperature. RNA was extracted from the supernatant using an RNeasy Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. For each extraction period, deionized-destilled (dd) H_2O was used as a negative control.

Detection of BToV and BCV

The author used nested RT-PCR for BToV and RT-PCR for BCV detection. Primers targeting BToV N, M, and S genes were designed based on reported BToV sequences (Duckmanton et al., 1998b; Smits et al., 2003). The primers, reference sequences, and the expected sizes of the fragments obtained in the first and nested round of amplification are shown in Table 1-1. The primers targeting the BCV N gene were as follows: forward: 5'- TGCCAGGATGATGGCGCGTG - 3' and reverse: 5' - AGA AGCACATCAGGGGATTC - 3'. Superscript II (Invitrogen Corp., Carlsbad, CA, USA) was used for reverse transcription using the reverse primer of each first round PCR primer. PCR was performed using TaKaRa Taq (TaKaRa, Tokyo, Japan). The mixture of reaction reagents was treated according to the manufacturer's instructions. The conditions of PCR were as follows: 30 cycles of denaturing at 94ºC for 1 min, annealing at 53ºC for 1 min, and extension at 72ºC for 1 min, followed by a final cycle of extension at 72ºC for 5 min. For each reaction, ddH_2O was used as a negative control. PCR products were detected by electrophoresis on a 2.0% agarose gel.

Detection of rotavirus

The author followed the methods of Okada and Matsumoto (2002) for detection of Rotavirus using the fecal supernatant, as in the samples for RNA extraction.

Genetic analysis of BToV PCR products

The nested RT-PCR products corresponding to the BToV N, M, and S open reading frames (ORFs) were directly sequenced by Dragon GenomicsCtr (TaKaRa Bio Inc., Mie, Japan) with the ABI Prism BigDye Terminator version 3.1 cycle sequencing kit and an Applied Biosystems 3730xl DNA analyzer (Applied Biosystems Inc., CA, USA). For the analysis of sequence relationships, BToV BRV-1, BRV-2 (BToV serotypes) and B145 were used as reference sequences, which had been detected in cattle from other countries. The accession numbers (National Center for Biotechnology Information) of the reference sequences are given in Tables 1-3~5. RT-PCR products were purified using a DNA purification kit (QIAGEN) and digested by several restriction enzymes (TOYOBO, Tokyo, Japan) according to the manufacturer's instructions. The size of the DNA fragments was estimated using a 1-kb plus DNA ladder (Invitrogen).

Phylogenetic analysis of the BToV S gene

Phylogenetic analysis of the deduced amino acid sequence of the BToV S gene was preformed using the neighbor-joining method (Saitou and Nei., 1987).

Results

Condition of BToV-positive samples

BToV gene products corresponding to the N, M, and S ORFs were detected in 2.1% (5/231), 3.5% (8/231), and 0% (0/231) of the samples, respectively, by first round PCR and were detected in 6.5% (15/231), 6.1% (14/231), and 5.6% (13/231) of samples by subsequent nested PCR (Table 1-2). Among the 15 samples that were positive for the BToV N gene, 14 were derived from diarrheic samples (8.4%) and 1 was derived from an asymptomatic sample (1.6%). The positive samples were collected in 4 of 12 prefectures in Japan (Fig. 1-1). Of these 14 positive diarrheic samples, 7 were also positive for BCV (Table 1-2). BCV was detected in 47 samples (20.3%) and rotavirus was detected in 15 samples (6.5%). These tests were performed 2 or 3 times to confirm the initial results.

Genetic diversity of PCR products

The nucleotide sequences of N and M fragments showed more than 97% identity, except for the N fragments of BRV-1 and BRV-2 (Tables 1-3 and 1-4). The nucleotide sequences of the BToV S gene fragment showed lower identity than those of N and M, even among the domestic samples

(Table 1-5). Representative sequence data have been deposited in the nucleotide database (DNA Data Bank of Japan) and assigned the following accession numbers: K-567 (N: AB270904, M: AB270905, S: AB270906), K-629 (N: AB270907), K-637 (M: AB270908, S: AB270909), K-639 (S: AB270910), K-645 (M: AB270911), K-674 (N: AB270912, M: AB270913, S: AB270914), K-676 (M: AB270915, S: AB270916), K-683 (M: AB270917, S: AB270918), K-684 (S: AB270919).

The author performed further analysis of the BToV S gene to characterize these samples. The author generated a phylogenetic tree of the deduced amino acid sequence of the BToV S gene, including previously reported BToV sequences, to investigate the phylogenetic relationships among these samples. The BToV S gene found in Japan was divided into three clusters (Fig. 1-2): cluster 1 (samples K-567, K-637 through K-642, K-644, and K-645), cluster 2 (samples K-674 and K-676), and cluster 3 (samples K-683 and K-684).

RFLP analysis of BToV-positive samples

The author established a method to rapidly confirm the phylogenetic cluster to which a sample belonged. Based on the nucleotide sequences, the author screened restriction enzymes and performed RFLP analysis of the

BToV S fragment. The three clusters could be differentiated using *Hae*III or *Acc*I. Within cluster-1, K-567 could be differentiated from K-637 through K-642, K-644, and K-645 by detection of a distinctive 142-bp fragment using *Acc*I (Fig. 1-3).

Discussion

The author performed an epidemiological analysis of BToV in Japan by looking for BToV-specific genes in 231 fecal samples collected in 2004 and 2005. The author performed nested RT-PCR to improve the detectability of BToV by using a primer located in the consensus sequence of the reported BToVs. Consequently, the author obtained 15 BToV-positive samples. Improvements in the detectability of BToV N, M, and S PCR products were observed in all samples by nested PCR, suggesting the usefulness of this method in detecting BToV. Sample K-629 had no M gene amplification, and this sample and sample K-643 were both negative for the S gene. However, since the N gene could be amplified from both samples, it is likely that nucleotide differences may exist in the primer annealing region used for M and S amplification and the corresponding sequences of these isolates.

Of 14 BToV-positive diarrheic samples, 6 were negative for BCV and rotavirus, a well known diarrheic agent in cattle. However, since infections with BCV, rotavirus, or other enteropathogens could have occurred before the samples were taken, it is impossible to determine whether BToV was the primary cause of diarrhea in these samples. However, there have been

several reports indicating the relationship of BToV and diarrhea in cattle. Further, the author detected BToV at a significantly higher rate in diarrheic samples than in asymptomatic samples. These results suggest that BToV may act as a risk factor for diarrhea in Japanese cattle.

The nucleotide sequences of the BToV S fragments had lower identity compared to the sequence identity of N and M fragments. In coronaviruses, which possess a similar structure to torovirus, a hypervariable region exists in the S gene (Wang et al., 1992). One amino acid substitution causes a change in virus neutralization activity in BCV (Yoo and Deregt, 2001). Thus, the S gene has been used to genetically characterize each strain of coronaviruses (Jackwood et al., 2005; Phillips et al., 2001; Yoo and Deregt, 2001). The BToV S molecule is thought to be located on the surface of the virus membrane and to have a similar function to the coronavirus spike (Horzinek et al., 1987).

Two BToV serotypes exist (BRV-1 and BRV-2; Duckmanton et al., 1998b), but further research of their antigenic properties is required because BToV cannot yet be propagated in cell culture. For the same reason, there are few reports characterizing BToV. In this study, the author genetically characterized BToV samples by phylogenetic analysis of the deduced amino acid sequence of the BToV S gene, including the

previously reported BRV-1, BRV-2 and B145 sequences. The detected BToVs were separated into three clusters. The cluster 2, which included samples K-674 and K-676, was located relatively closer to BRV-1. The cluster 1, the most frequently detected BToV cluster, was located distal to BRV-1, BRV-2 and B145. In addition, high divergence was observed in the N sequence between the domestic samples and BRV-1 and BRV-2, which were expected to possess high identity. These results raise the possibility that the predominant BToV in Japan may be different from BRV-1 and BRV-2 in antigenic properties. Therefore, it is important to identify the antigenic properties and to clarify their correlation with genetic properties.

The phylogenetic clusters were correlated with the geographic sources of the samples. The BToV-positive areas did not adjoin each other but were spread throughout Japan, spanning the BToV-negative areas. These results suggest that BToV is geographically differentiated in Japan.

Additionally, intertypic recombination occurs in torovirus (Smits et al., 2003). Therefore, it is important to continue the investigation of the genetic divergence of BToV. To support this, the author developed an RFLP method to distinguish the clusters indicated by the phylogenetic classification. Although both *Hae*III and *Acc*I discriminate among the clusters, the fragment patterns differ. From the predicted fragment pattern,

it is also possible to distinguish among BRV-1, BRV-2 and B145. The combination of these 2 enzymes will further support the polymorphism of novel BToV isolates in the future.

In this study, the author was able to examine the genetic divergence of BToV by analyzing the PCR products of the S gene, including previously reported BToVs. The overall detection rate of BToV was 6.5%, but the detection rate varied among geographic areas. It is not clear whether this rate is the consequence of indigenous BToV or overall lower invasion of BToV in Japan. Therefore, it is important to identify the retention of the BToV antibody to predict BToV prevalence.

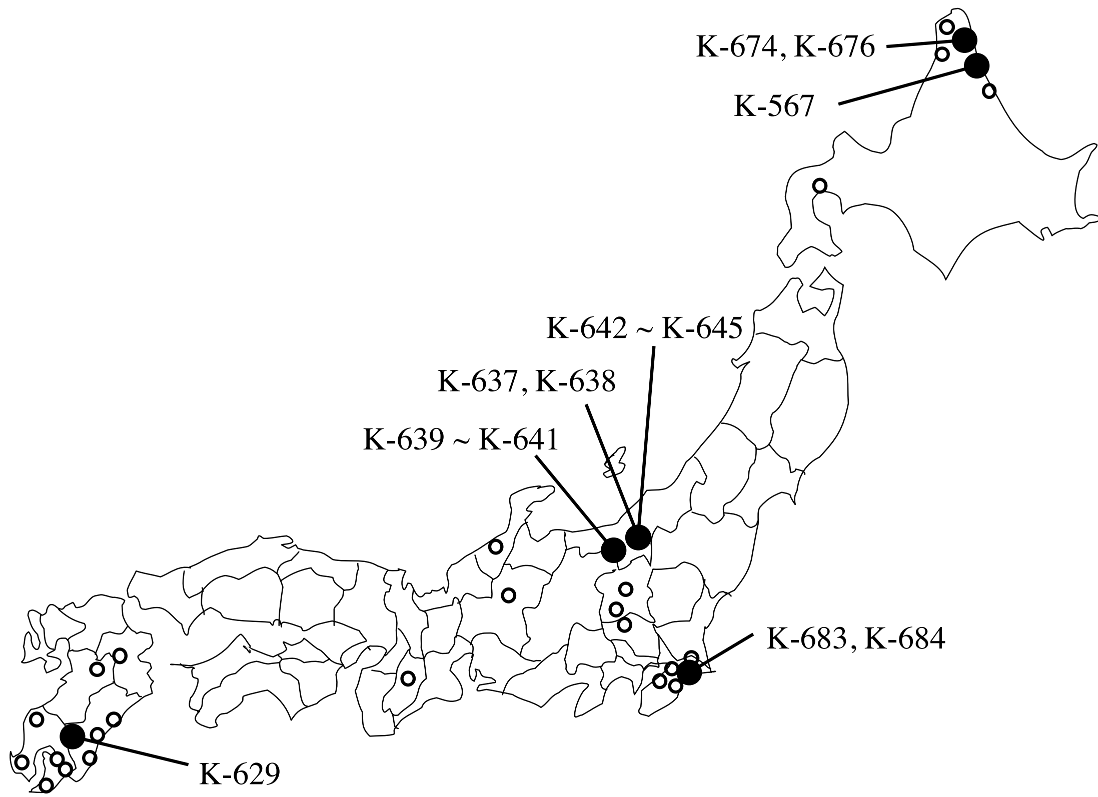


Fig. 1-1.

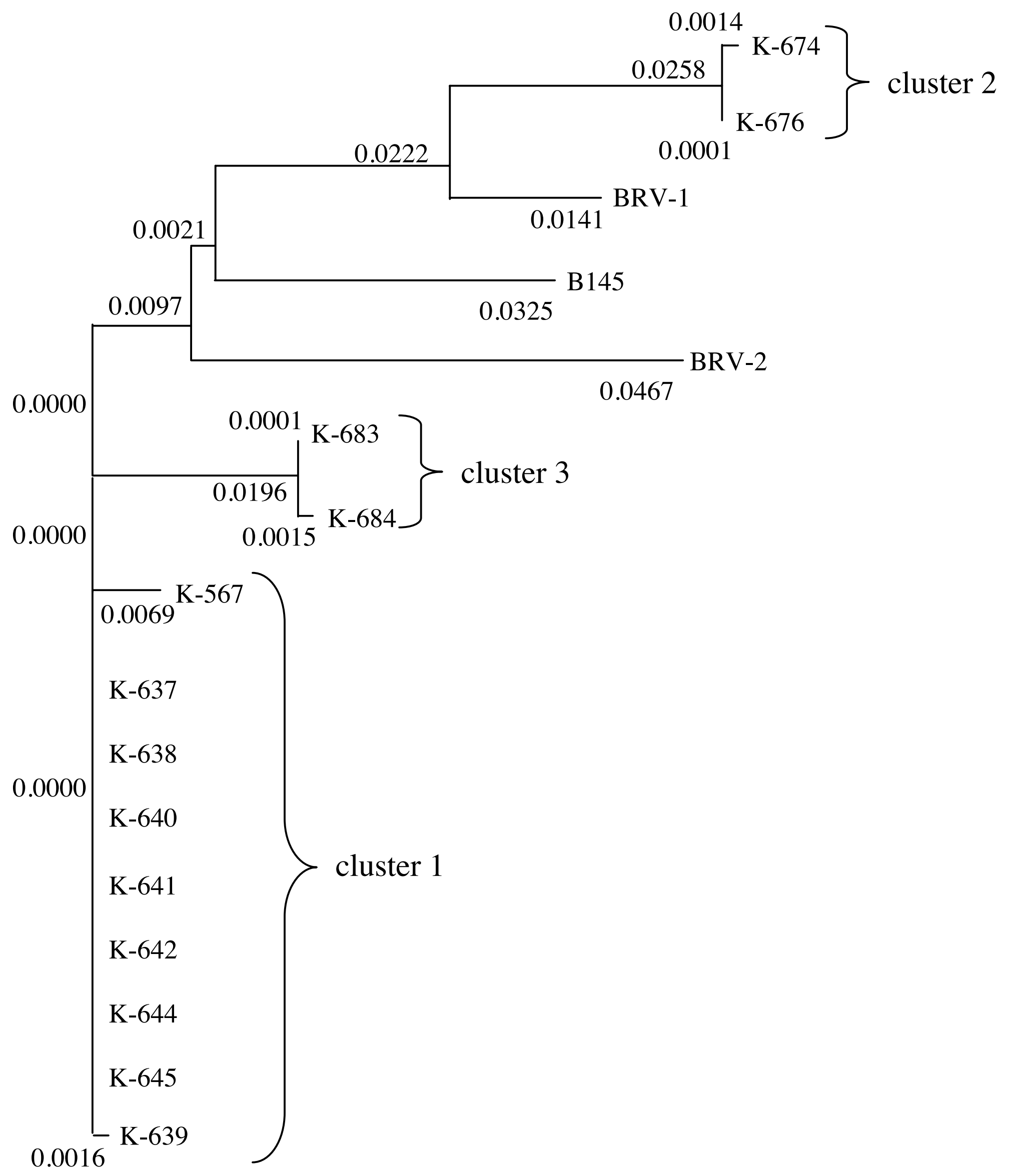
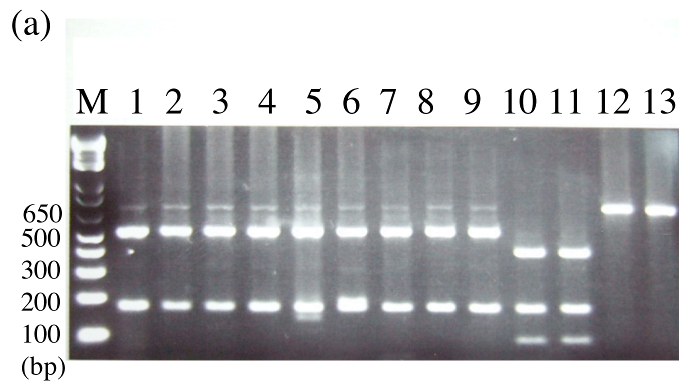


Fig. 1-2.

(a)



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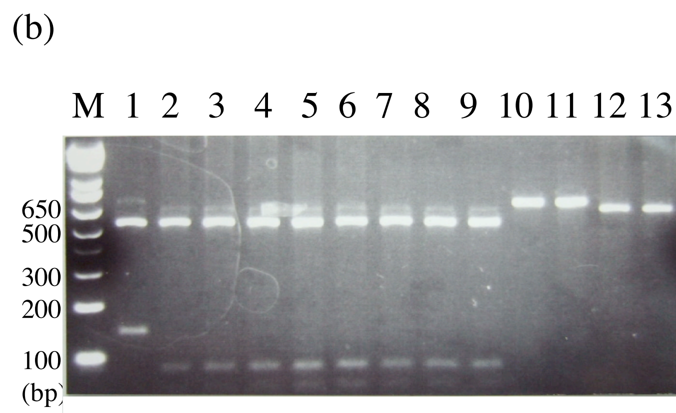


Fig. 1-3.

Table. 1-1. The oligonucleotide primer pairs used for RT-PCR

Target	Usage	Polarity	Position[1]	Primer sequence	Expected fragment size[2]	Reference sequences and content
BToV N	First step PCR	Forward	27775 bp ~ 27795 bp	5' - ATG AAT TCT ATG CTT AAT CCA - 3'	471 bp	AJ575389, AJ575388, AJ575387, AJ575386, AJ575385
		Reverse	28265 bp ~ 28245 bp	5' - AAT TCA AAG CCA CTT TTA TTG - 3'		
	Nested PCR[3]	Forward	27793 bp ~ 27813 bp	5' - CAA ATG CTA TGC CAT TTC AGC - 3'	395 bp	
		Reverse	28207 bp ~ 28187 bp	5' - TGG AAA CTT CAA CAG TGG CAT - 3'		
BToV M	First step PCR	Forward	25759 bp ~ 25779 bp	5' - TGT TTG AGA CCA ATT ATT GGC - 3'	682 bp	AJ575374, AJ575375, AJ575376, AY427798
		Reverse	26460 bp ~ 26440 bp	5' - TAC TCA AAC TTA ACA CTA GAC - 3'		
	Nested PCR	Forward	25797 bp ~ 25817 bp	5' - CCA AAC CCA TTT ACT GCT CAA - 3'	637 bp	
		Reverse	26413 bp ~ 26433 bp	5' - GTA TAA TCT GCA ACA CCT TGC - 3'		
BToV S	First step PCR	Forward	20957 bp ~ 20977 bp	5' - GTG TTA AGT TTG TGC AAA AAT - 3'	722 bp	AJ575373, AY427798, AF076621
		Reverse	21698 bp ~ 21678 bp	5' - TGC ATG AAC TCT ATA TGG TGT - 3'		
	Nested PCR	Forward	21025 bp ~ 21045 bp	5' - CAG AGG TGC CGT TGT TGT GTC - 3'	616 bp	
		Reverse	21660 bp ~ 21640 bp	5' - ACA TAG AGC GGT GTC TGT TGA - 3'		

1) Position with respect to the AY427798 strain.

2) Including primer length.

3) First round PCR products was directly used as template in nested PCR.

Table. 1-2. The condition of the BoTV-positive sample.

Sample name	Sampling month	Age	Fecal condition	BToV PCR result			Bovine rotavirus	Bovine coronavirus
				nucleocapsid	membrane	spike		
K-567	2004. Apr	1 week	diarrhea	+	+	+	-	-
K-629	2004. Apr	1 week	diarrhea	+	-	-	-	-
K-637	2004. Sep	adult[*)]	diarrhea	++	++	+	-	-
K-638	2004. Sep	adult	diarrhea	++	++	+	-	-
K-639	2004. Dec	adult	diarrhea	++	++	+	-	++
K-640	2004. Dec	adult	diarrhea	++	++	+	-	++
K-641	2004. Dec	adult	diarrhea	+	++	+	-	++
K-642	2005. Jan	adult	diarrhea	+	+	+	-	++
K-643	2005. Jan	adult	diarrhea	++	+	-	-	++
K-644	2005. Jan	adult	diarrhea	+	++	+	-	++
K-645	2005. Jan	adult	diarrhea	+	+	+	-	++
K-674	2005. Mar	adult	normal	+	+	+	-	-
K-676	2005. Mar	adult	diarrhea	+	+	+	-	-
K-683	2005. Mar	adult	diarrhea	+	++	+	-	-
K-684	2005. Mar	adult	diarrhea	+	++	+	-	-

(++) indicates the PCR-positive sample in 1st PCR and (+) indicates the PCR-positive sample in 2nd PCR.
*) between 1 and 6 years after birth.

Table. 1-3. Nucleotide and amino acid identities of N gene 369 base pair sequences

	Nucleotide identity (%)					
strain	group I	group II	group III	B145	BRV-1	BRV-2
group I		99.5	98.1	100	68.1	68.1
group II	100		97.6	99.5	68.1	68.1
group III	99.2	99.2		98.1	68.1	68.4
B145	100	100	99.2		68.1	68.1
BRV-1	68.8	68.8	68.8	68.8		97.6
BRV-2	69.6	69.6	69.6	69.6	96.8	
	Amino acid identity (%)					

The group I includes K-567, K-683 and K-684, group II includes K-629, K-637 ~ K645, the group III includes K-674 and K-676. Isolates included within a group have all completely identical sequences. The reference BoTV used for sequence comparison and accession numbers were as follow: B145 (AJ575388), BRV-1 (AY427798), BRV-2 (AF076621).

Table. 1-4. Nucleotide and amino acid identities of M gene 593 base pair sequences

	Nucleotide identity (%)								
strain	K-567	group I	K-645	K-674	K-676	group II	B145	BRV-1	BRV-2
K-567		99.3	99.2	98.7	98.5	99.2	99.2	94.6	93.3
group I	100		99.8	99	98.8	99.2	99.2	94.4	93.3
K-645	100	100		98.8	98.7	99	99	94.4	93.1
K-674	99.5	99.5	99.5		99.8	98.5	98.5	95.1	93.6
K-676	99	99	99	99.5		98.3	98.3	94.9	93.4
group II	99.5	99.5	99.5	99	98.5		99	94.3	93.3
B145	100	100	100	99.5	99	99.5		95.3	94
BRV-1	98	98	98	97.4	96.9	97.4	98		97.7
BRV-2	96.9	96.9	96.9	96.4	95.9	96.4	96.1	98.3	
	Amino acid identity (%)								

The group I includes K-637 ~ K644 and the group II includes K-683 and K-684. Isolates included within a group have all completely identical sequences. The reference BoTV used for sequence comparison and accession numbers were as follow: B145 (AJ575375), BRV-1 (AY427798), BRV-2 (AF076621).

Table. 1-5. Nucleotide and amino acid identities of S gene 594 base pair sequences

	Nucleotide identity (%)									
Strain	K-567	group I	K-639	K-674	K-676	K-683	K-684	B145	BRV-1	BRV-2
K-567		98.3	98.1	92.6	92.8	96.8	96.6	94.3	93.9	93.3
group I	99		99.8	92.3	92.4	96.5	96.3	94.1	93.4	93.1
K-639	98.5	99.5		92.1	92.3	96.3	96.1	93.9	93.3	92.9
K-674	91.4	92.4	91.9		99.8	91.6	91.8	92.4	96	90.6
K-676	91.9	92.9	92.4	99.5		91.8	91.6	92.6	96.1	90.4
K-683	98	99	98.5	91.9	92.4		99.8	93.4	92.8	93.1
K-684	97.5	98.5	98	92.4	91.9	99.5		93.3	92.6	93.3
B145	94.4	95.4	94.9	92.4	92.9	94.4	93.9		92.9	91.6
BRV-1	93.9	94.9	94.4	94.4	94.9	94.4	93.9	93.9		92.4
BRV-2	93.9	94.9	94.4	90.4	89.9	94.9	95.4	91.4	92.4	
	Amino acid identity (%)									

The group I includes K-637, K-638, K-640 ~ K642, K-644, K-645, which possesses completely identical sequences. The reference BoTV used for sequence comparison and accession numbers were as follow: B145 (AJ575373), BRV-1 (AY427798), BRV-2 (AF076621).

Figure legends

Fig. 1-1. Distribution of BToV-positive samples in Japan. Open circles (o) indicate BToV-negative areas tested in this study.

Fig. 1-2. Neighbor-joining phylogenetic tree showing the relationships among the deduced S amino acid sequences of BToV from Japan and from the reference strains described in Table 1-5. The numbers represent the distance to the nearest node.

Fig. 1-3. RFLP patterns of PCR-amplified BToV S gene products. (a) Digestion using *Hae*III. (b) Digestion using *Acc*I. Lane 1: K-567, 2: K-637, 3: K-638, 4: K-639, 5: K-640, 6: K-641, 7: K-642, 8: K-644, 9: K-645, 10: K-674, 11: K-676, 12: K-683, 13: K-684.

CHAPTER 2

Detection and characterization of bovine torovirus from the respiratory tract in Japanese cattle

Abstract

Bovine torovirus (BToV), a member of the *Coronaviridae* family, is a causative agent of diarrhea in cattle, but it may also possess tropism for the respiratory tract. However, no surveys concerning with the relation between respiratory symptoms and the detection of BToV have been conducted in wide range. Among 311 nasal samples, BToV gene products were detected in 7 samples (rBToV-1 to -7) derived only from calves with respiratory symptoms, suggesting that BToV may be a predisposing factor and/or causative agent for bovine respiratory disease. Regarding the degree of similarity between the spike and hemagglutinin-esterase coding regions, the rBToVs showed over 90.8% similarity with one another and 73.5–99.0% similarity with fecal tract-derived BToVs. rBToV-1, -2, and -3 were identical despite their being collected during different seasons; in comparison, rBToV-4 and -5 were distinct despite the fact that they were collected from the same herd, suggesting the existence of diversity among domestic rBToVs. One animal with a BToV-positive nasal sample also shed the virus in its feces, suggesting dual tropisms for BToV.

Introduction

BToV has become recognized as a causative agent of diarrhea in cattle since its discovery in 1982 (Duckmanton et al., 1998a; Koopmans et al., 1991; Liebler et al., 1992; Pohlenz et al., 1984; Scott et al., 1996). Furthermore, BToV may infect its host via respiratory and intestinal routes, similar to another member of the *Coronaviridae* family, BCV (Cho et al., 2001; Park et al., 2007). Indeed, the affinity of BToV for the tissues of the respiratory tract has been reported (Vanopdenbosch et al., 1991, 1992), although possible contamination of the reagents used renders the significance of this finding uncertain (Cornelissen et al., 1998). In addition, a study conducted at a single farm in the United States showed the presence of BToV in both fecal and nasal swabs from feedlot cattle (Hoet et al., 2002). However, no large-scale surveys of the relationship between respiratory symptoms and BToV have been conducted, primarily because toroviruses, with the exception of equine torovirus, are difficult to propagate via cell culture (Weiss et al., 1983). Consequently, amplification of the N gene by nested RT-PCR is considered the most practical method of detection for BToV in clinical specimens, as stated in chapter 1.

In this study, the author assayed the presence of BToV and BCV,

along with other recognized respiratory pathogens, in 311 nasal samples collected from Japanese cattle (107 dairy cattle and 204 beef cattle). Subsequently, the nucleotide sequences of the S and HE genes of BToV, which have been shown to be correlated with the antigenic properties of coronavirus (Clark, 1993; Gallagher and Buchmeier, 2001; Jackwood et al., 2005; Phillips et al., 2001; Yoo and Deregt, 2001), were compared to investigate the level of genetic diversity among the detected viruses and against those detected previously from fecal samples (Draker et al., 2006; Duckmanton et al., 1998b; Kuwabara et al., 2007; Smits et al., 2003; chapter 1).

Materials and methods

Specimens

Nasal swabs were collected from 205 cattle showing respiratory symptoms and 106 asymptomatic cattle raised on 42 farms located in 16 prefectures between March 2006 and June 2008; 264 of the cattle were less than 12 months old. The nasal swabs were diluted 1:10 in Dulbecco's modified Eagle's medium and centrifuged at 3000 × g for 5 min at room temperature; the supernatants were subsequently collected and subjected to RNA extraction followed by nested RT-PCR.

RNA extraction and Genetic analyses

The methods used for extraction of the RNA and for amplifcation of the N, S, and HE genes from BToV and the N gene from BCV by nested RT-PCR, and the primer sets used to target the N and S genes of BToV and the N gene of BCV are described in chapter 1. The primers used in the first round of PCR and for nested RT-PCR to target the BToV HE gene were as follows: first step forward, 5′-CGGCAACACCAGTAACACCAT-3′; first step reverse, 5′-TAACTAAAACTAATAACACC-3′; nested forward, 5′-GATTGGTGTGGGTTTGGT GA-3′; and nested reverse,

5′-ATATGCAGAGGAGGTTACATC-3′. The expected product was 1,094 bp long, corresponding to nucleotides 26,564–27,657 of AY427798.

Phylogenetic analysis

Nucleotide sequencing and phylogenetic tree analysis were performed as described in chapter 1.

Results and Discussion

The N gene of BToV and BCV was detected in 2.3% (7/311) and 10.9% (34/311) of the nasal samples, respectively (Table 2-1(a)). Seven BToV-N-positive samples, referred to as respiratory tract-derived BToV-1 (rBToV-1) through -7, were identified in 6 herds from 5 prefectures (Table 2-1(b)). Compared to the report from the U.S. feedlot, in which BToV was detected in almost all of the cattle during the first 21 days after arrival (Hoet et al., 2002), the frequency of BToV-positive cattle in this study was significantly lower. The reason of this difference is unclear at present as the sampling condition (population size, nature of farms, sampling period) between Hoet et al and the author's study differ considerably. Supporting this idea, Hoet et al. (2002) reported that newly arrived feedlot calves became infected with BToV soon after arrival and shed BToV in their feces and nasal secretions; however, secretion of the virus disappeared according to the calves acquired immunocompetence against BToV. Compared to the detection situation of BCV among the 311 samples, the detection of BToV was restricted to symptomatic calves. In addition, the detection rate was less than half that for BCV; however, the author's results support the notion that BToV is shed in nasal secretions as well as in feces, and that like BCV,

the virus is able to infect its host via oral and nasal routes.

Among the animals that developed clinical symptoms, BToV, BCV, *infectious bovine rhinotracheitis virus* (IBRV), *bovine viral diarrhea virus* (BVDV) and *bovine respiratory syncytial virus* (BRSV) was detected in 3.4% (7/205), 15.1% (31/205), 0.5% (1/205), 1.0% (2/205) and 2.0% (4/205) (Table 2-1(a)). The BToV-N-positive samples were collected over a relatively long period of time (year-round except during the warmest season) but all were calves that between the ages of 1 and 8 months developed clinical symptoms (Table 2-1(b)). Among the rBToVs, rBToV-1 was also positive for BCV while rBToV-5 was also positive for *Mycoplasma bovis*. These samples were additionally examined for several other pathogens known to be associated with respiratory disease (*M. bovis*, *Mannheimia haemolytica*, and *Pasteurella multocida*); however, negative results were obtained for the cattle that were positive for rBToV-2, -3, -4, -6, and -7 (data not shown). Therefore, although the etiological role of BToV in the development of respiratory disease is unclear, BToV may be a predisposing factor for and/or causative agent of bovine respiratory disease in young cattle. Additional field and laboratory studies are needed to elucidate the etiological significance of BToV in bovine respiratory disease.

The BToV S gene was detected in all of the N gene-positive samples except rBToV-7 (Table 2-1(b)). Regarding the S coding region, the respiratory tract-derived BToVs showed over 91.1% nucleotide similarity and more than 91.4% amino acid similarity with one another. Compared to the previously identified fecal tract-derived BToVs, rBToV showed 89.6–99.2% nucleotide similarity and 89.3–99.5% amino acid similarity (Table 2-2(a)). The BToVs used for the comparison were BRV-1 and -2 from North America; B145, B150, and B155 from Europe; and K-640, K-644, and Aichi/2004 from Japan (see Table 2-2 for the relevant accession numbers). Among the respiratory tract-derived samples, rBToV-1, -2, and -3 were completely homologous despite the fact that the samples were obtained from cattle raised in distinct areas and during different seasons. These tests were performed 1 or 2 times to confirm the initial results.

Phylogenetic tree analysis of the region revealed that rBToV-1, -2, and -3 were closely related to BToV cluster 2 (K-674 and K-676), which was classified among the fecal-derived BToVs from Japan (Fig. 2-1(a)). In contrast, rBToV-4 and -5 were distant from rBToV-1, -2, and -3 compared to B145 and BRV-1. rBToV-6 was clustered with K-640 and K-644 (cluster 1), as was Aichi/2004. Although two calves corresponding to

rBToV-4 and -5 samples were born in same farm and the nasal samples were collected at the same time, they were clustered on a separate branch; rBToV-4 was closer to K-683 and K-684 (cluster 3) than rBToV-5. The origin of this difference remains unclear until now.

In contrast to the N and S genes, HE was not detected in rBToV-1, -3, or -7. Regarding the HE coding region, the respiratory tract-derived BToVs showed over 90.8% nucleotide similarity and more than 91.1% amino acid similarity with one another. Compared to the fecal tract-derived BToVs, rBToV showed 73.5–99.0% nucleotide similarity and 72.0–99.1% amino acid similarity (Table 2-2(b)).

As shown by phylogenetic tree analysis of the HE region, rBToV-2 exhibited significant diversity, especially for BRV-1 compared to S, but it still retained a high correlation with K-674 and K-676 compared to B145 and the other dometic BToVs (Fig. 2-1(b)). Similar to S, divergence has also been detected between rBToV-4 and -5 but additionally, they were clustered on a separate major branch; rBToV-4 was clustered with K-683 and K-684, while rBToV-5 was clustered with K-640 and K-644. rBToV-6 was distant from the other rBToVs. Representative sequence data have been deposited in the nucleotide database (DNA Data Bank of Japan) and assigned the following accession numbers: AB371899–AB371906,

AB448741–AB448746.

As no comparative analysis of rBToVs has been performed, the author cannot compare the results with those for other rBToVs. On the other hand, previous research of the S region in domestic fecal derived BToVs (eBToVs) showed greater than 99% homology among the samples collected from a single herd at the same time as described in chapter 1. Accordingly, the existence of two distinct viruses in a single herd, as in the case of rBToV-4 and -5, has not yet been reported. rBToV-1, -2, and -3, however, were identical despite the data having been collected during different seasons. These results suggest diversity among domestic rBToVs in terms of their geographic and genetic characteristics. In contrast, Smits et al. (2003) reported a case of intertypic recombination between BToV and porcine torovirus within the HE region that was observed in B150 and B155. However, although HE exhibited a somewhat higher degree of diversity compared to S, none of the domestic BToVs were overwhelmingly diverse as the variety observed in B150 and B155 (Table 2-2(b)).

Among the members of the *Coronaviridae* family, porcine coronavirus is a well-known example of a virus with affinity for both the intestinal and respiratory tracts (porcine transmissible gastroenteritis virus [TGE] and

porcine respiratory coronavirus [PRCV]). The 2 viruses are antigenically related to each other, and PRCV is believed to have originated from TGEV by the deletion of the S gene, leading to a difference in tissue tropism (Laude et al., 1993). As the author's analysis of the S gene was incomplete, the possibility that rBToVs possess such distinct features compared to eBToVs cannot be excluded. However, considering the preservation of the HE coding region, the BToVs detected in the respiratory tract in this study did not show dramatic divergence with the eBToVs compared to the level of divergence observed between TGEV and PRCV but rather similar to the situation in bovine enteric/respiratory coronavirus; bovine enteric coronavirus and bovine respiratory coronavirus are not currently distinguishable on the basis of this fundamental property.

Of the 311 cattle tested, 16 also had fecal samples collected. Fecal samples from cattle sharing an origin with rBToV-2 that caused diarrhea were also positive for BToV N, S, and HE. In addition, the sequences of the N, S, and HE genes from these concurrently detected BToVs were identical (data not shown). This implies that the BToVs present in the respiratory and intestinal tracts in this case were identical and supports the idea that BToV possesses dual tropisms (to both the intestinal and respiratory tracts), similar to BCV. In conclusion, while the author

confirmed the possibility of respiratory tract transmission for BToV and a possible association with respiratory disease in young cattle, its etiological importance in cattle still remains obscure. Further investigation into the interactions between BToV and various tissues, viral persistence, and the immunological responses of infected cattle are needed to clarify the relationship between intestinal and respiratory disease and BToV infection. Isolating BToVs capable of being propagated by cell culture (preferably cytopathogenic) and investigating the immunological responses of infected cattle as well as their antigenic properties are of the utmost importance.

(a)

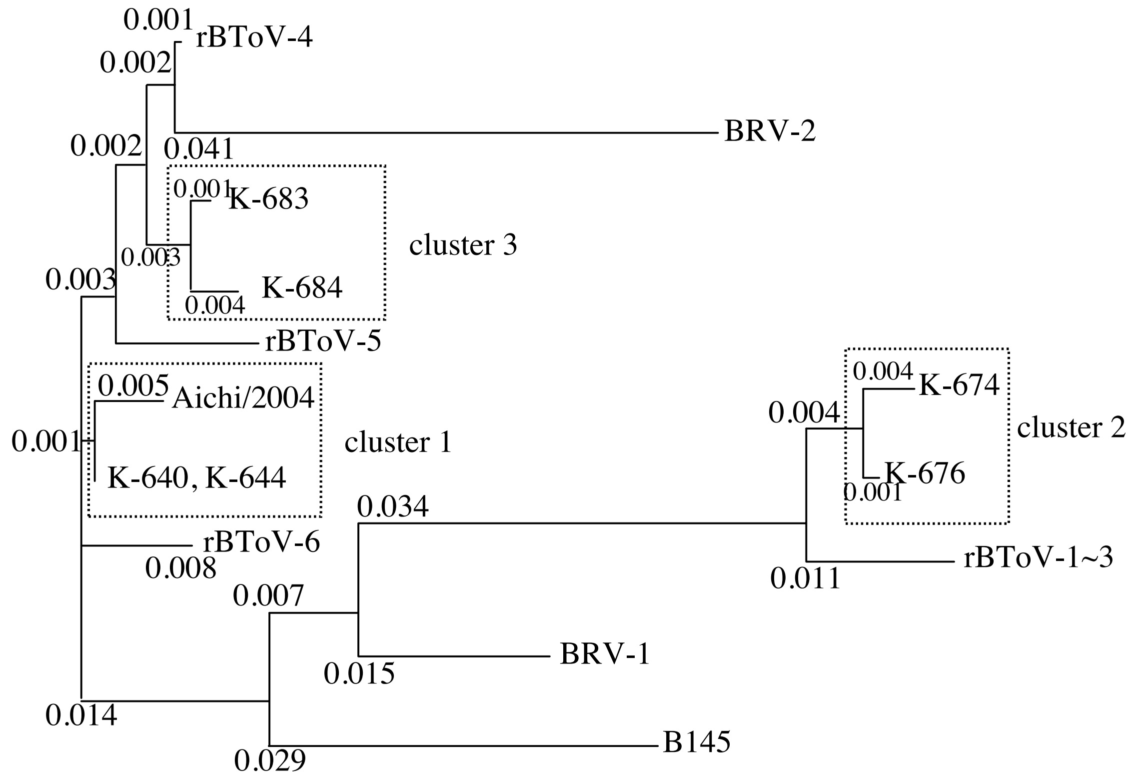


Fig. 2-1(a)

(b)

K-683
0.007
K-684
0.003
0.002
rBToV-4
0.002
K-640
0.004
K-644
0.002
rBToV-5
0.002
rBToV-6
0.008
0.034
B145
0.018
0.037
0.039
0.019
BRV-1
BRV-2
0.004
0.01
rBToV-2
0.006
K-674
0.01
K-676

Fig. 2-1(b)

Table. 2-1. Pathogen detection in the nasal samples
(a) Incidence of viral pathogens in 311 nasal samples

Respiratory symptom	Age	total	Pathogens detected				
			BToV	BCV	IBRV	BVDV	BRSV
+	1~12 month	174	7 (4.0%) *)	19 (10.9%)	0 (0.0%)	1 (0.6%)	4 (2.3%)
	over 12 month	31	0 (0.0%)	12 (38.7%)	1 (3.2%)	1 (3.2%)	0 (0.0%)
	total	205	7 (3.4%)	31 (15.1%)	1 (0.5%)	2 (1.0%)	4 (2.0%)
-	1~12 month	90	0 (0.0%)	3 (3.3%)	0 (0.0%)	2 (2.2%)	0 (0.0%)
	over 12 month	16	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
	total	106	0 (0.0%)	3 (2.8%)	0 (0.0%)	2 (1.9%)	0 (0.0%)
total		311	7 (2.3%)	34 (10.9%)	1 (0.3%)	4 (1.3%)	4 (1.3%)

*) No. (%) of positive cattle

Table. 2-1. Pathogen detection in the nasal samples
(b) the general condition of the BToV-positive cattle upon the collection of the nasal samples

Sample name	Sampling month	Prefecture	Age	breed	Other detected pathogens	BToV nested RT-PCR result		
						Nucleocapsid	Spike	Hemagglutinin-esterase
rBToV-1	2006. Dec	Gifu	8 month	beef	Bovine coronavirus	+	+	-
rBToV-2	2007. Apr	Gifu	7 month	beef		+	+	+
rBToV-3	2007. Jun	Akita	2 month	beef		+	+	-
rBToV-4	2008. Jan	Aichi	1 month	dairy		+	+	+
rBToV-5	2008. Jan	Aichi	2 month	dairy	Mycoplasma bovis	+	+	+
rBToV-6	2008. Mar	Nara	5 month	beef		+	+	+
rBToV-7	2008. Jun	Chiba	6 month	beef		+	-	-

Table. 2-2(a). Nucleotide and amino acid identities of the S gene (594 bp) sequences

strain	Nucleotide identity (%)													
	rBToV-1, 2, 3	rBToV-4	rBToV-5	rBToV-6	BRV-1	BRV-2	B145	K-640	K-644	K-674	K-676	K-683	K-684	Aichi/2004
rBToV-1, 2, 3	-	91.1	91.1	91.2	95.3	89.6	91.8	91.9	91.9	98.0	98.1	91.2	91.1	92.3
rBToV-4	91.9	-	98.7	94.9	92.1	93.1	93.1	96.1	96.1	91.6	91.4	98.8	99.0	96.6
rBToV-5	91.4	98.5	-	95.1	92.6	92.9	93.3	96.3	96.3	91.4	91.6	99.2	99.0	96.8
rBToV-6	91.4	98.0	97.5	-	94.9	92.8	93.4	96.5	96.5	91.8	91.9	95.3	95.1	97.1
BRV-1	94.4	94.4	98.5	95.9	-	92.4	92.9	93.4	93.4	96.0	96.1	92.8	92.6	93.4
BRV-2	89.3	95.9	94.4	94.4	92.4	-	91.6	93.1	93.1	90.6	90.4	93.1	93.3	93.1
B145	92.4	94.4	93.9	94.4	93.9	91.4	-	94.1	94.1	92.4	92.6	93.4	93.3	94.4
K-640	92.4	99.0	98.5	99.0	94.9	94.9	95.4	-	100.0	92.3	92.4	96.5	96.3	98.1
K-644	92.4	99.0	98.5	99.0	94.9	94.9	95.4	100.0	-	92.3	92.4	96.5	96.3	98.1
K-674	98.0	92.9	91.4	91.4	94.4	90.4	92.4	92.4	92.4	-	99.8	91.6	91.8	92.8
K-676	98.5	92.4	91.9	91.9	94.9	89.8	92.9	92.9	92.9	99.5	-	91.8	91.6	92.9
K-683	91.9	99.0	98.5	98.0	94.4	94.9	94.4	99.0	99.0	91.9	92.4	-	99.8	97.0
K-684	91.4	99.5	98.0	97.5	93.9	95.4	93.9	98.5	98.5	92.4	91.9	99.5	-	96.8
Aichi/2004	91.9	98.5	98.0	98.5	94.4	94.4	94.9	99.5	99.5	91.9	92.4	98.5	98.0	-
	Amino acid identity (%)													

The reference bovine toroviruses (BToVs) used in the comparison and their accession numbers are as follows: BRV-1 (AY427798), BRV-2 (AF076621), B145 (AJ575388 and AJ575379), B150 (AJ575380), B155 (AJ575381), K-640 and K-644 (AB270909), K-674 (AB270914), K-676 (AB270916), K-683 (AB270918), and K-684 (AB270919).

Table. 2-2(b). Nucleotide and amino acid identities of the HE gene (1051 bp) sequences

strain	Nucleotide identity (%)														
	rBToV- 2	rBToV-4	rBToV-5	rBToV-6	BRV-1	BRV-2	B145	B150	B155	K-640	K-644	K-674	K-676	K-683	K-684
rBToV-2	-	90.8	90.9	91.0	90.9	91.7	90.7	73.5	73.7	91.0	91.1	98.7	98.9	90.8	90.7
rBToV-4	91.1	-	99.0	98.7	88.6	89.7	98.6	73.9	73.9	98.9	99.0	90.9	91.1	99.1	99.0
rBToV-5	91.1	99.1	-	98.4	89.0	90.2	98.3	73.5	73.8	99.1	99.2	90.9	91.1	98.4	98.3
rBToV-6	92.0	98.6	98.3	-	89.0	90.0	98.2	73.7	74.0	98.7	98.8	90.8	91.0	99.0	99.0
BRV-1	90.6	90.6	90.3	90.9	-	98.2	89.0	70.6	70.9	89.1	89.2	91.4	91.6	88.9	88.8
BRV-2	90.6	92.0	92.0	91.7	97.7	-	90.1	71.3	71.6	90.3	90.4	92.1	92.3	90.0	9.9
B145	90.3	97.7	98.0	96.9	90.0	91.4	-	73.5	73.5	98.4	98.5	90.5	90.7	98.4	98.3
B150	73.4	72.0	72.3	72.3	69.7	69.7	71.7	-	99.3	73.3	73.4	73.1	73.1	73.8	73.8
B155	73.4	72.0	72.3	72.3	69.7	69.7	71.7	99.1	-	73.5	73.6	73.4	73.4	73.9	73.9
K-640	91.1	98.6	99.4	98.3	90.3	92.0	97.4	72.0	72.0	-	99.9	91.0	91.2	98.7	98.6
K-644	91.1	98.6	99.4	98.3	90.3	92.0	97.4	72.0	72.0	100.0	-	91.1	91.2	98.8	98.7
K-674	97.4	90.6	90.6	90.9	90.9	90.3	89.7	73.4	73.4	90.6	90.6	-	99.8	90.6	90.5
K-676	98.0	91.1	91.1	91.4	91.4	90.9	90.3	73.7	73.7	91.1	91.1	99.4	-	90.8	90.7
K-683	90.9	99.1	98.3	98.3	90.3	91.7	96.9	71.7	71.7	98.3	98.3	90.3	90.9	-	99.9
K-684	90.9	98.9	98.0	98.0	90.0	91.4	96.6	71.7	71.7	98.0	98.0	90.3	90.9	99.7	-
	Amino acid identity (%)														

Figure legends

Fig. 2-1. Neighbor-joining phylogenetic tree showing the relationships for (a) the deduced spike and (b) hemagglutinin-estrase amino acid sequences from Japanese bovine torovirus and the reference strains described in Table 2. The numbers represent the distance to the nearest node.

CHAPTER 3

Genetic and antigenic characterization of newly isolated bovine toroviruses from Japanese cattle

Abstract

Torovirus is a gastrointestinal infectious agent that has been identified in humans, cattle, pigs, and equines. Toroviruses, except equine torovirus, are difficult to propagate in cell culture; indeed, until 2007, only the Aichi/2004 strain of bovine torovirus (BToV) has been isolated among the human, bovine, and porcine toroviruses. In this chapter, 4 cytopathogenic BToVs were isolated from diarrheal feces of the cattle using the HRT-18 cell line and their genetic and antigenic properties were compared. The cytopathogenic features of BToV isolates in HRT-18 cells were similar to those of the Aichi/2004 strain. However, none of the isolates showed cytopathogenic effects in the HRT-18 cells of different origin, suggesting that one significant factor contributing to the cytopathogenicity of BToV depends on properties of the HRT-18 cells themselves. All BToVs isolated were able to agglutinate mouse, but not chicken, erythrocytes, while they lacked receptor-destroying enzyme activity. Analysis of the N-terminus of the spike gene showed that three isolates, but not the Gifu-2007TI/E strain, were phylogenetically located in cluster 1 and its analogs, and revealed high cross-reactivity with each other, as demonstrated by neutralization (NT) and hemagglutination-inhibition (HI) assays. The Gifu-2007TI/E

strain was classified close to cluster 2 and exhibited relatively low cross-reactivity with these viruses; however, the difference was not sufficient to classify BToVs into serotypes, suggesting that at least 2 subtypes distinguishable by the structure of the N-terminus of the spike gene and that both NT and HI tests may be exist.

Introduction

Several studies have established associations between toroviruses and gastrointestinal and respiratory symptoms (Beards et al., 1984; Jamieson et al., 1998; Woode et al., 1984). Indeed, the author's previous epidemiological research confirmed that BToV is involved in gastrointestinal and respiratory diseases in Japanese cattle (chapters 1 and 2). Additionally, the S and HE gene sequences of a number of field BToV isolates showed considerable variation. The products of these genes in coronaviruses are known to correlate closely with their antigenic properties (Clark et al., 1993). Therefore, the relationship between genetic and antigenic properties, and the antigenic variation of BToV, seems to be important for understanding the immunologic and pathogenic aspects of BToV infection, and in the development of an effective vaccine. The use of live virus from cultured cells would be the most appropriate method for investigating these aspects of BToV infection. However, only 1 report has described the detection of BToV in cultured cells: Kuwabara et al. (2007) isolated a cytopathogenic BToV strain, Aichi/2004, from HRT-18 cells.

In this chapter, the isolation of 4 novel cytopathogenic BToVs from cultured cells and their genetic and antigenic characterization are described.

Materials and methods

Samples

In total, 55 individual samples collected from Japanese cattle positive for the BToV N gene, as determined by the RT-PCR method described in chapter 1, were used for virus isolation. These samples included some BToV-positive fecal- and nasal-derived samples described in chapters 1 and 2. The details of the samples were as follows: fecal samples were collected from 48 cattle, 47 showing enteric symptoms and 1 asymptomatic, raised on 20 farms located in 12 prefectures between September 2004 and May 2009; nasal samples were obtained from 7 cattle with respiratory symptoms on 6 farms located in 5 prefectures between December 2006 and June 2008. Each sample was diluted 1:10 in Dulbecco's modified Eagle's medium (DMEM) and centrifuged (3000 × *g*, 5 min, room temperature). The supernatants were collected and used for virus isolation in HRT-18 cell cultures.

Virus isolation

Virus isolation was carried out using the human rectal tumor cell line, HRT-18. The reference Aichi/2004 strain of BToV was used as a positive

control. The HRT-18 cells and reference virus were kindly supplied by Dr. M. Kuwabara, Central Livestock Hygiene Service Center, Aichi Prefecture, Japan (Kuwabara et al., 2007). Confluent monolayers of HRT-18 cells, grown in test tubes, were inoculated with 0.1 ml of samples. 60 minutes after static absorption at 37°C, all cultures received 1.0 ml of DMEM, and were further incubated with rotation at 37°C for 7 days, and were observed for cytopathic effect (CPE). These procedures were further repeated 3 times. CPE-positive samples were cloned by 2 rounds of a limiting dilution method and tested for the presence of BToV, bovine coronavirus (BCV), and bovine rotavirus, as described previously (Okada and Matsumoto., 2002, chapter 1).

Measurement of hemagglutination (HA) and receptor-destroying enzyme (RDE) activity

The HA and RDE titers of isolated virus were measured by the microplate method using mouse (1.0%) and chicken (0.5%) erythrocytes, as described elsewhere (Sato et al., 1977).

Immunization of guinea pigs

Antisera against the isolates and reference strain were produced in

guinea pigs, as described elsewhere (Tsunemitsu et al., 1995). Briefly, concentrated viral suspensions were prepared from culture media by ultracentrifugation, emulsified with oil adjuvant consisting of liquid paraffin supplemented with 10% anhydrous mannitol-oleic acid ester, and used for immunization. The amount of virion contained in the final immunogens was estimated at $10^{7.0}$ median tissue-culture infective doses ($TCID_{50}$)/ml. Guinea pigs were injected intramuscularly twice with 0.5 ml and subsequently intraperitoneally with 2.5 ml of the respective immunogens at weekly intervals for 3 weeks. Two weeks after final inoculation, sera were collected and used in the experiments.

Genetic analyses

The isolates were subjected to RNA extraction, followed by nested RT-PCR. The methods used for RNA extraction and nested RT-PCR amplification of the BToV S gene, and primer sets used in the reaction are described in chapter 1. Additionally, the following primers were used to amplify the full-length open reading frame (ORF) of the S gene: fl-F1: ATTTTTGCTGTTGTTGTGAAG (corresponding to nucleotides 22,274–22,294 of AY427798), fl-F2: GTTGCAAGTYTATGAAACACC (corresponding to nucleotides 23,155–23,174 of AY427798), fl-F3:

TGCACTTCAATGGATTATTTT (corresponding to nucleotides 23,855–23,875 of AY427798), fl-F4: AACATCTTGGGCTCAGTTTA (corresponding to nucleotides 24,917–24,936 of AY427798), fl-R1: GCATGGAAACCTGATGTATT (corresponding to nucleotides 22,580–22,561 of AY427798), fl-R2: AAARCCCACACAACCAGGTA (corresponding to nucleotides 23,175–23,155 of AY427798), fl-R3: TGTGAAGCTGGTGACATAAA (corresponding to nucleotides 24,075–24,056 of AY427798), fl-R4: ATACCTATCGCAATAATGCTC (corresponding to nucleotides 25,073–25,053 of AY427798), and fl-R5: CAGTTGCTGATAACTGCTCA (corresponding to nucleotides 25,839–25,820 of AY427798). Nucleotide sequencing and phylogenetic tree analyses were performed as described in chapter 1.

Virus neutralization (VN) test

Antisera were serially diluted twofold with serum-free DMEM and incubated at 37°C for 60 min with an equal volume of the respective viral suspension containing 200 $TCID_{50}$/0.1 ml. Each serum–virus mixture was inoculated onto confluent monolayers of HRT-18 cells grown in test tubes. After 60 min of static absorption at 37°C, all tubes received 1.0 ml of DMEM and were incubated with rotation at 37°C for 7 days. Neutralization

antibody titers are expressed as the reciprocal of the highest serum dilution that inhibited 50% of the CPE.

Hemagglutination inhibition (HI) test

Antisera were treated with kaolin and mouse erythrocytes to remove nonspecific hemagglutinins before testing, according to methods described by Hasoksuz et al. (Hasoksuz et al., 1999). Serial twofold dilutions of antisera were made in a volume of 25 μl, and mixed with an equal volume of 8 HA units of each viral suspension. After a 60-min incubation at 37°C, 25 μl of a 1.0% mouse erythrocyte suspension was added to each. Settling patterns of erythrocyte were read 90 min after incubation at 22°C, and HI antibody titers were expressed as the reciprocal of the highest serum dilution that completely inhibited HA.

Antigenic-relatedness values

Antigenic similarities among the BToV isolates were indicated by the antigenic-relatedness values (R%), calculated from the results of cross-VN and HI tests using the following formula, as described previously (Hasoksuz et al., 1999; Shimizu et al., 1989):

$$R = 100 \times \sqrt{r1 \times r2}$$

where r1 and r2 are the heterologous titer of strain 1/homologous titer of strain 2, and the heterologous titer of strain 2/homologous titer of strain 1, respectively.

Results

Virus isolation

Four fecal samples of the 55 fecal and nasal samples investigated revealed CPE similar to that of the reference Aichi/2004 strain after serial passage in HRT-18 cell cultures. Agents causing CPE were identified as BToV by RT-PCR and immunofluorescence using antiserum prepared to the reference Aichi/2004 strain of BToV. The representative CPE produced in HRT-18 cells by BToVs is shown in Fig. 3-1. They were designated as the Miyagi-2006TI/E, Gifu-2007TI/E, Hokkaido-2008TI/E, and Gifu-2009TI/E strains. Notably, the strains could be isolated and caused CPE only in HRT-18 cells provided by Dr. M. Kuwabara, while BToV isolation was unsuccessful when cells maintained in the author's laboratory were used as a substrate. As shown in Table 3-1, the cytopathic BToVs were isolated from feces obtained from 4 cattle raised in 3 prefectures between February 2006 and March 2009; all 4 cattle had gastrointestinal problems, while their breeds and ages differed. Additionally, the BCV N gene was coincidentally detected in the original fecal materials of Gifu-2007TI/E and Gifu-2009TI/E strains, but BCV was undetectable after their serial passages in HRT-18 cell cultures.

HA and RDE activity

Four BToV isolates, as well as the reference Aichi/2004 strain, showed similar HA activity, with titers ranging from 16 to 128 to mouse erythrocytes at both 4°C and 37°C (Table 3-2). However, none of them agglutinated chicken erythrocytes at either temperature. Moreover, all of the isolates lacked RDE activity against both mouse and chicken erythrocytes.

Genetic analysis

As indicated in Table 3-3, the similarities of the 594-bp nucleotide sequences and 197 amino acid (aa) residues of the S gene N-terminal region were ≥91.6% and ≥91.9% among the isolates and the reference Aichi/2004 strain, respectively. These similarities were comparable with the results determined in chapter 1 and 2 in comparing BToVs derived from feces and nasal swabs. Interestingly, the nucleotide sequences of Hokkaido-2008TI/E and Gifu-2009TI/E strains were completely identical, although they were derived from the clinical specimens collected at geographically distinct areas and at different times (Table 3-3).

Analysis of the full-length ORF of the S gene showed that the ORF of

the isolates and the reference Aichi/2004 strain encoded a 1,584-aa residue protein, as did the registered sequences of the BRV-1 and B145 that were detected in the feces of cattle from North America and Europe, respectively (Draker et al., 2006; Smits et al., 2003). Among the 4 isolates and the reference Aichi/2004 strain, the nucleotide similarities of the S gene ORF were ≥95.4% for the nucleotide sequence and ≥96.1% at the amino acid level (Table 3-4). In a comparison with all registered sequences, the similarities of the nucleotide sequence and aa residues of the S gene ORF were ≥91.5% and ≥90.3%, respectively. Representative sequence data have been deposited in the DNA Data Bank of Japan and assigned the accession numbers AB526862 to AB526866.

Phylogenetic analysis of the 197-aa region of the S gene N terminal revealed that Miyagi-2006TI/E, the sequence of which was identical to that of K-637, grouped into cluster 1, which also included the Aichi/2004 strain. The Hokkaido-2008TI/E and Gifu-2009TI/E strains were located in a position close to cluster 1 and were most closely related to rBToV-6, detected in chapter 2 in a nasal swab collected from a Japanese cow. In contrast, Gifu-2007TI/E was distant from the other BToV isolates and close to cluster 2, which consisted of K-674 and K-676, originally detected in feces of Japanese cattle. Gifu-2007TI/E was most closely related to

rBToV-1–3, which were found in nasal swabs collected from Japanese cattle between December 2006 and June 2007 as described in chapter 2 (Fig. 3-2(a)). Phylogenetic analysis of the full-length aa sequence of the S gene ORF revealed that the BToV isolates, Miyagi-2006TI/E, Hokkaido-2008TI/E, and Gifu-2009TI/E, and the reference Aichi/2004 strain were relatively closer to one another than to Gifu-2007TI/E. Similarly to the analysis for the 197-aa region of the S gene N terminal, the Gifu-2007TI/E strain was located more distal to the other isolates than BRV-1 and B145 (Fig. 3-2(b)).

Cross-neutralization tests

The results of cross-VN tests with the 4 isolates and the reference BToV Aichi/2004 strain are shown in Table 3-5(a). Although some antigenic variation was evident among the viruses investigated, they were considered to be antigenically related to each other, with R values of 19.8–100%. Furthermore, the comparisons among strains Miyagi-2006TI/E, Hokkaido-2008TI/E, Gifu-2009TI/E, and reference BToV Aichi/2004 strains, which share over 98.5% sequence identity in the 197-aa region of the S gene N-terminal, yielded relatively high R values, 56.6–100%. In contrast, the Gifu-2007TI/E strain with lower similarity, <92.9% of the

197-aa region, appeared to be unique compared to the other viruses; R values between this strain and the other viruses were relatively low (19.8–31.3%; Table 3-5(a)).

Cross HI tests

The results of cross-HI tests well coincided with those of cross-VN tests (Table 3-5(b)). R values calculated from HI antibody titers ranged between 44.2% and 89.4% among the Miyagi-2006TI/E, Hokkaido-2008TI/E, Gifu-2009TI/E, and reference Aichi/2004 strains. In contrast, the Gifu-2007TI/E strain yielded relatively low R values, ranging from 14.0% to 19.8% between the other viruses.

Discussion

Toroviruses have been difficult to propagate in cell culture; indeed, until 2007, only one strain has been isolated among human, bovine, and porcine toroviruses (Kuwabara et al., 2007). In the present work, however, cytopathogenic BToVs were isolated from fecal samples collected from 4 cattle between February 2006 and March 2009. They all showed intestinal symptoms and some manifested respiratory signs, suggestive of that BToV played some roles as a predisposing factor.

The HRT-18 cell line used in this study was provided by Dr. Kuwabara, who succeeded in first isolating a cytopathogenic BToV strain, Aichi/2004 (Kuwabara et al., 2007). Unexpectedly, all of the BToVs isolated in this study lacked susceptibility to HRT-18 cells maintained in the author's laboratory and used previously. In contrast, the BCV strains isolated previously in the HRT-18 cells of the author's laboratory failed to propagate in or exhibited only weak susceptibility to those provided by Dr. Kuwabara. The fecal materials from which the Gifu-2007TI/E and Gifu-2009TI/E strains were isolated were coincidentally positive for BCVs, but only BToVs were propagated after repeated passage in cell culture. This seems to indicate that BToVs were preferentially propagated under the

particular conditions, including the source of the HRT-18 cells used. Additionally, in the virus isolation process, several bovine enteroviruses, which had been considered as being non-replicable in HRT-18 cells, were isolated from several samples besides 4 BToV isolated samples (the author's unpublished data). Based on these results, one significant factor that allowed the BToV isolation appears to be the particular properties of the HRT-18 cell line used. Although the cytological differences between the 2 lines of HRT-18 cells remain unclear, an unexpected cellular mutation that affects susceptibility to viruses might occur during long-term maintenance of the cells in the separate laboratories.

BToV, as well as certain other viruses, is known to possess HA activity to animal erythrocytes via hemagglutinin present on the surface of the viral envelope. BCV, another member of the *Coronaviridae* family having similar genomic structures to BToV, is well-known to show several HA and RDE reaction patterns with mouse and chicken erythrocytes (Fukutomi et al., 1999; Hasoksuz et al., 1999), while those of BToVs have not been investigated to date. In this study, 5 BToVs, 4 novel isolates and the Aichi/2004 strain, were compared for HA and RDE reaction patterns to mouse and chicken erythrocytes, and the author found that all viruses tested revealed similar HA and RDE properties to each other; they all

agglutinated only mouse erythrocytes at both 4°C and 37°C, but lacked RDE activity. Because the number and source of BToVs tested were limited to 5 fecal specimens, the presence of BToV strains with different HA and RDE properties cannot be ruled out. However, that the 5 BToVs analyzed here were isolated at distinct times and in different geographic areas may suggest that the HA and RDE properties observed are representative of most, if not all, BToVs.

So far, 33 fecal- and 6 nasal-derived BToVs have been sequenced directly from the biomaterials (Draker et al., 2006; Kirisawa et al., 2007; Smits et al., 2003 and the author's previous data [chapter 1 and 2]), and their identities in the 197-aa N-terminal sequence of the S gene have been estimated at >89%. Similarly, the BToV isolates investigated in this study showed ≥91.9% identity with each other, while the identity increased to ≥98.5% when the Gifu-2007TI/E strain was excluded from the comparison. The full-length sequences of the S gene ORF, however, have been reported for only 3 BToVs (BRV-1, BRV-2, B145) so far. Here, the sequences of the full-length S gene determined for the novel BToV isolates were likewise found to be well conserved among the strains as compared to 3 viruses mentioned above; sequence similarities, however, were somewhat lower than those determined for the 197-aa N-terminal region, except the

Gifu-2007TI/E strain. This strain showed lower sequence identities with the other 3 viruses in the 197-aa N-terminal region (91.9–92.9%) than those in the full-length S gene (96.1–96.3%).

The difference between porcine respiratory coronavirus and porcine transmissible gastroenteritis virus in tissue tropism has been considered as being attributable to the existence of a broad deletion sequence in the S gene of the former virus (Laude et al., 1993). No informative evidence on variation in the S gene capable of distinguishing rBToVs from eBToVs has been found. In chapter 2, the author compared the sequences of the N-terminal region of the BToV S gene amplified directly from nasal and fecal samples and found no significant variation in the region analyzed. However, the author's analysis was limited to the N-terminus of the S gene. The full-length S gene ORF of 4 isolates and foreign BToVs encoded 1,584 aa, with the exception of BRV-2, in which the ORF encoded 1,583 aa. Accordingly, BToV seems to be genetically well conserved among intestinally-derived strains. To examine the relationship between tissue tropism and S gene structures of BToVs, isolating BToVs from respiratory materials and comparing their complete S gene sequences with those of eBToVs would be of great interest.

The BToV isolates were phylogenetically classified into cluster 1 or its

analogs, with the exception of the Gifu-2007TI/E strain, which belonged closer to cluster 2. In chapter 1, the author found that BToVs belonging to cluster 1 or its surroundings were predominant. According to the S gene sequences reported by Kirisawa et al. (Kirisawa et al., 2007), however, 16 of 17 BToVs analyzed showed identity >99.0% with the Gifu-2007TI/E strain, suggesting that they are possibly placed around cluster 2. These findings suggest that BToVs of both genotypes and their surroundings are widespread in Japan.

All BToV isolates were antigenically related to each other, as revealed by both NT and HI tests, although the degree of cross-reactivity was different between the isolates. Moreover, the overall R values of both tests were well correlated with each other, although they were somewhat lower in the HI than NT tests. With respect to phylogenetic characteristics, the cross-reactivity between the Miyagi-2006TI/E and Aichi/2004 of cluster 1, and the its neighboring viruses Hokkaido-2008TI/E and Gifu-2009TI/E was remarkably high compared to the Gifu-2007TI/E strain, located around cluster 2. Unfortunately, no BToV belonging to cluster 3 was isolated in this study, but investigating antigenicity in this context would be of interest. R values below 25% are generally regarded as an indication of a significant antigenic difference between 2 viruses, and a difference greater than

20-fold in both directions (R < 5%) is used as a serotype criterion in several gastroenteric viruses (Hubálek, Z., 1982; Shimizu et al., 1989; Wyatt et al., 1982). Thus, it seems unlikely that BToVs comprise multiple serotypes, at least based on the results of NT and HI tests using 4 viruses in this study. BToVs seem to consist of a single serotype, although the serological properties of BToVs belongings to cluster 1 and surroundings and cluster 2 were markedly different. Nonetheless, the author's results suggest that at least 2 subtypes distinguishable by NT and HI tests, and probably related to the structures of N-terminus of the S gene, may be present in BToVs. This is based on the finding that the sequence diversity between the Gifu-2007TI/E strain and the other isolates was greater in the N-terminal region than in the full-length S gene.

The author believes this data to be the first report dealing with antigenic characterization of BToVs by both NT and HI tests, and the author could partially determine their serological characteristics. Antigenic and genetic investigations using more BToVs cultivatable in cell culture, especially those derived from respiratory specimens, are expected to yield more variable information for understanding serological properties of the BToVs.

The author's preliminary investigation suggests that the HE gene of the isolates consisted of a nonfunctional ORF that probably resulted from sequential insertion and/or deletion during the isolation process in tissue culture. Although the mechanism and frequency of these mutations remain unclear, the high mutability seems to be a unique characteristic for the HE gene, as compared to the coding regions of the N and S genes, which revealed no or less divergence between isolated viruses and the original fecal materials (data not shown). This is not always relevant to antigenic diversity of BToV, however, because isolates classified within cluster 1 and its surroundings showed strong cross-reactivity in NT and HI tests. The details of the frequency, mechanism, and significance of these mutations in the HE gene are now under investigation.

A few studies have been conducted to determine pathogenicity and antigenicity of BToV using infected diarrheal stools (Woode et al., 1985). In this chapter, the author successfully isolated 4 novel BToV strains in tissue cultures following the report of Kuwabara et al., who first reported a cytopathogenic BToV in 2007 (Kuwabara et al., 2007). The author believes the cytopathogenic viruses isolated will provide important tools for studying the pathological role and immunology of BToVs in enteric and respiratory diseases.

(a)

(b)

Fig. 3-1.

(a)

0.001
rBToV-4
0.002
BRV-2
0.002
0.041
0.001
K-683
cluster 3
0.003
0.002
K-684
0.004
rBToV-5
0.005
Aichi/2004
cluster 1
0.000
K-637, Miyagi-2006TI/E
0.052
Hokkaido-2008TI/E, Gifu-2009TI/E
0.004
K-674
0.005
cluster 2
0.043
K-676
rBToV-6
0.001
0.049
0.034
0.005
0.006
rBToV-1~3
BRV-1
0.014
0.015
0.006
B145
Gifu-2007TI/E
0.029

Fig. 3-2(a).

(b)

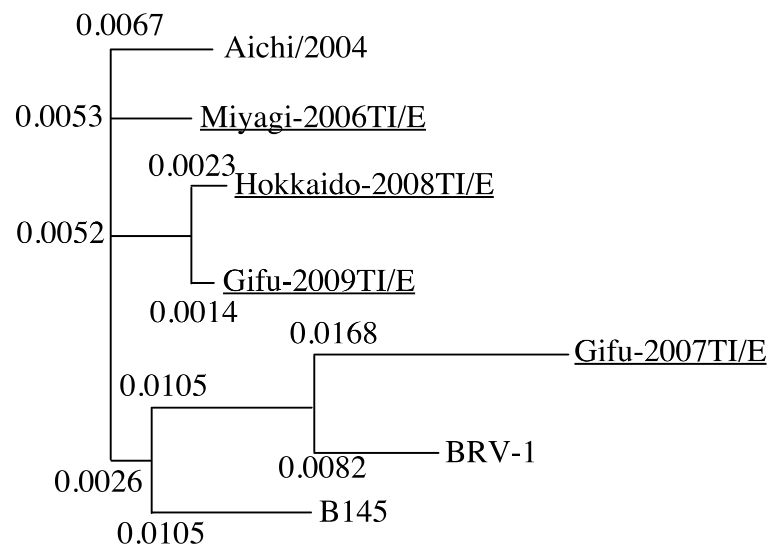


Fig. 3-2(b).

Table. 3-1. Origin and general properties of the BToVs isolated from

BToV strain	Origin of the sample				Host condition		
Isolated	Material	Sampling period	Prefecture	Other pathogens detected	Body condition	Age	Breed
Miyagi-2006TI/E	loose stool	Feb.2006	Miyagi		gastrointestinal symptom	calf	beef
Gifu-2007TI/E	muddy diarrhea stool	Apr.2007	Gifu	Bovine coronavirus	gastrointestinal and respiratory symptom	17 month	beef, female
Hokkaido-2008TI/E	watery diarrhea stool	Mar.2008	Hokkaido		gastrointestinal and respiratory symptom	1 month	beef, female
Gifu-2009TI/E	loose stool	Mar.2009	Gifu	Bovine coronavirus	gastrointestinal symptom	40 month	dairy, female

Table. 3-2. Hemagglutination (HA) and receptor-destroying enzyme (RDE) activities of BToV strains

Strain	HA titers				RDE titers *)	
	4°C		37°C			
	Mouse	Chicken	Mouse	Chicken	Mouse	Chicken
Miyagi-2006TI/E	32	<2	16	<2	<5	<5
Gifu-2007TI/E	64	<2	64	<2	<5	<5
Hokkaido-2008TI/E	128	<2	128	<2	<5	<5
Gifu-2009TI/E	32	<2	32	<2	<5	<5
Aichi/2004	32	<2	32	<2	<5	<5

*) Expressed as the reciprocal of the highest dilution of virus causing complete disappearance of HA patterns at 4°C after 2 hours incubation at 37°C

Table. 3-3. Nucleotide and amino acid identities of the S gene N-terminal 594 bp

	Nucleotide identity (%)				
Strain	Miyagi-2006TI/E	Gifu-2007TI/E	Hokkaido-2008TI/E	Gifu-2009TI/E	Aichi/2004
Miyagi-2006TI/E	-	91.6	96.3	96.3	97.6
Gifu-2007TI/E	92.9	-	91.6	91.6	92.4
Hokkaido-2008TI/E	99.0	91.9	-	100.0	97.5
Gifu-2009TI/E	99.0	91.9	100.0	-	97.5
Aichi/2004	99.5	92.4	98.5	98.5	-
	Amino acid identity (%)				

The reference bovine toroviruses (BToVs) used for sequence comparison and accession numbers were as follows: Aichi/2004 (AB285127), BRV-1 (AY427798), BRV-2 (AF076621), B145 (AJ575388).

Table. 3-4. Nucleotide and amino acid identities of the S gene full-length ORF

Strain	Nucleotide identity (%)							
	Miyagi-2006TI/E	Gifu-2007TI/E	Hokkaido-2008TI/E	Gifu-2009TI/E	Aichi/2004	BRV-1	BRV-2	B145
Miyagi-2006TI/E	-	95.4	98.4	98.5	98.5	95.8	93.1	97.1
Gifu-2007TI/E	96.2	-	95.4	95.5	95.7	96.4	91.5	95.0
Hokkaido-2008TI/E	98.7	96.1	-	99.9	98.2	95.8	93.0	97.1
Gifu-2009TI/E	98.8	96.1	99.6	-	98.2	95.9	92.9	97.1
Aichi/2004	98.7	96.3	98.5	98.5	-	95.9	93.0	97.1
BRV-1	97.0	97.5	96.8	96.9	97.2	-	93.2	95.8
BRV-2	91.9	90.3	91.9	91.8	92.0	91.6	-	92.9
B145	97.9	96.3	97.7	97.9	97.7	97.2	91.6	-
	Amino acid identity (%)							

The reference bovine toroviruses (BToVs) used for sequence comparison and accession numbers were as follows: Aichi/2004 (AB285127), BRV-1 (AY427798), BRV-2 (AF076621), B145 (AJ575388).

Table. 3-5. Result of virus (a) neutralization and (b) hemagglutination-inhibition tests using guinea pig antisera against BToV strain Aichi/2004 and the four newly isolated strains

(a)

Strain	Guinea pig anti-serum				
	Miyagi-2006TI/E	Gifu-2007TI/E	Hokkaido-2008TI/E	Gifu-2009TI/E	Aichi/2004
Miyagi-2006TI/E	**51,200 (100)** *)	10,240	40,960	20,480	12,800
Gifu-2007TI/E	32,000 (25.0)	**102,400 (100)**	32,000	12,800	8,000
Hokkaido-2008TI/E	12,800 (63.2)	6,400 (28.0)	**25,600 (100)**	12,800	6,400
Gifu-2009TI/E	25,600 (70.7)	6,400 (19.8)	40,960 (100)	**20,480 (100)**	6,400
Aichi/2004	20,480 (56.6)	12,800 (31.3)	20,480 (70.7)	12,800 (62.5)	**10,240 (100)**

*) Neutralization antibody titers and antigenic relatedness values (R%)

(b)

Strain	Guinea pig anti-serum				
	Miyagi-2006TI/E	Gifu-2007TI/E	Hokkaido-2008TI/E	Gifu-2009TI/E	Aichi/2004
Miyagi-2006TI/E	**20,480 (100)** *)	6,400	32,000	12,800	6,400
Gifu-2007TI/E	640 (14.0)	**10,240 (100)**	800	400	400
Hokkaido-2008TI/E	25,600 (88.4)	20,480 (17.7)	**51,200 (100)**	12,800	8,000
Gifu-2009TI/E	20,480 (69.9)	12,800 (17.7)	51,200 (89.4)	**16,000 (100)**	6,400
Aichi/2004	20480 (44.2)	12,800 (19.8)	32,000 (62.5)	12,800 (63.2)	**12,800 (100)**

*) Hemagglutination-inhibition antibody titers and antigenic relatedness values (R%)

Figure legends

Fig. 3-1. The condition of HRT-18 cells of 4 day after (a) inoculation of 3 times passaged BToV Miyagi-2006TI/E strain and (b) uninoculated control.

Fig. 3-2. Neighbor-joining phylogenetic tree showing the relationships for the deduced sequences of the spike gene. (a and b) N-terminal 197 amino acids (a) and full-length amino acid sequences (b) from bovine torovirus isolates and reference strains. The numbers represent the distance to the nearest node. The additional sequences refer to Table 3 for comparison. Their accession numbers are as follows: K-637 (AB270909), K-674 (AB270914), K-676 (AB270916), K-683 (AB270918), K-684 (AB270919), rBToV-2 (AB371899), rBToV-4 (AB448741), and rBToV-6 (AB448745).

CHAPTER 4

Clinical and virological responses of cattle experimentally infected with bovine torovirus

Abstract

Calves experimentally inoculated with bovine torovirus (BToV) isolates were observed for clinical manifestations of disease, excretion of the virus in feces and nasal secretions, and antibody responses. Although none of the calves exhibited severe clinical symptoms, all of the calves, including an uninoculated cohabitant calf, developed soft to watery feces, suggesting that experimental infection, including via contact exposure, had been established. These gastrointestinal symptoms were transient but concentrated between 4 and 10 days post-inoculation, which is consistent with the peak of fecal virus excretion. Although the clinical manifestations subsided after 14 days post-inoculation, virus excretion in the feces persisted discontinuously for a maximum of 8 weeks after inoculation. On the other hand, transient coughing was observed in only one calf. Although virus was also recovered from the animals' nasal secretions for a maximum of 4 weeks, the amount was significantly less than that in the fecal specimens. These data suggest that BToV possesses dual tropism for respiratory and intestinal routes. BToV appears to be more infectious and pathogenic toward the intestinal tract compared with the respiratory tract; however, the latter route may still be important in spreading the virus.

Circulating antibody levels began to rise beginning 7 days post-inoculation, which in part was associated with the development of tolerance to re-infection by BToV.

Introduction

Experimental inoculation studies using viral materials originating from the feces of BToV-infected calves have demonstrated that BToV infects the villous and crypt enterocytes of the jejunal and ileal regions of the host small intestine and spiral colon, resulting in severe diarrhea and dehydration (Woode et al., 1982; Pohlenz et al., 1984). In addition, several epidemiological reports have detected BToV transmission via aerosols, suggesting tropism for both the intestinal and respiratory tracts (Hoet et al., 2002; chapter 2). However, detailed experimental inoculation studies, particularly longitudinal analyses of viral shedding patterns and examinations of viral tropism for the respiratory tract, have not yet been conducted. This is due to the fact that BToV was, until 2007 (Kuwabara et al., 2007), unable to be propagated in cell culture.

In this chapter, the author evaluated the infectiveness and pathogenesis of BToV in terms of the clinical course, shedding of the virus in the intestinal and respiratory tracts, and the immune responses of experimentally inoculated calves using the cytopathogenic strain of BToV described in chapter 3.

Materials and Methods

BToV

BToV strain Hokkaido-2008TI/E, which had been passed 3 times through the human rectal tumor cell line HRT-18 as described in chapter 3, was used for experimental inoculation, as the standard virus for real-time RT-PCR, and to measure the antibody titers. The number of virions in the culture supernatant was estimated to be $10^{5.50}$ $TCID_{50}$/ml.

Calves and experimental inoculation

Three gnotobiotic calves (4 to 5 months old) were inoculated with 20 ml virus either orally (No. 1), nasally (No. 2), or both orally and nasally (No. 3). A fourth calf of the same age was raised in the same pen as a cohabitant (No. 4). The calves were monitored for clinical signs (appetite, depression, rectal temperature, and digestive and respiratory symptoms), viral excretion in the feces and nasal secretions, and antibody responses. Calf 3 was re-inoculated and monitored as in the first trial 3 months after the first inoculation.

Monitoring for viral excretion

Handling of the supernatants from the fecal and nasal samples and RNA extraction were done as described in chapters 1 and 2. A real-time RT-PCR assay based on the SYBR Green II system was carried out using a SYBR Premix Ex Taq Kit (Takara, Tokyo, Japan) to determine the viral content of each specimen. Reverse transcription was conducted as described to detect the N gene of BToV as described in chapter 1. A subsequent quantitative assay targeting the N gene was performed in a 25-μl reaction mixture containing 12.5 μl SYBR Premix Ex Taq II, 0.4 μM primers (Nrt-F, ACCCAACAACGTGCTAATGG, and Nrt-R, TGGAAACTTCAACAGTGGGCAT, corresponding to nucleotides 28,216–28,196 of AY427798), and 2 μl cDNA. The conditions were as follows: denaturation at 94°C for 30 s followed by 38 cycles of 94°C for 5 s, 62°C for 10 s, and 72°C for 15 s. BToV strain Hokkaido-2008TI/E was diluted ten-fold to create a standard curve for quantification.

Antibody titer measurement

The VN and HI antibody titers were determined as described in chapter 3.

Results

The inoculated calves (Nos. 1–3) and cohabitant (No. 4) did not develop general clinical signs (e.g., anorexia, depression, and fever) during the observation period, except that the rectal temperature of calf 2 rose 1°C above normal 13 and 14 days post-inoculation. Calf 1 developed intestinal symptoms (soft feces) 1 day post-inoculation that persisted up to post-inoculation day (PID) 14 except for on PID 3. In particular, watery diarrhea was observed on PIDs 7 and 13. The other calves, including the cohabitant, developed weak intestinal symptoms compared to calf 1. Calf 2 produced soft feces intermittently (PIDs 2, 5, 6, 10, and 14) and watery diarrhea on PID 13. Calf 3 produced soft feces on PIDs 1, 4, 6–10, 13, 17 and 3 week post-inoculation, while calf 4 did so on PIDs 2, 5, 6, 7, 10, 11 and 17. In contrast, respiratory symptoms were detected intermittently in calf 3 in the form of a nasal discharge on PID 2 and coughing on PIDs 3, 11, 13, 14 and 17 (Figs. 4-1(a) ~ (d)).

Viral excretion in the feces was first detected in the calves 1 and 2 on PID 3 and PID 4 in calves 3 and 4; it ultimately peaked above 10^7–10^8 copies on PIDs 4–7 then dropped but persisted intermittently for 8 weeks, except that viral excretion was not observed since 4 weeks after inoculation

in the feces of calf 2. Virus was start recovering from the nasal secretions on PID 3 in calves 2 and 3, PID 4 in calves 1 and 4 which peaked for 5 to 7 days around 10^4 copies, except that the peak of calf 4 was lower compared to others. In total, the virus excretion was significantly less than that in fecal specimens throughout the course of sampling except for PID 10 in calves 3 and 4, however, persisted for 4 weeks apart from calf 3 that viral excretion was not observed after PID 11.

The NT and HI antibody titers increased by PID 7 in calves 1 and 3 and by PID 14 in others, including the cohabitant; the highest level in each case was 64–512 (the level was 4–8 prior to inoculation).

Calf 3 was re-inoculated with BToV as before, 3 months after the first inoculation. This calf did not develop general clinical signs during the first 7 days after re-inoculation. Although the calf had soft feces on PIDs 2 and 7, the severity was reduced compared to that in the first trial, and respiratory symptoms were not detected. Viral excretion was significantly reduced compared to that in the first trial, especially in the fecal samples, which were below or around 10^2 copies throughout the course of sampling (Fig. 4-2).

Discussion

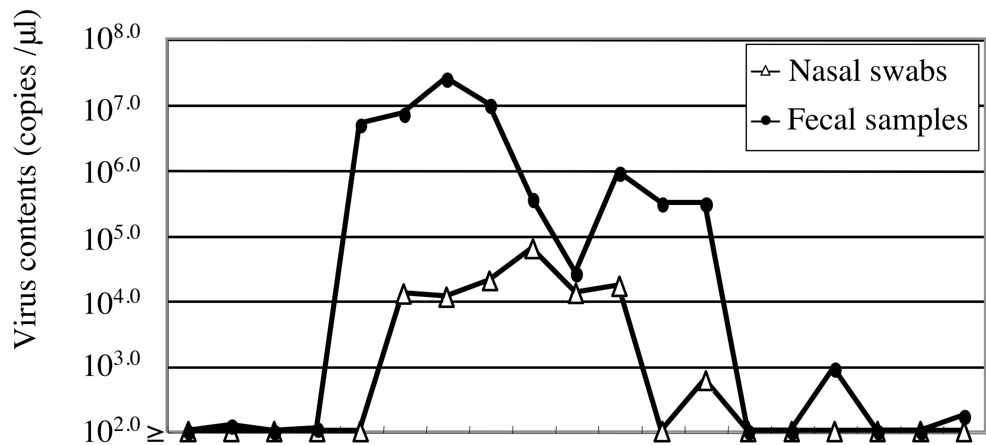
Four- to 5-month-old cattle were inoculated with BToV strain Hokkaido-2008TI/E orally (No. 1), nasally (No. 2), or both (No. 3) to evaluate viral pathogenicity. The inoculated calves and calf No. 4, which was raised in the same pen as a cohabitant, were the first to develop gastrointestinal symptoms and began shedding virus on PID 3, suggesting that experimental infection, including via contact exposure, had been established. The gastrointestinal symptoms were transient but concentrated on PID 4–10 in all calves, which is consistent with the peak of fecal virus excretion. Although the clinical manifestations subsided after PID 14, virus excretion in the feces persisted discontinuously for a maximum of 8 weeks after inoculation. These results indicate that the BToV isolate possessed both infectivity and pathogenicity for the bovine intestinal tract.

Compared with the gastrointestinal symptoms, respiratory symptoms were observed only in calf No. 3, and they were intermittent. Viral excretion as detected by nasal swabbing began from PID 3–4 in all calves; however, the amount excreted was significantly lower than in the feces. Virus detected on PID 0 in calf 3 is likely the inoculated virus itself. These results suggest that BToV is less infectious and pathogenic toward the

respiratory tract than toward the intestinal tract; however, the former route may still be important in spreading the virus, as similar viral shedding patterns were observed in the fecal and nasal specimens from calves inoculated by either route (calves 1 and 2) and the cohabitant (calf 4). Viral transmission under field conditions by these routes (intestinal and respiratory) to other calves was likely established over a few weeks, from the end of virus shedding until the calf acquired immunity. In fact, although the re-inoculated calf developed transient soft feces, both the nasal and fecal secretion of BToV was extremely low, suggesting that the calf developed tolerance to BToV, possibly through the acquisition of immunity during the initial inoculation procedure, which could be monitored in part by circulating antibodies beginning on PID 7. This observation is in agreement with epidemiological data showing that newly arrived feedlot calves became infected with BToV soon after arrival and shed the virus in their feces and nasal secretions, but that subsequent shedding of the virus ceased as the calves acquired immunocompetence against BToV (Hoet et al., 2002). Overall, the pattern of infection for BToV resembles that for BCV, another member of the *Coronaviridae* family that mainly causes diarrhea in cattle, but is also related to bovine respiratory disease; however, it is difficult to prove a relationship between a respiratory infection and

pneumonia through an experimental inoculation study (EI-Kanawati et al., 1996; Saif et al., 1986 and 1987).

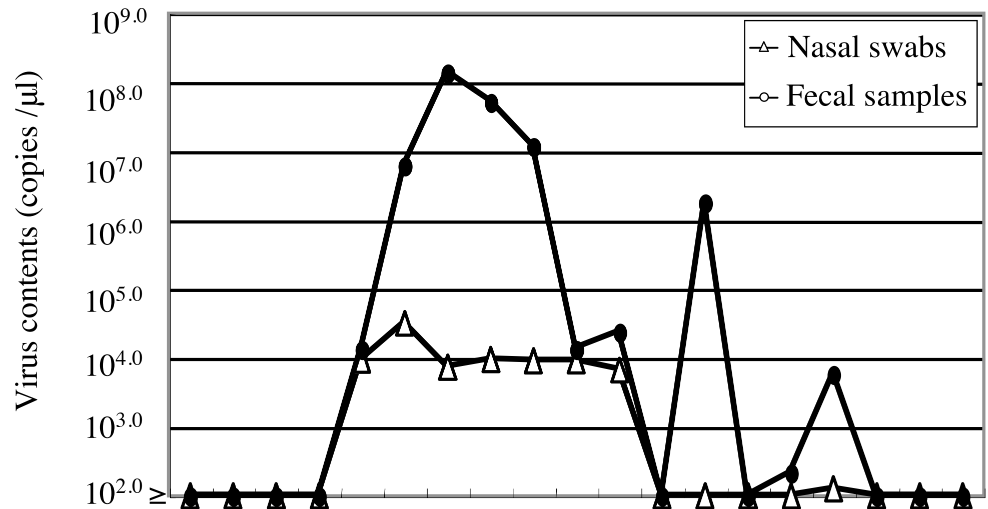
The experimental inoculation trial in this study demonstrates that BToV is pathogenic for the bovine intestinal and respiratory tracts, although the detailed pathogenicity (i.e., pathological changes in the respiratory passages) remains unclear. The fact that all of the calves tested in this study possessed antibodies against BToV at the time of inoculation might have influenced the pathogenicity; however, their titers were considerably low. Thus, it will be important for future studies to investigate the pathological changes that occur in intestinal and respiratory tissues under experimental inoculation conditions, preferably using colostrum-deprived newborn calves.



Time after inoculation		pre	0d PM	1 d	2 d	3 d	4 d	5 d	6 d	7 d	10 d	11 d	13 d	14 d	17 d	3 W	4 W	5 W	6 W	8 W
General clinical signs		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Temperature		39.1	39.4	38.8	39.0	39.0	39.0	39.3	38.7	39.2	39.2	39.2	39.5	39.1	38.9	39.5	38.9	39.2	39.1	38.7
Diarrhea [a)]		-	-	L	L	-	L	L	L	W	L	L	W	L	-	-	-	-	-	-
Respiratory symptoms [b)]		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Serum antibody titer	HI	4								16				32		64	64		64	32
	NT	8								64				64		128	128		64	64

a) L: loose stool, W: watery stool, b) C: coughing, R: rhinorrhea. - indicates nothing abnormal.

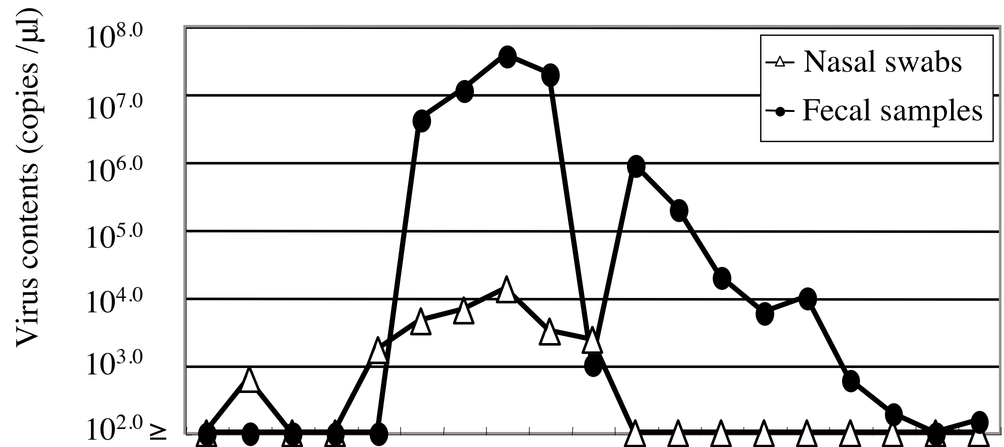
Fig. 4-1(a)



Time after inoculation		pre	0d PM	1 d	2 d	3 d	4 d	5 d	6 d	7 d	10 d	11 d	13 d	14 d	17 d	3 W	4 W	5 W	6 W	8 W
General clinical signs		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Temperature		39.1	39.5	38.9	39.3	39.2	39.0	39.1	39.1	39.2-	39.8	39.3	40.5	40.7	39.2	39.3	39.4	38.9	39.2	39.0
Diarrhea a)		-	-	-	L	-	-	L	L	-	L	-	W	L	-	-	-	-	-	-
Respiratory symptoms b)		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Serum antibody titer	HI	4								8				64		128	256		256	128
	NT	8								8				256		512	512		512	256

a) L: loose stool, W: watery stool, b) C: coughing, R: rhinorrhea. - indicates nothing abnormal.

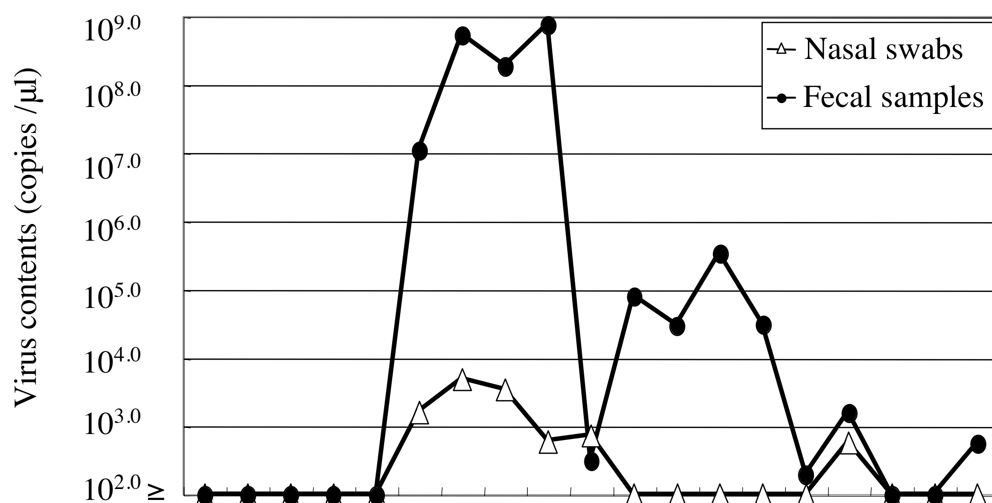
Fig. 4-1(b)



Time after inoculation		pre	0d PM	1 d	2 d	3 d	4 d	5 d	6 d	7 d	10 d	11 d	13 d	14 d	17 d	3 W	4 W	5 W	6 W	8 W
General clinical signs		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Temperature		38.9	39.3	39.2	39.1	39.2	39.4	39.3	39.3	39.2	38.9	39.3	39.7	39.4	39.2	39.0	39.1	38.8	38.6	392
Diarrhea a)		-	-	L	-	-	L	-	L	L	L	-	L	-	L	L	-	-	-	-
Respiratory symptoms b)		-	-	-	R	C	-	-	-	-	-	C	C	C	C	-	-	-	-	-
Serum antibody titer	HI	4								4				128		128	256		128	128
	NT	4								8				512		512	512		512	512

a) L: loose stool, W: watery stool, b) C: coughing, R: rhinorrhea. - indicates nothing abnormal.

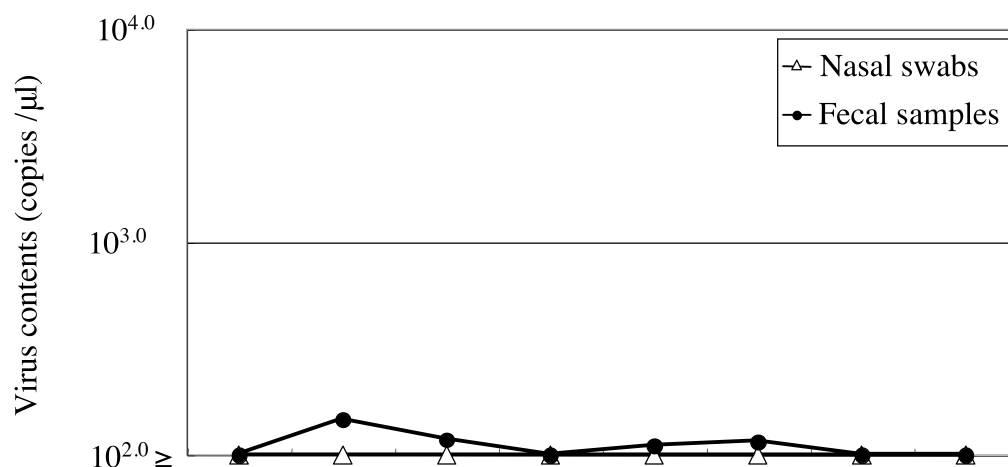
Fig. 4-1(c)



Time after inoculation		pre	0d PM	1 d	2 d	3 d	4 d	5 d	6 d	7 d	10 d	11 d	13 d	14 d	17 d	3 W	4 W	5 W	6 W	8 W
General clinical signs		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Temperature		38.7	39.2	39.1	39.0	39.0	39.3	39.2	39.2	39.3	39.1	39.3	39.1	39.3	38.9	39.1	39.1	39.3	38.8	39.2
Diarrhea [a)]		-	-	-	L	-	-	L	L	L	L	L	-	-	L	-	-	-	-	-
Respiratory symptoms [b)]		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Serum antibody titer	HI	4								4				64		128	128		256	128
	NT	8								16				256		256	256		512	256

a) L: loose stool, W: watery stool, b) C: coughing, R: rhinorrhea. - indicates nothing abnormal.

Fig. 4-1(d)



Time after inoculation	pre	0d PM	1d	2d	3d	4d	5d	7d
General clinical signs	-	-	-	-	-	-	-	-
Temperature	38.9	39.2	38.8	39.1	39.0	39.0	39.1	38.8
Diarrhea [a)]	-	-	-	L	-	-	-	L
Respiratory symptoms [b)]	-	-	-	-	-	-	-	-

a) L: loose stool, W: watery stool, b) C: coughing, R: rhinorrhea. - indicates nothing abnormal.

Fig. 4-2.

Figure legends

Fig 4-1. Clinical signs, antibody titers, and viral excretion in cattle inoculated (a) orally (calf No. 1), (b) intranasally (calf No. 2), or (c) orally and intranasally (calf No. 3) with BToV strain Hokkaido-2008TI/E. (d) The uninoculated cohabitant (calf No. 4).

Fig 4-2. Clinical signs and viral excretion in calf No. 3, which was re-inoculated orally and intranasally with BToV strain Hokkaido-2008TI/E.

CHAPTER 5

Seroepidemiological analysis of bovine torovirus in Japan

Abstract

Bovine torovirus (BToV) is a risk factor for diarrhea and in part, respiratory disease in cattle, similar to another member of the *Coronaviridae* family, bovine coronavirus. However, epidemiological data are limited because BToV was unable to be propagated via cell culture until recently. Consequently, no surveys of the relationship between clinical symptoms, especially respiratory tract disease, and the seroepidemiology of BToV have been conducted on a large scale. In this chapter, paired serum samples were collected between December 2005 and March 2010 from cattle in 17 prefectures (359 cattle from 48 herds, 111 cattle from 12 healthy herds, and 248 cattle from 36 herds with respiratory problems or diarrhea) and tested for hemagglutination inhibition antibodies against the Hokkaido-2008TI/E strain of BToV. Nearly all of the cattle possessed BToV-specific antibodies, although the levels varied. The antibody levels gradually decreased in most of the asymptomatic cattle; only three of 111 cattle (2.7%) showed a rise in antibody level in the second set of samples. In contrast, seroconversion was evident in 49 of 248 cattle (19.8%) from diseased herds, and especially among the herds with respiratory problems (42 of 121 cattle [34.7%]) although rising antibody

levels against other respiratory disease-related pathogens were observed coincidentally in these herds. These findings indicate that BToV is actively circulating among Japanese cattle, and that it may play an etiological role in bovine respiratory and intestinal diseases, mainly via co-infection with other pathogens.

Introduction

The global distribution of BToV was recently reported (Canada: Duckmanton et al., 1998; The Netherlands: Koopmans et al., 1991; Austria: Haschek et al., 2006; United States: Hoet et al., 2002, 2003; Germany: Liebler et al., 1992; South Korea: Park et al., 2008). In addition, several reports have suggested that BToV possesses infectivity for both the respiratory and intestinal tracts, although its pathogenesis in the respiratory tract is unclear (Hoet et al., 2002; chapters 3 and 4). However, seroepidemiological data are limited due to technical difficulties. Because of the difficulties involved in virus isolation, most seroepidemiological studies of BToV have been carried out by antibody detection using purified viral particles obtained by continuous passage in gnotobiotic and colostrum-deprived calves. Consequently, large-scale surveys of the relationship between clinical symptoms, especially respiratory tract disease, and the seroepidemiology of BToV have not been conducted.

In this study, the author conducted a seroepidemiological analysis of paired serum samples from Japanese cattle collected between December 2005 and March 2010 using the cytopathogenic BToV strain described in chapter 3 (Hokkaido-2008TI/E).

Materials and Methods

Serum samples for epidemiological analysis

Paired serum samples collected in 1- to 5-month intervals were obtained from 359 cattle from 48 herds, 111 cattle from 12 healthy herds, 121 cattle from 15 herds with respiratory problems, and 127 of 21 herds suffering from diarrhea between December 2005 and March 2010 in 17 prefectures (Table 5-2(a) ~ (c)).

Testing for antibodies.

Each serum sample was tested for hemagglutination inhibition (HI) antibodies against BToV Hokkaido-2008TI/E strain and BCV as described previously in chapter 3. BToV Hokkaido-2008TI/E strain was adopted for the reason that it seem to possess general serologic characteristics including the first isolated strain, Aichi/2004 as a result of the author's previous research (chapter 3). Herds in which over twice of antibody rising against BToV was detected were further tested for other antibodies as follows: IBRV, BVDV-1/-2, RSV, *Bovine parainfluenza virus 3* (PI3), *Pasteurella multocida* (P.M), *Haemophilus somnus* (H.S), and *Mannheimia haemolytica* (M.H) in the herds with respiratory problems; and bovine

Rotavirus A (types G6P1 and G10P11) in the herds suffering from diarrhea as described previously (Uchida et al., 2003; Kubota et al., 1992).

Results

Although the titer varied, most of the tested cattle had HI antibody titers of ≥4 to BToV (355/359, 98.9%) at the initial sampling. An antibody titer of 32-128 accounted for approximately half of the total (189/359, 52.6%), regardless of the herd and presence of symptoms (Table 5-1). A comparison of the geometric mean antibody titers (GMT) between paired serum samples for each group revealed that one of 12 herds among the healthy cattle (herd No.12), 10 of 15 herds with respiratory problems (herds No.1, 3, 4, 6, 7, 8, 10, 11, 14, and 15), and 8 of 21 herds suffering from diarrhea (herds No.2, 6, 7, 9, 12, 13, 16, and 20) showed rising antibody levels, although the exact level varied (Table 5-2(a) ~ (c)). Among the healthy herds and herds suffering from diarrhea, only a slight rise in antibody titer was observed (less than twofold). On the other hand, among the herds with respiratory problems, the GMT more than doubled in herds No.3, 4, 6, and 11; increased by about 5 times in herd No.7; and increased by 19 times in herd No.8. Notably, a greater than 30-fold increase was observed in herd No.15. Rising levels of antibodies for other pathogens associated with respiratory disease were observed coincidentally in these herds: BCV, PIV3, MH, and PM in herd No.3; MH in herd No.4;

BRSV and PIV3 in herd No.6; BVDV-1 and PIV3 in herd No.7; BRSV, PM, and HS in herd No.8; BVDV-2 in herd No.11; and BRSV in herd No.15.

Seroconversion in individual cattle, as determined by a greater than 4-fold increase in antibody titer, was observed in 52 of 359 cattle (14.5%), which is somewhat lower than for BCV (18.9%) (Table 5-3). Among the herds with respiratory problems, 42 of 121 cattle showed seroconversion (34.7%), which is about 13 times the rate in the healthy herds (3/111, 2.7%) and even higher than for BCV (20.7%). In contrast, 7 of 127 cattle showed seroconversion among the herds suffering from diarrhea (5.5%), which is considerably lower than in the herds with respiratory problems but roughly twice the rate in the healthy herds. The rate of seroconversion among calves was higher than that among adult cattle. In particular, seroconversion among those calves with respiratory symptoms exceeded 40%.

Discussion

In this study, the author conducted a seroepidemiological analysis of 359 paired serum samples, which revealed a seroprevalence of 98.9% against BToV. Among those herds consisting of animals aged around one month, healthy herds 5, 6, and 8 had relatively high antibody titers (GMT=152.2-215.3), which were most likely maternal antibodies, because a marked decrease in antibody titer was observed in the herds a few months later. On the other hand, some of the herds consisting of adult cattle (e.g., diarrhea-producing herd No.17) exhibited high antibody titers, which is assumed to be the result of contact with the virus because it is unlikely that maternal antibodies would be present in adult cattle. Consequently, BToV was proven serologically to be widely distributed in Japan, similar to data from Europe (86% in Switzerland in 1984 and 94.6% in The Netherlands in 1989) and the United States (88.5% in 1985), suggesting the worldwide spread of BToV infections (Koopmans et al., 1989; Weiss et al., 1984; Woode et al., 1985).

Although there are several reports of the detection of BToV in the respiratory tract (Hoet et al., 2002; chapter 2), the association between BToV infection and respiratory tract disease is incompletely understood. In

fact, farm cases of bovine pneumonia in which BToV is considered the main cause have not been reported until now. The author's previous experimental inoculation study indicated that although BToV is able to infect the respiratory tract, the pathogenicity of the virus in the respiratory tract is weak compared to that in the intestinal tract as described in chapter 4. Despite these findings, serological data in this study showed a considerable seroconversion rate among those herds with respiratory problems, which was even higher than that for BCV both in calves and adult cattle, in contrast to the situation in the other groups. However, rising levels of antibodies against other respiratory disease-related pathogens were observed coincidentally in these herds, suggesting that respiratory disease in which BToV is a primary factor is uncommon and that it occurs mainly via co-infection with other pathogens under field conditions. Indeed, epidemiological data indicate that calves shedding multiple pathogens, including BToV, are more likely to develop clinical disease compared to calves shedding a single pathogen (Hoet et al., 2003).

BToV has been shown to be a causative agent of diarrhea in cattle both in experimental inoculation and epidemiological studies (Pohlenz et al., 1984; Woode et al., 1982; chapters 1 and 4). In this study, however, a significant difference in shifting antibody titers between the asymptomatic

and diarrhea-producing herds was not observed compared to that in the herds with respiratory problems, although a difference in antibody titer was observed for individual and group GMT values. Generally, pathogen exposure leading to severe clinical disease occurs frequently in neonatal and young calves after shipping for several reasons, including a fragile constitution, co-infection with other pathogens, stress, and contact with a contaminated environment (including virus-shedding adult cattle) (Snodgrass et al., 1986). An early report from The Netherlands demonstrated that most cattle were infected with BToV before 7 to 12 months of age, suggesting that most of the adult cattle were immunized with BToV (Koopmans et al., 1989), which is generally considered to be an advantage compared with a primary BToV infection. Supporting this presumption, the author previously demonstrated a significant decrease in viral excretion in experimentally re-inoculated cattle following immunization by primary infection (chapter 4). The intestinal-symptom groups included in this study tended to be comprised of adult cattle, compared with the healthy herds and herds with respiratory symptoms and, accordingly, this may explain the low seroconversion rates for BToV among the herds with diarrhea. Previous epidemiological studies of the incidence of BToV in feces or nasal specimens have tended to show

strongly positive results for young calves (Hoet et al., 2003; Kirisawa et al., 2007; Park et al., 2008; chapter 2). On the other hand, the shift in BCV antibodies in the herds suffering from diarrhea was greater than that for the other groups both for individual and grouped GMT values, and different from the situation for BToV. These results suggest that BToV is more pathogenic toward young calves than adult cattle, compared with BCV.

Overall, the seroconversion rate was more than 7 times greater among the diseased herds (49 of 248 cattle [19.8%]), compared with the healthy herds, although the rate was somewhat lower in the herds with diarrhea. However, diarrhea is often correlated with pneumonia in calves, with diarrhea frequently preceding pneumonia and vice versa (Corbeil et al., 1984). In addition, the results of the author's previous experimental inoculation trial described in chapter 4 suggest that BToV possesses dual tropisms for the intestinal and respiratory tracts. Accordingly, it would appear that BToV plays a pathogenic role in bovine respiratory and intestinal diseases. The capacity of BToV to induce respiratory and/or intestinal disease may be affected by environmental stresses, the condition of the host and, most importantly, co-infection with other pathogens, which can result in severe secondary infections in calves, resulting in pneumonia and/or diarrhea under field conditions.

Further seroepidemiological research combined with pathogen detection is needed. In particular, the high rate of seroconversion in the herds with respiratory problems underscores the need for research into the relationship between BToV and the bovine respiratory disease complex.

Table. 5-1. Prevalence of hemagglutination inhibition (HI) antibodies against BToV at the initial sampling stage

HI antibody range	Origin of the cattle			Total
	Healthy herds	Herds with respiratory problems	Herds suffering from diarrhea	
<4	0*)	3	1	4
4 ~ 16	21	25	25	71
32 ~ 128	55	57	77	189
256 ~ 1024	35	36	23	94
<1024	0	0	1	1
Total	111	121	127	359

*) Number of the cattle

Table. 5-2(a). Serum specimens and antibody titers against BToV and BCV among healthy herds.

Herd No.	Sampling month	Prefecture	Host Information	Number of paired serum	Antibody GM titers (pre / post)	
					BToV	BCV
1	2008.4~6	Nara	beef, adult	4	64.0 / 32.0 (0*))	67.3 / 20.0 (0)
2	2008.8~9	Miyagi	beef, 1M	30	23.7 / 11.3 (0)	27.1 / 11.3 (0)
3	2008.3~7	Miyazaki	beef, 1M	4	76.1 / 38.1 (1)	53.8 / 4.0 (0)
4	2008.1~4	Miyazaki	beef, 1M	4	107.6 / 45.3 (0)	45.3 / 9.5 (0)
5	2007.12~2008.3	Miyazaki	beef, 1M	4	152.2 / 38.1 (0)	107.6 / 16.0 (0)
6	2008.1~4	Miyazaki	beef, 1M	4	215.3 /45.3 (0)	32.0 / 16.0 (0)
7	2008.9~10	Ibaraki	beef, 2~3M	19	265.5 / 137.7 (0)	15.4 / 21.4 (1)
8	2008.9~10	Kyoto	beef, 1~3M	5	168.9 / 97.0 (0)	194.0 / 16.0 (0)
9	2009.2	Gifu	beef, 1.5M	15	80.6 / 50.8 (0)	40.3 / 19.2 (0)
10	2009.8~9	Chiba	dairy, 2M	9	87.1 / 43.5 (1)	5.4 / 18.7 (5)
11	2009.10~11	Hokkaido	beef, 3~6M	3	32.0 / 25.4 (0)	16.0 / 32.0 (1)
12	2009.11~12	Hokkaido	dairy, 2M	10	64.0 / 74.7 (1)	16.0 / 18.7 (1)

*) Number of the cattle which shows ≥4-fold increase in HI antibody titers compared with their preliminary serum.

Table. 5-2(b). Serum specimens and antibody titers against BToV and BCV among herds with respiratory problems.

Herd No.	Sampling month	Prefecture	Host Information	Number of paired serum	Antibody GM titers (pre / post)	
					BToV	BCV
1	2008.2~3	Chiba	dairy, adult	28	389.9 / 463.7 (5*))	168.1 / 144.9 (1)
2	2008.9~10	Chiba	beef, adult	6	64.0 / 50.8 (0)	90.5 / 64 (1)
3	2008.10~12	Chiba	beef, 10M	5	8.0 / 24.3 (3)	2.0 / 7.0 (1)
4	2009.2~3	Gunma	dairy, 3~4M	5	48.5 / 128.0 (2)	16.0 / 12.1 (0)
5	2009.2~3	Chiba	dairy, adult	3	203.2 / 128.0 (0)	406.4 / 512.0 (1)
6	2009.1~3	Kyoto	dairy, 3~6M	6	64.0 / 194.0 (3)	35.9 / 55.7 (1)
7	2009.9~10	Hokkaido	beef, 4M	10	27.9 / 137.2 (9)	13.9 / 11.3 (2)
8	2009.7~9	Kagoshima	beef, calf	12	21.4 / 406.4 (10)	6.3 / 114.0 (11)
9	2009.7~8	Hokkaido	beef, 2~4M	10	90.5 / 68.6 (0)	24.3 / 42.2 (2)
10	2009.10~11	Aichi	dairy, 5M	5	16.0 / 27.9 (2)	42.2 / 32.0 (0)
11	2009.7~8	Tochigi	beef, calf	5	73.5 / 147.0 (2)	5.3 / 5.3 (1)
12	2009.5~6	Kagoshima	beef, 3M	6	114.0 / 50.8 (0)	57.0 / 25.4 (0)
13	2010.1~2	Hokkaido	beef, 2M	10	64.0 / 29.9 (0)	16.0 / 29.9 (3)
14	2009.12~10.1	Kagoshima	beef, 5~7M	5	111.4 / 168.9 (1)	10.6 / 9.2 (0)
15	2010.1	Chiba	dairy, adult	5	9.2 / 294.1 (5)	48.5 / 48.5 (1)

*) Number of the cattle which shows ≥4-fold increase in HI antibody titers compared with their preliminary serum.

Table. 5-2(c). Serum specimens and antibody titers against BToV and BCV among the herds suffering from diarrhea.

Herd No.	Sampling month	Prefecture	Host Information	Number of paired serum	Antibody GM titers (pre / post)	
					BToV	BCV
1	2005.12~2006.1	Chiba	dairy, adult	5	12.1 / 12.1 (1*))	34.8 / 30.3 (1)
2	2006.3	Gifu	beef, adult	10	48.5 / 52.0 (1)	48.5 / 48.5 (0)
3	2006.2~3	Mie	beef, neonatal	9	128.0 / 50.8 (0)	87.1 / 40.3 (0)
4	2006.3	Mie	beef, neonatal	5	168.9 / 55.7 (0)	111.4 / 36.8 (0)
5	2006.3	Mie	beef, neonatal	3	101.6 / 50.8 (0)	101.6 / 50.8 (0)
6	2006.4~6	Chiba	dairy, adult	5	36.8 / 64.0 (0)	55.7 / 111.4 (1)
7	2006.8~9	Gifu	dairy, adult	9	10.1 / 12.7 (1)	2.5 / 2.9 (1)
8	2007.1	Nagano	dairy, adult	5	36.8 / 27.9 (0)	2352.5 / 1351.2 (0)
9	2006.12~2007.2	Chiba	dairy, adult	2	22.6 / 32.0 (0)	181.0 / 362.0 (1)
10	2006.11~2007.2	Gifu	dairy, adult	5	64.0 / 42.2 (0)	4.0 / 84.4 (5)
11	2007.3~5	Gifu	beef, adult	4	26.9 / 26.9 (0)	2.4 / 16.0 (3)
12	2007.4~5	Chiba	dairy, adult	5	73.5 / 84.4 (0)	147.0 / 147.0 (0)
13	2006.4~6	Gifu	dairy, adult	6	32.0 / 45.3 (0)	25.4 / 71.8 (3)
14	2007.5~6	Niigata	dairy, adult	3	406.4 / 203.2 (0)	161.3 / 1625.5 (3)
15	2008.6	Chiba	dairy, adult	3	64.0 / 50.8 (0)	254.0 / 201.6 (0)
16	2008.7~9	Kanagawa	beef, 1M	11	32.0 / 43.9 (2)	9.7 / 23.4 (5)
17	2009.2~3	Hokkaido	beef, adult	6	574.7 / 312.1 (0)	63.5 / 131.3 (2)
18	2009.9~10	Tokushima	dairy, adult	6	256.0 / 256.0 (0)	512.0 / 456.1 (2)
19	2009.10~12	Chiba	beef, 3M	10	27.9 / 13.0 (0)	14.1 / 15.2 (3)
20	2009.4~5	Chiba	dairy, adult	5	9.5 / 13.9 (2)	9.5 / 48.5 (3)
21	2010.2~3	Chiba	dairy, adult	9	138.2 / 80.6 (0)	27.4 / 54.9 (2)

*) Number of the cattle which shows ≥4-fold increase in HI antibody titers compared with their preliminary serum.

Table. 5-3. Summary of the cattle in which antibody shifting was observed against BToV and BCV.

Origin	Age	Total number cattle	Antibody shifting BToV	BCV
Healthy herds	1~12 month	107	3[a] (2.8%)	8 (7.5%)
	over 12 month	4	0 (0.0%)	0 (0.0%)
	total	111	3 (2.7%)	8 (7.2%)
Herd with clinical signs				
Respiratory symptoms	1~12 month	79	32 (40.5%)	21 (26.6%)
	over 12 month	42	10 (23.8%)	4 (9.5%)
	total	121	42 (34.7%)	25 (20.7%)
Intestinal symptoms	1~12 month	38	2 (5.3%)	8 (21.1%)
	over 12 month	89	5 (5.6%)	27 (30.3%)
	total	127	7 (5.5%)	35 (27.6%)
Overall total	1~12 month	224	37 (16.5%)	37 (16.5%)
	over 12 month	135	15 (11.1%)	31 (23.0%)
	total	359	52 (14.5%)	68 (18.9%)

*) ≥4-fold increase in HI antibody titers compared with their preliminary serum.

General Discussion

In this thesis, the author carried out molecular epidemiological survey of bovine torovirus (BToV) infection among Japanese cattle by reverse transcription-polymerase chain reaction (RT-PCR) on fecal samples and nasal swabs, and further investigated genetic diversity of BToVs circulating in Japan in comparison with those reported in foreign countries. Besides the epidemiological analysis of BToV infection, the author made attempts to isolate BToVs in cell cultures and could obtain 4 cytopathogenic BToVs in HRT-18 cells. The isolates were virologically, antigenically, and genetically characterized. To investigate pathogenicity of BToV, then, cattle were experimentally exposed to the isolate and observed for clinical manifestations, virus excretions in feces and nasal secretions, and antibody responses. Finally, serum samples were collected from cattle with various conditions and tested for BToV antibodies against the isolate to predict seroprevalence in Japan.

The results of these investigations are summarized as follows.

Chapter 1: BToV specific gene was detected in 15 of 231 fecal samples collected in Japan in 2004 and 2005 and found at significantly

higher rate in samples obtained from diarrheic (14/167; 8.4%) than in asymptomatic (1/64; 1.6%) cattle, suggesting that BToV may act as one of risk factors causing diarrhea in cattle. The phylogenetic analysis of the spike (S) gene fragment revealed that BToVs detected in Japanese cattle were classified into 3 clusters, which were considerably correlated with the geographic origin of the samples. The areas where BToVs were detected did not adjoin one other but spread across a wide range, suggesting that BToV is conventionally distributed and geographically differentiated.

Chapter 2: Nasal samples collected from 311 cattle, 205 cattle with respiratory symptom and 64 from asymptomatic cattle, were tested for BToV by nested RT-PCR to amplify the nucleocapsid (N) gene. 7 samples derived from cattle with respiratory symptoms were positive in RT-PCR, but those from asymptomatic cattle were all negative, suggesting that BToV may be a predisposing factor and/or causative agent for bovine respiratory disease. The degrees of similarity of the S and hemagglutinin-estrase (HE) genes were over 90.8% within BToVs detected in nasal samples (rBToV-1 to -7) and 73.5–99.0% with feces-derived BToVs (eBToV). Phylogenetic analysis revealed that rBToV-1, -2, and -3 were closely related to one another and to BToV belonging to the cluster 2,

which was defined in feces-derived BToVs. In contrast, both rBToV-4 and -5 were genetically distant from rBToV-1, -2, and -3, while rBToV-6 was distinguished from other rBToVs and belonged to the analog of the cluster 1. These seem to indicate that genetic diversities are evident among rBToVs, but there are no definite divergences between rBToVs and eBToVs. Furthermore, one cattle showed BToV positive in both feces and respiratory secretion, suggesting that dual tropisms for BToV.

Chapter 3: Fifty-five fecal samples positive for the N gene of BToV were inoculated into HRT-18 cell culture derived from human rectal carcinoma and four cytopathogenic BToVs were successfully isolated. All isolates showed physicochemical characteristics similar to those of bovine coronavirus (BCV) and were able to agglutinate mouse, but not chicken erythrocytes, while they lacked receptor-destroying enzyme activity. Analysis of N-terminus of the S gene showed that the isolates, except one designated Gifu-2007TI/E strain, were phylogenetically located in cluster 1 and its analogs, and were antigenically almost identical with one another, as demonstrated by neutralization test (NT) and hemagglutination-inhibition (HI) test. On the other hand, the Gifu-2007TI/E strain was classified into the neighborhood of the cluster 2

and exhibited relatively low cross-reactivity with other 3 isolates in both NT and HI test. However, the antigenic differences between the Gifu-2007TI/E and other 3 strains were not sufficient to divide BToVs into apparent serotypes. The conclusions were that genetic and antigenic properties of BToVs vary moderately, and at least 2 subtypes distinguishable based on the difference in the structure of N-terminus of the S gene and by both NT and HI tests may exist.

Chapter 4: One calf each was inoculated via oral (No.1), nasal (No.2), and both routes (No.3), respectively, with the Hokkaido-2008TI/E strain of the isolate, and the other calf (No.4) was raised in the same pen as a cohabitant. All calves were monitored for clinical manifestations, virus excretions in feces and nasal secretions, and antibody responses. Although all calves revealed no severe clinical symptoms, calves No.1 and 3, and 2 developed soft feces on post inoculation day (PID) 1 and 2, respectively, that continued intermittently for approximately 2 weeks thereafter. The feces of calf No.4, a cohabitant, also became loose 2 days after cohabitation. The transient coughing was also evident in calf No.3. The virus excretion in feces first detected in all calves including cohabitant on PID 4, reached at the maximum level of 10^7 to 10^8 copies/mL, and persisted

discontinuously for a maximum of 8 weeks after inoculation. Although the viruses were also recovered from nasal secretions for a maximum of 4 weeks, their amounts were significantly smaller than those in fecal specimens, however, dual tropisms of BToV to these tracts were confirmed. Neutralizing and HI antibody titers increased by PID 7, and reached at the highest level of 64 to 512. Overall, it would appear that BToV is pathogenic for bovine intestinal and respiratory tracts, especially for the former tissues, however, both routes are important in spreading the virus.

Chapter 5: Paired serum samples were obtained from 359 cattle (111 cattle from healthy herds, 121 cattle from herds with respiratory problems, and 127 cattle suffering from diarrhea) and tested for HI antibody against the Hokkaido-2008TI/E strain of the isolate. Nearly all cattle possessed BToV antibodies with various levels in the initial samples, although the origin whether they were resulting from infection or derived from maternal antibody varied. The antibody levels gradually decreased in most of asymptomatic cattle with the lapse of time; only 3 of 111 cattle (2.7%) showed antibody rising. On the other hand, increase in antibody levels in the convalescent sera, as compared with the precritical samples, was evident in 49 of 248 cattle (19.8%) with diseases but especially remarkable

among the herds with respiratory problems although rising levels of antibodies against other respiratory disease-related pathogens were observed coincidentally in these herds. These appeared to indicate that BToVs are circulating widely among Japanese cattle and that BToVs may play some etiological roles, mainly by co-infection with other pathogens, in bovine respiratory and intestinal diseases.

BToV has been behind BCV, another member of *Coronaviridae* infecting cattle, in the research, because BToV are very difficult to propagate in cell cultures. In this thesis, the author could partially elucidate prevalence of BToV infection among Japanese cattle, etiological significance for cattle, virological properties, genetic and antigenic characteristics and variation of BToVs. In addition, 4 cytopathogenic BToVs were successfully isolated in HRT-18 cell cultures. The findings obtained in this study seem to contribute to understanding, at least in part, BToV and its infection in cattle. Especially, cytopathogenic viruses isolated in this study are expected to provide a useful tool for investigations on the pathological role and immunology of BToV in enteric and respiratory diseases in cattle.

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論文の内容の要旨

論文題目: Epidemiological Analysis of Bovine Torovirus
（牛トロウイルスに関する疫学的研究）

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コロナウイルス科はエンベロープを有する一本鎖（+）RNA ウイルスで、コロナウイルス属とトロウイルス属から構成される。コロナウイルス属に含まれるウイルスの多くは消化器官や呼吸器官に感染性を有するが、一部のウイルスは肝臓や腎臓、神経系など多様な指向性を示すことが知られている。一方、トロウイルス属は多型性のエンベロープを有する直径 120~140nm のウイルスで、コロナウイルス属と同様、ウイルス粒子の表面に棒状のスパイク蛋白が放射状に分散する。しかし、ヌクレオカプシドがらせん対称のドーナツ様構造を呈することから、コロナウイルス属と形態学的に区別される。トロウイルス属は宿主別にヒト、牛、豚及び馬トロウイルスに分類され、ヒトと牛、馬のトロウイルスは腸炎との関わりが報告されている。しかし、Bernevirus として他のトロウイルスとは区別される馬トロウイルスを除き、トロウイルス属のウイルスは分離が困難であることから、トロウイルス属に関する研究はコロナウイルス属に比べて遅れている。

牛トロウイルス（Bovine torovirus、以下 BToV）は 1982 年にアメリカで発生した子牛の集団下痢の糞便より初めて検出され、後に本件における集団下痢の原因ウイルスとして同定された。また、感染便を用いた実験感染により、牛の空回腸、結腸や盲腸の絨毛・陰窩細胞に感染し萎縮や壊死を起こすこと、その結果下痢を起こすことが確認された。しかし、BToV は牛のウイルス性下痢に関わる牛コロナウイルス（BCV）や牛ロタウイルスと異なり、培養細胞を用いたウ

イルス分離が極めて困難であることから、糞便の電子顕微鏡観察や ELISA 法、RT-PCR 法によるウイルス検出により浸潤調査が進められてきた。その結果、これまでに欧米を中心に BToV の検出が報告されている（1991: オランダ、1992:ドイツ、1998:カナダ、2002・2003: アメリカ、2006: オーストリア)。なかでも RT-PCR 法は、得られた遺伝子断片の配列解析により他のウイルスとの比較が可能となることから、遺伝子情報の蓄積・解析が進んでいる。Draker らは BToV 感染糞便より BToV ウイルスゲノムの全長を解読し、BToV は約 24.5kb のゲノムサイズを有し、RNA polymerase、spike (S)、membrane、hemagglutinin-esterase (HE)及び nucleocapsid (N)遺伝子より構成されることを示した。Smits らは S 及び HE 蛋白の相同性を指標に、野外株間での多様性について報告しており、コロナウイルス属において S 蛋白が抗原性状と深く関わっていることが知られていることから、BToV の抗原性状にも多様性の存在する可能性が考えられる。しかし、これまでに BToV の抗原性状の比較は行われていない。一方、日本を含むアジア地域では BToV の広域な浸潤調査は行われておらず、その感染実態や欧米で検出された BToV との異同については不明である。そこで、著者は国内で採取した牛由来材料を用いて BToV の浸潤状況調査を行うとともに、既報の BToV との遺伝子学的比較解析を行った。また、その過程で 4 株の細胞増殖性 BToV の分離に成功したため、それらの基礎的ウイルス性状及び抗原性状の解析を行うとともに、分離株を用いた野外での抗体調査を行った。

本研究は以下の 5 章より構成される。

第 1 章: 糞便を用いた BToV の疫学調査

2004 年から 2005 年にかけて 1 道 11 県より採取した牛の糞便 231 検体（下痢便 167 検体、正常便 64 検体）を用い、RT-PCR 法により BToV の N 遺伝子の検出により国内における BToV の検出状況と疾病との関連性を調べた。また、得られた BToV 陽性検体よりさらに S 遺伝子の塩基配列解析を行い、BToV の遺伝学的多様性について検討した。

糞便 231 検体のうち、1 道 3 県より得た 15 検体（6.5%）から BToV が検出され、国内で広範囲に BToV が浸潤していることが確認された。このうち、正常便由来のものが 1 検体（1/64、[1.6%]）であったのに対し、下痢便では 14 検体（14/167、[8.4%]）と明らかに高い検出率を示した。さらに下痢便由来の 14 検体のうち 7 検体からは BCV が同時に検出されたが、それ以外では BCV や RV、病原性大腸菌等の下痢に関する病原体は検出されず、BToV 感染と下痢との疫学的関連が示唆された。S 遺伝子の塩基配列解析を実施した結果、検体間で 91.6%～99.8%の相同性を有していたが、同じ地域で得られた検体の間では特に相同性が高かった。また、系統樹解析の結果、国内に分布する BToV は少なくとも 3 種類のクラスターに分類されることが明らかとなった。以上の結果から、国内において BToV は常在し、牛の下痢の一因として関与してい

る可能性が考えられた。また、地域毎に独自の進化が進んでいる可能性が示唆された。

第 2 章: 鼻汁を用いた BToV の疫学調査

BToV と同じく牛の下痢を引き起こす BCV は、消化器系と呼吸器系の双方の感染因子となることが知られている。Hoet らはオハイオ州の一農場において牛の鼻汁からの BToV の検出を報告しており、BToV においても呼吸器に対する侵襲性が予想される。しかし、BToV と呼吸器疾病との疫学的関連性については検討が行われていない。また、鼻汁より検出された BToV（rBToV）と糞便由来 BToV（eBToV）との間での遺伝学的関連性の有無についても報告が無い。以上のことから、著者は 2006 年から 2008 年にかけて 1 道 15 県より採取した牛の鼻汁 311 検体（呼吸器症状陽性牛 205 検体、健常牛 106 検体）より BToV-N 遺伝子の検出を試み、rBToV の検出状況と疾病との疫学的関連性を調べた。また、rBToV と eBToV の遺伝子解析を行い BToV の多様性について検討を行った。

鼻汁 311 検体のうち 7 検体（2.3%）から BToV が検出された。これら 7 検体は 5 県 6 農場に由来したが、いずれも呼吸器症状を示した若齢子牛より採取した検体であった。次いで、S 及び HE 遺伝子の配列解析を実施した結果、rBToV 間では S で 91.1%ー100%、HE で 90.8%ー100%の相同性を有し、既報の eBToV との間では S で 89.6%ー99.0%、HE で 70.6%ー99.0%の相同性を示した。系統樹解析の結果、3 検体は eBToV のクラスター2、また 1 検体はクラスター1 の近縁に位置づけられた。また他の検体はクラスター1 とクラスター3 の中間に位置づけられた。以上の結果から、BToV は BCV と同様に牛の消化器だけでなく呼吸器系組織にも感染性を有し、牛の呼吸器疾病との疫学的な因果関係が示唆された。一方、rBToV と eBToV との間には遺伝学的に本質的な差異は認められなかった。

第 3 章: 新規に分離した BToV 4 株のウイルス性状及び抗原性状と遺伝子性状との相関

BToV 遺伝子が検出された糞便をヒト直腸癌由来細胞（HRT-18 細胞）に接種・継代することにより 4 株の細胞増殖性 BToV の分離に成功した。分離ウイルスは、いずれの株もマウス赤血球に対して HA 活性を有していたが、鶏赤血球に対しては HA 活性を示さなかった。BToV 4 株の S 遺伝子について、既報の BToV と比較した結果、分離ウイルスは Gifu-2007TI/E を除いてクラスター1 とその近縁に位置づけられた。一方、Gifu-2007TI/E はクラスター2 の近縁に位置づけられた。次に、これらの分離ウイルスについて交差中和試験及び交差 HI 試験を行い、抗原関連値（R%）を指標に株間の抗原性状の相違を調べた。その結果、中和試験では R% : 19.8ー100%、HI 試験では R% : 14.0ー89.4%を示したが、Gifu-2007TI/E を除いた場合には前者で R% : 56.6ー100%、後者で R% : 44.2ー89.4%と高い交差性を有していた。このことから、Gifu-2007TI/E を除いた 3 株は互いに極めて強い交差性を有し、Gifu-2007TI/E 株はこれらとは抗原性が多少異なっ

たものの、血清型の違いとして定義づけるほどの相違はないことが考えられた。以上の結果から、中和及び HI 試験により識別される、少なくとも 2 種類の BToV 血清亜型が存在し、また、それは S 蛋白の相同性と関わっている可能性が示された。

第 4 章: BToV 分離ウイルスを用いた感染試験

BToV の感染試験に関する報告は少なく、呼吸器経路に対する感染性も実験感染では確かめられていない。そこで、細胞で増殖が可能な BToV 分離株を用いた感染実験を行い、消化器経路と呼吸器経路におけるウイルスの動態と抗体応答の推移を調べた。

HRT-18 細胞培養 Hokkaido-2008TI/E 株を 5 か月齢の牛に接種し、臨床観察をおこなうとともに糞便と鼻汁より排泄されるウイルスの定量を行った。その結果、実験感染牛に体温や元気、食欲に異常は認められなかったものの、攻撃後 4 日から 10 日にかけて軟便を呈した。消化器症状が観察され始めたのとほぼ同時期から、糞便より BToV の排出が観察され、特に攻撃後 4 日から 7 日には顕著なウイルス排出が確認された。同期間中、糞便に比べ少量ではあったが鼻汁からもウイルス排出が確認された。攻撃開始後 7 日より血清抗体の上昇が確認され、抗体価の推移は HI および中和試験で良く相関していた。これらの結果から、BToV は牛の消化器経路と呼吸器経路の双方に感染性を有し、また、HI 及び中和抗体を指標に感染の確認が可能であることが示された。

第 5 章: BToV の野外牛における血清疫学調査

BToV の抗体検出には、これまで感染牛の糞便材料が用いられており、実験手技が困難であったため、広範囲での血清疫学調査はおこなわれていなかった。そこで、分離ウイルスを用いて 2005 年から 2010 年にかけて 1 道 16 県より採取した健康牛群 12 群、臨床症状（消化器症状、呼吸器症状、発熱）を呈した牛群 36 群のペア血清について BToV に対する HI 抗体価の測定を行った。その結果、多くの個体は初回採血の時点で BToV に対する HI 抗体を保有していたが、健康牛群の多くは月齢を経るに従って抗体価の低下が見られたのに対し、疾病牛群の一部では臨床症状観察後に抗体の上昇が確認された。特に、呼吸器症状が見られた牛群でその程度は顕著であった。以上の結果から、BToV は牛の疾病に関わる一因子として広く浸潤している可能性が、血清学的にも示された。

BToV が初めて分離報告されたのは 2007 年で、牛の下痢症に関わる病原体として認識されたのはごく最近であり、本ウイルスの感染と病原性等については検討すべき課題が多く残されている。本研究は、今後重要性を増すであろう BToV の疫学研究と防除対策確立の一助となると考えられる。