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First detection of bovine group B rotavirus in Japan and sequence of its VP7 gene

Brief Report

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Summary. An epizootic outbreak of diarrhea occurred in adult cows on a dairy farm in Hokkaido, Japan. One colostrum-fed calf inoculated with pooled feces of the 5 affected cows, developed mild diarrhea, and shed rotavirus-like particles which reacted with antiserum to group B rotavirus in immune electron microscopy. Cell culture immunofluorescence tests, RNA-polyacrylamide gel electrophoresis and RT-PCR confirmed that this virus was bovine group B rotavirus, which was designated the Nemuro strain. Additional 2 colostrum-deprived calves inoculated with feces of the first calf also developed diarrhea and shed virus, suggesting that this group B rotavirus might be the etiological agent of the outbreak of adult cow diarrhea. The identities of the nucleotide (nt) and deduced amino acid (aa) sequences of the Nemuro VP7 gene were high (93–95% in nt and 96–97% in aa) and low (61–63% in nt and 49–61% in aa) compared to those of the published corresponding genes from 3 bovine and 2 other mammalian (human and rat) strains of group B rotaviruses, respectively. To our knowledge, this is the first report on the presence of bovine group B rotavirus in Japan.

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Rotaviruses are assigned to 6 groups (A to F) based on antigenic and genomic analysis [2, 19]. Each rotavirus group possesses its own cross-reactive anti-

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gens and shows similar double-stranded RNA (dsRNA) electrophoretic migration patterns, which vary among groups [19].

Group B rotaviruses (GBR) cause diarrhea in humans, pigs, cattle, lambs, and rats [2, 19]. Detection of GBR is difficult compared to that of group A rotavirus (GAR) because:1) serial propagation of GBR in cell culture was unsuccessful except for one porcine strain [22]; 2) the number of GBR in feces is usually very low [2, 19]; 3) antisera to GBR are available only in a few laboratories. Therefore, in contrast to GAR, which are a major cause of acute gastroenteritis in young animals and children [13], the prevalence and characterization of GBR remains unclear. Bovine GBR have been reported only in the United States and the United Kingdom [3, 4, 7, 16, 20, 23]. Recent reports have suggested that GBR might be more associated with adult cow diarrhea than with calf diarrhea [3, 20].

The GAR and group C rotaviruses (GCR) are classified into serotypes as defined by the outer capsid proteins, VP4 and VP7, which elicit the production of neutralizing antibodies [10, 26]. Sequence analysis of the amino acids of VP7 of GAR and GCR demonstrated a high degree of sequence similarity among the same serotypes, but a high degree of sequence divergence was observed among different serotypes [9, 12, 17, 25]. To date, only a few GBR VP7 genes have been analyzed and compared [3, 6, 18]. The objectives of this study were to investigate the occurrence of GBR infections in adult cows in Japan, to attempt to reproduce disease in colostrum-fed and colostrum-deprived calves with the GBR isolate, to determine the nucleotide sequence of the VP7 gene of the GBR isolate, and to compare it to the published corresponding sequences from bovine and other mammalian strains of GBR.

An epizootic outbreak of diarrhea in adult dairy cows occurred on a dairy farm in Hokkaido, Japan in the spring of 1997. About 10 of 40 milking cows in a herd developed diarrhea on the same day. The diarrheal feces were blackish liquid or semiliquid, and diarrhea continued for 1 to 2 days. Diarrheal feces and sera were obtained from 5 affected cows. Fecal samples were examined for rotavirus by electron microscopy (EM), polyacrylamide gel electrophoresis of viral dsRNA (RNA-PAGE) and reverse transcriptase PCR (RT-PCR) of the extracted RNA. These feces were also tested for GAR by latex agglutination tests (Rotascreen; Microgen Bioproducts LTD, Surrey, U.K.), and for *Salmonella* spp., *Coccidium* spp. and *Cryptosporidium* spp. by using standard techniques. Serum samples were collected from these cows at the acute and convalescent phases of the disease. These paired sera were tested for antibodies to bovine viral diarrhea virus, bovine coronavirus and reovirus by virus neutralization tests, and for antibodies to GAR and the isolate by indirect immunofluorescence (IF) tests [27].

Hyperimmune antisera to bovine GAR (Lincoln strain) and GBR (ATI strain) were prepared in gnotobiotic calves [1, 19, 21]. Hyperimmune antiserum to bovine GCR (Shintoku strain) was prepared in a guinea pig [27].

For RNA-PAGE, virus was partially purified from fecal suspensions by ultra-centrifugation through a 30% sucrose cushion, and viral dsRNA was extracted with phenol-chloroform. The dsRNA of a cell culture-adapted bovine GAR (41T strain) was also used as a control (unpublished data). The electrophoresis of the

extracted RNA was conducted in 10% polyacrylamide gels as described previously [27]. Gels were then strained with a silver stain kit (Wako Pure Chemical Industries, Osaka, Japan).

For EM, fecal suspensions were placed on carbon-coated collodion grids, negatively stained with 3% uranyl acetate, and examined with an electron microscope. For immune electron microscopy (IEM), 20 μl of the partially purified virus from the feces of calf 918 (see below) was incubated for 2 h at 37 °C with an equal volume of hyperimmune antiserum to bovine GAR, GBR or GCR (diluted 1:100) and examined for viral aggregation. For solid phase-IEM (SPIEM), carbon-coated collodion grids were floated on 25 μl of protein A solution (25 μg/ml) for 20 min, washed twice with phosphate-buffered saline (PBS), and refloated on 25 μl of antiserum to bovine GBR diluted 1:200 with PBS for 20 min. After washing twice with PBS, the grids were floated again on 25 μl of fecal suspensions for 60 min. The grids were washed twice with PBS and once with water, and then stained with 3% uranyl acetate.

Cell culture IF tests (CCIF tests) against the isolates were conducted with MA104 cells as described previously [24, 27] with slight modifications. Briefly, cell monolayers in Leighton tubes or 24-well plates were inoculated with calf 918 feces (see below) which were pretreated with trypsin (0, 10, 20 or 40 μg/ml) for 30 min at 37 °C. After adsorption for 1 h at 37 °C, the cells were washed once with Eagle's minimum essential medium (EMEM). EMEM supplemented with trypsin (0, 1, 2, or 4 μg/ml) was added, and the cells were incubated on a roller drum or as stationary cultures at 37 °C. After 20 h of incubation, the cells were fixed with acetone, and reacted with hyperimmune antiserum (diluted 1:100) to GAR, GBR or GCR or sera (diluted 1:10) of cows or calves for 30 min at 37 °C. After washing with PBS, the cells were stained with anti-bovine or anti-guinea pig IgG conjugated with fluorescein isothiocyanate (Zymed Laboratories, San Francisco, CA, U.S.A). Virus isolation was performed using roller tubes in the same manner as the CCIF tests except that incubation was for 3 days. Subsequent passages were carried out by using cell suspensions of the previous passage as the inoculum, and these cell suspensions were used for indirect IF tests and RT-PCR.

An RT-PCR for detection of bovine GBR was conducted with the primers reported by Chinsangaram et al. [7]. A pair of primers 9B3 and 9B4 resulted in 281 bp PCR products of the VP7 gene of bovine group B rotavirus. Viral dsRNA was extracted from fecal and cell suspensions using an Isogen LS kit (Wako Pure Chemical Industries, Osaka, Japan). The dsRNA of cell culture-adapted bovine GAR (41T strain) and bovine GCR (Shintoku strain) [27] were also used. The dsRNA was denatured by using dimethyl sulfoxide and heating, and the RT reaction was conducted with the primer 9B4 and Moloney murine leukemia virus RT (Life Technologies , Inc., Rockville, MD, U.S.A.) by incubation at 37 for 60 min. The PCR reaction with the primers and Taq polymerase (AmpliTaq Gold; Perkin-Elmer Corporation, Norwalk, CT, U.S.A.) was carried out by preheating at 94 °C for 10 min, and then 30 cycles of 1 min at 94 °C, 1 min at 48 °C and 2 min at 72 °C. The PCR products were electrophoresed on 1.2% agarose gels and visualized by ethidium bromide staining.

Both terminal sequences of the VP7 gene were determined with the synthetic primers by the procedure described previously [14, 25]. Confirmation of the terminal sequences was also provided by the use of a RACE kit (5′ RACE System; Life Technologies, Inc., Rockville, MD, U.S.A.). The full length VP7 gene was produced by RT-PCR with 5′ and 3′ end primers designed with the determined terminal sequences, and the PCR products were sequenced directly by cycle sequencing with an auto sequencer (ABI PRISM 377; Perkin-Elmer Applied Biosystems, Foster City, CA, U.S.A.). The DDBJ accession number for the VP7 gene of the GBR Nemuro strain is AB016818.

One 6-day-old colostrum-fed calf (918) and two 1- and 30-day-old colostrum-deprived calves (931 and 951) were used for viral inoculation tests. These calves were reared individually in isolation rooms and fed milk replacer twice a day. The older colostrum-deprived calf had recovered from a prior experimental oral challenge with a bovine GAR at 10 days of age. The calf 918 was inoculated orally (20 ml) with a 10% pooled fecal suspension of the 5 affected cows, which had been treated with 80 μg/ml of gentamicin (Schering-Plough Corporation, Osaka, Japan). The calves 931 and 951 were inoculated orally (20 ml) and nasally (5 ml) with a gentamicin-treated (calf 931) or filtered (calf 951) 10% suspension of diarrheal feces of the calf 918 collected at postinoculation day (PID) 3. After inoculation with fecal suspensions, feces and nasal swab specimens were collected daily for 14 days, blood samples were collected at inoculation and 14 to 21 days later, and clinical signs were observed through PID 14. Feces were examined for bovine GBR by EM, SPIEM, and RT-PCR, and nasal swab specimens were used for RT-PCR.

A few rotavirus-like particles were observed in the feces from 2 of 5 affected cows by EM, but no other viruses were observed. All fecal samples were negative for GAR by latex agglutination tests. All 5 fecal samples were positive for GBR by RT-PCR showing 281 bp PCR products (Fig. 1a). In contrast, the bovine GAR and GCR were negative in the RT-PCR. All fecal samples were negative for *Salmonella* spp. and *Cryptosporidium* spp., but *Coccidium* spp. was observed in one sample. Examination of paired sera from these cows showed no seroconversion ($\geq$ 4-fold increase) to bovine viral diarrhea virus, bovine coronavirus, reovirus and GAR. By contrast, the convalescent-phase sera from 4 of these cows were positive for GBR antibodies by CCIF tests.

Rotavirus-like particles were observed by EM in fecal samples at PID 2 and 3 from calf 918 inoculated with pooled feces of the 5 affected cows. In IEM, the particles were agglutinated by antiserum to GBR diluted 1:100, but not by antiserum to GAR or GCR diluted 1:100. In PAGE, the dsRNA migration pattern was characteristic of GBR (4-2-2-3 pattern) (Fig. 2). Using the fecal sample at PID 3 in CCIF tests, syncytia containing immunofluorescent cytoplasmic granules were observed in infected MA104 cells stained with antiserum to GBR (Fig. 3), but not antiserum to GAR or GCR. The fecal suspension at PID 3 contained 5 to 10 syncytium forming units per 0.1 ml. The syncytia usually contained 10 to 50 nuclei. Treatment of inocula with trypsin, supplement of the medium with trypsin, and rolling of cultures did not significantly effect the number of syncytia.

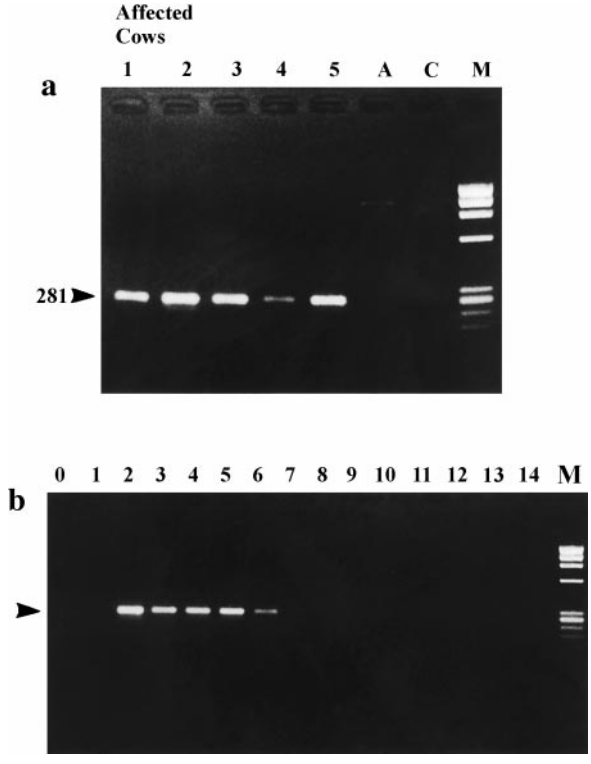

Fig. 1. RT-PCR detection of bovine GBR RNA in fecal samples of the 5 affected cows and the experimentally infected newborn calf (918). The RT-PCR products were visualized on 1.2% agarose gel stained with ethidium bromide. *M* Molecular weight marker. **a** *1–5* Feces of the 5 affected cows; *A* bovine GAR (41T strain); *C* bovine GCR (Shintoku strain). **b** Number of lanes indicates postinoculation day of the experimentally infected newborn calf (918)

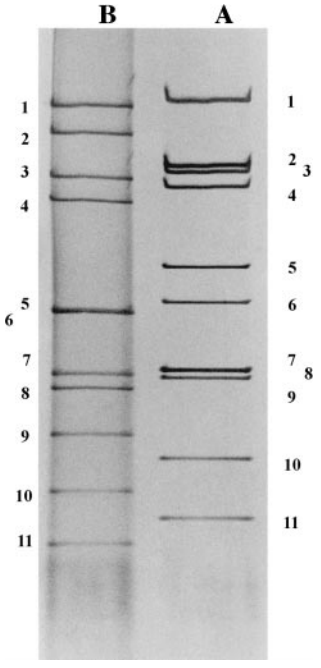

Fig. 2. Comparison of the electrophoretic migration patterns of rotavirus dsRNA. *A* Bovine GAR (41T strain); *B* dsRNA extracted from the feces of the calf 918 inoculated with pooled feces of the affected cows

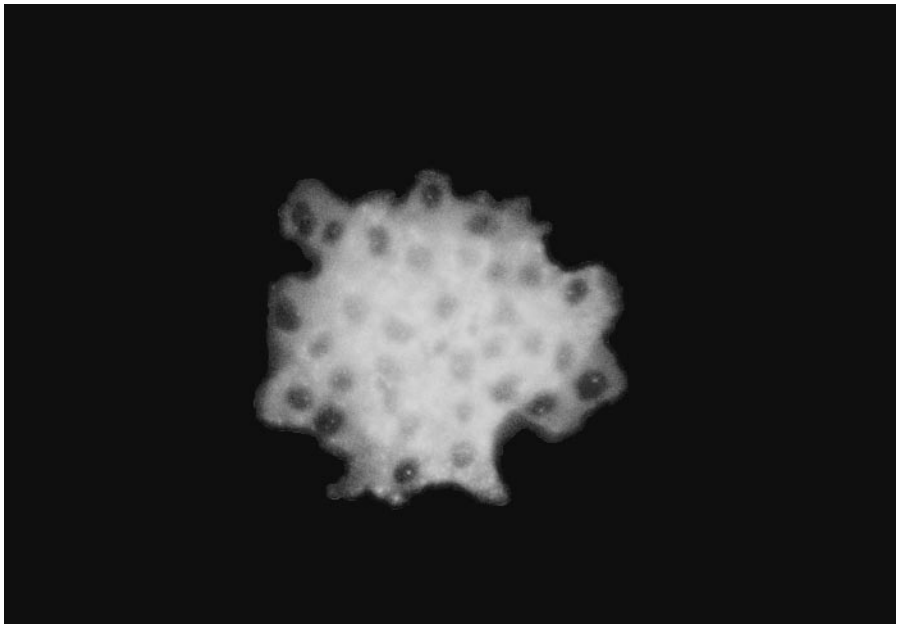

Fig. 3. Immunofluorescence in MA104 cell monolayers infected with the feces of the calf (918) inoculated with the pooled feces of the affected cows, and reacted with gnotobiotic calf anti-bovine GBR serum and then with FITC-conjugated anti-bovine IgG. Note syncytium with intracytoplasmic immunofluorescence

These feces were also positive for GBR by RT-PCR. This virus was designated the Nemuro strain of GBR. Attempts to serially passage the Nemuro strain of bovine GBR in MA104 cells were unsuccessful, as syncytia could not be detected after the second passage, and the amplified products were not detected after the third passage by RT-PCR (data not shown).

Calf 918 inoculated with pooled feces of the 5 affected cows developed mild diarrhea at PID3, and diarrhea persisted for 3 days. Diarrheal feces were pasty and yellowish. The virus was detected in feces by EM, SPIEM and RT-PCR for 2, 3 and 5 days, respectively (Fig. 4, Fig. 1b). The calves 931 and 951 developed diarrhea at PID 2 and 3, respectively, and diarrhea persisted for 2 days. Diarrheal feces were watery (calf 931 at 2 PID) or pasty and yellowish. The virus was detected in feces by EM, SPIEM and RT-PCR for 1–2, 2–4 and 5 days, respectively (Fig. 4). Virus shedding was not observed in nasal swabs from these calves by RT-PCR (data not shown). Depression and anorexia were not observed in any calves. All calves seroconverted to GBR at PID 21 by CCIF tests.

The full-length VP7 gene of the Nemuro GBR was 816 nucleotides (nt) long and contained one open-reading-frame encoding a polypeptides of 247 amino acids (aa). The number of nt differed from that of the other bovine ATI, Mebus and WD653 strains (811 nt), the human ADRV strain (814 nt), and the rodent IDIR strain (804 nt) of GBR. The number of aa corresponded to that of other bovine GBR (ATI, Mebus, WD653), and was 2 aa shorter and 1 aa longer than that of the ADRV and IDIR GBR, respectively. The nt identity of the Nemuro VP7

		↓ Postinoculation Day									
		0	1	2	3	4	5	6	7	14	21
Colostrum-fed calf (**918**; 6 days old) inoculated with pooled feces of the 5 affected cow	Virus from feces										
	EM	−	−	+	++	−	−	−	−	−	
	SPIEM	−	−	+++	+++	+	−	−	−	−	
	RT-PCR	−	−	+	+	+	+	+	−	−	
	Diarrhea	−	−	−	+	+	+	−	−	−	
	Antibody	−								−	+
Colostrum-deprived calf (**931**; 1 day old) inoculated with feces of the calf 918	Virus from feces										
	EM	−	−	+	+	−	−	−	−	−	
	SPIEM	−	+	+++	++	+	−	−	−	−	
	RT-PCR	−	+	+	+	+	+	−	−	−	
	Diarrhea	−	−	++	+	−	−	−	−	−	
	Antibody	−								−	+
Colostrum-deprived calf (**951**; 30 days old) inoculated with feces of the calf 918	Virus from feces										
	EM	−	−	−	+	−	−	−	−	−	
	SPIEM	−	−	−	++	+	−	−	−	−	
	RT-PCR	−	−	+	+	+	+	+	−	−	
	Diarrhea	−	−	−	+	+	−	−	−	−	
	Antibody	−								−	+

Fig. 4. Detection of bovine GBR by EM, SPIEM and RT-PCR in fecal samples from three experimentally infected newborn calves. Virus detection by EM and IEM: (−), not detectable; (+), <1–10 virus particles per grid square; (++), 11–100 virus particles per grid square; (+ + +), 101–1000 virus particles per grid square. Virus detection by RT-PCR: (−), no virus detectable; (+), virus detectable. Diarrhea: (−), normal feces; (+), pasty feces; (++), liquid feces

gene was 93–95% compared to the other 3 bovine strains, and 61–63% compared to the ADRV and IDIR strains. The deduced aa identity of the Nemuro VP7 gene was also high (96–97%) compared to the other 3 bovine strains, and low (49–61%) compared to the ADRV and IDIR strains. The 5′ termini of these bovine VP7 genes were conserved. By contrast, the 3′ terminus of the VP7 gene of the Nemuro strain was more similar to that of the ADRV and IDIR strains rather than that of the other bovine strains, which ended with "CCC". The hydropathicity profiles of the VP7 were similar between bovine and other mammalian GBR (data not shown). Multiple alignments of the deduced aa sequence of the VP7 from the 6 GBR were produced using the MEGALIGN program (DNASTAR Inc., Madison, WI, U.S.A.) as shown in Fig. 5. One potential N-linked glycosylation site located at aa residues 46–48 was conserved for all VP7. An additional one or two potential glycosylation sites were present in VP7 except for that of the WD653 strain. There was the putative signal cleavage site between Ala/Gly15 and Gln16 in the VP7 of all GBR, which was also present in the VP7 of GAR and GCR at aa residues 50–51 and 49–50, respectively [15, 25]. There were 7 cysteine residues in the VP7 of the Nemuro strain, and the locations of 5 were conserved in all GBR.

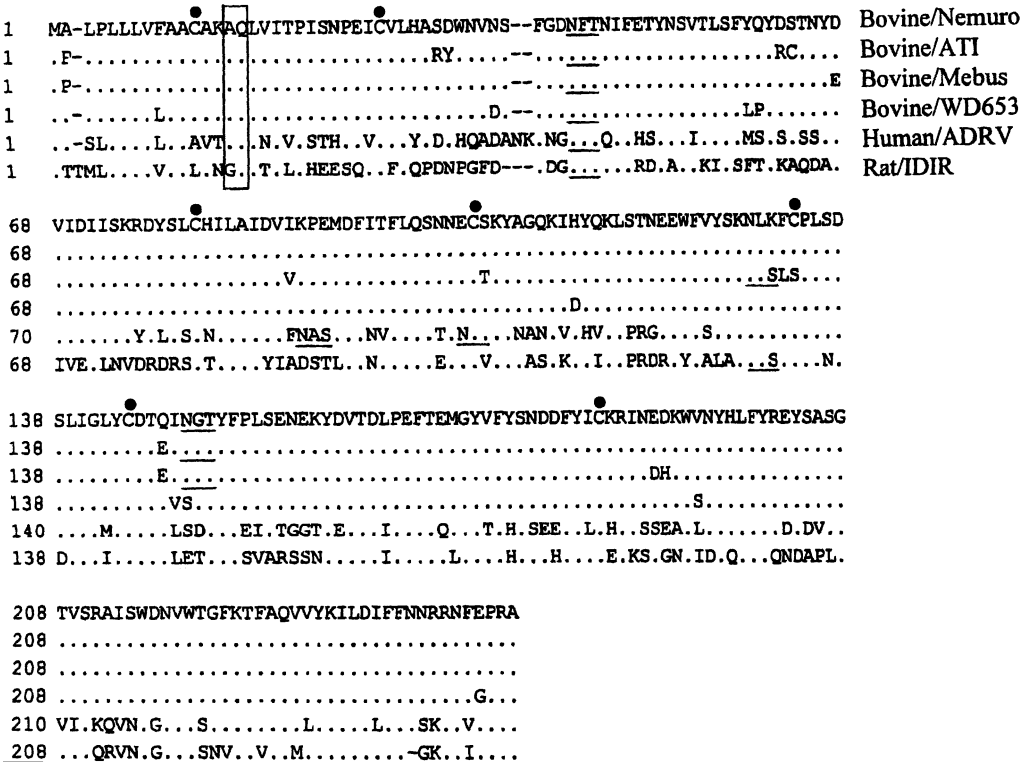

Fig. 5. Comparison of the deduced amino acid sequences of the VP7 from 6 GBR strains (Nemuro, ATI, Mebus, WD653, ADRV, and IDIR). Multiple sequence alignments were obtained using the MEGALIGN program (DNASTAR). Identical amino acids are indicated by a dot, and gaps are shown by dashes. Potential N-linked glycosylation sites are underlined, and the putative signal cleavage site is boxed. Cysteine residues recognized in the Nemuro strain are indicated by •. The VP7 sequences of the Mebus [3], ATI [3] WD653 [3], ADRV [6], and IDIR [18] strains were published previously

Bovine GBR have been reported in association of diarrhea in calves and adult cows [16, 20]. Recently, Chang et al. [3] reported that bovine GBR was detected more frequently from diarrheal feces of adult cows than from those of calves. In this study, no association with other pathogens was observed in the adult cow diarrhea, suggesting strongly that bovine GBR might cause diarrhea in adult cows similar to bovine coronavirus. Interestingly, human GBR infections have been also associated primarily with disease in adults [2, 11].

The pathogenicity of bovine GBR investigated only in gnotobiotic calves [3, 16, 19, 21, 23]. In the present study, colostrum-fed and colostrum-deprived calves were used for inoculation. All calves inoculated with the Nemuro strain of GBR developed transient, mild diarrhea. There were no distinct differences in the pathogenicity of the virus for newborn calves versus a 30-day-old calf. These results suggest that the bovine GBR Nemuro strain can cause diarrhea in a 30-day-old calf.

Saif et al. [21] reported that infection of gnotobiotic calves with bovine GBR led to shedding of fewer virus particles for a shorter period of time than GAR infection. Similar results were observed in the present study. Thus, the time of sampling and highly sensitive assays are critical for accurate diagnosis of bovine GBR infections. Recently, Chinsangaram et al. [7] have reported the use of RT-PCR for the detection of bovine GBR infections. In our study using RT-PCR with identical primers, the expected PCR products were observed from fecal samples of all 5 affected cows, and for 5 days in experimentally infected calves. Furthermore, GAR and GCR were negative in this RT-PCR, which suggests that this assay is suitable for diagnosis of bovine GBR infection.

Enterocyte syncytia are a characteristic of GBR infections [5, 16]. In the present study, syncytia containing immunofluorescent cytoplasmic granules were observed in infected MA104 cells stained with antiserum to GBR although they could not be maintained on serial passage. Similar results were reported for porcine GBR strains [24]. Recently, one porcine GBR strain has been adapted to growth in swine kidney cells by using a high concentration of pancreatin and rolling of cultures [22]. We used several concentrations of trypsin and rolling cultures, but the virus could not be adapted to growth in MA104 cells. Bovine or porcine cells and pancreatin instead of trypsin might be needed for the serial propagation of bovine GBR.

In GAR, the same VP7 serotype strains have a VP7 aa identity of 85% or higher, whereas the different VP7 serotypes show a high degree of sequence divergence in VP7 which is clustered in 9 variable regions [9, 17]. For GCR, we have demonstrated a similar relationship among VP7 types [12, 25]. There is no information about serotypes in GBR. But, if VP7 serotypes exist among GBR as for GAR and GCR, the Nemuro bovine strain and other bovine strains (Mebus, ATI, and WD653) might belong to the same VP7 serotype, based on the VP7 aa identity of 92–97%. Also, these bovine strains and the other mammalian strains (ADRV and IDIR) might be classified as different VP7 serotypes because the VP7 aa identity among them was only 48–61%. In the VP7 genes from different species origin and also the other genes of the human ADRV and rodent IDIR strains of GBR, more sequence substitutions were observed than for GAR and GCR [8, 18]. These observations suggest that the GBR strains of different species origin may have had a longer period of time to diverge from one another, compared with GAR and GCR [8]. Although VP7 aa identity was very low among GBR strains from different species, the fundamental structure of VP7 in GBR was similar as revealed by: 1) similar hydropathicity profiles; 2) the conserved putative signal cleavage site; 3) 5 of 6 to 8 cysteine residues in GBR were conserved; 4) 1 of 1 to 3 potential N-linked glycosylation sites was conserved. Comparative analysis of the VP6 genes from bovine and other mammalian GBR is in progress.

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