表２　検出されたロタウイルスのＧ及びＰ血清型

	P[4]	P[6]	P[8]	P[4]/P[6]	P[6]/P[8]	P[4]/P[6]/P[8]	UT	Total	(%)
G 1	2	1	18		6		1	28	32.6
G 2	6							6	27.3
G 3	1		33			2	1	37	43.0
G 4	1							1	1.2
G 1/2				1				1	1.2
G 1/3			7		3	1	2	13	15.1
Total	10	1	58	1	9	3	4	86	
%	11.6	1.2	67.4	1.2	10.5	3.5	4.7		

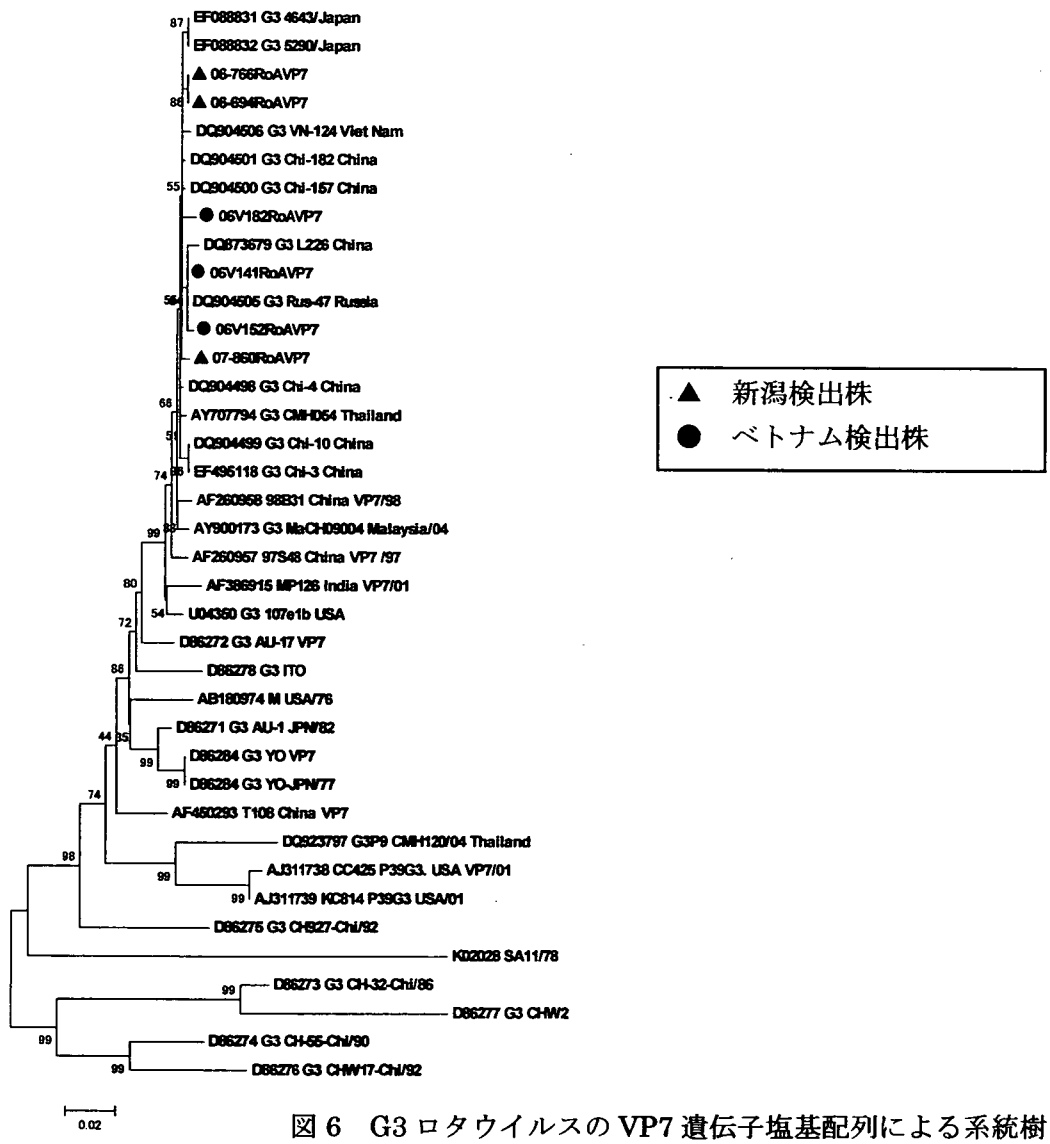

図６　G3 ロタウイルスの VP7 遺伝子塩基配列による系統樹
875bp　Nj 法による。

III

研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
西尾治	ノロウイルスの知識と感染予防	丸山務	ノロウイルス現場対策改訂版	幸書房	東京	2007	23-70
西尾治	Q&A	丸山務	ノロウイルス現場対策改訂版	幸書房	東京	2007	139-154
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IV

研究成果の刊行物・別刷

Human Sapovirus in Clams, Japan

Grant S. Hansman,* Tomoichiro Oka,* Reiko Okamoto,† Tomoko Nishida,† Shoichi Toda,† Mamoru Noda,‡ Daisuke Sano,§ You Ueki,¶ Takahiro Imai,§ Tatsuo Omura,§ Osamu Nishio,* Hirokazu Kimura,* and Naokazu Takeda*

Human sapovirus was detected in 4 of 57 clam packages by reverse transcription–PCR and sequence analysis. This represents the first finding of sapovirus contamination in food. Closely matching sequences have been detected in stool specimens from patients with gastroenteritis in Japan, which indicates a possible food-to-human transmission link.

Sapoviruses and noroviruses are etiologic agents of human gastroenteritis. Human noroviruses are the most important cause of outbreaks of gastroenteritis worldwide and can be transmitted by a variety of routes, including food (*1*). Sapovirus infections are mostly associated with sporadic gastroenteritis in young children; however, foodborne transmission routes are yet to be determined. The most widely used method of detection is reverse transcription–PCR (RT-PCR), which has a high sensitivity and can also be used for genetic analysis. Sapovirus strains can be divided into 5 genogroups; GI–GV infect humans; sapovirus GIII infects porcine species. Phylogenetic studies have also designated sapovirus clusters or genotypes to further describe strains.

The Study

The purpose of this study was to detect sapovirus in the clam *Corbicula japonica* (bivalve mollusk) and describe the genetic diversity of the strains. A total of 57 clam packages (30–60 clams per package) were collected from supermarkets or fish markets from 6 different areas in western Japan from December 8, 2005, to September 6, 2006. The samples were shucked, and the digestive diverticulum was removed by dissection and weighed. One gram of digestive diverticulum (10–15 clam/package) was homogenized with an Omini-mixer (Sorvall Inc., Newtown, CT, USA) in 10 mL phosphate-buffered saline. After centrifugation at 10,000× *g* for 30 min at 4°C, the supernatant was centrifuged at 100,000× *g* for 2 h (SW41 Rotor, Beckman Instruments, Inc., Fullerton, CA, USA). The pellet was resuspended in 140 μL distilled water and stored at –80°C until use.

RNA extraction and nested RT-PCR were performed as described (*2*). Briefly, for the first PCR, F13, F14, R13, and R14 primers were used; for the nested PCR, F22 and R2 primers were used. All RT-PCR products were analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. RT-PCR products were excised from the gel and purified by the QIAquick gel extraction kit (QIAGEN, Hilden, Germany). Nucleotide sequences were prepared with the terminator cycle sequence kit (version 3.1, Applied Biosystems, Warrington, England) and determined with the ABI 3130 sequencer (ABI, Boston, MA, USA). Nucleotide sequences were aligned with ClustalX, and the distances were calculated by Kimura's 2-parameter method, as described elsewhere (*2*). Nucleotide sequence data determined in this study have been deposited in GenBank under accession nos. EF104251–EF104254.

Four (7%) of 57 clam packages were contaminated with sapovirus (termed Shijimi1, Shijimi2, Shijimi3, and Shijimi4). Genetic analysis of the partial capsid gene showed that these 4 sequences shared >98% nucleotide similarity and >97% amino acid identity. Phylogenetic analysis grouped these 4 sequences in the same genotype, i.e., GI/1 (Figure). Similar sequences were found on the database (Figure). Strains from this cluster likely represent the dominant genotype worldwide (*3*). Three of 4 sapovirus-positive clam packages were collected from different areas and at different times (Figure). The clam packages that were contaminated with Shijimi1 and Shijimi3 were collected from the same area, but 6 weeks apart, which indicates an ongoing sapovirus contamination or resistance in the natural environment. The seasonality of sapovirus infection in Japan is unknown; however, as with norovirus, sapovirus infections may also peak during winter, although further epidemiologic and environmental studies are needed.

In a recent study, we detected sapovirus strains in 7 of 69 water samples, which included untreated wastewater, treated wastewater, and a river in Japan (*4*). Three of 7 sapovirus sequences detected in the water samples belonged to GI/1 and shared >97% nucleotide similarity with the sapovirus sequences detected in the clam packages. Additionally, sapovirus sequences belonging to GI/1 and sharing >99% nucleotide similarity, for example, Chiba/010598F strain (Figure), have been detected in stool specimens from children with sporadic gastroenteritis in Japan (*5*,*6*). The closely matching sapovirus sequences detected in the water, clams, and patients suggest that sapovirus contamination in the natural environment can

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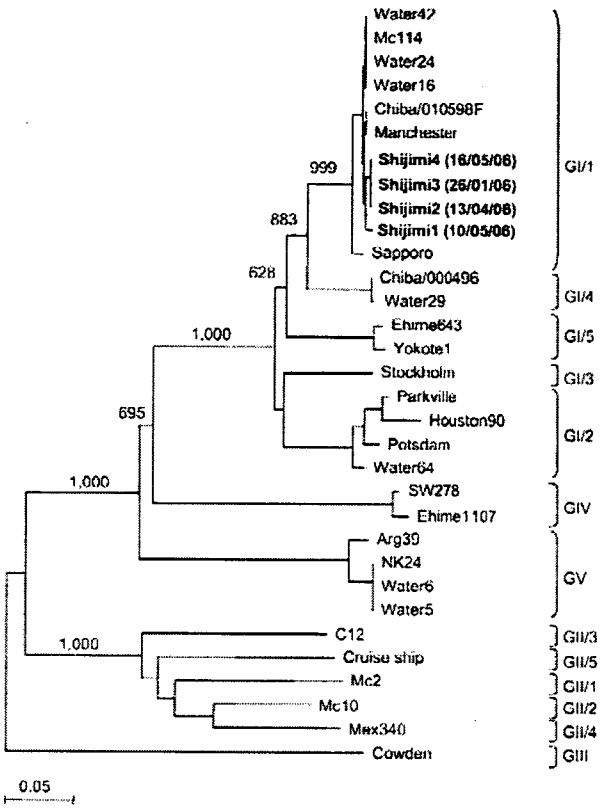

Figure. Phylogenetic analysis of sapovirus capsid sequences (≈300 nt) showing the different genogroups and clusters. Numbers on each branch indicate bootstrap values for the genotype. Bootstrap values of ≥950 were considered statistically significant for the grouping. The scale represents nucleotide substitutions per site. GenBank accession nos.: Mex340, AF435812; Parkville, U73124; Cowden, AF182760; Potsdam, AF294739; Sapporo, U65427; Stockholm, AF194182; SW278, DQ125333; water samples, DQ915088–DQ915094; and Yokote, AB253740. **Boldface** represents sequences detected in this study.

lead to foodborne infections in humans, although direct evidence is lacking. More important, a recent study found animal sapovirus in oysters and suggested that coinfection with human and animal sapovirus strains could result in genomic recombination and the emergence of new strains (*7*). At the same time, we recently described the first human sapovirus intergenogroup recombinant strain (*8*). Phylogenetic analysis of the nonstructural region (i.e., genome start to capsid start) grouped this sapovirus strain in GII, while the structural region (i.e., capsid start to genome end) grouped this strain in GIV.

A large number of studies have detected norovirus in oysters. In 2 recent studies, norovirus was detected in oysters (*Crassosterea gigas*) harvested from geographically isolated areas in Japan (*9,10*). We also screened the same oyster samples for sapovirus; however, all of the samples were negative for sapovirus. That sapovirus was detected in the clam samples, but not in the oyster samples, is of interest. In the past several years, increasing evidence has emerged that human noroviruses bind to histo-blood group antigens (HBGAs) (*11*). These carbohydrate epitopes are present in mucosal secretions and throughout many tissues of the human body, including the small intestine, and in oyster digestive tissues. A number of studies have found that different norovirus strains exhibit different binding patterns to HBGAs and oyster digestive tissues (*12,13*). In a recent study, we found that sapovirus GI and GV strains showed no such binding activity to HBGAs (*14*). These results suggest that human norovirus and sapovirus strains have different binding receptors or that human sapovirus may not concentrate in detectable levels in oysters.

Conclusions

Foodborne diseases are a major problem worldwide. We report what is, to the best of our knowledge, the first account of sapovirus contamination in food destined for human consumption. The report may represent a possible food-to-human transmission link, although direct evidence is lacking. In Japan, clams are usually boiled before they are consumed in soups. However, boiling to open the clam may not inactivate the virus (*15*); in addition, some areas in Japan do not boil clams before eating them. Further studies are needed to determine if boiling inactivates sapovirus and if the contaminated clams are indeed infectious. In conclusion, these novel results highlight the importance of sapovirus, in particular the GI/1 strains. A new awareness of sapovirus transmission routes is necessary and may help reduce sapovirus infections.

Dr Hansman is a scientist at the National Institute of Infectious Diseases, Japan. He studies viruses that cause gastroenteritis in humans, namely sapovirus and norovirus. His research interests include epidemiology, virus expression, and cross-reactivity.

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Journal of Virological Methods 147 (2008) 360–363
Journal of Virological Methods
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Short communication

Evaluation of immunochromatography and commercial enzyme-linked immunosorbent assay for rapid detection of norovirus antigen in stool samples

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Received 25 June 2007; received in revised form 11 September 2007; accepted 13 September 2007
Available online 23 October 2007

Abstract

The efficiency of immunochromatography and commercial enzyme-linked immunosorbent assay (ELISA) kit (Denka Seiken Co. Ltd., Tokyo, Japan) were evaluated for rapid detection of norovirus (NoV) from stool specimens. A total of 503 stool specimens collected from infants and young children who suffered from acute gastroenteritis were tested for NoV by the NoV-immunochromatography kit, Denka ELISA kit, and by a monoplex RT-PCR method. The NoV-immunochromatography revealed 78.9% sensitivity, 96.4% specificity, and 92.4% efficiency with the monoplex RT-PCR method. The Denka ELISA kit had a sensitivity of 90.4%, specificity of 96.4%, and an efficiency level of 95%. The findings indicate that the newly developed NoV-immunochromatography kit provides the specificity equal to that of the Denka ELISA kit, even through the sensitivity of detection was lower. However, the advantage of the NoV-immunochromatography kit is less time consuming and simpler. The data show that both the Denka ELISA and the NoV-immunochromatography kits may be used as an alternative method for screening of NoV in stool samples.

Keywords: Norovirus; Immunochromatography; Denka ELISA; RT-PCR; Gastroenteritis

Viral gastroenteritis is one of the most common illnesses in humans worldwide, and different agents such as rotavirus, astrovirus, adenovirus, and calicivirus have been associated with the disease (Clark and McKendrick, 2004). Norovirus (NoV) is one of the four members of the family *Caliciviridae*, which is a nonenveloped, positive-sense, single-stranded RNA virus. NoV is a leading cause of gastroenteritis worldwide and responsible for outbreaks in various epidemiological settings, including restaurants, schools, day-care centers, hospitals, nursing homes, and cruiser (McEvoy et al., 1996; McIntyre et al., 2002; Russo et al., 1997). Currently, based on the diversity of the capsid sequences, NoVs are grouped into five genogroups (G), of which GI, GII, and GIV have been found in humans (Kageyama et al., 2004; Zheng et al., 2006). Human NoV genogroups are subdivided further into at least 15 genotypes in GI, 18 genotypes in GII, and only one genotype in GIV (Kageyama et al., 2004; Okada et al., 2005; Vinje et al., 2004). Several epidemiological studies clearly indicated that NoV GII is the main causative agent among NoVs that cause acute diarrhea in humans (Hansman et al., 2004; Phan et al., 2006a,b; Tseng et al., 2007).

Noroviruses were first discovered by Kapikian et al. (1972) under electron microscopy (EM). More recently, application of reverse transcription-polymerase chain reaction (RT-PCR) and DNA sequencing techniques to detect and characterize NoV became the standard methods for detecting this pathogen. Although RT-PCR is used around the world as a standard tool for routine diagnosis of NoV infection, detection of viral agents with molecular techniques requires well-trained personnel and sophisticated equipments. Thus, a rapid and sensitive diagnostic test for NoV detection is required. Currently, a number

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doi:10.1016/j.jviromet.2007.09.007

of enzyme-linked immunosorbent assay (ELISA) kits for the detection of NoV in stool samples have been developed and commercialized (Burton-MacLeod et al., 2004; de Bruin et al., 2006; Richards et al., 2003). In addition to these laboratory diagnostic techniques, the immunochromatography diagnostic test is another alternative choice. The immunochromatography method is easy to perform and less time consuming to conduct the test. In this study, we evaluated the newly developed immunochromatography kit (Immuno-Probe, Co. Ltd., Saitama, Japan) in comparison with a new commercial ELISA kit (NV-AD; Denka Seiken Co. Ltd., Tokyo, Japan) to assess their sensitivities in detecting NoV antigen in stool samples. The monoplex RT-PCR was used as a "gold standard" method for this assessment.

A total of 503 stool samples were collected from infants and children with acute gastroenteritis, encompassing five different geographical settings in Japan (Maizuru, Tokyo, Sapporo, Saga, and Osaka) from July 2004 to March 2005. The presence of NoV GI and GII in fecal specimens was detected by RT-PCR using a protocol described previously (Yan et al., 2003). A forward primer G1-SKF (nt 5342–5261) 5′-CTGCCCGAATTYGTAAATGA-3′ was used in combination with the reverse primer G1-SKR (nt 5653–5671) 5′-CCAACCCARCCATTRTACA-3′, for the amplification of NoV GI. For NoV GII identification, a forward primer COG2F (nt 5003–5028) 5′-CARGARBCNATGTTYAGRTGGATGAG-3′ was used in combination with the reverse primer G2-SKR (nt 5367–5389) 5′-CCRCCNGCATRHCCRTTRTACAT-3′. All of the NoV positive samples were characterized further for their genotypes by direct DNA sequencing using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and analyzed the sequence on an automated sequencer (ABI 3100; Applied Biosystems). The sequences obtained were compared to those of NoV strains deposited in the GenBank using the BLAST program, and the genotypes were classified using the clustering determined previously by Kageyama et al. (2004).

To evaluate the sensitivity and specificity of this NoV-immunochromatography test, all of the 503 samples were tested for NoV antigen using the newly developed NoV-immunochromatography kit, which was kindly provided by the Immuno-Probe Company. The immunochromatography test was performed according to the manufacturer's directions. The NoV-immunochromatography strip used in this study was a nitrocellulose membrane coated with gold colloid conjugated with mouse monoclonal antibody (MAb 14-1) against GII/4 on right hand side of the adsorbent pad as shown in Fig. 1. From the previous study, the MAb 14-1 showed a broad range of cross-reactivity with several genotypes of the virus-like particles (VLPs of NoV) as tested by ELISA (Shiota et al., 2007). Therefore, the MAb 14-1 was selected and used as a capture antibody in this NoV-immunochromatography kit. The test line was coated with NoV polyclonal antibodies against NoV GII/3 and GII/4, while the control line was coated with antibody against mouse immunoglobulin. For a negative immunochromatography reaction, only a band of control was appeared on the immunochromatography strip, while a positive immunochromatography reaction both the control and test bands were appeared on the immunochromatography strip (Fig. 1).

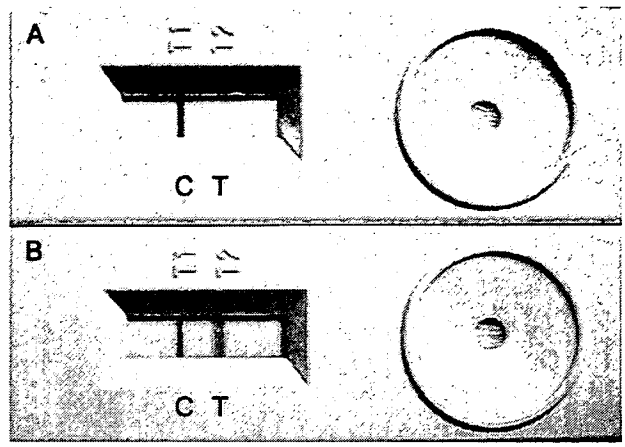

Fig. 1. Detection of NoV in a stool sample using the NoV-immunochromatography kit. The test is negative when only one band appears in the control area (A). The test is positive if two bands appear in the membrane (B). C represents the control band and T represents the test band.

An improved version of commercial ELISA kit, NV-AD (Denka Seiken Co. Ltd.), was evaluated using the same set of stool samples. The detection of NoV using the NV-AD Denka ELISA kit was slightly modified from the manufacturer's instruction. Briefly, 100 μl of a 20% stool suspension was mixed with 120 μl of sample extraction buffer. The reactions for the detection of NoV antigens were performed by mixing 100 μl of the NoV extract with 100 μl of peroxidase conjugated antibodies against NoV GI and GII in the wells which coated with antibodies against NoV GI and GII. After the wells were washed, 3,3′,5,5′-tetramethylbenzidine and hydrogen peroxide were added, and a colorimetric reaction was allowed to develop for 30 min at room temperature. The optical density (OD) of the colorimetric reaction was measured at 450 and 630 nm by an ELISA plate reader (Titertek Multiskan, USA). The sample with OD value greater than cut-off value was regarded as a positive reaction.

From a total of 503 fecal specimens collected from pediatric patients with diarrhea, 114 (22.7%) were found to be positive for NoV using the monoplex RT-PCR screening method (Table 1). Of these, majority of the samples (112; 98.2%) belonged to the NoV subgroup II (GII), while only 2 (1.8%) carried subgroup

Table 1
Comparison of NoV detection in stool samples between NoV-immunochromatography kit and commercial ELISA kit (Denka) with monoplex RT-PCR method

Test kit	Monoplex RT-PCR		
	Positive	Negative	Total (%)
Immunochromatography			
Positive	90	14	104 (20.7)
Negative	24	375	399 (79.3)
Denka ELISA			
Positive	103	14	117 (23.3)
Negative	11	375	386 (76.7)
Total (%)	114 (22.7)	389 (77.3)	503 (100)

Table 2
Comparison of the accuracy of the NoV genotype detection between NoV-immunochromatography and ELISA Denka kit with monoplex RT-PCR

Test kit	NoV genotypes determined by monoplex RT-PCR and sequence analysis				
	GI/1	GII/3	GII/4	GII/6	Total (%)
Immunochromatography					
Positive	1	13	75	1	90(78.9)
Negative	1	1	20	2	24(21.1)
Denka ELISA					
Positive	2	12	86	3	103(90.4)
Negative	–	2	9	–	11(9.6)
Total (%)	2(1.8)	14(12.3)	95(83.3)	3(2.6)	114(100)

I (GI) specificity. All of the NoV positive samples were characterized further for identification of their genotypes by sequence analysis of the capsid regions. It was found that both of the GI strains belonged to the GI/1 genotype (1.8%). Of the total 112 GII specimens detected, 95 (83.3%) were GII/4, 14 (12.3%) were GII/3, and 3 (2.6%) were GII/6 genotypes (Table 2).

To evaluate sensitivity, specificity, and efficiency of the newly developed NoV-immunochromatography kit, the results of 503 stool samples tested by NoV-immunochromatography were compared with those of the monoplex RT-PCR method. Of 114 samples that were positive by RT-PCR, 90 were positive by NoV-immunochromatography test, and 24 were negative. In addition, of 389 samples that negative by RT-PCR, 14 were positive by NoV-immunochromatography kit (Table 1). It was interesting to note that although this NoV-immunochromatography kit was developed for the detection of NoV GII/3 and GII/4, which were the major NoV genotypes circulating in humans, a cross-reactivity with some of GI/1 and GII/6 specimens were also observed (Table 2). The sensitivity and specificity of this NoV-immunochromatography test were 78.9 and 96.4%, respectively, and the overall efficiency compared to the monoplex RT-PCR method was 92.4%.

Based on the monoplex RT-PCR standard method, a total of 114 samples were positive for NoV detection. Of these, 103 samples were also positive by the Denka ELISA kit, while 11 samples showed discrepant results. In addition, from the 389 samples that negative by PCR, 14 were positive by the Denka ELISA kit (Table 1). Moreover, it was found that the Denka kit could detect four NoV genotypes (GI/1, GII/3, GII/4, and GII/6 as determined by sequence analysis) (Table 2). Overall, the sensitivity, specificity, and level of efficiency between the Denka ELISA kit and monoplex RT-PCR conventional method were 90.4, 96.4, and 95%, respectively. In comparison of the Denka ELISA assay and immunochromatography kit, the Denka ELISA showed a higher level of sensitivity than the immunochromatography kit (Denka kit: 90.4%, immunochromatography kit: 78.9%). However, specificity of the two kits was equal (96.4%).

Recently, large outbreaks of NoV occurred in various epidemiological settings in Japan. Although the main causative agent of these outbreaks was the GII/4 genotype, other genotypes were also detected and the predominant genotype was changed from one season to others (Morioka et al., 2006; Okada et al., 2005; Sasaki et al., 2006; Tokutake et al., 2006). When patients are diagnosed as severe diarrhea, rapid virus detection is essential for the intervention of appropriate treatment. For this reason, a new NoV-immunochromatography kit was developed to serve as a rapid method for identification of NoV directly from stool samples. In addition to the NoV-immunochromatography kit, the ELISA assay is another attractive supplementary method for screening of NoV in stool samples.

Previously, the sensitivity and specificity of immunochromatography for the detection of NoV in stool samples has been evaluated in our laboratory with a low sensitivity (72.7%) (Okame et al., 2003). The detection of NoV in stool specimens using the commercial RIDASCREEN ELISA kit (R-Biopharm AG, Darmstadt, Germany) was also reported with the sensitivity and specificity of 76.3 and 94.9%, respectively (Okitsu-Negishi et al., 2006). In the present study, the newly developed NoV-immunochromatography kit was evaluated and the results were compared with that of previous NoV-immunochromatography kit. It seems likely that the newly developed NoV-immunochromatography kit shows the sensitivity (78.9%) and specificity (96.4%) higher than that of the previous NoV-immunochromatography kit.

In this study, the new commercial ELISA kit (NV-AD) developed by Denka Seiken Co. Ltd. was also evaluated for sensitivity and specificity by comparing with the monoplex RT-PCR method using an identical set of stool samples that tested by the NoV-immunochromatography kit. It was clearly observed that the new Denka ELISA kit (NV-AD) showed the higher sensitivity (90.4%) than those of the NoV-immunochromatography test (78.9%) and RIDASCREEN ELISA kit (76.3%). When comparing of NoV-immunochromatography and RIDASCREEN ELISA kit, there was no significant difference in sensitivity and specificity. However, it should be pointed out that the advantage of NoV-immunochromatography kit is that it takes only 20 min which is much less time consuming to perform the test compare to 4 h by Denka ELISA and RIDASCREEN ELISA kits.

The detection limit of the NoV-immunochromatography assay, using the standard NoV strains, was found to be approximately 10^8 and 10^7 copies per gram of stool for NoV GII/3 and GII/4, respectively, without cross reaction with other diarrheal viruses (Immuno-Probe Co. Ltd., unpublished data). By using monoplex RT-PCR as a standard method, there were 24 samples that showed false negative results by the NoV-immunochromatography kit (Table 1). The discrepancy might be due to a low viral load in stool specimens or genetic variation that leads to antigenic change of NoV protein and fails to be recognized by MAb as reported by other commercial kits (Burton-MacLeod et al., 2004; de Bruin et al., 2006; Richards et al., 2003). Although the new NoV-immunochromatography kit was developed for the detection of GII/3 and GII/4 genotypes, which are the major genotypes detected in humans, it seems likely that this NoV-immunochromatography kit shows cross-reactivity with some of GI/1 and GII/6 from clinical samples (Table 2). Additionally, several NoV-VLP genotypes were used for testing this NoV-immunochromatography kit to determine a cross-reactivity with other NoV genotypes. It was found

that this NoV-immunochromatography kit could detect other genotypes of GII VLPs, including GII/1, GII/12, GII/13, and GII/14 genotypes. However, cross-reactivity with GI VLPs was not observed. In order to clarify this point, additional testing with several other NoV genotypes from clinical samples is essential.

In conclusion, the present study demonstrated that the NoV-immunochromatography kit or Denka ELISA kit could be used as an alternative method for detecting of NoV in stool specimens and may be practical for screening of NoV during outbreaks of food-borne and person-to-person transmitted gastroenteritis.

Acknowledgements

This research was supported by the Grants-in-Aid from the Ministry of Education, Culture, Sport, Sciences and Technology and the Ministry of Health, Labor and Welfare, Japan. We thank the Immuno-Probe Company (Saitama, Japan) for kindly provided the NoV-immunochromatography kit for this study.

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Infection, Genetics and Evolution 7 (2007) 542–546
Infection, Genetics and Evolution
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Short communication

Emergence of intragenotype recombinant sapovirus in Japan

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Received 9 November 2006; received in revised form 5 February 2007; accepted 13 February 2007
Available online 17 February 2007

Abstract

Sapovirus is an important causative agent of sporadic cases as well as of outbreaks of acute gastroenteritis in humans worldwide. A total of 603 fecal specimens collected from July 2005 to June 2006 from children with acute gastroenteritis in five localities in Japan (Maizuru, Tokyo, Sapporo, Saga, and Osaka) were screened for sapovirus by RT-PCR. It was found that 17 specimens were positive for sapovirus and it represented 2.8%. Interestingly, intragenotype recombinant sapovirus GI/1 emerged with 76.4% (13 of 17) and rapidly became the leading cause of acute gastroenteritis in Japan for the first time. The lower frequency of sapovirus GI/2 and GI/4 (each of 11.8%), which were the second prevailing genotypes, was also detected. A novel nomenclature of sapovirus was proposed, in which worldwide sapovirus strains were classified into seven genogroups. Of these, novel sapovirus genogroups VI and VII demonstrated the very low homologies, only 32.8–41.6% at the amino acid level and 43.6–49.9% at the nucleotide level, to those of sapovirus genogroups I–V. Of note, two distinct clusters of sapovirus were co-circulating in porcine. Interestingly, the worldwide sapovirus strains shared the 25 nucleotide-conserved region, covering the polymerase–capsid junction which differed according to each species due to multiple nucleotide substitutions. The finding suggests that the sapovirus recombination between human and animal hardly takes place in nature. This is also the first, to our best knowledge, demonstrating the emergence of the intragenotype recombinant sapovirus with its causing diarrheal illness in Japan.

Keywords: Sapovirus; Emergence; Genogroup; Recombination; Japan

1. The study

Viral gastroenteritis is a common disease with a high morbidity reported worldwide, especially in infants and the elderly. The mortality in children due to gastroenteritis is greater in developing than in developed countries. Acute gastroenteritis ranks consistently as one of the principal six causes of all deaths (Murray and Lopez, 1997; Parashar et al., 2003; Thapar and Sanderson, 2004). Sapovirus is recognized as a significant global enteropathogen, being a common cause of sporadic cases as well as of outbreaks of acute nonbacterial gastroenteritis in humans of all age in various epidemiological settings such as kindergartens, schools, and nursing home for the elderly (Chiba et al., 1979, 2000; Lopman et al., 2002; Akihara et al., 2005; Yan et al., 2005). Sapovirus is the distinct genus within the family Caliciviridae. The sapovirus genome contains two ORFs. The ORF1 encodes non-structural and capsid proteins while ORF2 encodes a small protein. Sapovirus has a typical "Star of David" configuration by electron microscopy. The prototype sapovirus is the Sapporo virus (Hu/SaV/Sapporo virus/1977/JP), which was originally discovered from an outbreak in a home for infants in Sapporo, Japan, in 1977 (Chiba et al., 1979).

A total of 603 fecal specimens were collected from sporadic cases of acute gastroenteritis in pediatric clinics, encompassing five localities (Maizuru, Tokyo, Sapporo, Saga, and Osaka) in Japan from July 2005 to June 2006. The ages of the subjects ranged from 2 months to 15 years, with a median of 26 months. The fecal specimens were diluted with distilled water to 10% suspensions, and clarified by centrifugation at 10,000 × *g* for 10 min. The supernatants were collected and the viral genomes were extracted by using a QIAamp Viral RNA kit (QIAGEN®,

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doi:10.1016/j.meegid.2007.02.004

Hilden, Germany). Using PCR with specific primers SLV5317 and SLV5749 as previously reported resulted in the identification of sapovirus (Phan et al., 2005). The polymerase region was also amplified to detect recombinant sapovirus using primers SR80 and JV33. Products were sequenced directly on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence analysis was performed using CLUSTAL X (Version 1.6). A phylogenetic tree with 100 bootstrap resamples of the nucleotide alignment datasets was generated using the neighbor-joining method with CLUSTAL X. The genetic distance was calculated using Kimura's two-parameter method (PHYLIP). SimPlot software (Version 1.3) was used to compare recombinant sapovirus sequences. Reference sapovirus strains and accession numbers used in this study were as follows: Cowden (AF182760), Manchester (X86560), Hou7-1181 (AF435814), Mex11859/99 (AY157857), Sapporo/82 (U65427), Plymouth (X86559), Dresden (AY694184), Houston/90 (U95644), 6728/05/Maizuru/JP (DQ395300), Bristol/98 (AJ249939), London/92 (U956445), Parkville (U73124), MEC151A (AY144337), C12 (AY603425), SK15 (AY646855), SW278 (DQ125333), 5863/Osaka/JP (DQ401095), NK24 (AY646856), JJ681 (AY974192), LL14 (AY425671), MM280 (AY823308), QW270 (AY826426), Lyon/598 (AJ271056) and JJ259 (AY826423).

Here sapovirus was detected in 17 out of 603 specimens tested, accounting for 2.8%. Fig. 1 reveals that sapovirus was divided into three distinct genotypes 1, 2, and 4 within genogroup I (GI). Of these, GI/1 was the most predominant genotype with 76.4% (13 of 17), followed by GI/2 and GI/4 with 11.8% of each (2 of 17). Thus, there was the changing epidemiology of sapovirus genotypes in Japan with the emergence of sapovirus GI/1 together with the sudden disappearance of predominant sapovirus GI/6 in the previous year (Phan et al., 2007). All sapovirus GI/1 isolates had great homologies (99–100%) each other. Obviously, they came from the same source of infection and very likely represented the same strain, the JP-6732. By BLAST, both capsid and polymerase sequences of the JP-6732 were highly identical (99–100%) to those of the novel intragenotype recombinant sapovirus 6728/Maizuru/JP (the GI/1b polymerase and the GI/1a capsid) (Phan et al., 2006a). Taken together, the results indicated that the JP-6732 was also recognized as a recombinant strain. In contrast, all sapoviruses belonging to the GI/2 and the GI/4, the genotypes remained the same no matter the polymerase or capsid regions were analyzed.

The novel intragenotype recombinant sapovirus was first noted in a 10-month old male child with acute gastroenteritis in Maizuru City in 2005 (Phan et al., 2006a) and no additional case was reported so far. Interestingly, this virus emerged and rapidly became the leading cause of acute gastroenteritis in Japan for the first time in this study. Sapovirus capsid contains the determinants which are important for the immune recognition (Chen et al., 2004, 2006). The emergence of recombinant virus with GI/1 capsid could be explained by the insufficient antibody protection from acquired viral immunity against sapovirus GI/1 due to the lack of a trigger of the previous sapovirus GI/1 infection in the previous year.

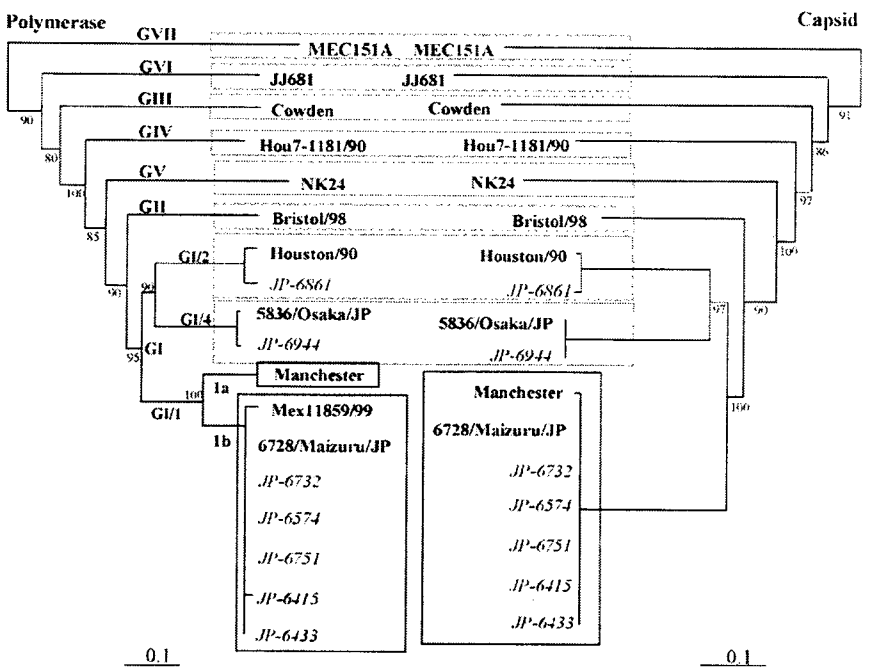

Fig. 1. Observation of changes of sapovirus subgenotypes (GI/1a and GI/1b) on the basis of phylogentic trees. The trees were constructed from nucleotide sequences of the capsid and polymerase regions of sapovirus isolates and reference sapovirus strains available in GenBank. The sapovirus isolates detected in the study are highlighted in italics. The scale indicates nucleotide substitutions per position. The numbers in the branches indicate the bootstrap values. The novel genogroup VI (known as the JJ681 virus cluster) and novel genogroup VII (known as the MEC151A virus cluster) were also shown.

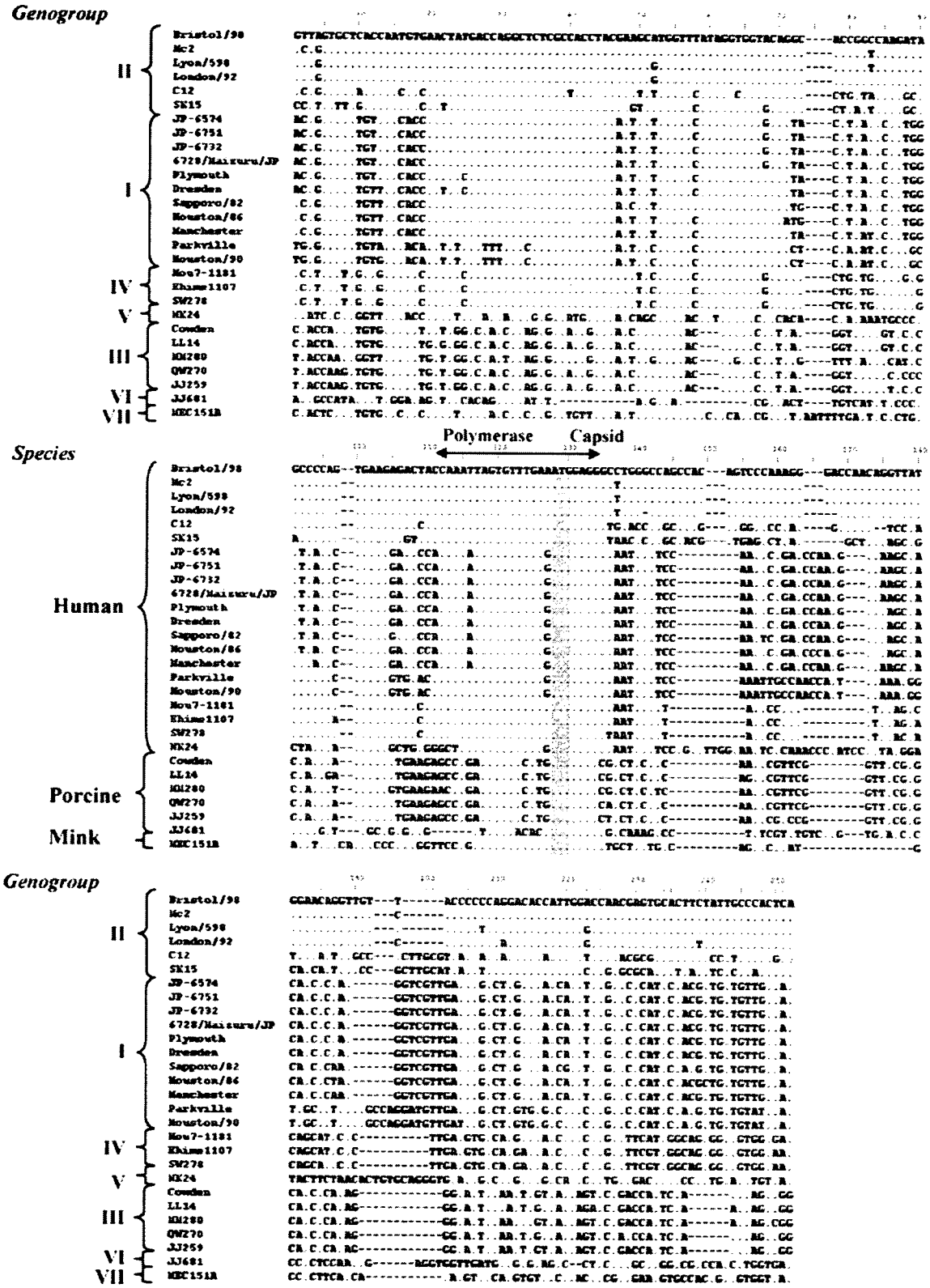

Fig. 2. Nucleotide alignment of worldwide sapovirus strains available in GenBank, showing the highly conserved region, covering polymerase and capsid junction which is indicated by a horizontal narrow. The dots represent conserved nucleotides. The shaded nucleotides represent the putative capsid start codons.

Table 1
The homology matrix of capsid sequences of representative sapovirus strains of seven sapovirus genogroups

Species	Genogroup	Strain	Homology (%) for strain						
			Manchester	Bristol/86	Cowden	Hou7-1181	NK24	JJ681	MEC151A
Human	I	Manchester		53.6	47.6	54.5	54.0	43.9	49.0
Human	II	Bristol/86	45.6		49.1	51.0	52.6	43.7	49.3
Porcine	III	Cowden	39.2	37.9		46.6	47.4	45.2	47.9
Human	IV	Hou7-1181	50.3	43.0	38.8		54.2	43.9	49.9
Human	V	NK24	49.7	47.5	41.8	47.2		43.6	49.2
Porcine	VI	JJ681	32.8	32.8	33.4	33.3	34.8		46.1
Mink	VII	MEC151A	41.6	39.4	38.9	40.0	41.5	34.1	

The nucleotide homology is shown in the upper right area and amino acid homology is shown in the lower left area.

Sapovirus capsid is well suited for the genotype classification (Farkas et al., 2004). The recombinant sapovirus belonged to two different subgenotypes GI/1a and GI/1b when the capsid- and polymerase-based groupings were performed. Obviously, the viral recombination event had a significant influence on the phylogenetic classification of sapovirus. This observation was in line with the previous report that the future sapovirus classification should rely on not only capsid sequence but also polymerase sequence (Phan et al., 2006b).

Sapovirus is divided into five genogroups with multiple genotypes (Farkas et al., 2004; Akihara et al., 2005). The homologies between sapoviruses within the same genogroup were 37.9–50.3% at the amino acid level and 46.4–54.5% at the nucleotide level (Table 1). Interestingly, porcine sapovirus JJ681 and mink sapovirus MEC151A in GenBank had the very low homologies, only 32.8–41.6% at the amino acid level and 43.6–49.9% at the nucleotide level, to those of sapovirus genogroups I–V. They did not belong to any cluster and therefore made novel genogroups VI and VII, respectively. Based on this novel nomenclature, two distinct genogroups of sapovirus, III (the Cowden virus cluster) and VI (the JJ681 virus cluster) were co-circulating in porcine. Interestingly, porcine Cowden strain was genetically closer to human sapoviruses (37.9–49.1%) than porcine JJ681 strain (33.4–45.2%).

To date, three types of recombinant sapoviruses have been reported as intergenogroup, intergenotype, and intragenotype recombinations (Katayama et al., 2004; Hansman et al., 2005; Phan et al., 2006a). Of these, intergenogroup recombination highlighted a possible route of zoonoses in humans (Hansman et al., 2005). Breakpoint analysis showed that the recombination site is at the polymerase–capsid junction (Katayama et al., 2004). Using CLUSTAL X the worldwide sapovirus strains shared the 25 nucleotide-conserved region, including the last 16 nucleotides of polymerase and the first 9 nucleotides of capsid (Fig. 2). This highly conserved region might represent the break and rejoin site for recombination during viral replication. Interestingly, this conserved region differed according to each species due to multiple nucleotide substitutions. This finding suggests that the recombination event occurs more capably within sapoviruses in the same species and the sapovirus recombination between human and animal hardly takes place in nature.

Acknowledgements

This study was supported by Grants-in-Aid from the Ministry of Education and Sciences and the Ministry of Health, Labor and Welfare, Japan.

We are grateful to Drs. Kunio Kaneshi, Yuichi Ueda, Shigekazu Nakaya, Shuichi Nishimura, Kumiko Sugita, Tuneyoshi Baba and Atsuko Yamamoto for fecal specimen collection.

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Journal of Medical Virology 79:1180–1186 (2007)

Anti-Norovirus Polyclonal Antibody and Its Potential for Development of an Antigen-ELISA

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Norovirus (NoV) capsid proteins were expressed as virus-like particles (VLPs) by using recombinant baculovirus in insect cells, which had 5 genotypes in genogroup I and 11 genotypes in genogroup II, and the VLPs were used as immunogens. Polyclonal antibody against the VLP of GII/3 genotype showed broad-range cross-reactivity, reacting not only with intra-genogroup strains, but also inter-genogroup strains, by antibody-ELISA using 16 kinds of VLPs. Furthermore, antigen-ELISA was conducted in sandwich enzyme-linked immunosorbent assay (ELISA) using the polyclonal antibody for capturing antigens, and three kinds of monoclonal antibodies against the VLP of GII/4 genotype for detecting antigens. This format successfully detected eight genotypes of NoV from clinical specimens and proved that polyclonal antibody, which has broad-range cross-reactivity, was capable of detecting various types of genotypes from clinical specimens. ***J. Med. Virol. 79: 1180–1186, 2007.*** © 2007 Wiley-Liss, Inc.

KEY WORDS: ELISA; genotype; immunochromatography; norovirus; polyclonal antibody

INTRODUCTION

Norovirus (NoV) is one of the major causative agents of nonbacterial gastroenteritis. NoV is in the family *Caliciviridae* and contains a single-stranded positive-sense RNA genome. The NoV genome is composed of three open reading frames (ORFs). The ORF2 encodes a capsid protein of 58 kDa. NoV can be divided genetically into five genogroups (GI, GII, GIII, GIV, and GV) based on genome sequence, but GI and GII are the common genogroups to infect humans. It is found that *C*-terminal P1 domain of NoV GI has the salt bridge which is not found in NoV GII [Parker et al., 2005]. Recently, NoV strains belonging to GI and GII were subdivided into at least 15 and 18 genotypes, respectively [Okada et al., 2005]. Of these, NoV GII/4 has been reported as the most prevalent genotype in causing acute gastroenteritis in both developed countries as well as developing countries. The illness is usually mild and self-limiting, but the virus is highly infectious and often transmitted from person to person in hospitals, hotels, schools, cruise ship, and restaurants [Vinje and Koopmans, 1996; Holtby et al., 2001; Billgren et al., 2002; Love et al., 2002; Marks et al., 2003]. Global outbreaks of gastroenteritis have been caused by plural strains of genogroups I and II [Noel et al., 1997; Otsu et al., 2003].

The effective control of NoV infection starts from the rapid identification of pathogens. Reverse transcription-PCR (RT-PCR) assays are now used as the common methods of NoV detection worldwide, however, these methods are time consuming, expensive, and require skillful techniques. The inability to propagate NoV in cell culture has inhibited the study of these viruses and the development of rapid immunological detection methods, such as enzyme-linked immunosorbent assay (ELISA) and immunochromatography (IC). In 1992, NoV capsid protein was expressed in the baculovirus expression system, and antigenically and immunologically indistinguishable virus-like particles (VLPs) were generated [Jiang et al., 1992; Green et al., 1993]. The

Grant sponsor: Ministry of Education, Culture, Sports, Sciences and Technology; Grant sponsor: Ministry of Health, Labor and Welfare, Japan.

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Accepted 24 April 2007
DOI 10.1002/jmv.20906
Published online in Wiley InterScience (www.interscience.wiley.com)

VLPs provided important reagents for use in developing immunologic techniques. To develop diagnostic ELISA, several researchers have generated monoclonal antibodies (MAbs) and polyclonal antibodies (PolyAb) against VLPs. Those MAbs were broadly reactive, recognizing GI or GII, or both GI and GII [White et al., 1997; Yoda et al., 2003; Parker et al., 2005]. On the other hand, the rabbit anti-VLP PolyAbs were highly specific for genotypes used as immunogens, especially when used in the antigen-ELISA [Hale et al., 1999; Kobayashi et al., 2000; Kamata et al., 2005]. A recent report revealed that some polyclonal antisera showed broad-range cross-reactivity: GI/11 antiserum cross-reacted strongly, not only with GI genotypes, but also GII genotypes. Likewise GII/1, GII/10, and GII/12 antisera showed broad-range cross-reactivity against GII genotypes [Hansman et al., 2006].

The final aim of this study was to construct an IC kit for the detection of broad-range genotypes of NoV. In the previous report, an IC technique specific for the NoVGII/4 genotype [Okame et al., 2003], which is most prevalent in sporadic cases and outbreaks of acute gastroenteritis [Marks et al., 2003; Kirkwood et al., 2005; Phan et al., 2005; Okame et al., 2006], was produced successfully. However, it was impossible to detect all of NoV genotypes [Okame et al., 2003]. In this study, 16 genotypes of VLPs were expressed and used to immunize mice then MAbs and PolyAb showing a broad reactivity were obtained. The performance of broad reactive PolyAb against GII/3 VLP, and MAbs against GII/4 VLP in antibody-ELISA was checked. Furthermore, antigen-ELISA was conducted in sandwich ELISA using the PolyAb for capturing antigen on the surface of the microwell, and mixing MAbs for detection of the captured antigen. The results showed that antigen-ELISA using PolyAb reacted with 1 genotype of GI VLP and 11 genotypes of GII VLP, and also detected distinct NoV strains in clinical specimens.

MATERIALS AND METHODS

Viruses and Stool Samples

All strains (GI/1:4656, GI/3:3634, GI/4:2876, GI/8:3006, GI/11:2258, GII/1:3101, GII/2:2840, GII/3:3229, GII/4:1207, GII/5:3611, GII/6:3612, GII/7:419, GII/12:2087, GII/13:3385, GII/14:2468, and GII/15:3625) used for VLPs, NoV positive samples, and other enteric virus samples used in testing, were isolated from infants and children with acute gastroenteritis from 1995 to 2003 in Japan [Phan et al., 2005; Okame et al., 2006]. All the stool samples were stored at −30°C until tested. The genotype classification of these NoVs was performed based on the method described by Kageyama et al. [2004].

Expression of VLPs

In the previous report, one VLP (strain 1207: GII/4) was expressed successfully [Okame et al., 2003]. Other 15 viral cDNA were cloned according to Gateway Technology and Baculovirus Expression protocol (Invitrogen Corp., Carlsbad, CA). For RT reaction, TX30SXN (GAC TAG TTC TAG ATC GCG AGC GGC CGC $CC(T)_{30}$) primer was used to synthesize cDNA, and PCR was conducted using specific primer pairs and KOD plus polymerase with a high fidelity (Toyobo, Osaka, Japan). For NoV GI strains, a sense primer attB1NVGI (GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CGA AGG AGA TAG AAC CAT GAT GAT GGC GTC TAA GG) was used, and for NoV GII strains, a sense primer attB1NVGII (GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CGA AGG AGA TAG AAC CAT GAA GAT GGC GTC GAA TGA) was used. For an antisense primer, attB2TX30SXN (GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC CTA GAC TAG TTC TAG ATC GCG AGC GGC CGC CC $CC(T)_{30}$) was used. PCR fragments were cut and purified from a 1.0% agarose gel. These were cloned into a donor vector, pDONR221 (Invitrogen Corp.), and recombinant pDONR221 was purified before transferring into a baculovirus transfer vector pDEST8 (Invitrogen Corp.). The recombinant pDEST8 was purified and used to transform DH10Bac competent cells (Invitrogen Corp.), producing recombinant bacmids containing the capsid gene.

Expression in Insect Cells

Recombinant bacmids were transfected into Sf9 cells using Effectene (Qiagen, Hilden, Germany). Sf9 cells were incubated for 5 days at 26°C, after the culture medium was clarified by centrifugation for 10 min at 3,000*g*, and the supernatant was stored as seed baculovirus. Tn5 cells were infected with the seed baculovirus at 26°C and harvested at 6-day postinfection. VLPs that secreted into the cell medium were separated from the cells by centrifugation for 10 min at 3,000*g*, and they were separated from seed baculovirus by further centrifugation for 30 min at 10,000*g*. The supernatant was concentrated by ultracentrifugation at 210,000*g* at 4°C for 2 hr (Beckman 45 Ti rotor), and resuspended in 100 μl of phosphate-buffered saline (PBS). VLPs were purified by CsCl equilibrium gradient ultracentrifugation at 125,000*g* at 10°C for 20 hr (Beckman SW-41Ti rotor). VLP formation was examined by electron microscopy.

Antibody Production

The P363-Ag-U1 myeloma cell line was used as the parent cell. CsCl-purifed 1207 VLP was used as an immunogen for preparing MAbs that were prepared as described previously [Kohler and Milstein, 1975] in which the MAbs were purified by using an Affigel Protein A MAPII kit (Bio-Rad, Richmond, CA). Rabbits were immunized subcutaneously with 100 μg of CsCl-purified 3229 VLP and Freund's complete adjuvant four times at 2-week intervals. One rabbit was used for one polyclonal antibody with a high titer specific for each NoV genotype. The serum was collected 1 week after the last injection. Rabbit immunoglobulin G (IgG) was