cases is continuously needed. It is also essential that food samplers not work when they are ill and that good hand-washing facilities be provided in all restaurants.

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Genotyping of *Norovirus* Strains Detected in Outbreaks between April 2002 and March 2003 in Osaka City, Japan

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Abstract: **Noroviruses (NVs) are the major cause of food- and waterborne nonbacterial gastroenteritis in Japan. Between April 2002 and March 2003, a total of 111 fecal specimens from 40 outbreaks of acute nonbacterial gastroenteritis in Osaka City, Japan were subject to NV detection. Seventy-two samples (64.9%) from 31 outbreaks (77.5%) were NV positive by a real time reverse transcription (RT)-PCR assay. To further determine the genotype of individual NV strains, we sequenced the capsid N-terminal/shell (N/S) domain of some representative strains from each outbreak. The 51 NV strains detected in this study were segregated into 15 genotypes (6 in genogroup I and 9 in genogroup II), and GII/5 genotype NV was a dominant outbreak genotype.**

Key words: ***Norovirus*, N/S domain, Genotype, Epidemiology**

Norovirus (NV) is a genus within the family *Caliciviridae* (www.ictvdb.iacr.ac.uk/Ictv), which has been previously termed Norwalk-like virus or small round structured virus. The NV prototype strain, Norwalk/68/US, has been entirely sequenced from cDNA clones derived from stool specimens, and its genome is a single-stranded, positive-sense RNA molecule of 7.5 kb that comprises three open reading frames (ORFs) (17, 19). NVs are the major cause of acute nonbacterial gastroenteritis worldwide, and illness occurs in people of all ages. NVs are transmitted not only by a fecal-oral route but also by direct person-to-person contact (11). There have been numerous outbreaks due to NV-contaminated foods, such as shellfish, salads, and deli sandwiches (6, 8, 12, 13), and due to NV-contaminated water (5, 26).

Since NVs have not yet been cultivated *in vitro*, electron microscopy (EM) or immuno-EM had been routinely used to detect NV particles in stool specimens in the laboratory. After the cloning and sequencing of Norwalk/68/US (17) and Southampton/91/UK (27), a reverse transcription-PCR (RT-PCR) assay was developed to target the RNA-dependent RNA polymerase gene in ORF1 of the NV genome (18, 28). Using sequence information of additional NV strains, different primer sets targeting the polymerase region have been used for the diagnosis of NV in fecal specimens from both outbreaks and sporadic cases (2, 9, 34, 35). Based on the sequence information obtained from the polymerase region, the NV strains can be divided into two genogroups, genogroup I (GI) and genogroup II (GII), each comprising a large number of genetically diverse strains (1, 10, 30).

A classification system has been proposed for NVs, in which the sequence of ORF2 (the gene for the major capsid protein) of the strains is compared with that of reference strains (3, 11, 22, 33). A recent study indicated that NV GI and GII strains consist of at least 14 and

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Abbreviations: EM, electron microscopy; G, genogroup; N/S, N-terminal/shell; NV, *Norovirus*; ORF, open reading frame; RT-PCR, reverse-transcription polymerase chain reaction.

17 genotypes, respectively (21). ORF2 encodes the major structural capsid protein, including a shell domain (S) and a protruding (P) domain (31). Several reports suggested a good correlation between the clustering NV strains by the sequence of the 5′ end of ORF2 and antigenic grouping confirmed by patient immune response against NVs (4, 23, 24, 30). Therefore, it may be possible to predict antigenic types by phylogenetic analysis of the capsid region.

Previously, we described classification of NV strains detected in Osaka City, Japan by probe types, based on hybridization of the amplified viral genomes with six NV-specific DNA probes (13–15). However, we often came across NV strains to which none the probes would hybridize, making their classification difficult. In this report, we describe the characterization of the NVs in fecal specimens from 31 nonbacterial gastroenteritis outbreaks occurring in Osaka City, Japan, between April 2002 and March 2003.

Materials and Methods

Outbreaks and specimens. Fecal specimens were collected from 40 outbreaks of acute nonbacterial gastroenteritis, including 22 outbreaks associated with oysters, in Osaka City, Japan, between April 2002 and March 2003. A total of 111 fecal specimens were examined by real time RT-PCR.

RNA extraction. A 10% stool suspension was prepared as described previously (13). Viral RNA was extracted from 140 μl of the suspension with a QIAamp viral RNA Mini kit (Qiagen, Valencia, Calif., U.S.A.) according to the manufacturer's instructions. RNA was eluted with 60 μl of diethyl pyrocarbonate-treated water and kept at −80 C until use in RT-PCR.

Real time RT-PCR. Real time RT-PCR was carried out as described by Kageyama et al. (20). Viral RNA (15 μl) was added to 15 μl of the mixture containing 100 mM Tris-HCl (pH 8.3), 150 mM KCl, 6 mM $MgCl_2$, 1 mM of each deoxynucleoside triphosphate, 10 mM dithiothreitol, 75 pmol of random hexamer (pdN6; Amersham Pharmacia Biotech, Piscataway, N.J., U.S.A.), 30 U of RNase inhibitor (TaKaRa Shuzo, Kyoto, Japan), and 7.5 U of avian myeloblastosis virus reverse transcriptase XL (Life Science Inc., St. Petersburg, Fla., U.S.A.). RT was performed at 42 C for 60 min, and the enzyme was inactivated at 70 C for 15 min. cDNA was stored at −20 C.

The real time quantitative PCR was carried out in 50 μl reactions containing 4 μl of cDNA, 25 μl of *Taq*Man Universal PCR Master Mix (Applied Biosystems, Branchburg, N.J., U.S.A.), 5 pmol each of primers COG1F and COG1R and 15 pmol of RING1 (a)-*Taq*Man probe (TP) and 5 pmol RING1 (b)-TP fluorogenic probe for GI NV detection, or 5 pmol each of primers COG2F and COG2R and 5 pmol RING2-TP for GII NV detection. PCR amplification was performed with an ABI7700 sequence detector (Applied Biosystems) under the following conditions: 2 min at 50 C and 10 min at 95 C, then 40 cycles of 95 C for 15 sec and 56 C for 1 min. Data were corrected by using internal standards as described by Kageyama et al. (20).

Sequencing of the N/S domain. When more than 2 samples from an outbreak appeared to be positive by real time RT-PCR, several samples, including these 2, were selected for sequencing analysis. To amplify the N-terminal/shell (N/S) domain of the capsid region, PCR was carried out with primers G1SKF and G1SKR for GI NV strains or G2SKF and G2SKR for GII NV strains as described by Kojima et al. (25). After purification of the amplicon with a QIAquick PCR purification kit (Qiagen), the nucleotide sequences were determined with the BigDye Terminator Cycle sequence kit and ABI 310 sequencer (Applied Biosystems).

Phylogenetic analysis. Capsid sequences of the reference strains of NV were obtained from GenBank. These strains and accession numbers are shown in Table 1. Phylogenetic analysis was performed as described by Katayama et al. (22). Briefly, the sequences of N/S domain (GI, 290 nt; GII, 278 nt) were aligned by using Clustal X (version 1.81) with parameters provided in Clustal W1.6. A phylogenetic tree was constructed by the neighbor-joining method, and the genetic distances were calculated according to the Kimura two-parameter method. Reliability of the tree was estimated by 1,000 bootstrap replications, and bootstrap values of 950 or higher were considered statistically significant for the grouping (7).

Probe typing. The polymerase region was amplified by RT-PCR, using SR primers (G1 sets: SR33, SR48, SR50, and SR52; G2 sets: SR33, SR46, OC0281B) as described in Ando et al. (1). PCR products were analyzed by Southern hybridization with probes P1-A, P1-B, P2-A, P2-B, SOV, and 96065 (13), and strains were classified according to the probe to which they hybridized.

Nucleotide sequences and accession numbers. The nucleotide sequences of the N/S shell domains determined in this study were submitted to DNA Databank of Japan with the accession numbers AB186057 to AB186107.

Results

Diagnosis and Epidemiology of NV Associated Outbreaks

Fecal samples from 40 outbreaks of acute nonbacte-

Table 1. Reference strains and genetic classification of NVs

Genotype[a]	Reference strains (GenBank accession no.)	Genetic classification			
		Katayama[b]	Vinje[c]	Ando[d]	Green[e]
GI/1	Norwalk/68/US (M87661)	GI/1	GI/1	GI/1	GI/1
GI/2	Southampton/91/UK (L07418)	GI/4	GI/2	GI/2	GI/2
	Whiterose/96/UK (AJ277610)				
GI/3	DesertShield/90/SA (U04469)	GI/9	GI/3	GI/3A	GI/3
	Birmingham/93/UK (AJ277612)			GI/3B	
	LittleRocks/94/US (AF414405)				
	Stav/95/NO (AF145709)				
GI/4	Chiba/87/JP (AB022679)	GI/7	GI/4	GI/4	GI/4
	Valetta/95/MA (AJ277616)				
GI/5	Musgrove/89/UK (AJ277614)	GI/6	GI/6	GI/5	GI/5
	AppalachicolaBay/95/US (AF414406)				
	KU83aGI/99/JP (AB058545)				
GI/6	BS5/97/GE (AF093797)	GI/2	NA[f]	NA	GI/6
GI/7	Winchester/94/UK (AJ277809)	GI/8	GI/5	NA	GI/7
GI/8	Sindlesham/95/UK (AJ277615)	GI/3	GI/7	NA	GI/6
	WUG1/00/JP (AB081723)				
GI/9	SzUG1/99/JP (AB039774)	GI/5	NA	NA	NA
GII/1	Hawaii/71/US (U07611)	GII/7	GII/3	GII/1A	GII/1
	Girlington/93/UK (AJ277606)				
GII/2	Melksham/94/UK (X81879)	GII/4	GII/4	GII/2	GII/2
	SnowMountain/76/US (U70059, U75682)				
GII/3	Toronto/TV24/91/CA (U02030)	GII/2	GII/1	GII/3	GII/3
	Mexico/89/MX (U22498)				
	Arg320/95/AR (AF190817)				
GII/4	Bristol/93/UK (X76716)	GII/1	GII/2	GII/4	GII/4
	Lordsdale/93/UK (X86557)				
GII/5	Hillingdon/90/UK (AJ277607)	GII/5	GII/5	GII/5	GII/5
	WhiteRiver/290/94/US (AF414423)				
GII/6	Seacroft/90/UK (AJ277620)	GII/8	GII/7	GII/6	GII/6
	Miami/292/94/US (AF414410)				
GII/7	Leeds/90/UK (AJ277608)	GII/3	GII/6	GII/7	GII/7
	Gwynedd/273/94/US (AF414409)				
GII/8	Amsterdam/98/NL (AF195848)	GII/10	NA	GII/8	NA
GII/9	Idaho Falls/378/1996/US (AY054299)	NA	NA	GII/9	NA
	VA9207/97/US (AY038599)				
GII/10	Erfurt/00/DE (AF427118)	NA	NA	NA	NA
	KU5GII/00/JP (AB058575)				
GII/12	Chitta/96/JP (AB032758)	GII/6	GII/8	GII/1B	GII/1
	Wortley/90/UK (AJ277618)				
GII/14	Fayetteville/1998/US (AY113106)	NA	NA	NA	NA
	Kashiwa47/00/JP (AB078334)				
GII/15	Saitama KU82GII/99/JP (AB058588)	NA	NA	NA	NA
GII/17	Alphatron/98/NL (AF195847)	GII/9	NA	NA	NA
	Fort Lauderdale/560/98/US (AF414426)				

[a] Kageyama et al. (21).
[b] Katayama et al. (22).
[c] Vinje et al. (33).
[d] Ando et al. (3).
[e] Green et al. (11).
[f] Not assigned.

Table 2. Description of outbreaks in which NVs were detected in Osaka City, Japan, between April 2002 and March 2003

Outbreak no.	Mo/yr	Source	Attack rate ill/risk	No. of specimens	No. of NV-positive	Probe type	Genotype
02065	Apr/02	UK[a]	2/5	2	1 (GI)	P1A	GI/7
02172	Sep/02	Oyster	5/28	5	5 (GI, GII)	P1A	ND[b]
						P2B	GII/5, GII/3, GII/12
02189	Nov/02	UK	2/3	2	2 (GII)	P2B	GII/12
02198	Dec/02	UK	UK/25	3	3 (GII)	P2B	GII/4
02202	Dec/02	UK	4/5	1	1 (GII)	P2B	GII/4
03006	Jan/03	Oyster	1/1	1	1 (GII)	P2B	GII/3
03008	Jan/03	Oyster	28/35	1	1 (GII)	—	GII/5
03009	Jan/03	Oyster	3/3	2	2 (GI, GII)	P1A	GI/7
						P1B	ND
						P2B	GII/5
03011	Jan/03	Oyster	1/1	1	1 (GI)	—	GI/7
03012	Jan/03	Oyster	77/295	12	9 (GI, GII)	—	GI/7
						P1B	ND
						P2B	GII/5, GII/3, GII/15
03017	Jan/03	UK	10/13	9	8 (GII)	P1B	GII/6
03020	Feb/03	Oyster	3/3	1	1 (GI)	P1A	GI/4
						SOV	ND
03021	Feb/03	Oyster	2/2	2	2 (GII)	P2B	GII/3, GII/1
03022	Feb/03	Oyster	5/5	4	4 (GI, GII)	—	GI/7
						SOV	GI/2
						P2B	GII/3, GII/15
03024	Feb/03	UK	6/15	5	3 (GII)	P2B	GII/5
03026	Feb/03	Oyster	26/331	2	2 (GI, GII)	P1A	GI/4
						SOV	ND
						P2B	GII/5
03027	Feb/03	Oyster	2/2	1	1 (GII)	—	GII/5
03028	Feb/03	Oyster	3/3	1	1 (GII)	P2B	GII/5
03034	Feb/03	Oyster	3/3	3	3 (GI, GII)	P1A	GI/7, GI/4
						SOV	ND
						UT[c]/GI	GI/8
						P2B	GII/5, GII/3
03035	Feb/03	Oyster	6/6	2	1 (GII)	P2B	GII/15
03036	Feb/03	Oyster	3/5	1	1 (GII)	P2B	GII/3
03037	Feb/03	UK	3/15	3	2 (GI)	P1A	GI/9
03039	Feb/03	Oyster	5/9	4	3 (GI, GII)	P1A	GI/4
						P2B	GII/14, GII/8
03040	Mar/03	UK	5/9	4	2 (GII)	P2B	GII/8
03042	Mar/03	UK	20/47	1	1 (GII)	—	GII/8
03047	Mar/03	UK	2/8	1	1 (GI, GII)	P1A	GI/1
						P2B	GII/8
03048	Mar/03	UK	UK	1	1 (GII)	—	GII/6
03050	Mar/03	UK	27/62	7	4 (GII)	P2B	GII/15
03053	Mar/03	UK	11/50	3	1 (GII)	P2B	GII/3
03054	Mar/03	Oyster	3/6	2	2 (GII)	P2B	GII/5
03055	Mar/03	UK	157/283	2	2 (GII)	P1B	GII/6

[a] Unknown.
[b] Not determined.
[c] Untype.

rial gastroenteritis were tested for NV by real time RT-PCR. Seventy-two of 111 fecal specimens (64.9%) from 31 outbreaks (77.5%) were positive for NV (Table 2). GII NV was detected from 28 outbreaks, including 8 outbreaks also positive for GI. The 31 NV-positive outbreaks occurred in different settings, including restaurant, party, hotel, and home. The most common viral transmission mode in these outbreaks was ingestion of

contaminated oysters (54.8%). The NV-positive gastroenteritis outbreaks in Osaka City occurred mostly between January 2003 and May 2003 (83.9%).

All NV-positive specimens were tested for the probe type. Twenty-six of the 31 NV-positive outbreaks could be classified as 2 P1A, 2 P1B, 13 P2B, and 9 mixed probe types (Table 2). P2B strains were detected in 8 of 9 mixed probe-type outbreaks. In total, the P2B type was detected in 21 outbreaks (67.7%) and was, therefore, a predominant probe type during the 2002–03 seasons in Osaka City. The probe type could not be determined for 5 outbreaks (03008, 03011, 03027, 03042, and 03048), nor for one specimen each in outbreaks 03012 and 03022; all of these specimens were NV-positive by real time RT-PCR, but could not be amplified by RT-PCR using G1 or G2 primer sets. In addition, a single specimen from outbreak 03034, which was positive by RT-PCR using the G1 primer set, did not react with any probes (represented as UT/GI in Table 2).

Phylogenetic Analysis and Genotyping of NVs

To analyze the genetic relationships among the NV strains from the 31 outbreaks, the nucleotide sequence encoding the N/S domain of the capsid protein was determined. A total of 58 NV-positive specimens, including 18 GI and 51 GII NV-positive, were sequenced. Any strains from a single outbreak having identical nucleotide sequence in this region were considered to be identical strains.

The 51 nucleotide sequences (14 GI, 37 GII) of the N/S domain were aligned with the reference strains described in Table 1, and the genotype of each strain was determined. Phylogenetic trees based on the N/S domain were constructed by the neighbor-joining method for GI and GII NVs (Fig. 1, A and B). The bootstrap values of each genetic cluster was greater than 999, except for the GI/6 (Hesse cluster) type in GI NVs. The 51 NV strains were classified into 15 genotypes (6 GI and 9 GII genotypes) based on reference strains, and the number of each genotype was according to Kageyama's report (21). The genotypes of the 31 outbreaks strains were classified as follows: 2 GI/7, 1 GI/9, 5 GII/5, 3 GII/3, 3 GII/6, 2 GII/4, 2 GII/8, 2 GII/15, 1 GII/12, and 10 mixed genotype outbreaks.

In the outbreaks caused by mixed genotype NVs, GI/7 NV was detected in 4 outbreaks, and GII/5 NV in 5 outbreaks. Therefore, GI/7 NV was detected in a total of 6 outbreaks (19.4%), making it a dominant outbreak genotype in GI NV, and GII/5 NV was detected in 10 outbreaks (32.3%) and a dominant outbreak genotype in GII NV. In 9 of the outbreaks involving the GII/5 NV, oyster was the causal food.

In 7 outbreaks, strains of a single probe type were classified into multiple genotypes: 03021/P2B strains were of the GII/1 and GII/3, 03022/P2B strains were GII/3 and GII/15, 03034/P1A strains were GI/4 and GI/7, 03039/P2B strains were GII/8 and GII/14, and 02172/P2B, 03012/P2B, and 03034/P2B strains were of the GII/3 and GII/5 genotypes. The strain 03034-2/GI, which did not hybridize with any probes, was classified into the GI/8 genotype. Within each outbreak, the NV strains classified into the same genotype shared identical nucleotide sequence, except for NVs from outbreaks 02172 (02172-1 and 02172-2 in the GII/3 genotype) and 02198 (02198-1 and 02198-2 in the GII/4 genotype).

The relationship between probe types and genotypes of the NV outbreak strains detected in this study is as follows: P1A probe reacted with 4 genotypes (GI/1, GI/4, GI/7, and GI/9), SOV probe reacted with the GI/2 genotype, P1B reacted with the GII/6 genotype, and P2B reacted with 8 genotypes (GII/1, GII/3, GII/4, GII/5, GII/8, GII/12, GII/4, and GII/15).

Discussion

Molecular epidemiological studies of NV infections have been based on the phylogenetic analysis of the polymerase and capsid regions. The RNA polymerase region, which is relatively conserved among NV strains, has been used for detection of a wide variety of field strains, and most epidemiological studies of NV infection have been based on the sequence in this region (1, 28, 34, 35). We also reported epidemiological studies of NV infection in Osaka City, Japan targeting the polymerase region (13–15).

In general, good correlation has been reported between phylogenetic analyses of the polymerase region and capsid region (30, 33). However, recent studies indicated that phylogenetic analysis of the polymerase region sequence did not facilitate the classification of strains into genotypes (22), and a system has been proposed for the identification of NVs in which the capsid sequences are compared to those of reference strains. Ando et al. (3) used sequences encoding the capsid N-terminal 94 amino acids to divide G1 NVs into 5 "genetic clusters" and GII NVs into 10 clusters. Vinje et al. (33) demonstrated that the NVs could be divided into 7 "phylogenetic groups" within GI and 5 within GII using the capsid N-terminal region sequence (GI; 278 nt, GII; 249 nt). Katayama et al. (22) demonstrated that the NVs could be divided 9 "genotypes" within GI and 10 within GII using the capsid N/S domain. Furthermore, Green et al. (11) demonstrated that the NVs could be divided 7 "genetic clusters" within GI and 7 within GII using the complete capsid

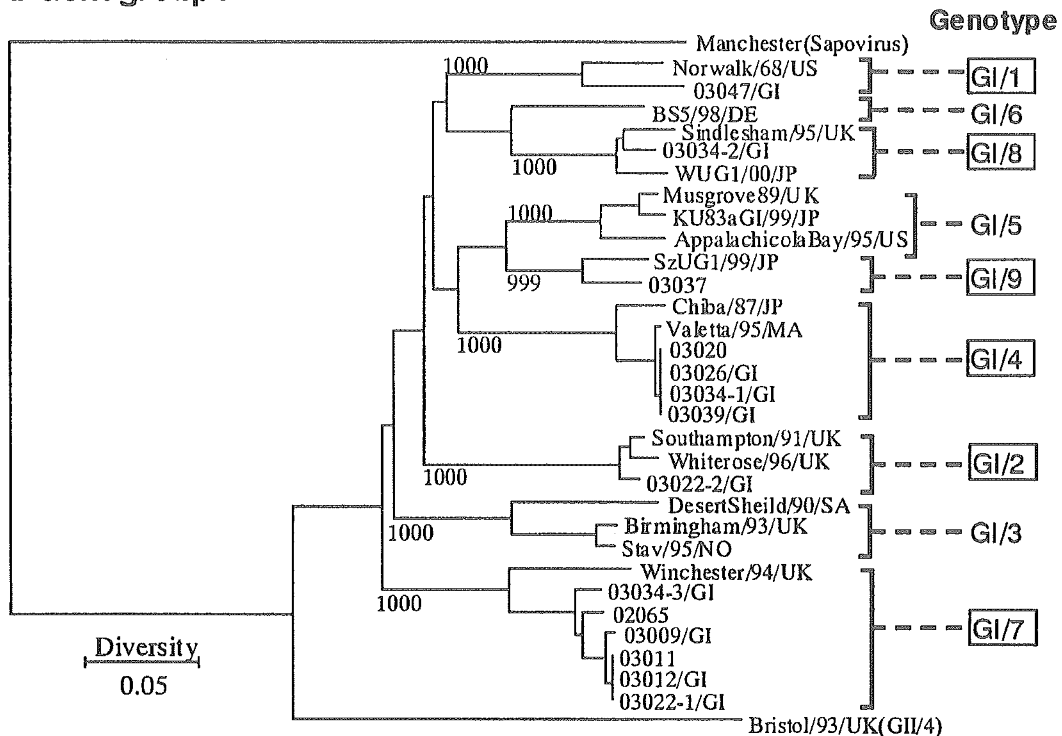

Fig. 1. Phylogenetic trees of published NV sequences and outbreak strains from this study based on the N/S domain region (GI, 290 nt; GII, 278 nt). Genogroup I NVs and Bristol/93/UK(GII/4) (A), genogroup II NVs and Norwalk/68/US(GI/1) (B) are reconstructed using Manchester *sapovirus* as the outgroup. The numbers on each branch indicate the bootstrap value for the clusters supported by that branch. The genotypes that include an outbreak strain are boxed.

region. The genetic classification described in these four reports is summarized in Table 1. The comparison indicates that their constellations of genotypes or genetic clusters are almost identical except for the names. The phylogeny based on the ORF2 region is related to antigenic types, as indicated by solid phase immuno-EM for 8 genetic types (33). The phylogenetic analysis of the N/S domain (22) was well correlated with antigenic analysis using recombinant virus-like particles and their antisera (23, 24). Therefore, the 5′ end of ORF 2 region (N/S domain) may be suitable for the molecular typing of NV strains.

In this study, a phylogenetic analysis of the N/S domain incorporating new outbreak strains, NV reference strains from previous reports, and additional reference strains, GI NVs were segregated into 9 genotypes, as reported by Katayama et al. (22), but GII NVs were segregated into 14 genotypes (Fig. 1, A and B). Kageyama et al. reported that the NV could be divided into 14 genotypes within GI and 17 genotypes within GII based on the capsid N/S domain (21).

The P2B outbreak strains, the predominant probe type in this season, were characterized into 8 genotypes (Table 2). However, there was no predominant genotype of NV outbreak strains; multiple genotypes of NV were prevalent in Osaka City, Japan. The outbreaks in which mixed NV genotypes were detected mainly occurred by consumption of oysters. It may be that concurrent infections with more than one strain occurred by ingesting the contaminated oysters. Similar results of coinfection have been reported previously (21, 32), and various types of NVs have been detected from oysters in Japan (29). Ninety percent of GII/5 NV outbreaks were associated with consumption of oysters, indicating that the GII/5 NV was closely related to oysters in this season. The 9 GII/3 NV strains, classified as P2B, detected in this study, in Fig. 1B, were closely related to Arg-320/95/AR which might be occurred a genetic recombination between ORF1 and ORF2 (15, 16, 33).

In summary, we applied a recently developed quantitative real time PCR a method (20) to detect NV genomes from stool specimens in Osaka City, Japan. This method is useful for routine diagnosis, because of

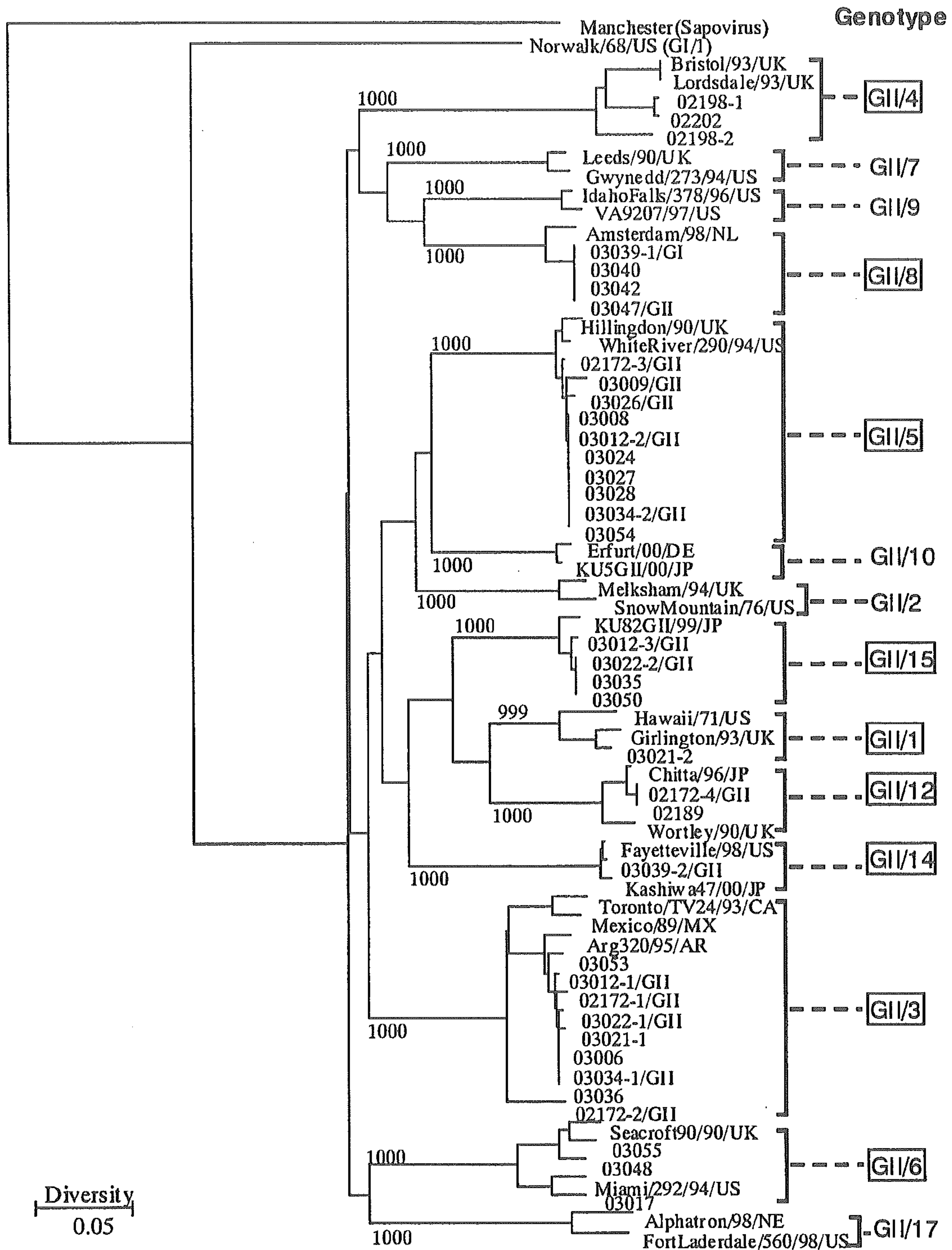

Fig. 1. B

its broad reactivity and high sensitivity compared to our previous diagnostic method using RT-PCR and hybridization. Application of genotyping methods has provided information on disease transmission for epidemiological investigations of public health significance. Further molecular phylogenetic studies of NVs will contribute to an understanding of the epidemiology of NV infection.

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Expression of sapovirus virus-like particles in mammalian cells

Brief Report

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Summary. Sapovirus (SaV) is an etiological agent of acute gastroenteritis in human and swine. SaV can be divided into five genogroups, GI to GV. Virus-like particles (VLPs) morphologically similar to native SaV have been expressed for GI, GII, GIII and GV strains in insect cells, although only low expression levels were observed for GII strains. In this study, we report the successful expression of SaV GII VLPs using cultured mammalian COS-7 and 293T cells. Our results demonstrated that this mammalian expression system was able to express and form SaV VLPs.

*

Sapovirus (SaV), a member of the family *Caliciviridae*, is an agent of gastroenteritis [9, 15]. Based on the capsid gene sequence, SaV strains are divided into five genogroups, GI to GV, of which GI, GII, GIV, and GV strains infect humans, while GIII strains infect swine [4]. Only GIII strains can be propagated in cultured cells [1, 5], whereas human SaV strains are noncultivable. The SaV GI, GIV, and GV genomes are predicted to encode three ORFs, whereas SaV GII and GIII have two ORFs. The SaV ORF1 encodes non-structural proteins and the capsid protein (VP1), and ORF2 and ORF3 encode proteins of yet unknown functions [3].

The expression of the recombinant major capsid (rVP1) gene leads to the self-assembly of virus-like particles (VLPs), which are morphologically similar to native SaV. To date, 7 VLPs have been successfully formed in insect cell systems with four GI strains, Sapporo [16], Houston/90 [13], Parkville [2], and Mc114 [10, 11], one GII strain, C12 [11], one GIII strain, PEC [8], and one GV strain, NK24 [11]. In contrast, no VLP formation in insect cells was reported for two SaV GII

strains: Bristol/98 [18] and Mc10 [14]. Although GII VLPs were generated with C12 strain, its yield was lower when compared with that of GI Mc114 and GV NK24 VLPs (Hansman et al., unpublished data). This promoted us to investigate alternative expression systems for GII VLP formation. In this study, we report GII Mc10 VLP formation using cultured mammalian cells.

To construct the Mc10 expression plasmid, a DNA fragment corresponding to the putative capsid gene (nt 5174–7458: GenBank Accession No. AY237420) was amplified with a plasmid, pUC19/SaV Mc10, which contains a full-length Mc10 genome [17] as the template. A sense primer, 5′-ggatcc**TAATACGACTCACTA TAGGG**AACAGCCACCATGGAGGGCCTAGGCCAACCACAGCCACAG-3′ containing a *Bam*HI site indicated as lower case, the T7 promoter sequence as bold, and Mc10 specific sequence as underlined, and an antisense primer, 5′-agatctT_{29}CCAAGAAAGCAC-3′, containing a *Bgl*II site indicated as lower case and Mc10 specific sequence as underlined, were used. PCR was performed in 100 μl of the reaction mixture containing 500 ng of the plasmid, 40 pmol of each primer, KOD polymerase buffer, 0.2 mM of each dNTPs, 1 mM $MgSO_4$, and 2 U of KOD-Plus-DNA polymerase (Toyobo, Osaka, Japan). After initial denaturation at 94 °C for 5 min, 25 cycles consisting of denaturation at 94 °C for 30 s, primer annealing at 55 °C for 30 s, and primer extension at 72 °C for 3 min were performed, followed by a final extension at 72 °C for 15 min. The PCR products were purified using PCR Purification Kit (QIAGEN, Hilden, Germany), and cloned into pCR-Blunt II-Topo® vector (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The resultant plasmid was digested with *Bam*HI and *Bgl*II (New England Biolabs, Beverly, MA), and the fragment was purified from an agarose gel using Gel Extraction Kit (QIAGEN). This fragment was cloned into a BamHI and BglII digested vector prepared from pUC19/SaV Mc10 full-length plasmid [17]. The resultant plasmid was designated as SaV Mc10 T7 capsid-genome end/pUC19. *E. coli* DH5α cells (Toyobo, Japan) were used for the transformation and propagation of the plasmid.

COS-7 and 293T cells purchased from RIKEN Cell Bank (Tsukuba, Japan) and American Tissue Culture Center (ATCC), respectively, were cultured in DMEM medium (Invitrogen), supplemented with 100 units/ml of penicillin G, 100 μg/ml of streptomycin and 10% fetal calf serum (Invitrogen). The cells were plated onto 6 well dishes (BD Falcon, Franklin Lakes, NJ) at ~60% confluence and incubated at 37 °C for 16 to 24 h, and then, the cells were washed twice with PBS and infected with a recombinant vaccinia virus encoding T7 RNA polymerase at the 0.1 PFU (plaque forming unit) per cell [6], and incubated at 37 °C for 1 h. Then, SaV Mc10 T7 capsid-genome end/pUC19 (1 μg per well) was transfected into COS-7 and 293T cells using Effectene Transfection Reagent (QIAGEN) according to the manufacturer's protocol. The cells were incubated at 37 °C for 48 h, and the medium was removed. The cells were collected by scraping with 300 μl of OPTI-MEM (Invitrogen). The cell suspension was subjected to freezing and thawing three times. The cell debris was removed by centrifugation (2,300 × g, 5 min at 4 °C). VLPs from the cell lysate were pelleted by centrifugation in a Beckman Coulter SW32-Ti rotor (31,000 rpm, 2 h at 10 °C), resuspended in 500 μl of OPTI-MEM at 4 °C, and the debris was removed by low-speed

centrifugation (2,300 ×g, 5 min at 4 °C). The supernatant was layered onto a 5 to 30% (wt/vol in PBS) sucrose gradient and centrifuged at 25,000 rpm for 2.5 h at 10 °C in a Beckman Coulter SW41 rotor. After fractionation, each aliquot was centrifuged at 45,000 rpm for 3 h at 4 °C in a Beckman Coulter TLA55 rotor. The resulting pellet was resuspended in 20 μl of PBS and stored at 4 °C. SDS-PAGE, Western blot analysis, and electron microscopy (EM) were performed as previously described [11].

Two mammalian cell lines, COS-7 and 293T cells, were used for the expression of Mc10 rVP1. Both COS-7 and 293T cells formed VLPs, however VLPs were mostly found in cell lysate, and the VLP expression level was slightly higher in COS-7 than 293T cells (data not shown). Based on this result, we used COS-7 cells for further expression experiments. VLPs were separated from other components by sedimentation in sucrose, which appeared in the range of 22.9–17.8% (wt/vol) (Fig. 1). The protein(s) composing these particles were found to be a single protein with 60 kDa which corresponds well to the predicted Mc10 VP1 (558 amino acids) by Western blot analysis using hyperimmune rabbit antisera raised against *E. coli*-expressed N-terminal VP1 (aa1721 to 1950) [17] (Fig. 1). EM analysis showed that the purified VLPs were approximately 41–46 nm in diameter with the typical "Star of David" structure, as seen with native SaV (Fig. 2). Approximately 10 μg of VLPs could be purified from COS-7 cells grown in 10 × 6 well plates.

The major capsid (VP1) gene of SaV is fused to the non-structural genes on ORF1, and the subgenomic RNA was recently identified in the cells infected with porcine GIII SaV strain [1, 7]. The tri-peptide, MEG or MEA, conserved among human SaV strains or porcine SaV strains [19] is likely to be the putative VP1 start on the subgenomic RNA. Therefore, the VP1 may be produced by either translation as part of ORF1 and then cleavage, or translation from subgenomic

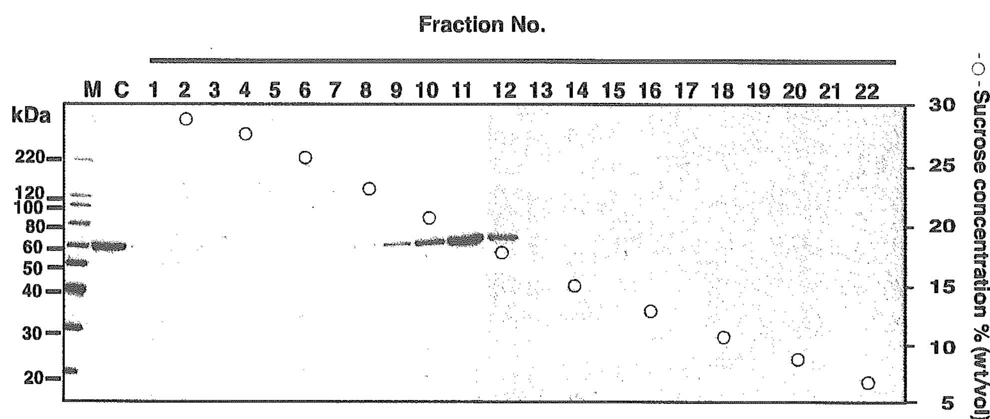

Fig. 1. Western blot analysis of Mc10 VLPs assembled in COS-7 cells. COS-7 cells were transfected with the plasmid (Mc10 T7 capsid-genome end/pUC19) and incubated for 48 hrs. The cell lysate was prepared and separated by centrifugation at 25,000 rpm for 2.5 h at 10 °C in a Beckman Coulter SW41 rotor in 5 to 30% (wt/vol) sucrose density gradient. Each fraction (1 μl) was analyzed by Western blotting with rabbit polyclonal antibodies raised against *E. coli* expressed N-terminal-deleted Mc10 VP1 (aa 1721 to 1950 of the ORF1) [17]. Lanes: *M*, molecular weight marker; *C*, cell lysate; *1* to *22*, fractions

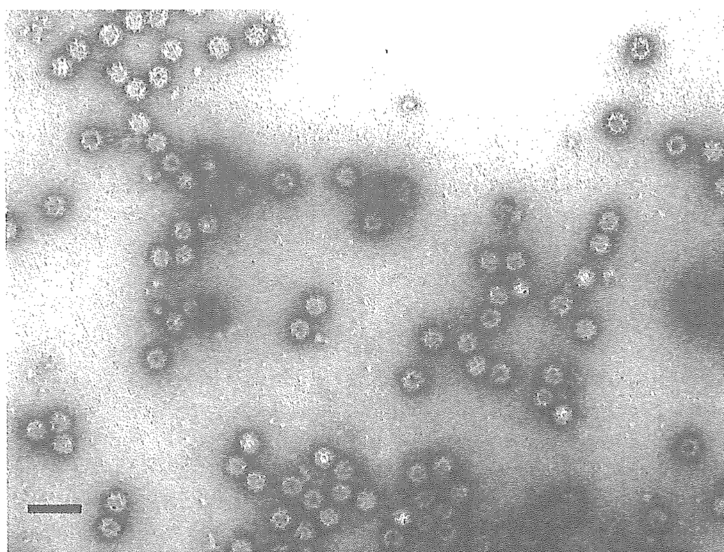

Fig. 2. Electron micrograph of purified Mc10 VLPs expressed in COS-7 cells. Purified VLPs were stained with 4% uranyl acetate (pH4) and examined by an electron microscope (JEM-1220; JOEL, Japan) operating at 80 kV. The bar indicates 100 nm

RNA, or by both. The expression from the putative VP1 start has been shown to form the VLPs in this study and in other studies [2, 8, 11, 13, 16], whereas evidence to form VLPs from ORF1 remains unclear. We have recently identified the Mc10 ORF1 cleavage map; NH_2-p11-p28-p35(NTPase)-p32-p14(VPg)-p70(Pro-Pol)-p60(VP1)-COOH [17]. 60 kDa protein corresponding to VP1 was immunoprecipitated with VP1-specific antibody. Thus, it will be interesting to determine whether or not VLP formation occurs when ORF1 is expressed in the mammalian cells.

We previously attempted to express Mc10 rVP1 in an insect expression system, but we did not observe the formation of VLPs [14]. Similarly, we reported the formation of VLPs with another SaV GII strain (C12 strain) in insect cells [11], however the expression level was lower when compared to GI and GV strains (Hansman et al., unpublished data). Using this mammalian expression system, we were able to prepare Mc10 VLPs. Thus, this mammalian expression system may also be useful for the expression of the C12 strain. To our knowledge, this is the first report on the preparation of SaV VLPs in mammalian cells. More importantly, the expression level was sufficient to prepare antisera (data not shown). The difficulty in expression of GII VLPs in insect cells suggests different requirements for GII VLP formation. SaV GI, GII, and GV VLPs have been used to reveal the antigenic diversity among these genogroups [11, 12]. Therefore, the mammalian expression system described in this study provides an alternative tool to prepare SaV VLPs in order to further analyze antigenic and immunogenic differences among SaVs.

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