purified from sera using Hi Trap™ rProtein A FF (GE Healthcare Bio-Science Corp., Piscataway, NJ) and its concentration was determined using the Coomassie Plus Protein Assay Reagent (Pierce, Biotechnology, Inc., Rockford, IL).

ELISA for Titration of MAbs and PolyAb

Ninety-six-well plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with 90 ng of VLP/well in 60 µl of 0.1 M carbonate buffer (pH 9.6) for 1 hr at 37°C. The wells were blocked with 1% BSA in PBS containing 0.1% Tween 20 (PBS-T). The plates were incubated overnight at 4°C. After the wells were washed three times with PBS-T, for the titration of MAbs, 60 µl of twofold serially diluted MAbs in PBS-T containing 1% BSA were added to each well and the plate was incubated for 1 hr at 37°C. After washing three times with PBS-T, 60 µl of a 1:4,000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (Biosource International, Camarillo, CA) were reacted for 1 hr at 37°C as the second antibody. For the titration of PolyAb, 60 µl of twofold serially diluted PolyAb were added to each well from a starting dilution of 1: 1,600 in PBS-T containing 1% BSA and the plate was incubated for 1 hr at 37°C. After washing three times with PBS-T, 60 µl of a 1:65,000 dilution of biotinylated goat anti-rabbit IgG were added to each well and the plate was incubated for 1 hr at 37°C. After washing three times with PBS-T, a 1:2,000 dilution of avidin-peroxidase conjugate was added to each well and the plate was incubated for 1 hr at 37°C. Titration of MAbs and PolyAb followed the same protocol. After washing three times with PBS-T, 60 µl of substrate *o*-phenylenediamine containing 0.012% H_2O_2, 0.2 M citrate-phosphate buffer (pH 5.0) were added to each well and left in the dark for 20 min at room temperature. The reaction was stopped by the addition of 60 µl of 2 M H_2SO_4 to each well and the optical density (OD) at 492 nm (620 nm as reference) was determined with a Labsytems Mutiskan MCC microplate reader (Thermo Electron Corporation, Waltham MA). For this experiment, Tn5 cell lysate was included as a negative control. A sample with OD > 0.2 and a sample/negative control ratio >2.0 was considered positive. Each assay was conducted in duplicate. For the antigen-ELISA, CsCl-purified NoV GII/3 and NoVGII/4 VLPs were used as the positive control.

Antigen-ELISA for Detection of NoV From Clinical Specimens

Ninety six-well plates (Maxisorp, Nunc) were coated with 1 µg of rabbit anti-NoVGII/3 PolyAb/well in 60 µl of 0.1 M carbonate buffer (pH 9.6) for 4 hr at 37°C. The wells were blocked with 1% BSA in PBS. The plates were incubated overnight at 4°C. Sixty microliters of a final 10% stool suspension were added to each well and incubated for 2 hr at 37°C. The wells were washed three times with PBS-T, and sixty micrograms of each MAb (MAb 49-6, 67-9, and 70-4) /well in 60 µl of 1% BSA in PBS-T were added and incubated for 2 hr at 37°C. After washing three times with PBS-T, 60 µl of a 1:10,000 dilution of peroxidase-conjugated goat anti-mouse IgG (Biosource International) were added and titration followed the same procedure with ELISA. For ELISA detecting NoV in the stool, CsCl-purified NoVGII/3 VLP was used as the positive control.

RESULTS

ELISA Titer of MAb to NoVGII/4 VLP and PolyAb to NoVGII/3 VLP

The culture supernatant of hybridomas, which reacted with both GII/3 and GII/4, was cloned by limiting dilution, and ascitic fluids were prepared by injection of hybridomas into mice and used for this study. The titers of the MAbs were examined by ELISA against 16 VLPs and the number of cross-reactivities among different genotypes was observed (Table I). MAb 49-6 cross-reacted (equal to fourfold lower than homologous VLP titer) with GII/1, GII/2, GII/3, GII/5, GII/12, GII/14, and GII/15 genotypes and moderately (eightfold to sixteenfold lower than homologous VLP titer) with the GI/4 genotype. MAb 67-9 also cross-reacted with GII/1, GII/2, GII/3, GII/5, GII/12, GII/14, and GII/15 genotypes and moderately with GI/4, GII/6, and GII/7. MAb 70-4 cross-reacted with GII/1, GII/3, GII/6, GII/7, GII/13, and GII/14 genotypes, but showed no cross-reaction with GI genotypes.

An antibody-ELISA using PolyAb raised against GII/3 VLP was used to determine cross-reactivity (Table I). The highest titer was detected in PolyAb against homologous GII/3 VLP. A broad cross-reactivity was observed not only with intra-genogroup strains, but also inter-genogroup strains. GII/3 PolyAb had titers against GII strains fourfold to sixteenfold lower than homologous VLP titer, and also showed titers against GI strains eightfold lower than homologous VLP titer. Both PolyAb and MAbs did not react with Tn5 lysate, which was infected without baculovirus.

ELISA for NoV Detection From Stool Samples

To determine the utility of the MAbs and PolyAb, antigen-ELISA using anti-GII/3 PolyAb as a capture antibody, and three MAbs as detector antibodies was developed. At first, reactivity was checked against NoV VLPs. As shown in Figure 1, the antigen-ELISA reacted with many genotypes, except GI/3, GI/8, and GI/11. The highest limit of detection was found in GII/4 (2.4 ng/ml), and a twofold to fourfold lower limit of detection than the highest reaction was found in GII/1, GII/3, GII/5, GII/12, and GII/13 genotypes. An eightfold to sixteenfold lower limit of detection against the highest reaction was observed in GII/2, GII/6, GII/7, GII/14, and GII/15 genotypes. The GI/1 and GI/4 genotypes showed a lower limit of detection compared to the highest reaction, with a 32-fold and 512-fold lower reaction, respectively. The OD values of each genotype decreased in a dose-dependent manner.

TABLE I. ELISA Titers of Three MAbs and Anti-GII/3 PolyAb Against VLPs

	VLPs															
	GI/1	GI/3	GI/4	GI/8	GI/11	GII/1	GII/2	GII/3	GII/4	GII/5	GII/6	GII/7	GII/12	GII/13	GII/14	GII/15
MAbs																
49-6	32	0	128	0	0	4096	2048	1024	**2048**	4096	16	8	1024	16	1024	1024
67-9	128	0	512	4	4	4096	8192	4096	**4096**	1024	512	512	2048	8	8192	8192
70-4	0	0	0	0	0	4096	16	4096	**8192**	32	4096	4096	0	4096	1024	16
Anti-GII/3 PolyAb	2048	2048	2048	2048	2048	1024	4096	**16384**	2048	2048	2048	2048	2048	1024	2048	1024

Homologous titers are shown in boldface. Three MAbs and anti-GII/3 PolyAb did not react with Tn5 lysates, which infected without baculovirus.

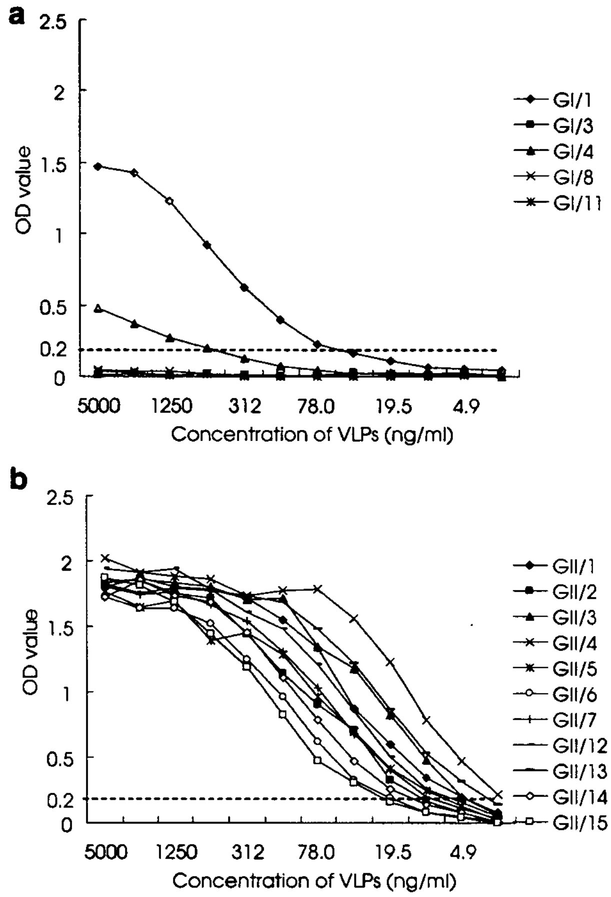

Fig. 1. Detection limit of antigen-ELISA against NoVGI VLPs (**a**) and against NoVGII VLPs (**b**) using anti-GII/3 PolyAb as a capture antibody and three MAbs as detector antibodies. VLPs were diluted twofold from a starting concentration of 5,000 ng/ml. Dashed lines on horizontal axes indicate cut-off OD values.

Evaluation of the antigen-ELISA against clinical specimen reactivity was tested with known positive NoV by RT-PCR from 1995 to 2003 in most of the 10 stool samples from each genotype, NoV negative samples, and other enteric viruses such as sapovirus, rotavirus (G3, G4, and G9 genotypes), adenovirus, and astrovirus. Among fecal specimens with NoVGII strains, antigen-ELISA detected NoV from fecal specimens containing GII/1, GII/2, GII/3, GII/4, GII/5, GII/6, GII/12, and GII/13 genotypes. As shown in Table II, stool specimens containing GII/3 and GII/4 strains showed high OD values and GII/2, GII/5, and GII/6 strains had both high and low OD values. The OD values of GII/12 and GII/13 strains were relatively low, although these two genotypes showed a high limit of detection rate against VLPs. On the other hand, none of stool samples containing NoV GI strains, negative stool samples and stool samples containing other enteric viruses became positive.

DISCUSSION

VLPs have been used as a potent tool in NoV studies. Even the similar molecular weights of 58 kDa, Alps are

TABLE II. Detection of Human Viruses From Clinical Specimens by Antigen-ELISA

Strain	$OD_{492\ nm}$	Virus and genotype	Strain	$OD_{492\ nm}$	Virus and genotype
4710	0.167	NoVGII/1	4714	**1.635**	NoVGII/5
4721	**0.857**	NoVGII/1	2176	0.037	NoVGII/6
2703	**1.981**	NoVGII/2	2295	**0.476**	NoVGII/6
2776	**0.939**	NoVGII/2	3578	**0.941**	NoVGII/6
2839	<0	NoVGII/2	3591	**0.822**	NoVGII/6
2840	0.055	NoVGII/2	3615	0.043	NoVGII/6
2842	**0.548**	NoVGII/2	4403	0.167	NoVGII/6
4681	**1.499**	NoVGII/2	4511	**0.870**	NoVGII/6
2288	**2.264**	NoVGII/3	4815	**0.646**	NoVGII/6
2290	**1.823**	NoVGII/3	2309	**0.438**	NoVGII/12
3583	**2.250**	NoVGII/3	3636	**0.329**	NoVGII/12
3606	<0	NoVGII/3	4381	**0.378**	NoVGII/12
4407	**2.357**	NoVGII/3	3308	**0.291**	NoVGII/13
4616	**2.498**	NoVGII/3	4531	**0.275**	NoVGII/13
4665	0.106	NoVGII/3	4464	<0	SVGI
5189	**2.352**	NoVGII/3	4509	0.026	SVGI
5192	**2.412**	NoVGII/3	4457	<0	SVGII
5196	**2.089**	NoVGII/3	4527	<0	RV
2431	**1.981**	NoVGII/4	4525	0.038	ASV
3271	<0	NoVGII/4	4528	0.061	ASV
3604	**0.874**	NoVGII/4	4462	<0	AdV
4801	**0.448**	NoVGII/4	4523	0.042	AdV
2439	**1.348**	NoVGII/5	5163	<0	Normal stool
2907	<0	NoVGII/5	5169	<0	Normal stool
3600	<0	NoVGII/5	5175	<0	Normal stool
3611	**1.648**	NoVGII/5	5187	<0	Normal stool

Positive reactions are shown in boldface. SV, Sapovirus; RV, rotavirus; ASV, astrovirus; AdV, adenovirus.

antigenically distinguishable according to different NoV genotypes since the epitopes located in capsid gene of different NoV genotypes are different [Parker et al., 2005]. Many genotypes of VLPs including 5 genotypes in genogroup I and 11 genotypes in genogroup II were obtained, and the reactivity of the generated antibodies with the VLPs was investigated. The final aim was to construct an IC kit to detect different NoV genotypes. The use of a broad-reactive antibody is desirable for IC kit construction fitting many genotypes in comparison with the mixture of antibodies specific for each genotype. In this study, MAbs and PolyAb against GII/4 and GII/3, respectively, were generated. MAb 70-4 reacted with intra-genogroup strains, detecting 7 of 11 genotypes in GII. MAb 49-6 and MAb 67-9 reacted with inter-genogroup strains, reacting strongly with 8 of 11 genotypes in GII and reacting weakly with 2 of 5 genotypes in GI. The similar observation was also found when Western blot analysis was performed with these VLPs (data not shown). Although MAbs recognizing GI or GII, or both GI and GII genotypes were reported previously, the cross-reactivity was tested with a low number of genotypes in each genogroup [Parker et al., 2005]. This report is the first to show titers against a lot of genotypes, 5 in GI and 11 in GII. The NS14 monoclonal antibody, which was known as GII specific, detected GII/2, GII/4, GII/6, GII/12, and GII/14 strains [Kitamoto et al., 2002] and the MAb 67-9 reacted with them. Moreover, MAb 67-9 recognized GII/1, GII/3, GII/4, GII/5, GII/7, and GII/15. It is unknown whether or not 67-9 and NS14 detect the same epitope. Further characterization of epitopes using a small fragment of recombinant capsid proteins is needed to make this clear.

Rabbit anti-GII/4 VLP antibody was produced and IC was developed by using this antibody [Okame et al., 2003]. It was specific only for GII/4 strains, which were used as an immunogen that showed high sensitivity and specificity against GII/4 strains. This kind of reactivity has been observed in many laboratories, as it is specific only for genetic subgroups used for immunization [Hale et al., 1999; Kobayashi et al., 2000; Kamata et al., 2005]. In a recent report, however, the GII/1 polyclonal antibody cross-reacting strongly with other GII genotypes, and the GI/11 polyclonal antibody cross-reacting strongly with both GI and GII genotypes were obtained, and they showed the possibility of detecting a broad-range of genotypes in clinical specimens [Hansman et al., 2006]. The rabbit anti-GII/3 VLP also showed broad cross-reactivity, detecting not only GII genotypes, but also GI genotypes. The titer was from fourfold to sixteenfold lower than the homologous VLP titer and this was twofold lower than in the previous report. Although five types of GII/3 antisera were generated and none of them had broad-range cross-reaction in the previous study, it was interesting that the GII/3 polyclonal antibody, which shared 96–98% nucleotide identity with those five strains, showed broad cross-reactivity.

A sandwich ELISA using the anti-GII/3 polyclonal antibody as a capture antibody and mixed MAbs as detecting antibodies was performed, and detected successfully eight genotypes of NoV from clinical specimens. To date, three commercial ELISA kits have been available and they use broadly reactive MAbs or a polyclonal antibody in a pooled way, since the rabbit anti-VLP antibodies were specific for a genotype used as an immunogen [Burton-MacLeod et al., 2004; Dimitriadis and Marshall, 2005]. On the other hand, this result is the first to report detection of many genotypes of NoV from clinical samples using only anti-GII/3 PolyAb as a capture antibody in the sandwich ELISA. This suggested that a polyclonal antibody that showed a wide range of cross-reaction in antibody-ELISA, such as anti-GI/11 antisera in the previous report, is capable of detecting distinct genotypes in clinical specimens. The range of OD values in antigen-ELISA differed among both inter-genotypes and intra-genotypes. This difference of OD values did not seem to concord with the limit of detection rate. For example, the OD value of GII/12 and GII/13 against NoV in stool specimens was low, but the limit of detection showed a high rate. This might be due to the variation in concentration of NoV virus particles and difference in the existence of substance work as an inhibitor of binding between NoV VLPs and clinical stool specimens. The other reason was that some mutations occur exactly where the MAbs bind.

In conclusion, these results proved that the polyclonal antibody, which has broad-range cross-reactivity, is capable of detecting many types of genotypes from clinical specimens and it showed its potential for the use of antigen-ELISA and IC. However, the reactivity differed among genotypes, and GI strains cannot be detected in this format. Further generations of MAbs and PolyAb against GI strains and specific genotypes are necessary for the detection of NoV from clinical specimens containing many kinds of genotypes.

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Genetic Heterogeneity, Evolution, and Recombination in Noroviruses

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Norovirus is one of the most common causes of nonbacterial gastroenteritis in humans. A total of 603 fecal specimens collected from sporadic pediatric cases of acute gastroenteritis in Japan from 2004 to 2005 were tested for the presence of norovirus by RT-PCR. It was found that 51 (8.5%) specimens were positive for norovirus. The norovirus genotypes detected in this study were GII/1, GII/2, GII/3, GII/4, GII/6, and GII/7. Of these, GII/3 was the most predominant (52.9%), followed by GII/4 (37.2%) and others. It was noticed that four distinct types of recombinant noroviruses were co-circulating and the variant norovirus GIIb suddenly emerged to be the leading strain in Japan for the first time. A novel norovirus nomenclature was proposed, in which worldwide noroviruses were classified into seven distinct genogroups (I–VII). Norovirus GI and GII consisted of 16 genotypes with 32 subgenotypes and 23 genotypes with 34 subgenotypes, respectively. Of note, human and porcine noroviruses had a close genetic relationship within GII. Interestingly, multiple short amino acid motifs located at N terminus, S domain, P1 domain, P2 domain, and C terminus of capsid gene correctly defined the phylogenetic norovirus genogroups, genotypes, and subgenotypes. Another interesting feature of the study was the identification of eight hitherto unreported recombinant noroviruses. It was noteworthy that three different types (intergenogroup, intergenotype, and intersubgenotype) of recombination in noroviruses were also found. This is the first report to demonstrate the existence of intergenogroup and intersubgenotype recombinations in noroviruses and highlights a possible route of zoonoses in humans because porcine, bovine and murine noroviruses belong to genogroups II, III, and V, respectively. ***J. Med. Virol. 79:1388–1400, 2007.*** © 2007 Wiley-Liss, Inc.

KEY WORDS: norovirus; recombination; genogroup; genotype; subgenotype

INTRODUCTION

Viral gastroenteritis is one of the most common illnesses in humans worldwide and has a great impact on people. The mortality among children due to acute gastroenteritis is greater in developing than in developed countries. Annual mortality associated with acute gastroenteritis was estimated to be 2.1 million in 2000 [McEvoy et al., 1996; Parashar et al., 2003; Okitsu-Negishi et al., 2004; Thapar and Sanderson, 2004; Parashar and Glass, 2006]. Among different types of diarrheal viruses, norovirus (NoV) is also considered to be a significant global enteropathogen, being a major cause of acute gastroenteritis in infants and young children worldwide [Noel et al., 1999; Inouye et al., 2000; Lopman et al., 2002; Dai et al., 2004]. The transmission routes of this virus are classified into food-borne, water-borne, air-borne, person-to-person spread and might be by other unknown modes [Marks et al., 2000; McIntyre et al., 2000; Kageyama et al., 2004; Lopman et al., 2004]. NoV is highly infectious and spreads by ingestion of contaminated food such as oysters and water. These characteristics make NoV a major public health concern [Katayama et al., 2002].

NoV is the distinct genus within the family *Caliciviridae*. NoV is one of the leading agents of acute gastroenteritis worldwide and causes outbreaks in various epidemiological settings such as restaurants, schools, day care centers, hospitals, nursing homes, and cruise ships [Chiba et al., 1979; Matson, 1994; McEvoy et al., 1996; Vinje et al., 1997; Marks et al., 2000; Buesa et al., 2002; Kageyama et al., 2004; Maunula and Von Bonsdorff, 2005]. The prototype strain of NoV is the

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Norwalk virus, which was originally discovered in an outbreak of acute gastroenteritis in an elementary school in Norwalk, Ohio, USA in 1968 [Kapikian et al., 1972]. NoV contains a positive sense single-strand RNA genome surrounded by an icosahedral capsid. The NoV genome contains three ORFs (ORF1, 2, and 3). The ORF1 encodes non-structural proteins, including NTPase, protease, and RNA-dependent RNA polymerase, ORF 2 encodes the capsid protein (VP1), and ORF3 encodes a small capsid protein (VP2). The NoV capsid protein is divided into N-terminal region, shell (S) domain, protruding (P) domain, and C-terminal region. The P domain is in turn subdivided into P1-1, P2, and P1-2 domains. Of these, P2 locates at the exterior surface of the capsid and predicted to bear antigenic determinants of immunological response of the host [Green et al., 2001; Nilsson et al., 2003].

To date, NoV is still uncultivable by standard culture with different cell lines. However, expression of either VP1 alone or both VP1 and VP2 using recombinant baculoviruses formed the virus-like particles that are morphologically and antigenically similar to the native virion [Jiang et al., 1995]. The lack of a virus culture system has been a significant obstacle to the study of NoV, but recent advances in cloning and sequencing of NoV have enabled its genomic characterization and therefore genetic analysis becomes the principal method to classify NoV. NoV is genetically and antigenetically diverse. RNA recombination is one of the major driving forces of NoV evolution [Worobey and Holmes, 1999]. A recombinant NoV can be defined as one that clusters with two distinct groups of NoV strains when two different regions of the genome are subjected to phylogenetic analysis. The first naturally occurring recombinant NoV was the prototype Snow Mountain virus [Hardy et al., 1997]. Later several recombinant NoV strains causing sporadic cases and outbreaks of acute gastroenteritis were reported [Jiang et al., 1999; Schreier et al., 2000; Phan et al., 2006a,b].

In this study, the occurrence of NoV infection in children with acute gastroenteritis in five different localities in Japan during 2005 and 2006 was determined and NoV was characterized further according to genogroup and genotype. The study also provided genetic insights as well as described the genetic diversity in worldwide NoV strains.

MATERIALS AND METHODS

Fecal Specimens

A total of 603 fecal specimens were collected from sporadic cases of acute gastroenteritis in pediatric clinics in five localities (Maizuru, Tokyo, Sapporo, Saga, and Osaka) in Japan from July 2005 to June 2006. The case definition of diarrhea used in this study was as at least three passings of unformed (loose and watery) stool a day. Acute gastroenteritis was defined as the occurrence of diarrhea and other symptoms such as vomiting, fever, and abdominal pain. 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 stored at −30°C until use for the detection of NoV.

Extraction of Viral Genome

The viral genomes were extracted from 140 μl of 10% fecal suspensions using a spin column technique according to the manufacturer's instructions (QIAGEN®, Hilden, Germany).

Reverse Transcription

For reverse transcription (RT), 4 μl of extracted viral genome was added to 4 μl of a reagent mixture consisting of 5× first strand buffer (Invitrogen, Carlsbad, CA), dNTPs (10 mM/μl) (Roche, Mannheim, Germany), DTT (Invitrogen), superscript reverse transcriptase III (Invitrogen), random primer (hexa-deoxyribonucleotide mixture) (Takara, Shiga, Japan), RNase inhibitor (Toyobo, Osaka, Japan), and MilliQ water. The total reaction mixture was 8 μl. The RT step was carried out at 50°C for 1 h, followed by 99°C for 5 min and then held at 4°C [Phan et al., 2005].

Polymerase Chain Reaction

Using multiple polymerase chain reaction (PCR) with two specific primer sets as previously reported resulted in the identification of NoV [Phan et al., 2005]. Primers G1SKF (CTGCCCGAATTYGTAAATGA) and G1SKR (CCAACCCARCCATTRTACA), and COG2F (CARGAR BCNATGTTYAGRTGGATGAG) and G2SKR (CC RCC NGCATRHCCRTTRTACAT) [where B was C, G or T; H was A, C or T; N was any base; R was A or G, and Y was C or T] that amplify both polymerase and capsid genes of NoV were used. PCR was carried out with 1 μl of cDNA in 10 μl of the reagent mixture containing 10× Taq DNA polymerase buffer (Promega, Madison, WI), dNTPs (2.5 mM/μl), primers (33 μM), Taq DNA polymerase (5 U/μl) (Promega) and MilliQ water. PCR was performed at 94°C for 3 min followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 60 sec, and a final extension at 72°C for 7 min, and then held at 4°C.

Electrophoresis

The PCR products were electrophoresed in a 1.5% agarose gel, followed by staining with ethidium bromide for 20 min and then visualized under ultraviolet (UV) light. The results were recorded by photography.

Nucleotide Sequencing and Phylogenetic Analysis

The nucleotide sequences of PCR products (DNA) positive for NoV were determined with the Big-Dye terminator cycle sequencing kit and an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Inc., Foster City, CA). Sequence analysis was performed using CLUSTAL X software (Version 1.6). Phylogenetic tree with 100 bootstrap resamples of the nucleotide sequence alignment data sets 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 the recombinant NoV sequences [Lole et al., 1999]. The nucleotide sequences of NoV strains detected in the study had been submitted to GenBank and the assigned accession numbers were EF028229–EF028234. Reference NoV strains and accession numbers used in this study were as follows: SaitamaT35a (AB112132), Chiba/030335 (AJ865512), Chiba/020247 (AJ865511), CG2 (DQ220749), CG18 (DQ220766), CG1 (DQ220748), Winchester (AJ277609), CU050335 (DQ522122), SaitamaKU4a (AB058535), Chiba/991013 (AJ865475), Mc124 (AY237412), Ehime/C3 (AY641756), Oy1-2/020115 (AB097920), OC03012 (AB186073), Chiba/030541 (AJ865500), Taipei-73 (DQ263720), Taipei-82 (DQ263721), K (AF406999), R2 (AF406993), 0937 (AY237483), SaitamaKU8 (AB067547), Chiba/030546 (AJ865502), SaitamaT2 (AB112103), Potsdam (AF439267), Chiba/020251 (AJ865492), CG6 (DQ220753), Saitama-T66e (AB112321), Birmingham (AJ277612), Honolulu (AF414403), Stav (AF145709), VA98115 (AY038598), DesertShield (U04469), 21B/04/Korea (DQ004637), Water-1B/04/Korea (DQ004658), SaitamaKU19a (AB058525), Chiba/030100 (AJ844469), IF2036/Iraq (AY675555), Boxer (AF538679), Otofuke (AB187514), CG3 (DQ220750), SaitamaT25 (AB112100), Hokkaido/C-40 (AY672744), Southampton (L07418), WestChester (AY502016), Norwalk/68 (M87661), KY-89 (L23828), Chiba/030308 (AJ865496), BS5 (AF093797), VA497 (AF538678), WUG1 (AB081723), Sindlesham (AJ277615), Chiba/040001 (AJ865506), Wisconsin (AY502008), Babbacombe (AM263418), Musgrove (AJ277614), AppalachicolaBay (AF414406), SzUG1 (AB039774), Kagoshima (AY356540), Thirtlehall (AJ277621), Chiba (AB022769), Valetta (AJ277616), NewOrleans (AF414402), Aichi8A (AB021995), CS-E1/02/USA (AY502009), Karachi/1001/90 (AB126940), CG20 (DQ220768), Katrina-17/05/US (DQ438972), Saitama T53 (AB112260), IF1998/Iraq (AY675554), SaKaeo-53/Thai (AY646860), Leeds (AJ277608), IdahoFall/96/US (AY054299), SaitamaU25 (AB039780), Amsterdam (AF195848), QW48/02/US (AY823303), 918/97/JP (AB074893), VA34/98/NL (AY077644), 43/97/JP (AB074892), K5/JP (AB221132), QW170/03/US (AY823306), QW101/03/US (AY823304), Girllington (AJ277606), Chitta (AB032758), Hawaii (U07611), Fayette/99/USA (AY502014), Neustrelitz (AY772730), Hillington (AJ277607), MOH (AF397156), Hokkaido133 (AB212306), S63 (AY682550), Mc37 (AY237415), Snow Mountain (U70059), Melksham (X81879), Kagoshima/C15/01/JP (AY353923), E3/97/Crete (AY682552), Fayetteville/98/US (AY113106), SaitamaU17 (AB039779), Miami/292 (AF414410), Saitam U3 (AB039776), Seacroft (AJ277620), Ueno7k (AB078337), C14/02/AU (AY845056), Picton/03/AU (AY919139), Kunming/146/CN (DQ304651), 5017/04/JP (AB242257), Toronto (U02030), Arg320 (AF190817), Chiba/030486 (AJ865562), Minato6 (AB233471), M7/99/US (AY130761), Chiba/991118 (AJ865516), Minato33 (AB233473), Chiba/021050 (AJ865554), Lordsdale (X86557), Camberwell (AF145896), MD145 (AY032605), Erfurt/007/00/DE (AF312518), FarmingtonHills (AY502023), Hunter284E/04O/AU (DQ078794), 95/96US (AF080549), Murine/US (AY228235), Alphatron (AF195847), Jena (AJ011099), CH126/NL (AF320625), J23/99/US (AY130762), Mex7076/99 (AF542090), and Chiba/040502 (AJ844470).

RESULTS

Detection of Norovirus Infections

A total of 603 fecal specimens collected from children with acute gastroenteritis from five different localities in Japan during July 2005 and June 2006 were examined for the presence of NoV. NoV was detected in 51 out of 632 (8.5%) specimens tested. The highest prevalence of NoV was found in children with the age range of 12–23 months (62.7%) and the lowest prevalence of NoV was found in children aged less than 6 months (5.9%). It was also found that children younger than 3 years old had a high rate of NoV infection (92.1%). NoV was detected almost year-round, except for July, September and October. The NoV infection was found highest in December (37.3%), followed by November and January (each of 17.6%). The lowest NoV detection rate fell into August (2%).

Distribution of Norovirus Genotypes With Emergence of GII/3

The partial nucleotide sequences of capsid gene of NoV detected in this study were compared to each other as well as to those of NoV reference strains available in GenBank by BLAST. It was found that based on the sequence analysis of the capsid gene of these NoV strains, NoV was divided into six distinct genotypes 1, 2, 3, 4, 6, and 7 within genogroup II (GII). Of these, the GII/3 (known as the Toronto virus cluster) was the most predominant genotype with the prevalent rate of 52.9%, followed by 37.2% of GII/4 (the Lordsdale virus cluster) and 3.9% of GII/6 (the Seacroft virus cluster) (Table I).

Existence of Multiple Recombinant Noroviruses

To further characterize the NoV genome and to verify the changing epidemiology of NoV genotypes, their RNA polymerase genes were also analyzed. By BLAST, the sequences of both capsid and polymerase genes of the strain 7107JP (belonging to the GII/1) and the strain 7297JP (belonging to the GII/7) were highly identical (98–99%) to those of the recombinant NoV Picton/03/AU (the GIIb polymerase and the GII/1 capsid) [Bull et al., 2005] and the recombinant NoV Kunming/146/CN (the GII/6 polymerase and the GII/7 capsid) [Phan et al., 2006a], respectively. Within 27 GII/3 NoVs, the sequences of both capsid and polymerase genes of 5 (18.5%) and 22 (81.5%) strains were similar with those of the recombinant NoV 5017/04/JP (the GII/4 polymerase

TABLE I. Distribution of Norovirus Genotypes Circulating in Children With Acute Gastroenteritis in Five Different Localities in Japan

Date of fecal specimen collection	Number of fecal specimens tested	Norovirus positives (%)	Genogroup II					
			Genotype 1	Genotype 2	Genotype 3	Genotype 4	Genotype 6	Genotype 7
June 2005–July 2006	603	51 (84.6)	1 (2)	1 (2)	27 (52.9)	19 (37.2)	2 (3.9)	1 (2)

and the GII/3 capsid) [Phan et al., 2006b] and the new variant C14/02/AU (the GIIb polymerase and the GII/3 capsid) [Bull et al., 2005], respectively (Table II). Taken together, the results clearly indicated that these NoVs were also recognized as recombinants. In contrast, all NoV strains belonging to the GII/2, the GII/4, and the GII/6, the genotypes remained the same no matter that the polymerase or capsid regions were analyzed.

A Novel Nomenclature for Noroviruses

In an attempt to understand the molecular basis of NoV genetic heterogeneity and diversity, the N/S domain sequences of the capsid gene of NoV strains detected in this study and worldwide NoV reference strains were used for genetic analysis. Phylogenetic analysis identified seven distinct genogroups from I to VII (Fig. 1A). The genogroups I, IV, VI, and VII were found exclusively in humans. The genogroups III and V infected only in bovine and murine, respectively. It should be noted that genogroup II consisted of both the human NoV strains and the porcine NoV strains. The porcine NoVs made three distinct clusters and represented genotypes 21–23 within GII. These NoVs shared the higher identity (74–76%) to human NoV strain Miami/292/US of the genogroup II than other animal (bovine and murine) NoVs (58–63%). NoV GI and GII were further subdivided into 16 genotypes with 32 subgenotypes and 23 genotypes with 34 subgenotypes, respectively (Fig. 1B,C). NoV GIII in bovine was classified into only two genotypes 1 and 2, representing by the Jena strain and the CH126/98/NL strain, respectively. It was found that the nucleotide sequence divergence between different genotypes in the same genogroup was 12–26%. The sequence variation in different genogroup was considerably higher, ranging from 34% to 44% (Table III).

Alignment of the Amino Acid Sequences of Norovirus Capsid Gene

Direct inspection of the amino acid sequence alignment of NoV capsid gene revealed that there are multiple short signature sequences of capsid, which correctly defined the phylogenetic NoV genogroups, genotypes and subgenotypes, were found. For differentiation within NoV genogroups, four amino acids at positions 101–104 of S domain and two amino acids at positions 522–523 of C terminus formed an identification code specific for each genogroup, for example, NGWV and SR for genogroup I; NSYA and SS for genogroup IV. Interestingly, the signature motif of C terminus also could distinguish two distinct genotypes 1 and 2 within NoV genogroup III, SL and SF for GIII/1 and GIII/2, respectively (Fig. 2A). For differentiation within genotypes of NoV genogroup I, seven short amino acid motifs including one motif located at P1-1 domain and six motifs located at P2 domain were found, for example, the motif 2 at amino acids 294–296 formed identification codes TQFG for GI/1 and SGQ for GI/4; the motif 5 at amino acids 333–336 formed identification codes TQFG for GI/1 and SPTT for GI/16. Of note, these motifs can differentiate some subgenotypes of NoV genogroup I, for example, the motif 6 at amino acids 340–343 formed an identification code TQSN for only GI/4c (Fig. 2B). For differentiation within genotypes and subgenotypes of NoV genogroup II, there were seven signature sequences located at different regions of capsid gene, including N terminus, S domain, P1-1 domain, P2 domain and P1-2 domain, for example, the motif 3 at amino acids 290–293 formed an identification code VNAH for GII/2 and INQK for GII/12; the motif 6 at amino acids 396–402 formed identification codes NQWALPN and DQWALPS for GII/1a and GII/1b, respectively (Fig. 2C).

Identification of Multiple Novel Recombinations in Noroviruses

In the study, eight novel recombinant NoV strains were detected. These novel recombinant NoVs were further divided into three different types (intergenogroup, intergenotype and intersubgenotype) of recombination. Of these, intergenogroup and intersubgenotype recombinations were first found in this study. In the intergenogroup recombination, the strain Mex7076/99 together with the J23/99/US strain formed the NoV genogroup VI based on the capsid gene sequence; however; the strain Mex7076/99 clustered into the genogroup II when the polymerase-based grouping was performed (Fig. 3A). In the genogroup II, the polymerase sequence of the strain Mex7076/9 had the closest march (77%) with that of the strain Virginia and also represented the novel genotype. In the intergenotype recombination, six novel recombinant NoVs were identified (Fig. 3B). Of these, two strains belonged to the genogroup I and four strains belonged to the genogroup II. These NoV strains clustered into two distinct genotypes when their polymerase- and capsid-based groupings were performed. Interestingly, another NoV strain, the Miami292, belonged to two different subgenotypes, the subgenotype GII/6a (known as the SaitamaU17 virus cluster) and the subgenotype GII/6b

TABLE II. Characteristics of Multiple Recombinant Noroviruses, Including Newly Identified Recombinant Noroviruses, Found in the Study

Strain	Detection	Type of recombination	Polymerase		Capsid		Number (%)
			Genogroup/genotype	Representative	Genogroup/genotype	Representative	
C14/02/AU-like recombinant	Previously reported	Intergenotype	GIIb	Oberhausen	GII/3	Toronto	22 (43.1)
Picton/03/AU-like recombinant	Previously reported	Intergenotype	GIIb	Oberhausen	GII/1	Hawaii	1 (2)
Kunming/146/CN-like recombinant	Previously reported	Intergenotype	GII/6	SaitamaU3	GII/7	Leeds	1 (2)
5017/04/JP-like recombinant	Previously reported	Intergenotype	GII/4	Lordsdale	GII/3	Toronto	5 (9.8)
SaitamaT25	Novel, in this study	Intergenotype	GI/11	SaitamaKU8	GI/14	Otofuke	—
SaitamaKU4a	Novel, in this study	Intergenotype	GI/13	SaitamaT35a	GI/7	Winchester	—
Hokkaido133	Novel, in this study	**Intergenotype**	GII/19	Minato6	GII/5	MOH	—
SaitamaT66e	Novel, in this study	Intergenotype	GII/19	Minato6	GII/3	Toronto	—
CS-E1/02/USA	Novel, in this study	Intergenotype	GII/5	MOH	GII/11	Karachi/1001/90	—
Neustrelitz	Novel, in this study	Intergenotype	GII/5	MOH	GII/15	Fayette/99/USA	—
Miami292	Novel, in this study	**Intersubgenotype**	GII/6b	SaitamaU3	GII/6a	SatamaU17	—
Mex7076/99	Novel, in this study	**Intergenogroup**	GII/novel	None	GVI	J23/99/US	—

Boldface represents three different representative types of recombination in noroviruses.
Number of different kinds of recombinant noroviruses in 51 noroviruses detected.

(known as the SaitamaU3 virus cluster), when its polymerase- and capsid-based grouping was performed, respectively. This kind of phenomenon was recognized as an intersubgenotype recombination.

Norovirus Recombination in ORF1/ORF2 Overlap

Intergenotype recombination. To localize the potential recombination site of intergenotype recombination, the representative recombinant strain Hokkaido133 was used to analyze. When the nucleotide sequence of the Hokkaido133 was compared with that of the MOH using SimPlot, an apparent region of genetic recombination was found at the polymerase-capsid overlap (Fig. 4A). Before this junction, the homology of the Hokkaido133 and the MOH was distinctly different. After this junction, however, they were highly homologous. Using CLUSTAL X, the Hokkaido133 shared a high level of nucleotide identity (96%) in the capsid region and a low level of the nucleotide identity (74%) in the polymerase region with the MOH. The results demonstrated that the nucleotide sequences of polymerase regions in these two strains were notably different, but their capsid sequences were identical.

Intersubgenotype recombination. It was found that the capsid sequence of the Miami292 had the closest march to that of the SaitamaU17 (95%). However, the Miami292 had the higher identity to the SaitamaU3 (96%) than the SaitamaU17 (90%) when the polymerase- and capsid-based grouping was performed. Up stream to this overlap, the SimPlot analysis showed a sudden drop in the nucleotide identity for the SaitamaU17 but not for the Miami292 and the SaitamaU3. When the nucleotide sequence of the Miami292 was compared with those of the SaitamaU17 and the SaitamaU3 using the SimPlot software, region of genetic recombination was found at the overlap of ORF1 and ORF2 (Fig. 4B).

Alignment of the deduced sequences of norovirus polymerase and capsid regions. To establish the evidence of NoV recombinant site at ORF1/ORF2 overlap, the nucleotide sequence alignment of NoV polymerase and capsid regions using CLUSTAL X interestingly found that the worldwide NoV strains covering all genogroups, except for the genogroups VI and VII (their data were not available in GenBank) shared the 13 nucleotide-highly conserved region including the last 13 nucleotides of polymerase and the first 9 nucleotides of capsid (Fig. 4C). Obviously, this region contained the partial 8 nucleotide–polymerase/capsid overlap.

DISCUSSION

Overall, the prevalent rate of NoV infection in children with acute gastroenteritis in Japan was 8.5% in all age groups of the subjects included in the study. However, the prevalent rate was increased up to 92.1% in children less than 3 years old. The highest

TABLE III. The Homology Matrix of Capsid Sequences of Seven Norovirus Genogroups

Genogroup	Strain	Norwalk/68	Lordsdale	Jena	Alphatron	Murine/USA	J23/99/USA	Chiba/040502
Homology (%)								
I	Norwalk/68		47	56	47	47	48	47
II	Lordsdale	56		46	54	46	60	55
III	Jena	60	54		45	46	47	45
IV	Alphatron	57	67	57		48	55	55
V	Murine	56	54	59	57		47	47
VI	J23/99/USA	57	67	56	66	55		56
VII	Chiba/040502	57	67	56	68	57	66	

The nucleotide homology of N/S domain sequences is shown in the lower left area and the nucleotide homology of full capsid gene sequences is shown in the upper right area.

infection of cases was in the 12–23 months age group, and the incidence decreased with increasing age over 24 months. Quite possibly, 12–23-year-old children might lack antibody protection to NoV, whereas by the time they have reached the age of 24 months they have begun to acquire viral immunity. The result also indicated that NoV is an important enteropathogen in causing diarrheal illness in children in Japan. In some reports, NoV was predominant in cold season, and several studies did not find a seasonal correlation [Bon et al., 1999; White et al., 2002; Oh et al., 2003; Reuter et al., 2005]. The finding was in line with the surveillance on pediatric cases of viral gastroenteritis in Japan, which demonstrated that the main peak of NoV infection was in the period of November, December, and January [Inouye et al., 2000; Iritani et al., 2002, 2003; Phan et al., 2006b].

The genetic analysis revealed that the NoV strains in this study belonged to only one distinct GII. This result confirmed that NoV GII was the dominant group associated with acute gastroenteritis in Japan. According to the previously published reports, NoV belonging to the Lordsdale virus cluster (known as the GII/4) was the most predominant genotype in sporadic cases as well as in outbreaks of gastroenteritis in children not only in Japan but also in many other countries which conduct molecular epidemiological studies on NoV infection [White et al., 2002; Oh et al., 2003; Lopman et al., 2004; Phan et al., 2006b]. However, the changing pattern of genotype distribution of NoV infection in children with acute gastroenteritis has been demonstrated in the study. It is surprisingly noted that NoV GII/3 (know as the Toronto virus cluster) was the most prevalent with a high frequency (52.9%) compared to the lower frequency NoV GII/4 (37.2%) and NoV GII/6 (3.9%), which were the second and third prevailing genotypes, respectively. To verify this unusual phenomenon, the polymerase regions of these NoV strains were further characterized. Interestingly, four different kinds of recombinant NoVs were found to be co-circulating in association with acute gastroenteritis in Japan. Within a total of 27 recombinant NoVs with the GII/3 capsid, 81.5% were the C14/02/AU-like recombinant variants with the GIIb polymerase which were recently reported to cause outbreaks as well as sporadic cases of acute gastroenteritis throughout European countries and Australia [Buesa et al., 2002; Bull et al., 2005; Maunula and Von Bonsdorff, 2005; Reuter et al., 2005]. Obviously, recombination in NoV gave rise to novel viral strains capable of emergence in human population. In Japan, this GIIb variant was first recovered from a male patient aged 2 years who developed symptoms of acute gastroenteritis in Saga City in 2003 [Phan et al., 2006b]. The rapid emergence in the number of the variant strain from 4% in 2003–2004 to 81.5% in 2005–2006 indicated that this variant was still virulent in causing the illness in Japan. Further surveillance of diarrheal viruses should be conducted to determine whether this recombinant variant will be dominant in Japan in the coming year.

To gain further insights into the genetic variability of NoV, capid gene of NoVs were used for genetic analysis.

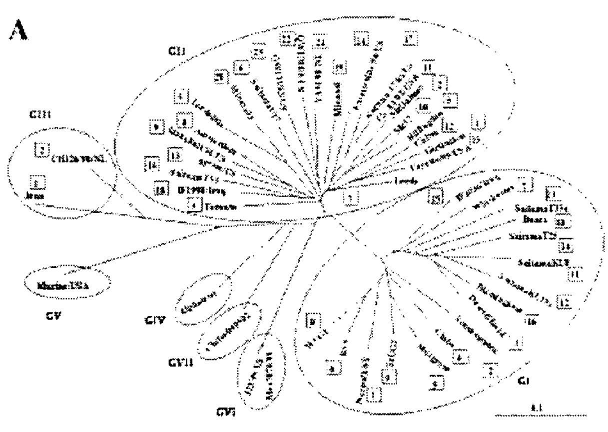

Fig. 1. Novel nomenclature of norovirus. The trees were constructed from N/S domain nucleotide sequences of capsid gene of worldwide norovirus strains. Reference strains of norovirus were selected from GenBank under the accession numbers indicated in the text. The scale indicates nucleotide substitutions per position. **A**: Schematic presentation of seven distinct norovirus genogroups from I to VII. Number in the square indicates norovirus genotype. **B**: Novel classification of norovirus strains within the genogroup I. Norovirus strain was classified into multiple genotypes and subgenotypes. **C**: Novel classification of norovirus strains within the genogroup II. Norovirus strain was classified into multiple genotypes and subgenotypes. The Japanese norovirus detected in the study was highlighted in italics. * indicated the genotypes or subgenotypes containing the Japanese norovirus detected in the study.

The N/S domain of the capsid gene is predicted to be well suited for the genotype classification of circulating NoV strains [Katayama et al., 2002]. This domain has been widely used to determine the molecular epidemiology of NoV genotypes [Kageyama et al., 2004; Okada et al., 2005; Seto et al., 2005; Phan et al., 2006a]. The alignment of a N/S domain nucleotide sequence from a number of NoVs detected in different parts of the world demonstrated seven distinct genogroups, which were further divided into multiple genotypes and subgenotypes. Of note, NoV GII/4 was classified into different subgenotypes represented by different NoV variant strains. It is helpful to describe the evolution of NoV GII/4 and to explain the predominance of NoV GII/4 around the world. Another interesting finding of this study was the discovery of a wide range (up to 16) of short amino acid motifs, which could differentiate NoV strains into phylogenetic genogroups, genotypes and subgenotypes. These motifs were exclusively present in only one cluster and consistently changed according to each cluster. Of these, as many as nine signature motifs were located at hypervariable region

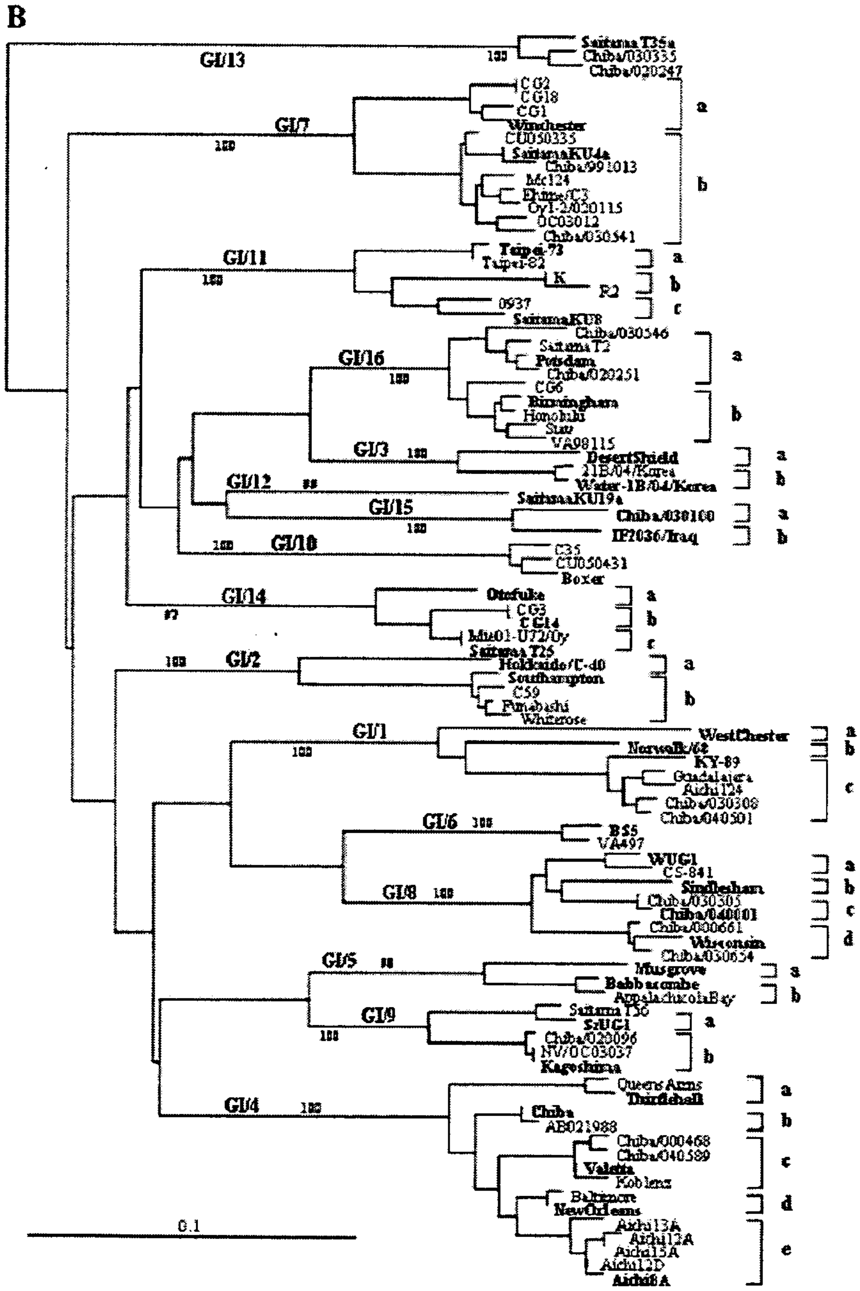

Fig. 1. (*Continued*)

P2 of capsid gene. Three signature motifs located at the N/S domain should be noted. The identification of these motifs in capsid genes of NoVs are important because it may help further studies to identify novel epitopes located in capsid genes of NoVs and then may help to explain the different antigenicity for different genogroups, genotypes and subgenotypes of NoVs.

Based on the novel scheme of the NoV genogroup II, the NoV strains 7029JP and 6931JP detected in the study, which belonged to the genotype 6 (known as the SaitamaU3 virus cluster), did not group any genetic cluster and therefore made a novel subgenotype GII/6c. Interestingly, nineteen NoV GII/ 4 strains did not group into the prototype Lordsdale virus cluster (known as the subgenotype GII/4b), but they together with the variant Farmington Hills virus and the variant Hunter virus made two distinct subgenotype GII/4e (57.9%; 11 of 19) and subgenotype GII/4f (42.1%; 8 of 19), respectively. It was found that the genogroup II contained both human NoV strains and the porcine NoV strains from Japan and the United States. The porcine NoVs made three distinct genotypes 21–23 and shared the higher identities, ranging from 74% to 76%, at the nucleotide level to human NoV strain Miami/292/US than the bovine and murine NoVs with only 58–63%. Altogether, in view of NoV evolution, a genomic relation might exist between human and porcine strains.

RNA recombination plays a key role in virus evolution and it shapes a good deal of the virus diversity [Worobey and Holmes, 1999]. Even though there are now several

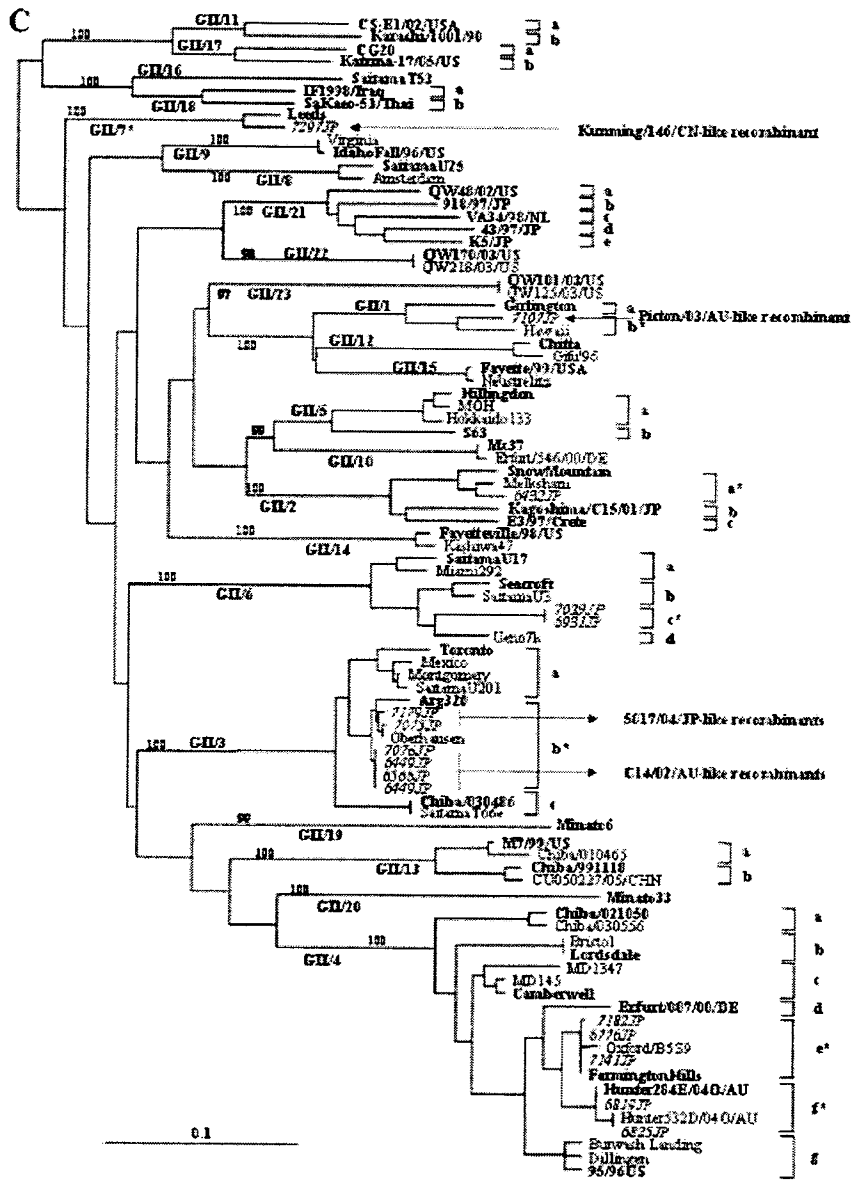

Fig. 1. (*Continued*)

molecular epidemiological studies on intergenotype recombinant NoV infection worldwide, reports documenting other recombination in NoV are not available. Remarkably, two novel types of recombination in NoV, intergenogroup recombination and intersubgenotype recombination were found. These results are noteworthy because this is the first report of intergenogroup recombination and intersubgenotype recombination. These findings warned the thread that zoonoses could occur in humans and highlighted a possible route of zoonoses because porcine, bovine and murine NoVs belong to genogroups II, III, and V, respectively. Another interesting finding was the identification of multiple hitherto unreported intergenotype

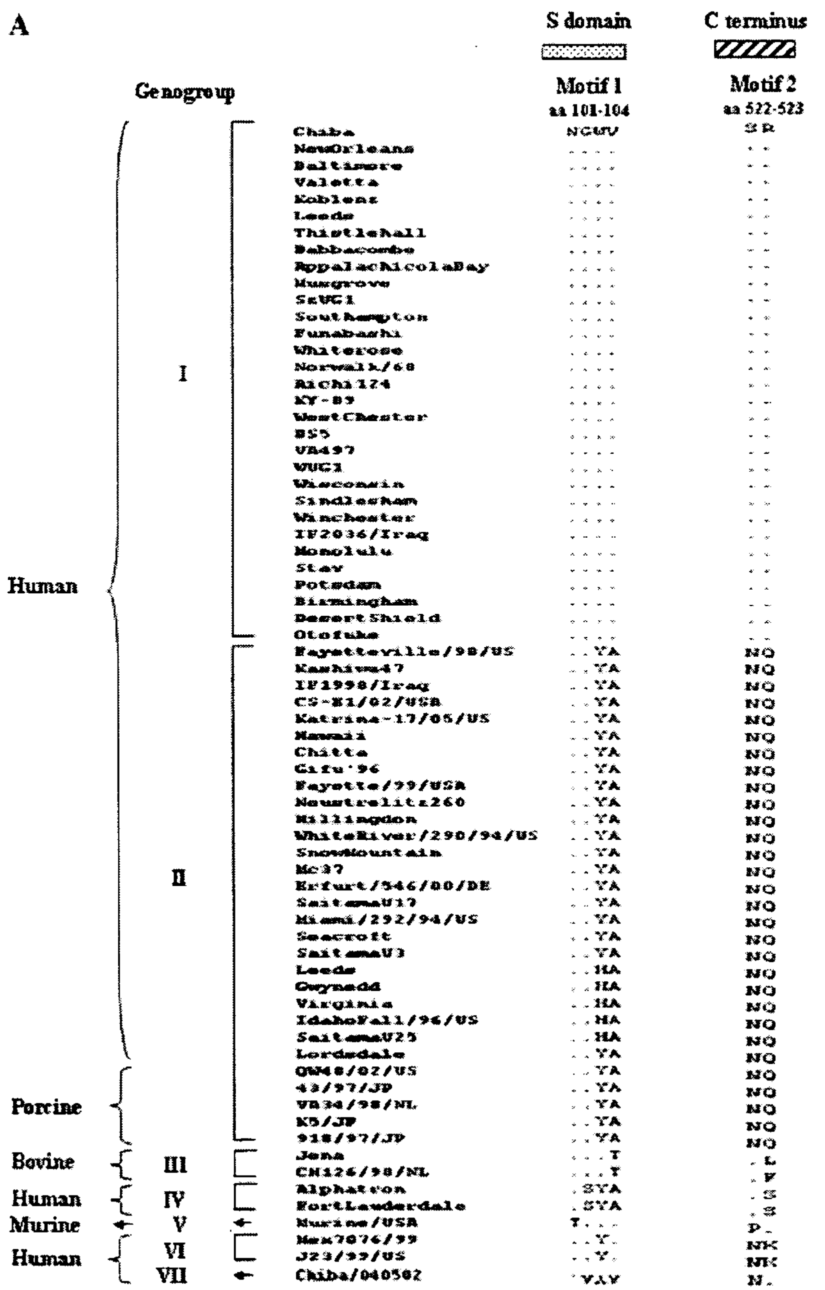

Fig. 2. Identification of 16 short amino acid motifs located at N terminus, S domain, P1 domain, P2 domain, and C terminus of norovirus capsid gene. A: The motifs correctly defined the phylogenetic norovirus genogroups. B: The motifs correctly defined the phylogenetic norovirus genogroup I. *, the norovirus strains used could be divided into subgenotypes. C: The motifs correctly defined the phylogenetic norovirus genogroup II. *, the norovirus strains used could be divided into subgenotypes.

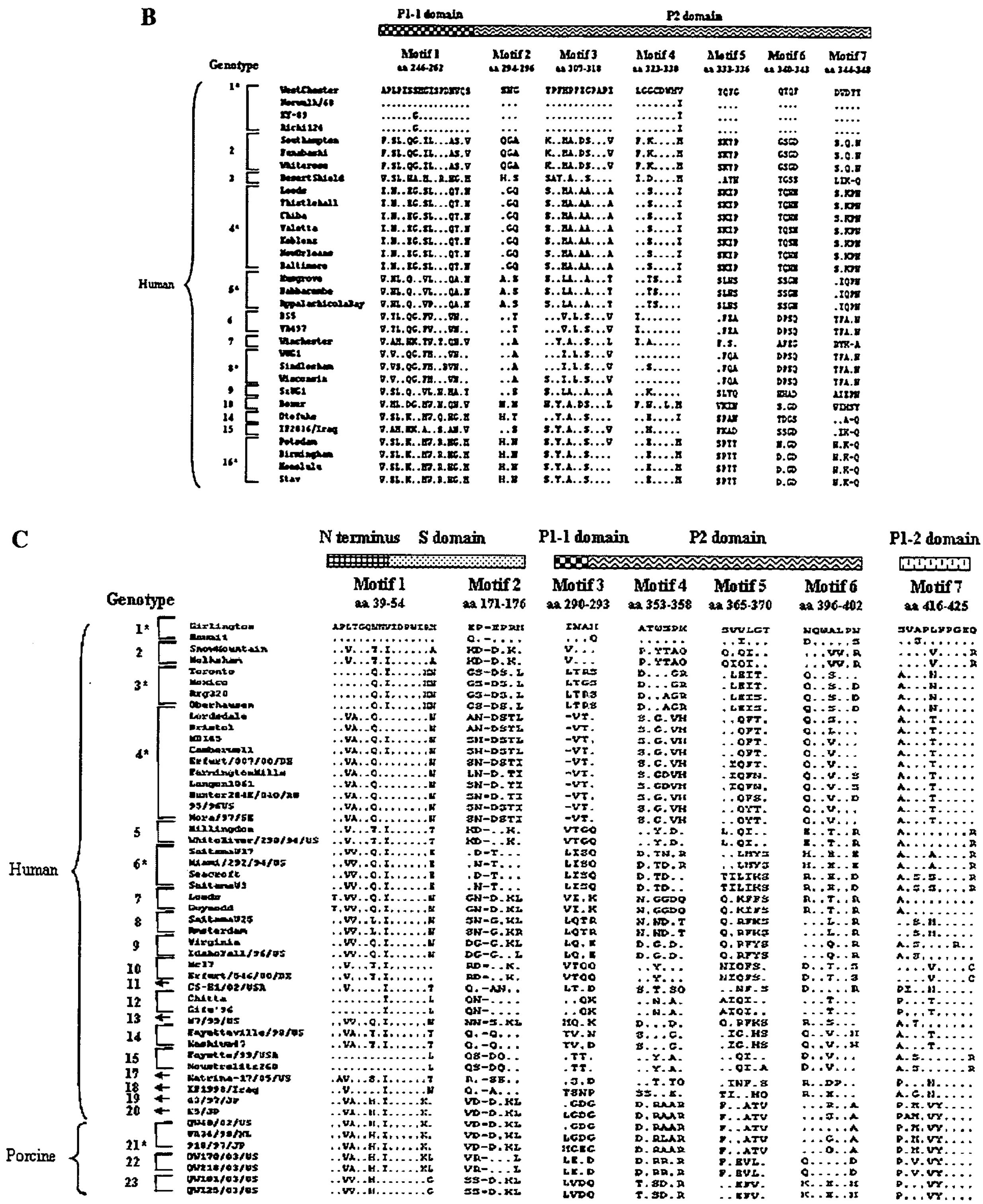

Fig. 2. (*Continued*)

recombinant NoVs in this study. These results provide evidence that recombination in NoV is not rare and these NoV strains fulfilled theoretically all prerequisites for recombination.

Breakpoint analysis of intergenotype recombinant NoVs previously reported suggested that the recombination site occurred at ORF1/ORF2 overlap [Hardy et al., 1997; Jiang et al., 1999; Katayama et al., 2002;

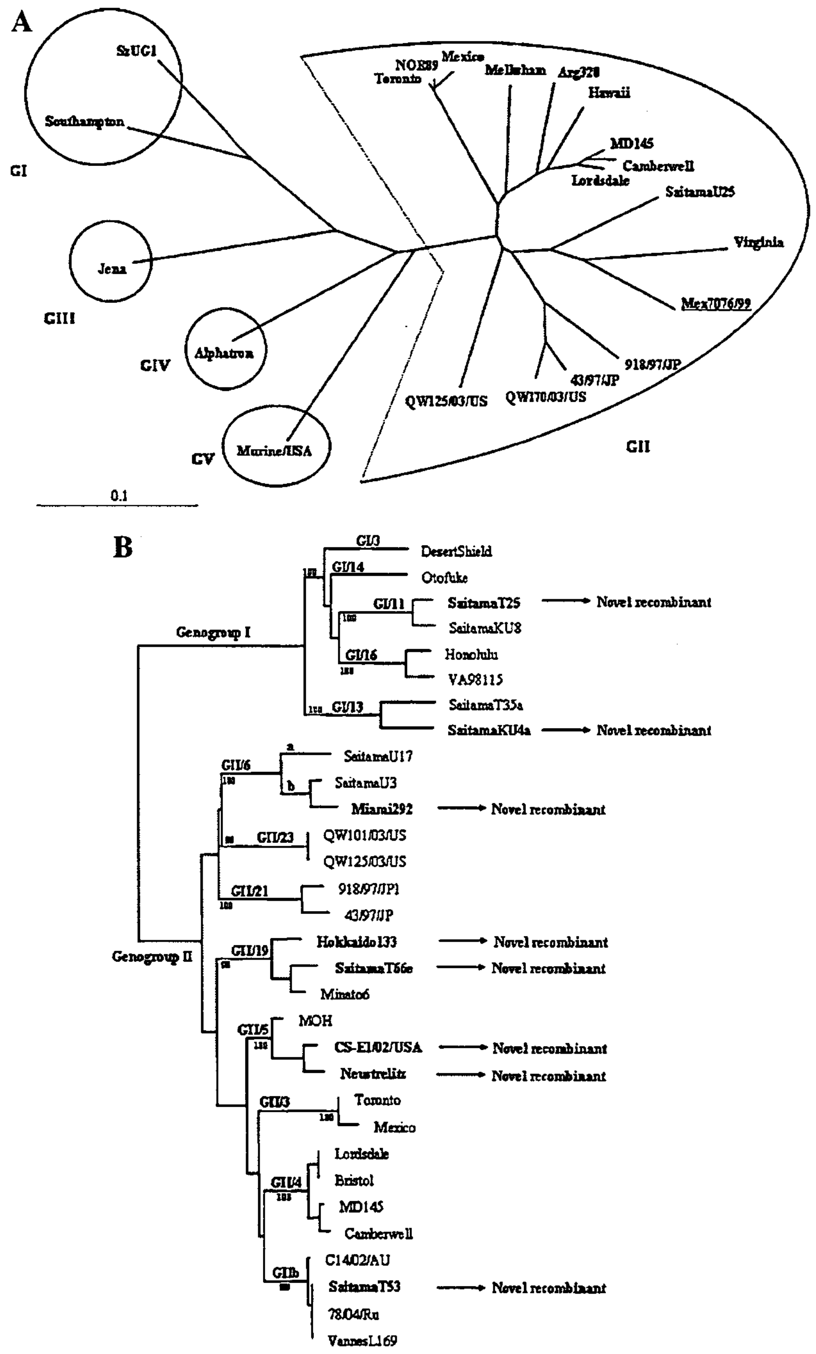

Fig. 3. Identification of novel recombinant G9 rotavirus. **A**: Nucleotide identity comparison and bootscanning plots of the complete sequence of VP7 gene of the CIT-254 as a query strain with those of the R136 (known as G9) as a reference strain, the 87H140 (known as G3) as a reference strain, and the LL4260 (known as G5) as an outgroup strain. **B**: Observation of changes of norovirus genogroups and genotypes on the basis of phylogenetic trees of nucleotide sequences. The trees were constructed from partial nucleotide sequences of polymerase region of the worldwide norovirus strains. Reference strains were selected from GenBank under the accession numbers indicated in the text. The scale indicates nucleotide substitutions per position. A: Schematic presentation of distinct norovirus genogroups. The intergenogroup recombinant Mex7076/99 was highlighted in underline. B: Identification of seven recombinant norovirus strains within the genogroup I and genogroup II.

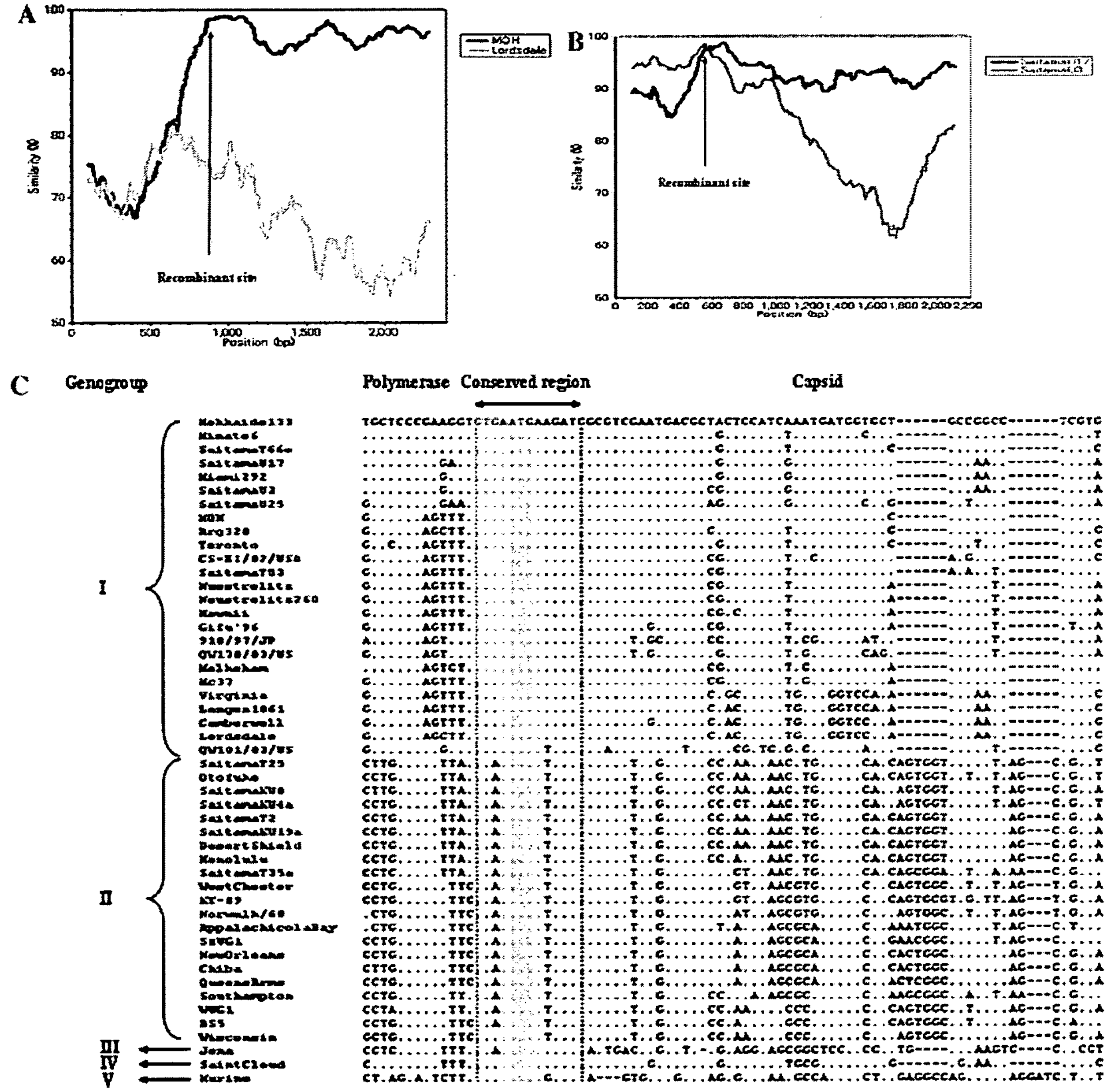

Fig. 4. Norovirus recombination in ORF1/ORF2 overlap. A: The Simplot analysis of the norovirus representative strain of the intergenotype recombination, the Hokkaido133 as the query strain, the reference strain MOH and the reference strain Lordsdale. The low and high homologies with the polymerase and capsid regions among them were found. B: The Simplot analysis of the norovirus strain of the intersubgenotype recombination, the Miami292 as the query strain, the reference strain SaitamaU17 and the reference strain SaitamaU3. The high and low homologies with the polymerase and capsid regions among them respectively were found. C: Direct inspection of the nucleotide sequence alignment of the norovirus polymerase and capsid regions. The 13 nucleotide-highly conserved region covering the partial ORF1/ORF2 overlap was found. The sequence data of the ORF1/ORF2 overlap of the genogroup VI and the genogroup VII were not available in GenBank.

Bull et al., 2005]. In the study, using Simplot the multiple novel intergenotype and intersubgenotype recombinations were also at this junction. Direct observation of the nucleotide sequence alignment of NoV polymerase and capsid genes revealed that the worldwide NoV strains, regardless different genogroups or different genotypes, shared the conserved region, covering this junction. This conserved region suggested that the recombination event occurred when two parental strains come into physical contact when both of them co-infected one cell. This conserved region might represent the break and rejoin site for recombination during viral replication.

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ORIGINAL ARTICLE

Detection and Genetic Characterization of Norovirus in Oysters from China and Japan

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SUMMARY

A total of 225 oysters from China and Japan were collected during October 2005 to September 2006 and were then tested for the presence of norovirus by RT-nested PCR. The detection rate of norovirus was different between China and Japan, accounting for 14.6% (19 of 130) and 25.3% (24 of 95), respectively. In China, norovirus in oyster was detected continuously from July to February with the highest prevalence in August, October and November (each of 21%, 4 of 19). On the other hand, norovirus in Japan was found year-round with highest prevalence in March and October (each of 20.8%, 5 of 24). Norovirus strains detected were subjected to further characterization by sequence analysis. It was found that the norovirus strains belonged to only two distinct genotypes, the GII/3 (known as the Mexico virus cluster) and the GII/4 (known as the Lordsdale virus cluster). In China, the norovirus GII/4 was the most predominant, accounting for 78.9% (15 of 19). In contrast, it was interesting that both the norovirus GII/4 and the norovirus GII/3 were co-predominant with a prevalence of 50% (12 of 24) in Japan. Another interesting feature of the study was that the norovirus GII/4 strains in oysters from both countries were grouped into two distinct variant clusters known as the Farmington Hills variant and the Hunter variant. More than 10^2 copies of norovirus were detected in 41 of 43 oysters. This study provided additional evidence of the presence of norovirus in oysters and is also the first report to demonstrate the existence of norovirus variants in oysters. (Clin. Lab. 2007;53:405-412)

KEY WORD

norovirus, oysters, Japan, China

INTRODUCTION

Acute gastroenteritis has been demonstrated as a major cause of morbidity and mortality of children in both developed and developing countries. The mortality among children due to acute gastroenteritis is greater in developing than in developed countries (1, 2). It has been well established that virtually every child becomes infected with a rotavirus at least once by 3 years of age. Despite much progress in the understanding of the pathogenesis and of management with the widespread use of oral rehydration therapies, acute gastroenteritis consistently ranks as one of the top causes of death worldwide (1-4). Norovirus is one of the leading agents of acute gastroenteritis worldwide and causes outbreaks in various epidemiological settings such as restaurants, schools, day care centers, hospitals, nursing homes, and cruise ships (5-10). Norovirus is the distinct genus within the family *Caliciviridae*. Norovirus contains a positive sense single-strand RNA genome surrounded by an icosahedral capsid. The norovirus genome contains three ORFs (ORF1, 2 and 3). The ORF1 encodes non-structural proteins, the ORF 2 encodes the capsid protein, and the ORF3 encodes a small capsid protein (11).
The transmission modes of norovirus are classified into food-borne, water-borne, air-borne, person-to-person spread

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and might be by other unknown routes (5-8, 10, 12). Norovirus is highly infectious and spreads by ingestion of contaminated food such as oysters and water. These characteristics make norovirus a major public health concern. Oysters are filter feeders, and hence concentrate the viruses from contaminated water. As a result, consumption of virus-contaminated oysters represents a significant threat to oyster consumers as well as an economic threat to the seafood industry. Recently, oysters were reported to be responsible for viral outbreaks and sporadic cases of acute gastroenteritis around the world, and the number of cases of norovirus-related food poisoning has been increasing (13-16). For diarrheal disease control to be successful, continuous monitoring of the norovirus contamination of oysters is needed. Moreover, study of the molecular epidemiology of norovirus provides knowledge on the diversity of the specific types found in oysters, which is important in an effort to design suitable and effective norovirus control strategies.
Thus, in this study we determined the occurrence of norovirus contamination in oysters from China and in Japan during 2005-2006, characterized norovirus detected according to genogroup and genotype, and quantified the norovirus genome in these oysters.

MATERIALS AND METHODS

Oyster samples
A total of 225 oysters including 130 from Fujian Sheng, China and 95 from four different localities (Miyagi, Ibaraki, Mieken, and Nagoya) in Japan were collected during October 2005 to September 2006. The intestines of the oysters were removed by dissection and then weighed. Virus extraction and concentration were performed by using the modified method previously reported (17). The supernatants were collected and stored at –30 °C until use for the detection of norovirus.

Extraction of viral genome
The viral genomes were extracted from 140 µl of intestine suspensions using a spin column technique according to the manufacturer's instructions (QIAGEN®, Hilden, Germany).

Reverse transcription
For reverse transcription (RT), 4 µl of extracted viral genome was added to 4 µl of a reagent mixture consisting of 5X first strand buffer (Invitrogen, Carlsbad, CA, USA), dNTPs (10 mM/µl) (Roche, Mannheim, Germany), DTT (Invitrogen), superscript reverse transcriptase III (Invitrogen), random primer (hexa-deoxyribonucleotide mixture) (Takara, Shiga, Japan), RNase inhibitor (Toyobo, Osaka, Japan), and MilliQ water. The total reaction mixture was 8 µl. The RT step was carried out at 50 °C for 1 h, followed by 99 °C for 5 min and then held at 4 °C (18).

Table 1: Detection of norovirus in oysters from China and Japan during 2005-2006.

Country	Total of samples	Norovirus positives (%)
China	130	19 (14.6%)
Japan	95	24 (25.3%)

Polymerase chain reaction
Using nested polymerase chain reaction (PCR) with specific primers as previously reported resulted in the identification of norovirus (18). The RT-PCR was performed by using primers COG1F and G1SKR, and COG2F and G2SKR for amplification of the partial capsid genes of norovirus. The second amplification was performed using the first PCR product as the template with primers G1SKF and G1SKR, and G2SKF and G2SKR. These primers specifically generated five different sizes of amplicons of 330 bp and 344 bp for norovirus genogroup I and norovirus genogroup II, respectively. PCR was carried out with 1 µl of cDNA in 10 µl of the reagent mixture containing 10x Taq DNA polymerase buffer (Promega, Madison, WI, USA), dNTPs (2.5 mM/µl), primers (33 pM), Taq DNA polymerase (5 U/µl) (Promega) and MilliQ water. PCR was performed at 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s, and a final extension at 72 °C for 7 min, and then held at 4 °C.

Electrophoresis
The PCR products were electrophoresed in a 1.5% agarose gel, followed by staining with ethidium bromide for 20 min and then visualized under ultraviolet light. The results were recorded by photography.

Quantitation of norovirus gemone
The norovirus capsid genes in oysters were quantified by using real-time PCR as previously described (14). The real-time PCR mixture contained 4 µl of cDNA, 17.5 µl of TaqMan universal PCR master mix (Applied Biosystem), a 100 pM concentration of each primer, and fluorogenic probes. The following PCR protocol was used: 2 min at 50 °C and 10 min at 95 °C, 50 cycles of 95 °C for 15 s, and 56 °C for 60 s.

Nucleotide sequencing and phylogenetic analysis
The nucleotide sequences of PCR products (DNA) positive for norovirus were determined with the Big-Dye terminator cycle sequencing kit and an ABI Prism 310 Genetic Analyzer (Applied Biosystems Inc.). Sequence analysis was performed using CLUSTAL X software (Version 1.6). A phylogenetic tree with 100 bootstrap resamples of the nucleotide sequence alignment data sets was generated using the neighbor-joining method with