TABLE I. Prevalence of NoV and SaV Infections in Pediatric Patients Hospitalized With Acute Gastroenteritis in Chiang Mai, Thailand From 2002 to 2004

Year	No. of samples collected	No. of samples positive for virus (%)	
		NoV (GII)	SaV
2002	43	6 (14.0)	0 (0.0)
2003	45	5 (11.1)	2 (4.4)
2004	160	24 (15.0)	1 (0.6)
Total	248	35 (14.1)	3 (1.2)

and GII/16 (Fig. 1). It was observed that GII/4 was the most predominant genotype and accounted for the majority (22 of 35; 62.8%) of NoV isolates detected in the present study. In addition to the GII/4, NoV GII/3 was detected as the second most common genotype and accounted for 20% (7 of 35) of the NoV isolates. The prevalence rates of GII/1 and GII/7 genotypes were equal at 5.7% (2 of 35 isolates). Moreover, a relatively low frequency of GII/2 and GII/16 genotypes at 2.9% (1 of 35) was observed.

Detection and Genotype Distribution of Sapoviruses

Of 248 samples obtained from infants and young children hospitalized with diarrhea, 3 (1.2%) were found positive for SaV by the RT-PCR screening method (Table I). The detection rate of SaV was definitely much lower (1.2%; 3 of 248) than that of NoV (14.1%; 35 of 248). All of the SaV positive samples were identified as a single SaV infection, with none being a mixed infection between NoV and SaV. The age of patients ranged from 8 months to 1-year-old. Of these, all three SaV isolates were directly sequenced and phylogenetically analyzed based on partial nucleotide capsid regions. The phylogenetic tree shown in Figure 2 was generated based on the clustering methods described by Phan et al. [2007b]. From the phylogenetic tree, great genetic diversity of SaV genotypes was observed. All three SaV isolates were clustered exclusively with other human SaV reference strains and classified further into two distinct genogroups. One of the three SaV isolates was clustered with GIV genogroup, while the other two SaV isolates belonged to two distinct genotypes of the GI cluster, GI/1 and GI/2 genotypes, respectively.

DISCUSSION

Based on early antigenic analyses, and more recently extensive sequence analysis, the circulations of both NoV and SaV in nature have been shown to be highly variable. Genetic analysis of NoV in Chiba, Japan from 1999 to 2004 demonstrated that 31.8% of samples collected from sporadic or outbreak cases were positive for NoV. Phylogenetic analysis of these NoV isolates showed a great genetic diversity and at least 13 and 16 genotypes were identified in GI and GII genogroups, respectively [Okada et al., 2005]. Recently, molecular epidemiological studies of SaV have been conducted in several countries, including Australia, Japan, Thailand, UK, USA, and Vietnam, and it was found that the rates of detection and overall prevalence of SaV infections varied from one country to another, with usually much less frequency than NoV infections [Hansman et al., 2004, 2006; Blanton et al., 2006; Gallimore et al., 2006; Nguyen et al., 2007; Phan et al., 2007a].

In Thailand, the first NoV and SaV epidemiological study was conducted in Chiang Mai, during 2000 and 2001 [Hansman et al., 2004]. This surveillance study indicated that NoV and SaV detection rates were 8.6% and 4.8%, respectively. Based on the clustering methods determined previously by Kageyama et al. [2004], NoV GII/4 genotypes was the most predominant genotype. The other co-circulating strains belonged to GI/3, GI/7, GI/8, GII/7, GII/8, and GII/10. For SaV detection, the most prevalent genotype was GI/1, followed by GII/1. Interestingly, one isolate of SaV was found to be an intragenogroup recombinant strain [Hansman et al., 2004; Katayama et al., 2004]. However, the study conducted by Guntapong et al. [2004] from 2002 to 2003 reported a higher prevalence of NoV and SaV infection at 17.5% and 15.9%, respectively. The most prevalent genotype of NoV was GII/4, while the other co-circulating strains were GII/1, GII/3, GII/6, and GII/16. However, the NoV GI genogroup was undetectable from this surveillance study. The majority of SaV strains were GI/1, while one was GV and another represented a novel genotype in the GII cluster. In this study, the prevalence and distribution of NoV and SaV genotypes were investigated in children hospitalized with diarrhea in Chiang Mai, Thailand in three consecutive years from 2002 to 2004. It was found that NoV and SaV circulated in this area with the prevalence rates of 14.1% and 1.2%, respectively. Of these, over 60% of NoV detection was the GII/4 genotype. These results are consistent with the previous findings in Thailand, in which the prevalence rates of NoV were higher than SaV infections, and NoV GII/4 was the most predominant genotype circulating in this country. In 2000 and 2001, GII/4 was accounted for 33.3% of all the NoV genotypes detected [Hansman et al., 2004]. Interestingly, the following years of 2002 and 2003 NoV GII/4 detection rate was increased dramatically to 64.3% [Guntapong et al., 2004]. This data also correlate with our result that during 2002–2004, 62.8% of NoV detection was identified as GII/4 genotype. The accumulated data from previous studies and this study indicate that NoV GI was first detected in Thailand during 2000 and 2001. However, in the three consecutive years of 2002–2004, NoV GI disappeared completely and has remained undetectable. For SaV detection, the prevalence rate is much lower (1.2%) than those of previous reports (4.8% and 15.9%). In addition, it should be noted that although the surveillance data between this study and the study reported by Guntapong et al. [2004] were conducted in the same periods, the vast genetic diversity between the two studies has been observed. For SaV, two different genotypes of GI/1 and GI/2 together with one of the

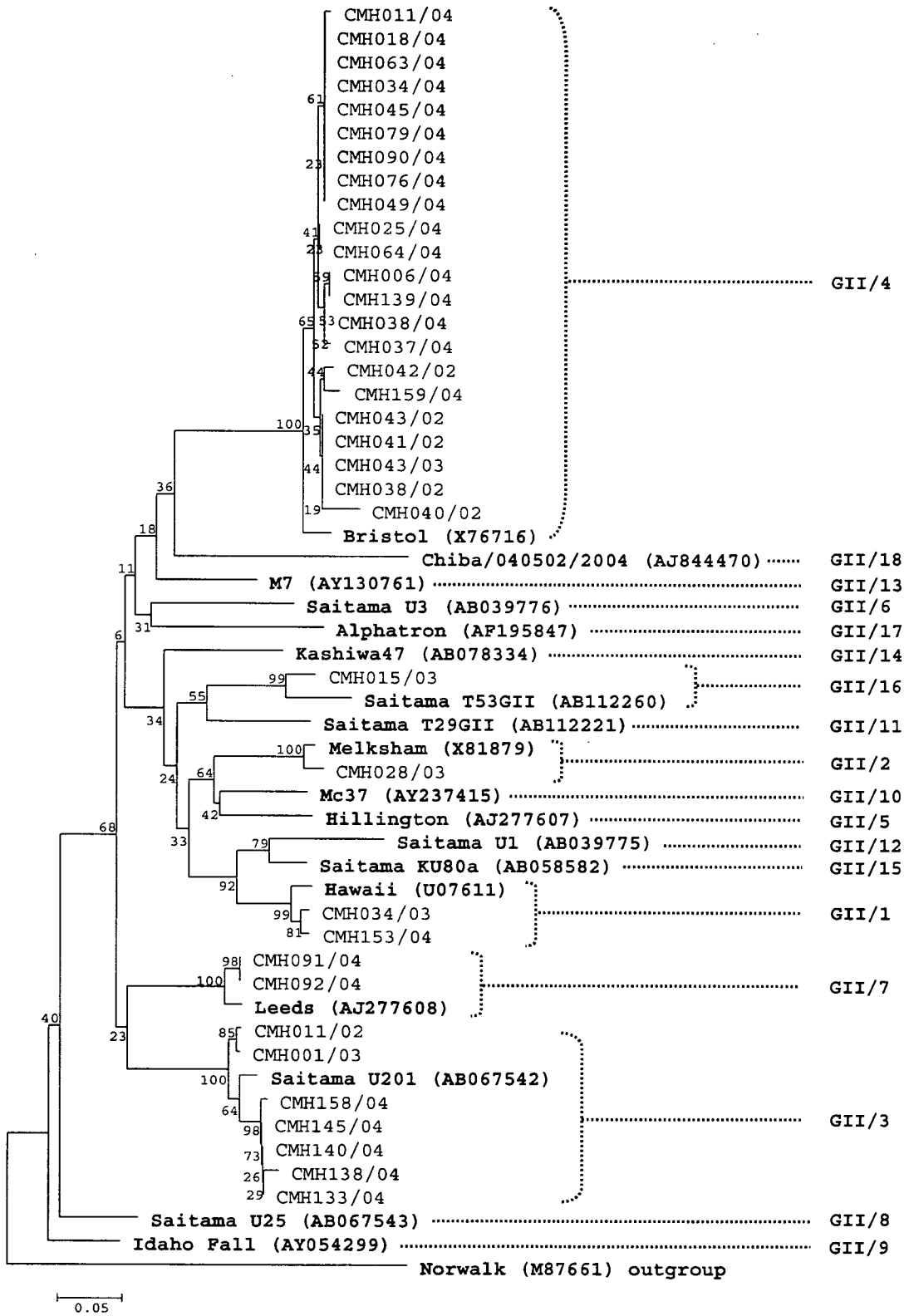

Fig. 1. Phylogenetic analysis of partial capsid sequences of NoV detected in pediatric patients hospitalized with acute gastroenteritis in Chiang Mai, Thailand from 2002 to 2004. The tree was constructed on the basis of the neighbor-joining method and the numbers on each branch indicate the bootstrap values. The NoV outgroup and reference strains of GII/1–GII/18 are presented in boldface and GenBank accession numbers are given in parentheses.

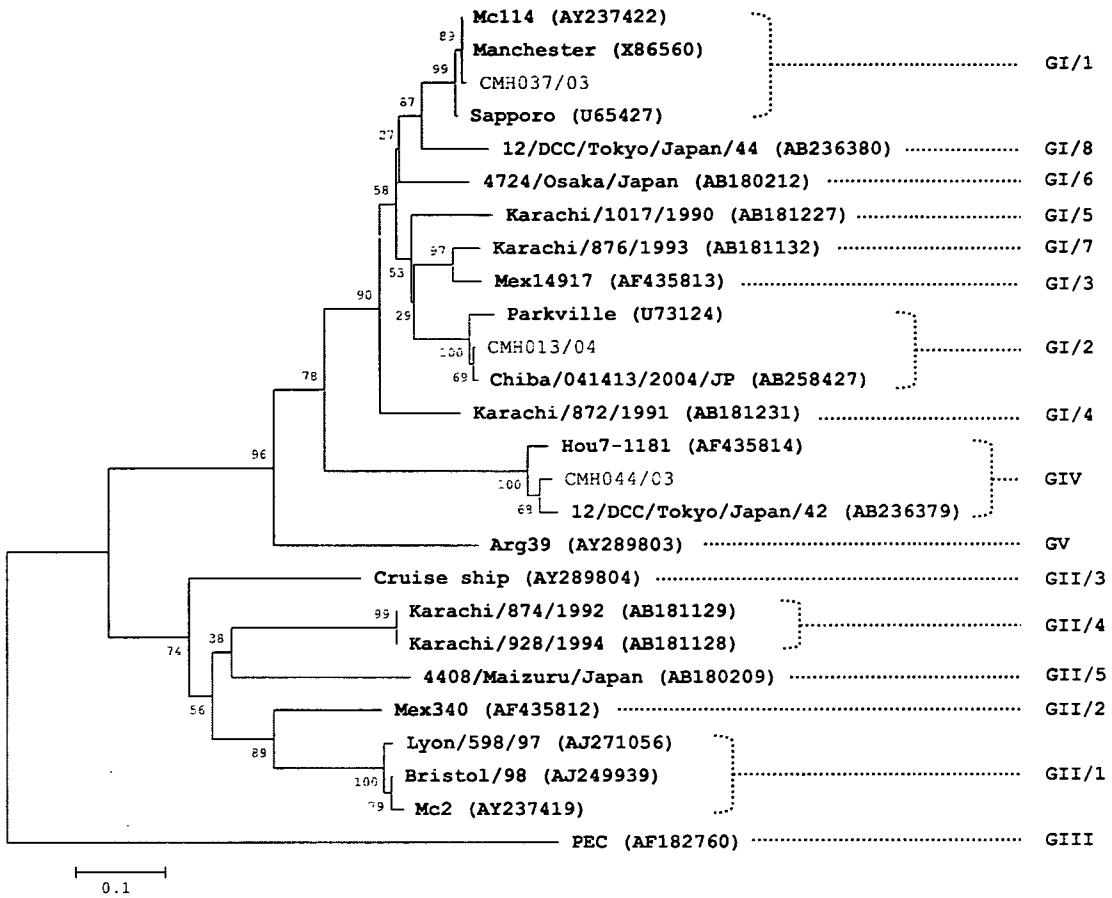

Fig. 2. Phylogenetic analysis of partial capsid sequences of SaV detected in pediatric patients hospitalized with acute gastroenteritis in Chiang Mai, Thailand from 2002 to 2004. The tree was constructed on the basis of the neighbor-joining method and the numbers on each branch indicate the bootstrap values. The SaV reference strains of GI to GV are presented in boldface and GenBank accession numbers are given in parentheses.

SaV GIV genogroup were isolated in this study, while previous studies identified the circulation of SaV GI, GII, and GV strains. The discrepancy of SaV strains detected by the two studies might be due to the study sites, that is, Guntapong et al. [2004] study was done in Sa Kaeo, Chanthaburi, Songkhla, Nong Khai, and Tak while our study was conducted in Chiang Mai city.

In summary, this study demonstrated that human caliciviruses are important enteric viruses causing acute gastroenteritis in hospitalized children in Chiang Mai, Thailand from 2002 to 2004. The great genetic diversities of NoV and SaV have also been observed.

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Clin. Lab. 2007;53:567-570

SHORT COMMUNICATION

Multiple Recombinant Noroviruses in Japan

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SUMMARY

A total of 417 fecal specimens collected from sporadic pediatric cases of acute gastroenteritis in Japan from 2004 to 2005 were tested for noroviruses by RT-PCR. Noroviruses were detected in 44 of 417 (10.1%) fecal specimens tested. Of these, the GII/3 was the most predominant genotype with a prevalence rate of 56.8%, followed by 34% of the GII/4 and others. Phylogenetic analysis reveals that multiple recombinant noroviruses, which were both dependently and independently introduced from four different continents (Asia, America, Europe, and Oceania), emerged to cause acute gastroenteritis among Japanese children. Of these, "new variant" noroviruses suddenly emerged to become the leading strain in Japan for the first time. This report is also the first indication of the existence of multiple recombinant noroviruses co-circulating in Japan. (Clin. Lab. 2007;53:567-570)

KEY WORDS

Recombination, norovirus, Japan

Noroviruses are 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 ages in various epidemiological settings (1). Noroviruses are now classified as a distinct genus within the family *Caliciviridae*. Noroviruses have a positive sense single-strand RNA genome surrounded by an icosahedral capsid and their genome contains three open reading frames (ORFs). ORF1 encodes non-structural proteins; ORF2 encodes the capsid protein, and ORF3, the smallest, encodes a protein of unknown function that has been suggested to be a minor component of the virion (2). In this study, the occurrence of norovirus infection in children with acute gastroenteritis in Japan from 2005 to 2006 was determineed and noroviruses were further characterized according to genogroup and genotype.

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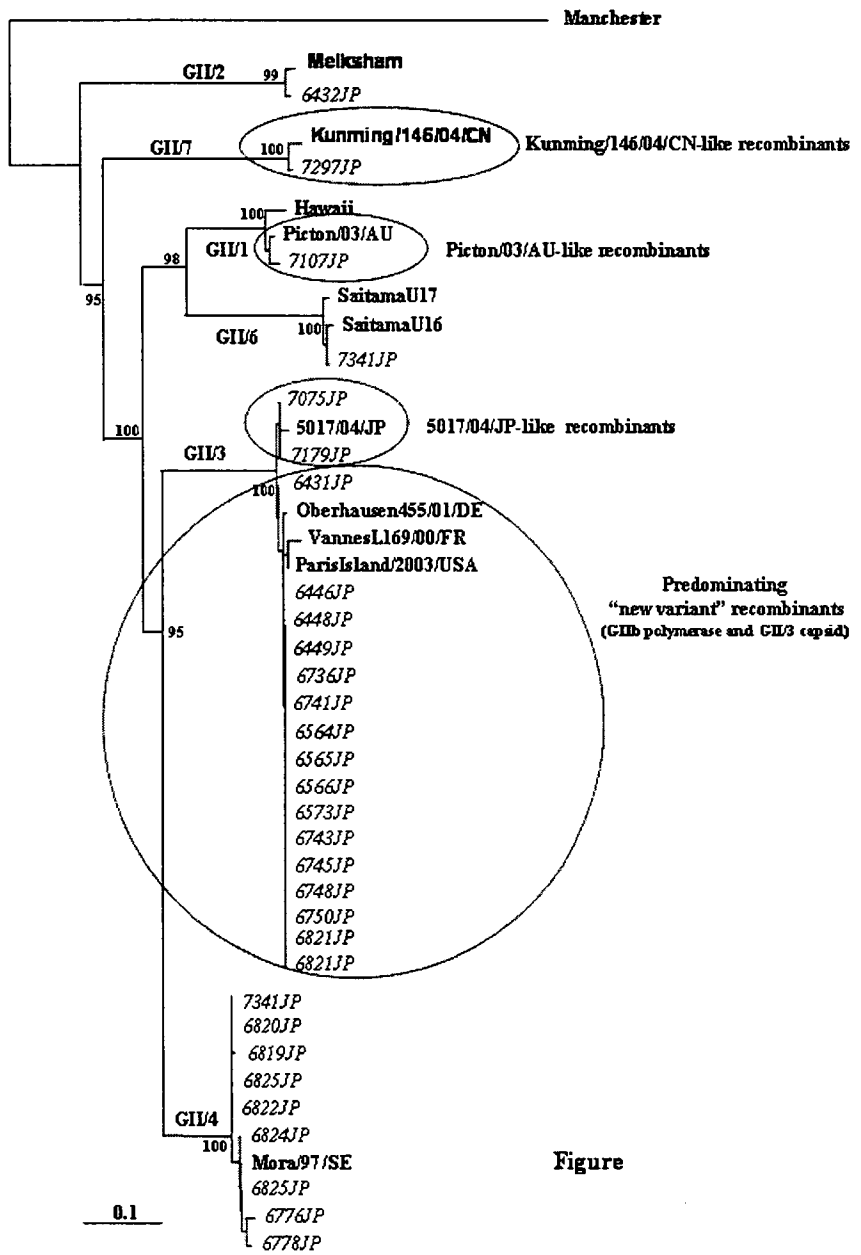
A total of 417 fecal specimens were collected from 417 different individuals of sporadic cases of acute gastroenteritis in pediatric clinics in two localities (Maizuru and Osaka) in Japan from July 2005 to June 2006, and were then tested for the presence of noroviruses. Norovirus genome was extracted applying a RNA extraction QIAGEN® kit (QIAGEN, Hilden, Germany). Using PCR with specific primers G1SKF and G1SKR and COG2F and G2SKR that amplify both the polymerase and capsid genes of noroviruses, resulted in the identification of noroviruses (3). These primers specifically generated two different sizes of amplicons of 330 bp and 387 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 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 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. The nucleotide sequences of PCR products (DNA) positive for noroviruses 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



Figure

Figure: Diversity of norovirus genotypes in Japan. A phylogenetic tree was constructed from partial nucleotide sequences of capsid genes of the representative norovirus strains detected in the study and the closely related reference strains available in GenBank. The norovirus strains detected in the present study are highlighted in italics. The scale indicates nucleotide substitutions per position. The numbers in the branches indicate the bootstrap values. The Manchester strain was used as an out-group strain for phylogenetic analysis. The nucleotide sequences of norovirus strains detected in the study had been submitted to GenBank and the assigned accession numbers were EF028229-EF028234. Reference norovirus strains and accession numbers used in this study were as follows: Manchester (X86560), Melksham (X81879), Paris Island/2003/USA (AY652979), Oberhausen 455/01/DE (AF539440), Hawaii (U07611), Picton/03/AU (AY919139), Kunming/146/04/CN (DQ304651), Mora/97/SE (DQ104023), VannesL169/00/France (AY773210), 5017/04/JP (AB242257), SaitamaU161 (AB039778), and SaitamaU17 (AB039779).

1.6). The ORF2-based 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).

Here, noroviruses were detected in 44 of 417 (10.1%) fecal specimens tested. The Figure reveals that these noroviruses were divided into six distinct genotypes 1, 2, 3, 4, 6, and 7 within genogroup II (GII) based on the recent norovirus capsid region classification schemes described by Kageyama et al., 2004 (4). Of these, the GII/3 (known as the Toronto virus cluster) was the most predominant genotype with a prevalence rate of 56.8% (25 of 44), followed by 34% (15 of 44) of the GII/4 (known as the Lordsdale virus cluster), and others (each 2.3%; 1 of 44). In contrast, the GII/4 was the leading genotype with a prevalence rate of 77.7%, followed by 15.8% for GII/3 in the previous year, 2004-2005 (5). The results clearly indicated that there was a changing epidemiology of norovirus genotypes in Japan.
Comparison of the sequences found in this study to those in the literature was performed. 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 similar to those of the recombinant norovirus Picton/03/AU (the GIIb polymerase and the GII/1 capsid) and the recombinant norovirus Kunming/146/04/CN (the GII/6 polymerase and the GII/7 capsid), respectively (6, 7). Within 25 GII/3 noroviruses, the sequences of both capsid and polymerase genes of 3 and 22 strains were similar to those of the recombinant noroviruses 5017/04/JP (the GII/4 polymerase and the GII/3 capsid) and the "new variants" Oberhausen455/01/DE, VannesL169/00/FR, 78/04/Ru, and ParisIsland/2003/USA (the GIIb polymerase and the GII/3 capsid), respectively (6, 8, 9). Taken together, the results indicated that these noroviruses were also recognized as the recombinant strains. In contrast, in all noroviruses belonging to the GII/2, the GII/4, and the GII/6, the genotypes remained the same no matter how the polymerase or capsid regions were analyzed.

RNA recombination plays a key role in virus evolution and it shapes a good deal of the virus diversity. This event has great impact on epidemiological research, vaccine design as well as viral control programs. The "new variant" norovirus was first noted in Finland in 2001 and then spread throughout Europe (10, 11). This new variant virus (the GIIb polymerase and the GII/3 capsid) was first detected in Saga City, Japan in 2003-2004 in only one case (9). Interestingly, this virus emerged and rapidly became the leading cause of acute gastroenteritis in Japan for the first time in this study. This report is also the first indication of the existence of multiple recombinant noroviruses co-circulating in Japan and it increases the evidence for the worldwide distribution of recombinant noroviruses.

Norovirus capsid is predicted to be well suited for genotype classification (4, 12). In this study, the recombinant norovirus strains belonged to many different genotypes including some novel ones when the capsid- and polymerase-based groupings were performed. Obviously, the viral recombination event had a significant influence on the phylogenetic classification of norovirus strains. This observation was in strong agreement with the previous report that the future norovirus classification should rely not only on the capsid sequence but also on the polymerase sequence (7).

It was found that the polymerase and capsid sequences of the majority of the recombinant noroviruses in both localities in Japan were 100% identical with each other and had the closest match (99%) with other strains from Europe (Oberhausen455/01/DE from Germany, VannesL169/00/FR from France, 78/04/Ru from Russia), North America (Paris Island/2003/USA from the United States) (6, 8). However, two other recombinant noroviruses exclusively found in two localities had the highest similarities (97%-99%) with other strains from Asia (Kunming/146/04/CN from China) or Oceania (Picton/03/AU from Australia) (6, 7). We conclude that these recombinant noroviruses might have arrived both dependently and independently from four different continents, perhaps by water, oysters, or through unprotected trade and travelers. Obviously, recombinant norovirus infections are becoming rather common and are very likely to be more so.

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Characterization of a Broadly Reactive Monoclonal Antibody against Norovirus Genogroups I and II: Recognition of a Novel Conformational Epitope▽

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Norovirus, which belongs to the family *Caliciviridae*, is one of the major causes of nonbacterial acute gastroenteritis in the world. The main human noroviruses are of genogroup I (GI) and genogroup II (GII), which were subdivided further into at least 15 and 18 genotypes (GI/1 to GI/15 and GII/1 to GII/18), respectively. The development of immunological diagnosis for norovirus had been hindered by the antigen specificity of the polyclonal antibody. Therefore, several laboratories have produced broadly reactive monoclonal antibodies, which recognize the linear GI and GII cross-reactive epitopes or the conformational GI-specific epitope. In this study, we characterized the novel monoclonal antibody 14-1 (MAb14-1) for further development of the rapid immunochromatography test. Our results demonstrated that MAb14-1 could recognize 15 recombinant virus-like particles (GI/1, 4, 8, and 11 and GII/1 to 7 and 12 to 15) and showed weak affinity to the virus-like particle of GI/3. This recognition range is the broadest of the existing monoclonal antibodies. The epitope for MAb14-1 was identified by fragment, sequence, structural, and mutational analyses. Both terminal antigenic regions (amino acid positions 418 to 426 and 526 to 534) on the C-terminal P1 domain formed the conformational epitope and were in the proximity of the insertion region (positions 427 to 525). These regions contained six amino acids responsible for antigenicity that were conserved among genogroup(s), genus, and *Caliciviridae*. This epitope mapping explained the broad reactivity and different titers among GI and GII. To our knowledge, we are the first group to identify the GI and GII cross-reactive monoclonal antibody, which recognizes the novel conformational epitope. From these data, MAb14-1 could be used further to develop immunochromatography.

Norovirus is the major cause of nonbacterial epidemic gastroenteritis (11) and belongs to the family *Caliciviridae* containing five distinct genera, *Vesivirus*, *Lagovirus*, *Norovirus*, *Sapovirus*, and *Becovirus* (33). Norovirus has been identified as the cause of 73% to more than 95% of gastroenteritis outbreaks in the United States and approximately half of those worldwide (1).

Norovirus is classified into five genogroups (genogroup I [GI] to genogroup V [GV]) by genetic diversity: viruses in genogroups I, II, and IV (GI, GII, and GIV, respectively) are associated with diarrhea in humans, with GII also able to infect pigs; genogroups III and V (GIII and GV) are associated with bovines and mice, respectively (19). Moreover, norovirus GI and GII are the main causative agents in humans and subdivided further into at least 15 and 18 genotypes (GI/1 to GI/15 and GII/1 to GII/18), respectively (30).

Because the lack of a cell culture system for norovirus has hindered immunological and structural study, the recombinant virus-like particles (rVLPs), which are morphologically and antigenically similar to native norovirus virions, were expressed by using the baculovirus expression system (12, 16, 37).

Norovirus is composed of 180 molecules (90 dimers) of the single major capsid protein, VP1, which has two principal domains. One is the shell (S) domain, which is highly conserved among animal caliciviruses. The other is the protruding (P) domain, which is divided into three subdomains: N-terminal P1, P2, and C-terminal P1 domains. The P2 domain is the most protruding and diverse domain (37). In addition, the internally located N-terminal domain participates in a network of interactions through domain swapping to assist the assembly of the shell domain into an icosahedral scaffold (6).

Several laboratories have generated polyclonal antibodies by using recombinant VP1 as antigens. The rabbit anti-rVLP polyclonal antibody was highly specific for genotypes used as immunogens (13, 18, 21). This specificity has hindered the development of immunological diagnosis. We previously developed the immunochromatography test for detection of norovirus infection by using the anti-rVLP polyclonal antibody (31); however, this method showed the immunogen's genotype specificity.

Monoclonal antibodies are a useful tool for detecting various kinds of noroviruses, and they are more stable than polyclonal antibodies for use in a rapid immunological assay. The previously reported broadly reactive monoclonal antibodies

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TABLE 1. Capsid fragment primers

Primer[a]	Sequence (5′ to 3′)[b]	Position (nucleotide)[c]	Polarity
1207cacc-418	CAC CGC TCC TGC CGT TGC CCC C	1252–1270	Sense
1207cacc-427	CAC CGG TGA GCA ACT TCT TTT C	1279–1296	Sense
1207-528	CTA TGT GTA GAA CTG GTT GAC CC	1553–1575	Antisense
1207-534	CTA CCC CGC TCC ATT TCC CAT	1582–1602	Antisense
1207-541	GGG CCA TTA TAA CGC ACG TC	1604–1623	Antisense

[a] The numbering of the sense primers indicates the nucleotide sequences of the N-terminal (first) norovirus residue contained within a construct generated with a particular primer. The numbering of the antisense primers indicates the nucleotide sequences of the C-terminal (last) norovirus residue contained within a construct generated with a particular primer.

[b] Four bases used for directional cloning are shown underlined.

[c] The 1207 sequence was assigned accession number DQ975270 in GenBank.

could be classified into two groups by their epitope properties. The first group recognizes the intergenogroup cross-reactive linear epitopes on the S or P domain, NS14, 1B4, and 1F6 (20, 35, 46, 47). The other group recognizes the intragenogroup cross-reactive conformational epitopes, NV3901 and NV3912 (35, 46). In addition, gaining information about the location of norovirus-specific epitopes is essential for designing diagnostic tools (i.e., enzyme-linked immunosorbent assay [ELISA] and immunochromatography), identifying the neutralizing epitope, and developing antivirals and an effective vaccine.

In this study, we describe characterization of a novel monoclonal antibody, which shows broad reactivity with both GI and GII norovirus rVLPs. These findings could be applied for further development of the rapid immunochromatography test, because immunochromatography using this novel antibody has demonstrated high performance in detecting norovirus infection (28).

MATERIALS AND METHODS

Antigens (rVLPs). Sixteen rVLPs were previously expressed by the baculovirus expression system and confirmed by electron microscopy (31, 32). The sequences were genetically classified based on the method described by Kageyama et al. (17). Within GI, five genotypes of rVLPs were generated, including genotypes 1 (strain 4656 [sequence accession number EF547392]), 3 (strain 3634 [EF547393]), 4 (strain 2876 [EF547394]), 8 (strain 3006 [EF547395]), and 11 (strain 2258 [EF547396]). For GII, 11 genotypes of rVLPs were generated, including genotypes 1 (strain 3101 [EF547397]), 2 (strain 2840 [EF547398]), 3 (strain 3229 [EF547399]), 4 (strain 1207 [DQ975270]), 5 (strain 3611 [EF5473400]), 6 (strain 3612 [EF5473401]), 7 (strain 419 [EF5473402]), 12 (strain 2087 [EF5473403]), 13 (strain 3385 [EF5473404]), 14 (strain 2468 [EF5473405]), and 15 (strain 3625 [EF5473406]).

Production of monoclonal antibody. The P363-Ag-U1 myeloma cell line was used as the parent cell. CsCl-purified GII/4 rVLP (r1207) was used as an immunogen for preparing the monoclonal antibody, as previously described (22).

ELISA for titration of the monoclonal antibody. Plates with 96 wells (Maxisorp; Nunc, Roskilde, Denmark) were coated with 90 ng of rVLP/well in 60 μl of 0.1 M carbonate buffer (pH 9.6) for 1 h at 37°C. To compare the reactivities of ELISAs with different pHs, two coating buffer solutions with different pH conditions were used. Phosphate-buffered saline (PBS) with a pH of 7.4 was used, and carbonate buffer with a pH of 9.6 was used only for GII/3 rVLP r3229 and GII/4 rVLP r1207. The wells were blocked with 1% bovine serum albumin 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 titration of the monoclonal antibody, 60 μl of a twofold serial dilution was added to each well, starting with a 1:100 dilution of the monoclonal antibody in PBS-T containing 1% bovine serum albumin, and the plate was incubated for 1 h at 37°C. After the wells were washed three times with PBS-T, 60 μl of a 1:4,000 dilution of horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) (Biosource International, Camarillo, CA) was allowed to react for 1 h at 37°C as the second antibody. After the wells were washed three times with PBS-T, 60 μl of substrate *o*-phenylenediamine containing 0.012% H_2O_2 and 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 adding 60 μl of 2 M H_2SO_4 to each well, and the optical density at 492 nm (OD_{492}) was determined (using OD_{600} as the reference) with a Labsystems Multiskan MCC microplate reader (Thermo Electron Corporation, Waltham, MA). For this experiment, Tn*5* cell lysate was included as a negative control. A sample that which had an OD of ≥0.2 and signal/noise ratio of ≥2.0, was considered positive. Each assay was conducted in duplicate.

Fragment construction. The pET 100 directional TOPO vector (Invitrogen Corp., Carlsbad, CA) was used to express the capsid fragments with a His tag. The primers used in this study are shown in Table 1. PCR-amplified fragments of r1207 were generated using the primer pairs indicated by the names of the constructs. The template used for the PCR was the previously reported plasmid containing the complete capsid sequence of r1207 (31). PCR fragments were directly cloned into the pET 100 directional TOPO vector. The plasmids were transformed into *Escherichia coli* One Shot TOP10 (Invitrogen Corp., Carlsbad, CA). Positive transformants were identified by PCR. The plasmids from positive transformants were transformed further into *E. coli* BL21 Star cells (Invitrogen Corp., Carlsbad, CA). To express the r1207 capsid fragments, overnight cultures of *E. coli* BL21 cells, transformed with each plasmid, were diluted to a ratio of 1:20 in fresh Luria-Bertani broth supplemented with 100 μg/ml of ampicillin. The cells were grown at 37°C until the culture reached a certain cell density (when the OD_{600} was 0.5 to 0.7). Expression was induced by adding 1.0 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) (Invitrogen Corp., Carlsbad, CA), and cultures were grown for an additional 3 h. The cells were pelleted by centrifugation for 15 min at 3,000 × *g* at 4°C. The supernatant was removed, and the cell pellet was suspended in a 1/20 volume of lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, and a protease inhibitor cocktail [complete, Mini, EDTA-free] [1 tablet/10 ml] [Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany]) and gently shaken at 4°C for 30 min. Following that treatment, Triton X-100 and lysozyme were added to concentrations of 1% and 0.2 mg/ml, respectively, and gently shaken at 4°C for 20 min. Finally, the cells were centrifuged for 30 min at 12,000 × *g* at 4°C, after which the protein was found in the insoluble fraction.

This fraction was resuspended in 20 mM of Tris (pH 8), 500 mM of NaCl, and 8 M of urea, filtered, and loaded onto a HisTrap column (GE Healthcare Bio-Science Corp., Piscataway, NJ) equilibrated in 20 mM of Tris (pH 7.4), 500 mM of NaCl, and 8 M of urea. On-column renaturing was performed with 8 to 0 M urea gradient solutions. The elution was performed with a 0 to 1 M imidazole gradient. The peak fractions were pooled, and the solvent displaced PBS (pH 7.4) from the PD10 column (GE Healthcare Bio-Science Corp., Piscataway, NJ).

Fragment analysis. Analysis of proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis was done by the method of Laemmli et al. (24) with slight modifications. Briefly, 15% polyacrylamide resolving gels and a 5% acrylamide stacking gel were used. Capsid fragments were suspended in electrophoresis sample buffer containing 4% sodium dodecyl sulfate, 10% mercaptoethanol, 125 mM of Tris-HCl (pH 6.8), 0.01% bromophenol blue, and 10% glycerol. Samples were boiled for 5 min. Separated proteins were transferred onto a 0.45 μm polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA) in a semidry transfer (CB-09A; ATTO, Tokyo, Japan) at a constant current of 2 mA/cm^2 for 30 min. The blotted membrane was washed with PBS-T and blocked with 5% skim milk in PBS-T overnight at 4°C. The membrane was washed with PBS-T and then incubated overnight at 4°C with an antibody against the five-histidine tag (QIAGEN, Hilden, Germany) and antinorovirus monoclonal antibody diluted to 1/10,000 and 1/1,000, respectively, with 0.5% skim milk in PBS-T. The blot was washed with PBS-T and incubated with a 1/10,000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (Tago, Burlingame, CA). The blot was then reacted with peroxidase substrate solution (diaminoben-

TABLE 2. Site-directed mutagenesis primers

Primer	Sequence (5′ to 3′)[a]	Position (nucleotide)[b]	Polarity
F425G	CTG CCG TTG CCC CCA CT**G** **G**CC CGG GTG A	1256–1283	Sense
F425G antisense	TCA CCC GGG **CC**A GTG GGG GCA ACG GCA G	1256–1283	Antisense
P(CCG)426F(GCG)	CGT TGC CCC CAC TTT C**GC** GGG TGA GCA ACT TCT TTT C	1260–1296	Sense
P(CGG)426F(CGC)	GAA AAG AAG TTG CTC ACC CG**C** GAA AGT GGG GGC AAC G	1260–1296	Antisense
L(CTT)526A(GCT)	GGT CAA CCA GTT CTA CAC A**GC** TGC CCC CAT GGG AAA TGG	1557–1595	Sense
L(AAG)526A(AGC)	CCA TTT CCC ATG GGG GCA **GC**T GTG TAG AAC TGG TTG ACC	1557–1595	Antisense
A(GCC)527K(AAG)	GGT CAA CCA GTT CTA CAC ACT **TAA** **G**CC CAT GGG AAA TGG AGC	1557–1598	Sense
A(GGC)527K(CTT)	GCT CCA TTT CCC ATG GG**C** **TTA** AGT GTG TAG AAC TGG TTG ACC	1557–1598	Antisense
P(CCC)528A(GCC)	CCA GTT CTA CAC ACT TGC C**GC** CAT GGG AAA TGG AGC G	1563–1599	Sense
P(GGG)528A(GGC)	CGC TCC ATT TCC CAT GG**C** GGC AAG TGT GTA GAA CTG G	1563–1599	Antisense
G(GGA)530A(GCA)	CAC ACT TGC CCC CAT GGC AAA TG**C** AGC GGG *GTA GAA GG*	1572–1602	Sense
G(TCC)530A(TGC)	*CCT TCT* ACC CC**G** CTG CAT TTG CCA TGG GGG CAA GTG TG	1572–1602	Antisense

[a] Mutant nucleotides are shown in boldface type, and vector nucleotides are shown in italic type.
[b] The 1207 sequence was assigned accession number DQ975270 in GenBank.

zidine; SIGMA, St. Louis, MO) to detect the antigen-antibody complexes on the blot.

Sequence analysis. The ClustalX multiple-sequence alignment program (version 1.83) was used for multiple alignment of constructed rVLP sequences and other genogroups (40). The capsid subdomains were determined based on previously reported data from Prasad et al. (37).

Mutational analysis. Specific residues in the capsid fragment, 418 to 534, were altered using the QuikChange XLII site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Some mutagenesis primers were engineered by the QuikChange Primer Design Program (Stratagene, La Jolla, CA) as shown in Table 2. Generated mutants were purified and analyzed by using the same protocols as those for fragment construction and analysis. Mutant clones were confirmed by sequencing.

Structural analysis. The crystal structure of the prototype Norwalk virus capsid protein (PDB code 1IHM) was used to built homology models for r1207 (37). The initial sequence-to-structure alignments and the refined three-dimensional models of r1207 with minimized side chain conformations were obtained using the T-Coffee and SWISS MODEL (29, 38). The figures were made by using PYMOL (http://pymol.sourceforge.net).

Nucleotide sequence accession numbers. Newly determined sequences were submitted to GenBank under accession numbers DQ975270 and EF547392 through EF547406.

RESULTS

Cross-reactivity of the novel monoclonal antibody. The ELISA comparison of the reactivities of the novel monoclonal antibody by using different pH conditions for the coating buffer (pH 7.4 and 9.6) showed that a high pH condition (pH 9.6) could not affect the result of ELISA. The novel monoclonal antibody (MAb14-1) obtained from a mouse immunized with r1207 (GII/4 rVLP) showed broad reactivity against various genotypes of rVLPs by ELISA (Table 3). All the different rVLP norovirus genotypes (GI/1, 3, 4, 8, 11 and GII/1 to 7 and 12 to 15) used in this study were recognized by MAb14-1. However, only a weak affinity to the GI/3 genotype was observed (data not shown). The titers of MAb14-1 were almost the same as those against GII rVLPs, and quite different from those against GI rVLPs (Table 3).

Minimal binding region on the capsid with monoclonal antibody MAb14-1. To determine the binding domain of the VP1 capsid region against MAb14-1, five fragments were constructed with the His tag: full VP1 (amino acid positions 1 to 541), full VP1 except for the N-terminal subdomain (positions 46 to 541), P domain (positions 222 to 541), P domain except for the C-terminal P1 domain (positions 222 to 417), and N-terminal P1 domain (positions 222 to 275). Only fragments not containing the C-terminal P1 domain showed nonreactivity for MAb14-1 (Fig. 1B,. top five schematic fragments). This result suggested that the C-terminal P1 domain might contain the specific epitope of MAb14-1.

In addition, to determine the minimal binding region of MAb14-1, five capsid fragments were constructed by deletion of both terminal regions of the C-terminal P1 domain. It was found that MAb14-1 showed predictable reactivity for the C-terminal P1 domain, while the N-terminal deletion (amino acid positions 418 to 426 from 418 to 541) induced abolition of reactivity for MAb14-1 and the C-terminal deletion mutants with amino acids 418 to 534, 418 to 528, and 418 to 525 deleted induced rise, decline, and abolition of antigenicity, respectively (Fig. 1A and B, bottom five schematic fragments). These results implied that the minimal binding region is probably from amino acid positions 418 to 534 (Fig. 1) and suggested that nine amino acid residues (positions 418 to 426 [A region]) on the N-terminal domain, and three amino acids (526 to 528 [B region]) and six amino acids (529 to 534 [C region]) on the C-terminal domain were important regions for the antigenicity of MAb14-1.

Epitope for monoclonal antibody 14-1. Alignment of the minimal binding regions on rVLPs and other genogroups

TABLE 3. Titers of newly developed MAb14-1 with various rVLPs by ELISA

Monoclonal antibody	Isotype	Titer for rVLP (100)[a]															
		Genogroup I genotype					Genogroup II genotype										
		1	3	4	8	11	1	2	3	4	5	6	7	12	13	14	15
MAb14-1	IgG1	512	<1	8	64	16	8,192	2,048	4,096	**8,192**	2,048	4,096	4,096	1,024	8,192	2,048	2,048

[a] The homologous titer is shown in boldface type.

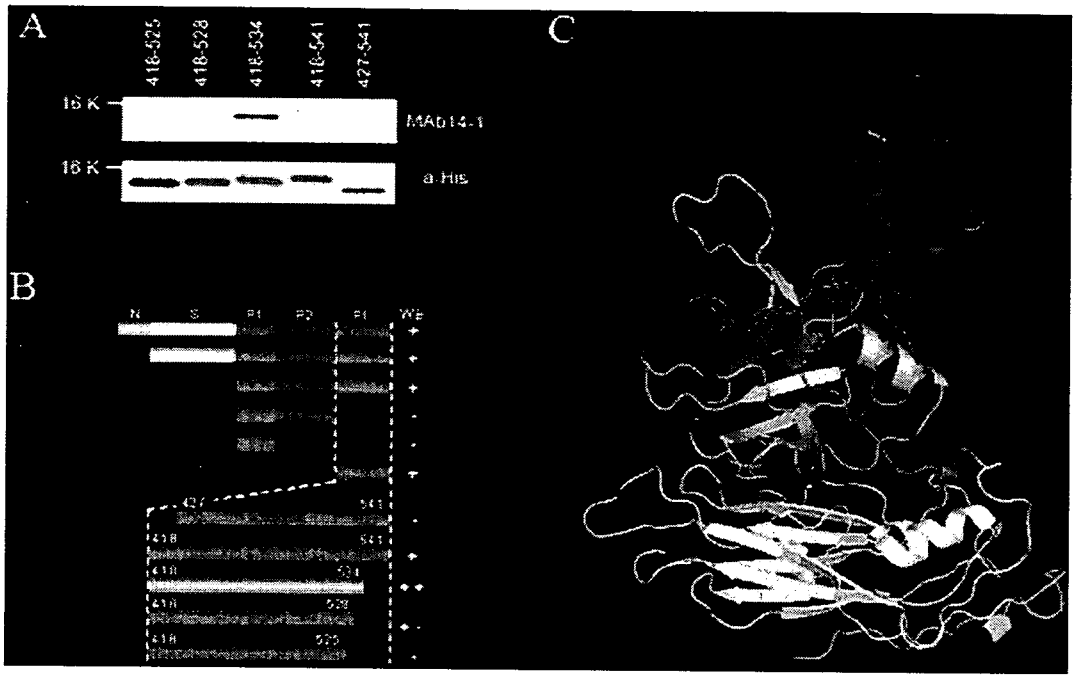

FIG. 1. (A) Reactivities of several capsid fragments for MAb14-1 and anti-His_5 antibody (a-His) by Western blotting. 20 K, 20,000. (B) Process of mapping the minimal binding region shown in silver on the map of VP1. WB, Western blotting. Symbols; ++, increase in antigenicity; +, same antigenicity as for r1207; +−, decline in antigenicity; −, abolition of antigenicity. (C) Prediction structure of r1207 (a part of the C-terminal P1 domain prediction structure could not be created through lack of structural data from 1IHM [Protein Data Bank identification code for Norwalk virus capsid protein]). The N-terminal domain (amino acid positions 0 to 45) (green), S domain (positions 46 to 221) (yellow), N-terminal and C-terminal P1 domains (positions 222 to 275 and 418 to 541) (red), and P2 domain (positions 276 to 417) (blue) are indicated in panels B and C.

showed that the deleted terminal regions had genus-specific residues (such as A418 and P419) and genogroup-specific residues (such as V421 and F425), but these regions did not have GI/3-specific single point mutations (Fig. 2).

To confirm the relationship between both terminal regions of highly conserved residues (amino acid positions 418 to 426 [A region] and 526 to 534 [B and C regions]) and antigenicity, genus-specific and GII-specific residues were changed to alanine and GI-specific amino acids, respectively (Fig. 2). The six mutations induced several kinds of changes in antigenicity, whereas other mutations did not affect antigenicity. These effects were classified into four different groups: (i) disappearance of the antigenicity (point mutation of L526A within the B region), (ii) severely attenuated antigenicity (F425G in the A region), (iii) significant reduction in reactivity (P426F in the A region, A527K in the B region, and P528A in the C region), and (iv) moderate reduction (G530A in the C region) (Fig. 3). These results confirmed that three regions affected antigenicity as predicted by alignment analysis.

To confirm that the relationship between these amino acids affected the antigenicity and structure of r1207, prediction of antigen structure was performed by using the registered Norwalk virus (GI/1) capsid structure (Fig. 1C and 3B). This showed that antigenic residues were contiguous with each other (Fig. 3B). The insert region from amino acid positions 427 to 525 made both the terminal regions proximate; however, they did not have direct interactions via charged residues. The interaction between each terminal region and the insert region (positions 427 to 525) was not observed except for hydrophilic interaction. Moreover, a GI/3-specific single point mutation, close to both terminal regions, was not identified in our study (Fig. 2).

DISCUSSION

In this study, a newly developed monoclonal antibody (MAb14-1) was identified as being a broadly reactive monoclonal antibody, which recognized GI (GI/1, 4, 8, and 11) and GII (GII/1 to 7 and 12 to 15) rVLPs with a weak affinity to GI/3. This recognition range is the broadest of the existing monoclonal antibodies (Table 4) (14, 20, 35, 39, 46, 47). MAb14-1 shows low affinity to GI, but this result was also observed in a previous report by Yoda et al. (46). Therefore, we determined the broad reactivity of MAb14-1 after Yoda's observation.

In this study, the coating buffer with pH 9.6 was used for the antibody titration. Previous and recent studies repeatedly showed that rVLPs disassemble completely into soluble capsid proteins when a pH value is equal to or higher than 8.9; therefore, rVLPs no longer exist at pH 9.6 (2, 45). From this observation, the same experiments using a coating buffer with pH 7.4 were performed only for r3229 and r1207; however, different conditions showed the same results. Therefore, the data on titer and broad reactivity of the antibody can be compared with not only several laboratory results using rVLPs but also with results using recombinant capsids (or fragments) (Table 4). To explain this reactivity at the molecular level, we demonstrated precise determination of the epitope by using

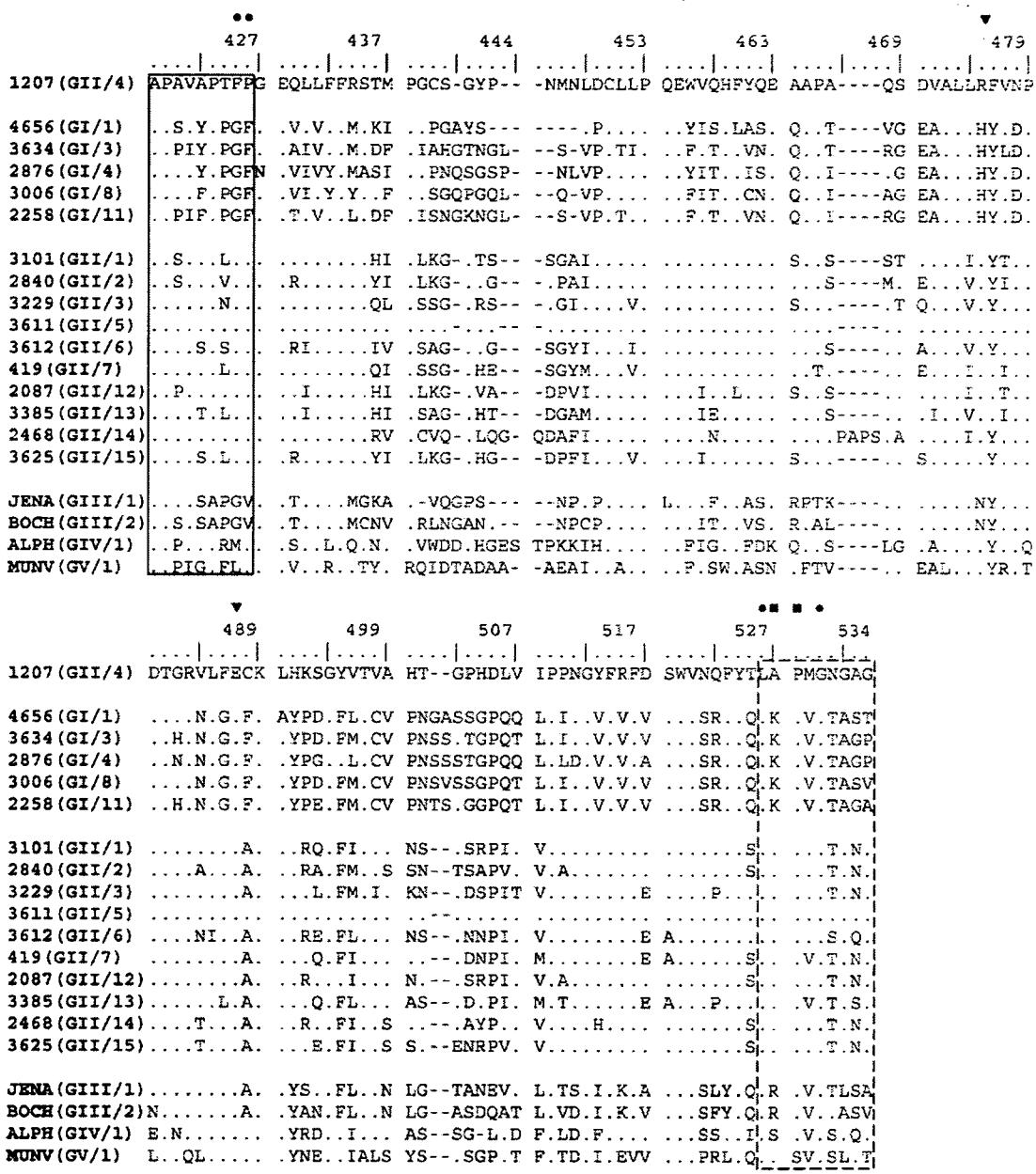

FIG. 2. Alignment of the amino acid sequence of the minimal binding region on noroviruses for MAb14-1. The MAb14-1-specific residues (●), identical components of the previously reported conformational epitope of NV3901 and NV3912 (▼), and the amino acid positions shared between the epitopes of MAb14-1, NV3901, and NV3912 (■) are indicated (37). The solid-line and dashed-line boxes represent the N-terminal antigenic region (A region) and C-terminal antigenic region (B and C region), respectively, on the minimal binding region. Dots indicate identical amino acid residues, and dashes indicate gaps. JENAGIII/1 (Bo/Jena/1980/DE [GenBank accession number AJ011099]), BOCHGIII/2 (Bo/CH126/1998/NL [[GenBank accession number AF320625]), ALPH GIV/1 (Hu/Alphatron/1998/NL [GenBank accession number AF195847]), and MUNV GV/I (Mu/Murine norovirus-1/US [GenBank accession number AY228235]).

fragment analysis, single point mutants, and structure prediction of antigen.

The results of the fragment analysis for VP1 showed that the epitope for MAb14-1 exists on the C-terminal P1 domain, which is more conserved than the N-terminal P1 and P2 domains based on sequence identity among noroviruses. This location of the epitope may be the reason for the same reactivity under different pH conditions because particle (P domain on the surface of the particle) or a single capsid protein did not relate to the accessibility of the epitope on the P domain for MAb14-1.

The fragment analysis for the C-terminal P1 domain and structural analysis showed that almost the whole C-terminal P1 domain generated the conformation of the minimal binding region. Both terminal antigenic regions (amino acid positions 418 to 426 and 526 to 534) on the minimal binding region approached each other via the insertion region (positions 427

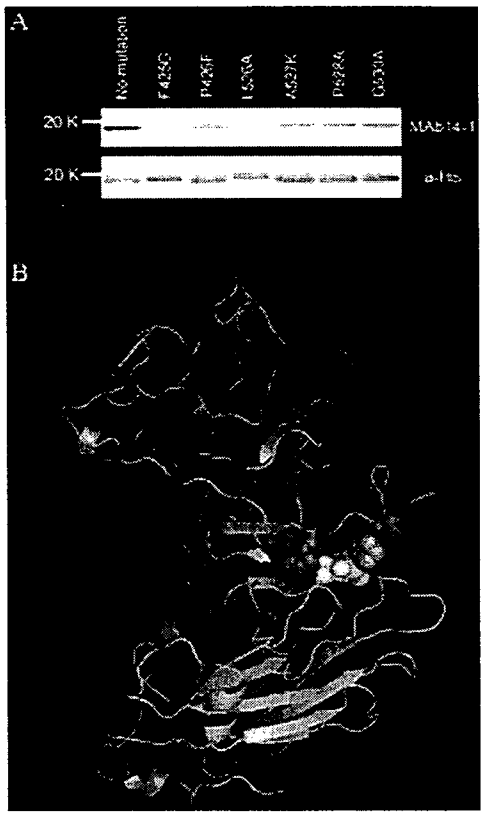

FIG. 3. (A) Reactivities of the six point mutations by Western blotting analysis. 20 K, 20,000; a-His, anti-His_5 antibody. (B) Position of each point mutation on the r1207 prediction structure. The phenylalanine at position 426 (blue), proline at position 427 (red), leucine at position 526 (yellow), alanine at position 527 (green), proline at position 528 (orange), and glycine at position 530 (magenta) are shown.

to 525). This motif forms the conformational epitope and may explain the broad reactivity, because MAb14-1 was generated by immunization of GII/4, which is the most sophisticated strain for immune response, implying a potential evolutionary selection.

The components of the epitope for MAb14-1 were determined by mutational analysis. It was found that the components comprise six amino acids and are classified into four major groups, groups 1 to 4, by the following reactivities.

(i) Not only was L526 conserved in all rVLP sequences but also the same conserved residues in other genogroups of norovirus were observed. More interestingly, L526 was even conserved among other caliciviruses, suggesting that this leucine residue might be influence the calicivirus-specific reactivity of MAb14-1 (7).

(ii) There were two interpretations of the role of F425, which is conserved among GII. One of them was the generation of high-titer GII-specific antigenicity for MAb14-1. A previous study by Chakravarty et al. also supported this observation (5). The other interpretation was the generation of genus-specific antigenicity, due to the existence of a GI-specific phenylalanine close to GII-specific phenylalanine. For confirmation of this interpretation, site-directed mutagenesis on the GI capsid needs to be performed in the future.

(iii) Three residues, P426, A527, and P528, gave the same result in inducing a significant reduction in reactivity, but their roles were probably different from each other. P426 possibly constructed the epitope directly. Parker et al. previously reported that K527 (GI) directly interacted with E487, generating the GI-specific structure (35). As a result, K527 (GI) may induce a low titer of MAb14-1 for GI. Our results also supported the previous observations reported by Parker et al. (35) and Chakavraty et al. (5), in that the difference between K and A induced a difference of antigenicity between GI and GII. P528, which is conserved among all noroviruses, except for

TABLE 4. Cross-reactivities of representative previously reported broadly reactive monoclonal antibodies with various norovirus capsids by Western blotting and/or ELISA

Monoclonal antibody	Isotype	Reactivity[a] of rVLP or recombinant capsid protein with:																				Recognition domain	Minimal binding region	Reference(s)
		Genogroup I genotype[b]							Genogroup II genotype[b]															
		1	2	3	4	6	8	11	1	2	3	4	5	6	7	8	12	13	14	15	17			
MAb14-1	IgG1	−	+	+/−[c]			+	+	+	+	+	+	+	+	+	+	+	+				C-terminal P1	418–534	This study
NV23	IgG1	+	+		+					+		+		+			+		+					20
NS14	IgG1	−	−		−					+		+		+			+		+			C-terminal P1	473–494[d]	20, 34
NV3901 and NV3912	IgG1	+	+		+					−		−		−			−		−			C-terminal P1	454–520[e]	14, 20, 35
1B4	IgG1	−	+	+	−	+		+	+	+	+		+	+		+	+				+	S	31–70[f]	46, 47
1F6	IgG2	−/−[g]	−	+/−[h]	−	+		−	+	+	+		+	+		+	+				+	S	31–70[f]	46, 47
MAb5		+	+		+					+	+	+		+					−					39

[a] Symbols: +, reactive; −, not reactive. Blanks mean the reactivity was unconfirmed. Homologous reactivities are shown in boldface type, especially NS14 against mixture of recombinant norovirus and recombinant Snow Mountain agent (SMA).
[b] Genetic classification based on the method described by Kageyama et al. (17).
[c] MAb14-1 has weak reactivity for GI/3.
[d] Amino acid numbers correspond to the sequence of Norwalk virus (GenBank accession number M87661).
[e] Amino acid numbers correspond to the sequence of Houston virus related to Lordsdale virus (GenBank accession number X86557).
[f] Amino acid numbers correspond to the sequence of the immunogen used to generate the specific antibodies: recombinant norovirus 36 for 1B4 and 1F6 (GenBank accession number AB028244).
[g] +/− indicates positive for KU4bGI (GenBank accession number AB067549) but negative for Gifu'96 (GenBank accession number AB0456039).
[h] +/− indicates positive for Desrt Shield (GenBank accession number U04469) but negative for Stav/95/Nor (GenBank accession number AF145709).

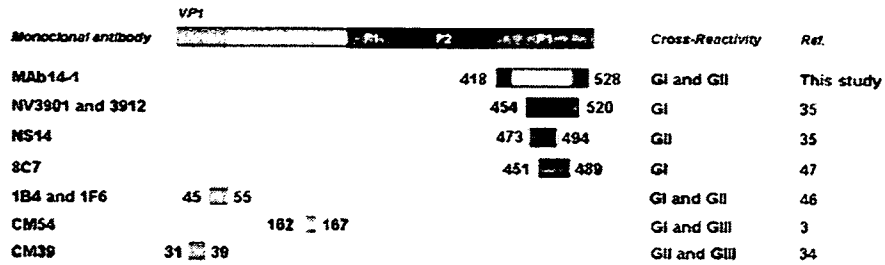

FIG. 4. Location map of the norovirus cross-reactive monoclonal antibody binding sites (being or containing an epitope) on VP1. The blank (amino acids positions 427 to 525) on the binding site for MAb14-1 means that it is not the region for a binding site but is necessary for generating a binding site structurally. Amino acid numbers correspond to the sequences of the immunogens used to generate the specific antibodies: Southampton virus for CM54 (GenBank accession number L07418) Jena virus for CM39 (GenBank accession number AJ011099) Norwalk virus for NV3901 and NV3912 (GenBank accession number M87661), SMA for NS14 (GenBank accession number U70059), recombinant Norwalk virus capsid protein (NV 96-908) for 8C7 (GenBank accession number AB028247), and recombinant genogroup II virus capsid protein (NV 36) for 1B4 and 1F6 (GenBank accession number AB028244). Ref., reference.

murine norovirus, is the component of the epitope and induces GI and GII cross-reactivity of MAb14-1.

(iv) G530 is the critical component of the epitope. Functional change of protein was usually ignored in the change from G to A, because there is not much difference in character between G and A (35). Nevertheless, our results showed that the change was important. It is suggested that a slight difference from G to A generates moderate effect on reactivity when glycine is the main component of the epitope.

These mutational analyses elucidated the character of the epitope residues, explaining GI and GII cross-reactivity of the epitope and difference in titer among GI and GII. High conservation of the six amino acids among GII explains high GII-specific titer of MAb14-1. Genus-specific residues generate tolerant reactivity for GI. GI-specific residues induce low reactivity of MAb14-1 for GI. These results imply that the epitope for MAb14-1 is the genus-specific epitope. To investigate this possibility, the reactivity of MAb14-1 for GIII-V rVLPs needs to be elucidated further.

Our results could not explain the low affinity to GI/3 for MAb14-1 because we could not find appropriate GI/3-specific mutations in the minimal binding region. Two possible explanations for this were proposed. First, the epitope on GI/3 may be inhibited by a conformational change derived from the remote amino acid residue(s) in the minimal binding region. Second, other domains, such as N, S, N-terminal P1, and P2 domains, may shield or mask the epitope, as in previous reports about human immunodeficiency virus or picornavirus (4, 8, 23, 26, 36, 44). To confirm these hypotheses, we need to conduct further investigation including crystallography studies.

The fragment, sequence, structural, and mutational analyses identified the epitope formed by the six amino acids and excluded any other amino acids composing the epitope. The structural sequence of these six amino acids generates a linear region; therefore, we can consider this epitope to have potential as a linear epitope with the binding property for the monoclonal antibody. Moreover, in a previous finding on the linear epitope, five amino acid residues were essential for antibody binding, which supports our supposition (10).

In comparison with the location of a previously reported cross-reactive epitope on VP1 (3, 34, 35, 46, 47), our identified epitope is obviously a novel conformational one (Fig. 4). However, a previously reported GI cross-reactive conformational epitope for monoclonal antibodies, NV3901 and NV3912, and the novel identified epitope in this study shared two amino acid positions, 527 and 528, but the MAb14-1 showed both GI and GII cross-reactivity (Fig. 2) (35). In addition, previous studies reported that broadly reactive monoclonal antibodies, GI and GII cross-reactive antibodies, NS14, 1B4 and 1F6, have linear epitopes (35, 46, 47). Therefore, MAb14-1 had more advantages than previously reported broadly reactive monoclonal antibodies did. In other words, we were the first to identify the GI and GII cross-reactive monoclonal antibody, which recognizes the novel conformational epitope.

With the absence of an appropriate cultivation system, we are not able to use neutralization methods to determine the neutralizing antibody for norovirus. The potentially neutralizing monoclonal antibodies were indirectly determined by Vance's study using histo-blood group antigens assumed to be a receptor for norovirus infection and the putative neutralizing antibodies detecting P2 epitopes (15, 25). Recently, Batten et al. (3) and Oliver et al. (34) reported that anti-human norovirus monoclonal antibody could detect the bovine norovirus, while the opposite was also true. Until now, several neutralizing epitopes have been reported for caliciviruses, except for norovirus (9, 27, 41–43). These previous findings suggest that there are genus-specific neutralizing epitopes on caliciviruses. If this suggestion were true, a broadly reactive monoclonal antibody, such as MAb14-1, which has potential for detecting other caliciviruses, may neutralize calicivirus infection. The use of MAb14-1 may be contribute towards antiviral and vaccine development.

In conclusion, to our knowledge, we were the first group to determine the conformational epitope on the norovirus capsid for GI (GI/1, 4, 8, and 11) and GII (GII/1 to 7 and 12 to 15) cross-reactive novel monoclonal antibody, which showed a weak affinity to GI/3. From these data, MAb14-1 could be applied further for the development of the immunochromatography test.

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Development of a rapid immunochromatographic test for noroviruses genogroups I and II

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Abstract

Norovirus (NoV) is known to cause acute gastroenteritis in children worldwide. Although reverse transcription-PCR (RT-PCR) method is considered to be the "gold standard" for diagnosis of this viral infection, it requires skillful personnel and well-equipped laboratory. In this study, a rapid and easily performable diagnostic kit was developed using immunochromatographic method with rabbit polyclonal antibodies raised against recombinant virus-like particles (rVLPs) of most prevalent genotypes, genogroup II genotypes 3 and 4. This kit was evaluated for reactivity to rVLPs and detection of natural viruses in stool samples collected from children with diarrhea in comparison to the results obtained by RT-PCR. In the prospective assessment, the kit showed agreement rate of 84.1%, sensitivity of 69.8% and specificity of 93.7%. Genotyping of the RT-PCR positive samples by sequence analysis revealed that some heterogeneous genotypes were also detected while some in homogeneous genotypes occasionally showed false negative records resulting in lower sensitivity. No cross-reactivity with other common viral pathogens was observed. Taken together with the result of the detection limit of viral load as small as approximately 10^{6-7} copies/g of stool, the current immunochromatography test is justified for screening for NoV infection with simple laboratory support.

Keywords: Norovirus; Immunochromatography; Rapid detection test; Recombinant virus-like particles

1. Introduction

Norovirus (NoV) is a distinct genus in the family *Caliciviridae* and one of the major causative agents of non-bacterial acute gastroenteritis in children worldwide (Okitsu-Negishi et al., 2004). It contains a single strand RNA genome and is composed of three open reading frames (ORFs) (ORF1, -2, and -3). The ORF2 encodes the capsid protein (VP1), and classification of NoV is performed generally based on the sequence analysis of this capsid gene (Kageyama et al., 2003). NoV is highly infectious and causes not only sporadic acute gastroenteritis but also outbreaks in semi-closed communities such as hospitals, day care centers, and evacuees in natural disasters (Centers for Disease Control and Prevention, 2005; Lopman et al., 2002; Tsugawa et al., 2006). The most prevalent genotypes in sporadic cases in Japan was reported to be genogroup II, genotype 4 (GII/4), followed by GII/3 (Okame et al., 2006), although global outbreak cases contain various genotypes (Kirkwood et al., 2005; Noel et al., 1997; Seto et al., 2005) and numerous new recombinant viruses were also detected (Phan et al., 2006; Tsugawa et al., 2006; Vidal et al., 2006).

Many attempts have been made to establish diagnostic methods for NoV infection. Traditionally, electron microscopy has been used to screen clinical stool samples, which requires skillful personnel and a well-equipped laboratory (Atmar and Estes, 2001). After the successful sequencing of the full gene of NoV, RT-PCR with modified primers has been widely used as a gold standard in many reference laboratories (Atmar and

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Estes, 2001), although this method also takes time and sophisticated machines. Genetic engineering progress has enabled researchers to produce recombinant virus-like particles (rVLPs) by baculovirus expression system (Jiang et al., 1992). They are similar morphologically and antigenically to the native virion, and contributed very much to the establishment of immunological tests such as enzyme-linked immunosorbent assay (ELISA) (De Bruin et al., 2006; Dimitriadis et al., 2006; Gonzalez et al., 2006; Okitsu-Negishi et al., 2004). This is relatively easy to perform but still requires at least 4 h to obtain the result, which is not suitable for managing outbreaks in hospitals or on the spot diagnosis in a clinical setting.

Immunochromatography is one of the representative methods in rapid diagnosis and widely used to detect various infectious diseases, such as influenza virus, rotavirus, and adenovirus (Bon et al., 2007; Fujimoto et al., 2004; Hara et al., 2006). The assays often complete within 30 min, and only limited equipment such as centrifuge machines and micropipettes are needed. Until now, only one report of immunochromatography for NoV (Okame et al., 2003) has been published, which pointed out difficulties in obtaining good sensitivity and specificity due to diverse genotypes with distinct antigenecities of NoV.

The aim of this study was to develop an immunochromatography test using polyclonal antibodies (Poly Abs) raised against the prevailing genotypes of NoV, namely GII/3 and GII/4. Basic evaluation was performed by comparison of results of Poly Ab in ELISA with those obtained by immunochromatography, and clinical evaluation was conducted using stored/freshly collected stool samples based on the results of RT-PCR. Clinical applicability of the immunochromatography test is also discussed with regards to the detection limit, agreement rate, sensitivity and specificity.

2. Materials and methods

2.1. Expression of rVLPs

The expression of 15 rVLP (GI/1 strain 4656, GI/3 strain 3634, GI/4 strain 2876, GI/8 strain 3006, GII/1 strain 3101, GII/2 strain 2840, GII/3 strain 3229, GII/4 strain 1207, GII/5 strain 3611, GII6 strain 3612, GII/7 strain 419, GII/12 strain 2087, GII/13 strain 3385, GII/14 strain 2468, GII/15 strain 3625) has been reported previously (Okame et al., 2003; Okitsu-Negishi et al., 2006). The baculovirus expression system with Gateway Technology (Invitrogen Japan, Tokyo, Japan) was used to produce recombinant bacmids, and the processes from the transfection of bacmids into Sf9 insect cells to purification of rVLPs were performed essentially according to the method of Hansman et al. (2004). Protein concentration of each rVLP was measured by the BCA Coomassie protein assay (Pierce Biotechnology, Rockford, IL) and 150 μg/ml was prepared as stock solution.

2.2. Polyclonal antibody production

Rabbits were immunized subcutaneously with 100 μg of CsCl-purified rVLP GII/3 or GII/4 in Freund's complete adjuvant four times at 2-week intervals. Small volume of blood samples were taken from the rabbits to confirm the absence of high titer of antibody against Tn5 cell which was used for generating recombinant protein to eliminate backgrounds by the ELISA. And then, all the serum was collected 1 week after the last injection. Rabbit IgG was purified from sera using Hi Trap rProtein A FF (Amersham Biosciences, Piscataway, NJ) and used as Poly Ab.

2.3. ELISA for titration of Poly Ab against 15 genotypes of rVLP

Ninety-six-well Maxisorp plates (Nunc, Roskilde, Denmark) were coated with 90 ng of each rVLP per well in 60 μl of 0.1 M carbonate buffer for 1 h 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, 60 μl twofold serially diluted Poly Ab from a starting dilution of 1:400 in PBS-T containing 1% BSA was added to each well and the plate was incubated for 1 h at 37 °C. After washing three times with PBS-T, 60 μl of a 1:10 000 dilution of Horse radish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) was added to each well and the plate was incubated again for 1 h at 37 °C. Having washed three times with PBS-T, 60 μl of substrate *o*-phenylenediamine containing 0.012% H_2O_2, 0.2 M citrate–phosphate buffer was 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 Labsystems Mutiskan MCC microplate reader. For this experiment, the lysate of Tn5 cell was included as negative control. A sample of which sample–negative OD > 0.2 and sample/negative ratio >2.0 was considered positive.

2.4. Conjugation of Poly Ab with colored latex

The Poly Abs raised against rVLP GII/3 and GII/4 were conjugated separately to carboxyl-modified colored latex particles with water-soluble carbodiimide. Briefly, 100 mg of EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, Sigma, St. Louis, MO) were added to 50 mg of carboxyl-modified colored latex particles suspended in 10 mM of boric acid buffer. After mixing at 4 °C, 6.9 mg of polyclonal antibody raised against rVLP GII/3 or GII/4 was added to perform coupling reaction. After being washed with the boric acid buffer, the latex was finally suspended to make up 5.0% of the solution.

2.5. Immunochromatography

2.5.1. Test principles

This test kit included two antibodies; one was against rVLP either GII/3 (Immunochromatography-GII/3) or GII/4 (Immunochromatography-GII/4), the other was against general rabbit IgG which was applied on the control line. The former was applied in a conjugated pad with colored latex and also in the test line as a capture antibody for complex of virus in the

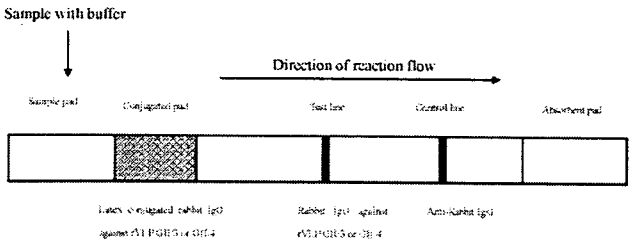

Fig. 1. Diagram of the immunochromatography for NoV.

sample and latex-conjugated antibody. All the lines were painted onto the Hiflow plus nitrocellulose membrane (Millipore, Billerica, MA). The test strip was assembled in the order shown in Fig. 1; sample pad, conjugated pad, nitrocellulose membrane and absorbent pad. All the pads were overlapped to enable migration of the complex of NoV and antibody along the test strip.

2.5.2. Test procedures

The stool sample was diluted with distilled water to 10% suspension and clarified by centrifugation at 10 000 × *g* for 10 min. Fifty microlitres of the diluted stool sample and 50 μl of the reaction buffer (0.2 M NH_4Cl buffer containing 0.15 M NaCl and 0.5% Tween 20) were put into the well of Nunc-Immuno Module (Nunc, Roskilde, Denmark) and mixed well by gentle pipeting. Then, the sample pad of the test strip was inserted in the well to let the reaction mixture come up along the strip by capillary filling. When NoV existed in the sample, it bound with the polyclonal antibody in the conjugate pad and the complex was captured by the immobilized polyclonal antibody applied on the test line and a pink band was formed. The immobilized anti-rabbit IgG combined with the latex-conjugated rabbit polyclonal antibody independently of the NoV antigen, and confirmed that the assay had been performed correctly. It takes 15 min to obtain the result of the assay. Examples are shown in Fig. 2a for a positive sample and Fig. 2b for a negative sample.

2.6. Detection limit of immunochromatography

The rVLPs stock solutions were diluted serially twofold with the above-mentioned reaction buffer and used to determine the detection limit of the immunochromatography for purified antigenic protein. Similarly, clinical stool samples with known viral copy number by real-time PCR (Kageyama et al., 2003) (GII/3: JP3472 5.1×10^9 copies/g of stool, JP3500 2.5×10^{10} copies/g of stool, JP3583 2.3×10^{10} copies/g of stool, JP3590 7.1×10^9 copies/g of stool, JP3607 1.7×10^8 copies/g of stool, JP4933 2.1×10^9 copies/g of stool; GII/4: JP3102 7.7×10^9 copies/g of stool, JP3109 5.3×10^8 copies/g of stool, JP3296 4.9×10^9 copies/g of stool, JP3303 5.0×10^9 copies/g of stool), which were kindly provided by Dr. Osamu Nishio, The National Institute of Infectious Diseases, Japan, were used for determination of detection limit of viral load in clinical samples. Each assay for rVLPs was conducted in triplicate, whereas the mean value of detection limit of viral load for each genotype was expressed for each type of immunochromatography.

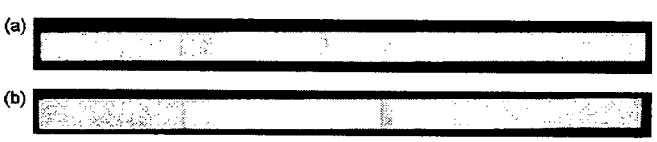

Fig. 2. (a) A representative photograph of a positive sample. (b) A representative photograph of a negative sample.

2.7. Cross-reactivity of immunochromatography among 15 genotypes of rVLP and 10 genotypes of NoV in clinical samples

In order to investigate whether the immunochromatography system possessed the same property in terms of cross-reactivity observed in ELISA, concentrated rVLP of each genotype as well as various genotypes of NoV isolated from clinical stool samples were tested by the immunochromatography. Seven hundred and fifty nanograms per milliliter of each genotype rVLP was prepared by diluting the stock solution with reaction buffer and 100 μl of the solution was applied to the well of Nunc-Immuno Module. The results were recorded as +, ±, and − according to the density of the test line. Assay for each rVLP was conducted in triplicate. For checking the cross-reactivity of NoV in clinical samples, 10 genotypes, GI/1, GI/4, GII/1, GII/2, GII/3, GII/4, GII/5, GII/6, GII/13, and GII/14 were used from the stored stool samples collected among children with diarrhea between 1995 and 2003 in Japan. Two samples positive for astrovirus were also selected from this group of stored samples for the assessment of cross-reactivity with enteric viruses.

2.8. Prospective assessment of immunochromatography in the peak season of NoV infection

In order to evaluate the immunochromatography with no interference which may arise due to repeated freezing and thawing of the samples as well as the effect of centrifugation performed for other analyses, prospective assessment was conducted using freshly collected samples. A total of 107 stool samples were obtained from children with acute gastroenteritis visiting one pediatric clinic in Kyoto, Japan in the winter from December 2005 to March 2006, during which period NoV infection reaches its peak in Japan (Okame et al., 2006).

2.9. RT-PCR and sequencing

The viral genomes were extracted from 140 μl of 10% stool suspension using a QIAamp spin-column technique according to the manufacturer's instructions (QIAGEN, Hilden, Germany). Subsequently, two sets of RT-multiplex PCR were performed to detect NoV (GI and GII), astrovirus, sapovirus, rotavirus and adenovirus (Yan et al., 2003, 2004). NoV-negative samples were investigated by two sets of monoplex PCRs for NoV GI and GII. Four samples were further analyzed by semi-nested PCR using a set of primers, G2SKF and G2SKR for NoV GII (Kageyama et al., 2003). Positive PCR products were subjected to sequencing to determine the genotypes of NoV using Big-Dye terminator cycle sequencing kit and an ABI prism 210 Genetic Analyzer (Applied Biosystem,