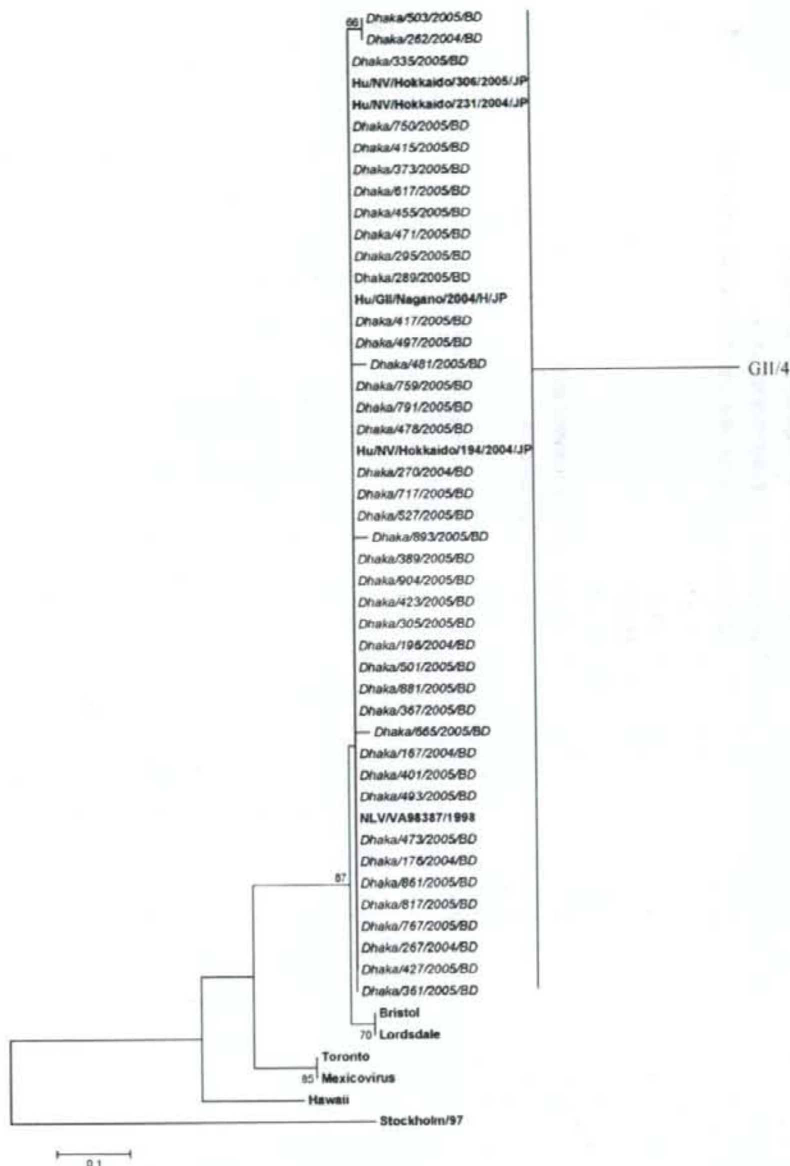

Fig. 1. Phylogenetic tree of nucleotide sequences of Bangladeshi norovirus. The tree was constructed from partial amino acid sequences of capsid region of norovirus. Reference strains of norovirus were selected from DDBJ/GenBank under the accession number indicated in bold. Bangladeshi norovirus was highlighted in italic. Stockholm/93 strain was used as an out-group strain for phylogenetic analysis. The scale indicates nucleotide substitutions per position. The numbers in the branches indicate the bootstrap values. The GenBank accession numbers of this study are: DQ889461–DQ889501.

We found that NoV-infected persons developed symptoms of severe vomiting and watery diarrhea and typically remain symptomatic for 2–3 days.

Although the importance of viral gastroenteritis as a prime cause of morbidity and mortality in developing countries is well recognized, very few studies were conducted to evaluate the role of viral agents in childhood diarrhea in Bangladesh.

In conclusion, this is the first epidemiological research of norovirus in Bangladesh.

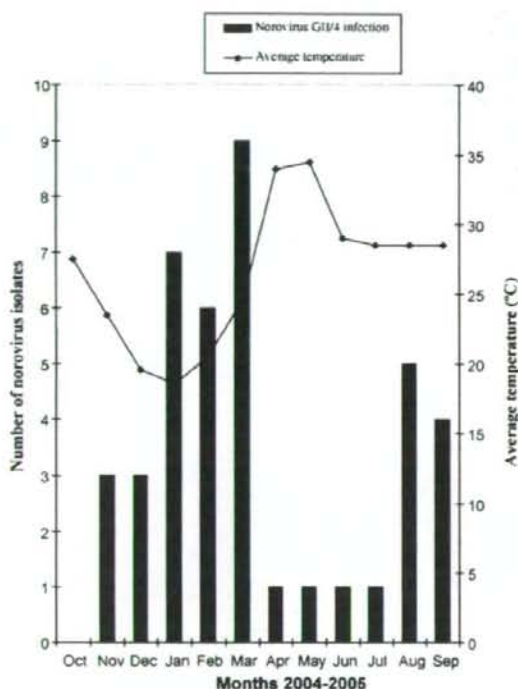

Fig. 2. Seasonal pattern of norovirus infection in infants and children with acute gastroenteritis in Dhaka City, Bangladesh during October 2004–September 2005. Average temperature data were obtained from the Metrological Institute, Dhaka.

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Genetic Diversity of Noroviruses and Sapoviruses in Children Hospitalized With Acute Gastroenteritis in Chiang Mai, Thailand

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Human caliciviruses, including norovirus (NoV) and sapovirus (SaV), are recognized as common pathogens that cause acute viral gastroenteritis in children and adults throughout the world. To gain an overview of molecular epidemiology of human caliciviruses in children hospitalized with acute gastroenteritis in Chiang Mai, Thailand, from 2002 to 2004, NoV and SaV were detected and characterized molecularly for identification of their genotypes. From a total of 248 fecal specimens collected, 35 (14.1%) were positive for NoV GII genogroup. Among the 35 NoV GII, GII/4 was the most predominant genotype (22 strains), followed by GII/3 (7 strains), GII/1 (2 strains), GII/7 (2 strains), GII/2 (1 strain), and GII/16 (1 strain). In addition, only three specimens (1.2%) were positive for SaV, each of which was classified into two different genogroups. One isolate was clustered with GIV genogroup, while the other two belonged to two distinct genotypes of the SaV GI cluster, GI/1 and GI/2 genotypes. This study demonstrated that human caliciviruses are important enteric viruses that caused acute gastroenteritis in the hospitalized children in Chiang Mai, Thailand from 2002 to 2004. Moreover, a great genetic diversities of NoV and SaV were observed. ***J. Med. Virol. 79:1921–1926, 2007.*** © 2007 Wiley-Liss, Inc.

KEY WORDS: noroviruses; sapoviruses; acute gastroenteritis; Chiang Mai; Thailand

INTRODUCTION

Viral gastroenteritis is one of the most common illnesses in humans worldwide, and different agents such as rotavirus, astrovirus, adenovirus, and calicivirus have been associated with the disease [Clark and McKendrick, 2004]. Among different types of viruses that cause diarrhea, rotavirus is the most common, and is a major cause of severe gastroenteritis in infants and young children worldwide [Parashar et al., 2006]. Recently, however, human caliciviruses have emerged as significant etiologic agents of diarrheal disease across all age groups. Norovirus (NoV) and sapovirus (SaV) are two of the four genera of the family *Caliciviridae*, which is a nonenveloped, positive-sense, single-stranded RNA [Green et al., 2001]. These viruses are a leading cause of gastroenteritis worldwide and are responsible for outbreaks in various epidemiological settings, including restaurants, schools, day-care centers, hospitals, nursing homes, and cruise ships [McEvoy et al., 1996; Russo et al., 1997; McIntyre et al., 2002; Akihara et al., 2005].

Extensive molecular epidemiological studies of calicivirus infection in humans have been conducted. Application of RT-PCR and DNA sequencing techniques for the detection and characterization of NoV and SaV became the standard tests for detecting these pathogens [Yan et al., 2004]. These detection techniques have enhanced markedly our understanding of the epidemiology of NoV and SaV infections. Thousands of NoV and SaV strains have been identified, named, and classified into genogroups and genetic clusters.

Currently, based on the diversity of capsid sequences, NoVs are grouped into five genogroups (GI–GV), of which three have been found in humans; GI, GII, and GIV [Kageyama et al., 2004; Zheng et al., 2006]. Human NoV genogroups are subdivided further into at least

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15 genotypes in GI, 18 genotypes in GII, and only 1 genotype in GIV [Kageyama et al., 2004; Vinje et al., 2004; Okada et al., 2005]. Several epidemiological studies clearly indicated that NoV GII is the main causative agent among NoV, which causes acute diarrhea in humans [Hansman et al., 2004; Phan et al., 2006a,b; Tseng et al., 2007]. SaVs can be divided into five genogroups (GI–GV), of which GI, GII, GIV, and GV are known to infect humans, whereas SaV GIII infects porcine species [Farkas et al., 2004; Wang et al., 2006; Hansman et al., 2007].

In Thailand, very few molecular epidemiological studies of NoV and SaV have been conducted and various genotypes were circulated in different epidemiological settings [Guntapong et al., 2004; Hansman et al., 2004; Veeravigrom et al., 2004]. The frequency of NoV and SaV detection rates ranged from 8.6%–17.5% and 4.8%–15.0% of diarrheal disease in hospitalized cases. However, only two studies have been characterized further for their genotypes by sequence and phylogenetic analyses. The study carried out in Chiang Mai during 2000 to 2001 demonstrated that out of 105 specimens collected, 8 and 4 were found to be a single infection with NoV and SaV, while 1 was a mixed infection [Hansman et al., 2004]. The other study was conducted during 2002–2003. The stool specimens were collected from five different geographical areas in Thailand (Sa Kaeo, Chanthaburi, Songkhla, Nong Khai, and Tak). Of the 80 specimens examined, 11 and 9 of NoV and SaV single infections were identified, respectively, and three were found to be mixed infections [Guntapong et al., 2004].

With the aim of having an overview of molecular epidemiology of human caliciviruses in children hospitalized with acute gastroenteritis in Chiang Mai, Thailand, the prevalence of NoV and SaV infections were examined from 2002 to 2004. The genotypes of NoV and SaV were identified by sequence and phylogenetic analyses.

MATERIALS AND METHODS

Specimens Collection

A total of 248 fecal specimens were collected from pediatric patients aged less than 5-years-old, who were hospitalized with acute gastroenteritis at McCormick Hospital, Chiang Mai, Thailand. The study period was from March 2002 to December 2004.

RNA Extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The RNA genome of NoV and SaV were first extracted from 10% fecal suspension supernatant using the QIAamp viral RNA Mini Kit (Qiagen, Hilden, Germany). The presence of the NoV (GI and GII) and SaV in fecal specimens was detected by RT-PCR using the protocol described previously [Yan et al., 2004]. A forward primer, G1-SKF (nt 5342–5361) 5′-CTGCCCGAAT-TYGTAAATGA-3′, was used in combination with the reverse primer G1-SKR (nt 5653–5671) 5′-CCAACC-CARCCATTRTACA-3′, for the amplification of NoV GI, which specifically generated a PCR amplicon of 330 bp. For NoV GII identification, a forward primer, COG2F (nt 5003–5028) 5′-CARGARBCNATGTTYAGRTGGA-TGAG-3′, was used in combination with the reverse primer G2-SKR (nt 5367–5389) 5′-CCRCCNGCAT-RHCCRTTRTACAT-3′, which showed a PCR product size of 387 bp. For SaV detection, a 434 bp fragment was generated using forward primer SLV5317 (nt 5317–5339) 5′-CTCGCCACCTACRAWGCTTGGTT-3′ and reverse primer SLV5749 (nt 5727–5749) 5′-CGGRCYT-CAAAVSTACCBCCCCA-3′. All of the virus positive samples were analyzed further for their genotypes by nucleotide sequence and phylogenetic analyses.

Sequence and Phylogenetic Analyses

The PCR amplicons were purified with a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI) and sequenced by using the BigDye Terminator Cycle Sequencing kit (Perkin Elmer-Applied Biosystems, Inc., Foster City, CA) on an automated sequencer (ABI 3100; Perkin Elmer-Applied Biosystems, Inc.). The primers used for amplification of the partial capsid genes were also used as sequencing primers. The sequences obtained were compared to those of NoV and SaV strains deposited in the GenBank using the BLAST program. The genotypes of NoV and SaV were classified by using the clustering methods determined previously by Kageyama et al. [2004] and Phan et al. [2007b].

Nucleotide Sequence Accession Numbers

The partial nucleotide sequences of the capsid gene were deposited in GenBank under the accession number EF600759–EF600793 for NoV strains and EF600794–EF600796 for SaV strains.

RESULTS

Detection and Genotype Distribution of Noroviruses

Between March 2002 and December 2004, a total of 248 fecal specimens were collected from pediatric patients hospitalized with diarrhea, and 35 (14.1%) were found positive for NoV by using the RT-PCR screening method (Table I). Of these, all of the positive samples were identified as NoV GII genogroup, and none was a mixed infection between NoV and SaV. The age of the patients, who were infected with NoV, ranged from 4 months up to 3-years-old. All of the NoV positive samples were characterized further for their genotypes by sequencing of the partial capsid regions. The genotypes were classified according to the phylogenetic clustering method described by Kageyama et al. [2004]. A total of 35 NoV GII isolates were clustered exclusively with other GII reference strains and these GII strains were classified further into six distinct genotypes, including GII/1, GII/2, GII/3, GII/4, GII/7,

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

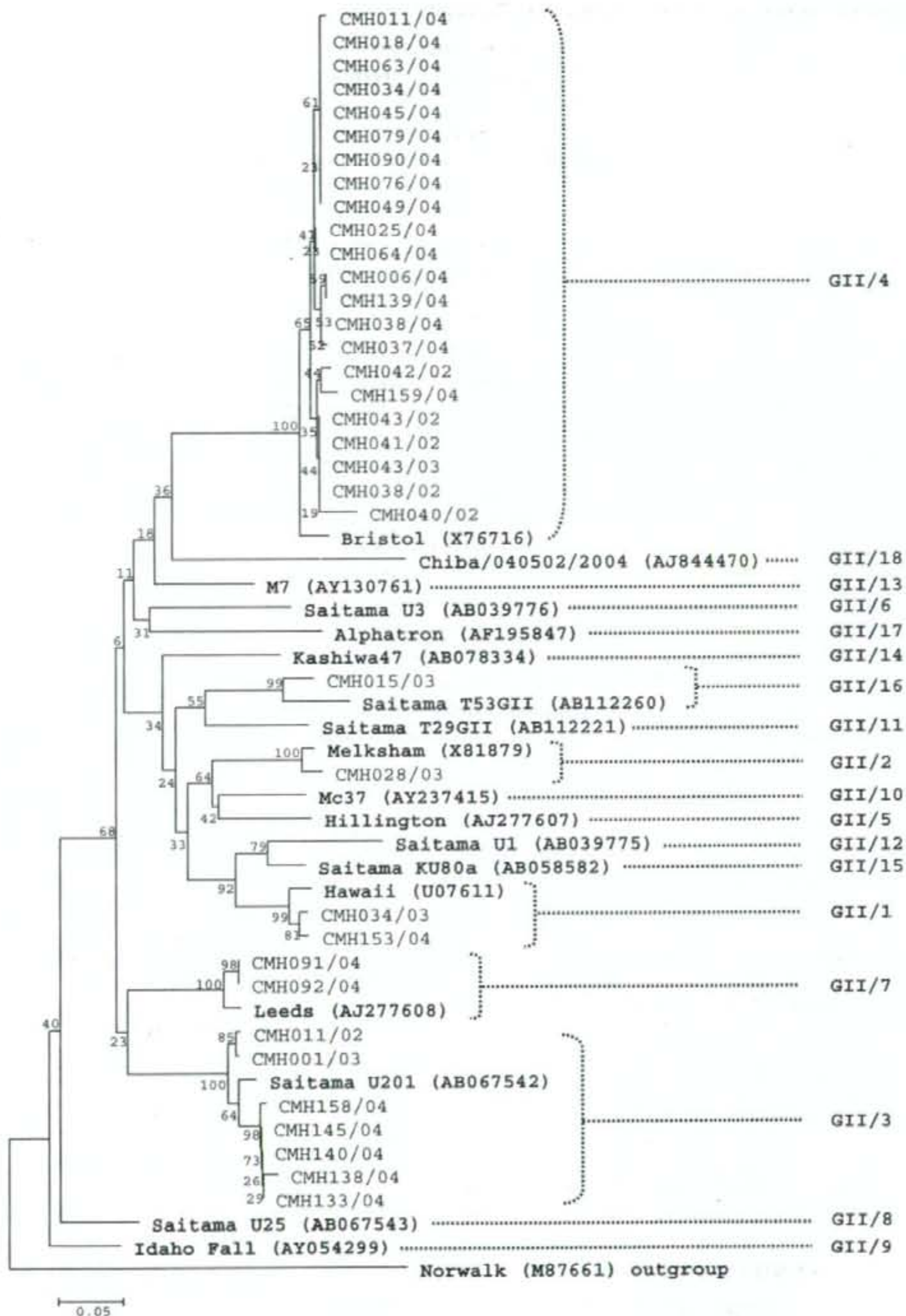

Fig. 1. Phylogenetic analysis of partial capsid sequences of NoV detected in pediatric patients hospitalized with acute gastroenteritis in Chiang Mai, Thailand from 2002 to 2004. The tree was constructed on the basis of the neighbor-joining method and the numbers on each branch indicate the bootstrap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

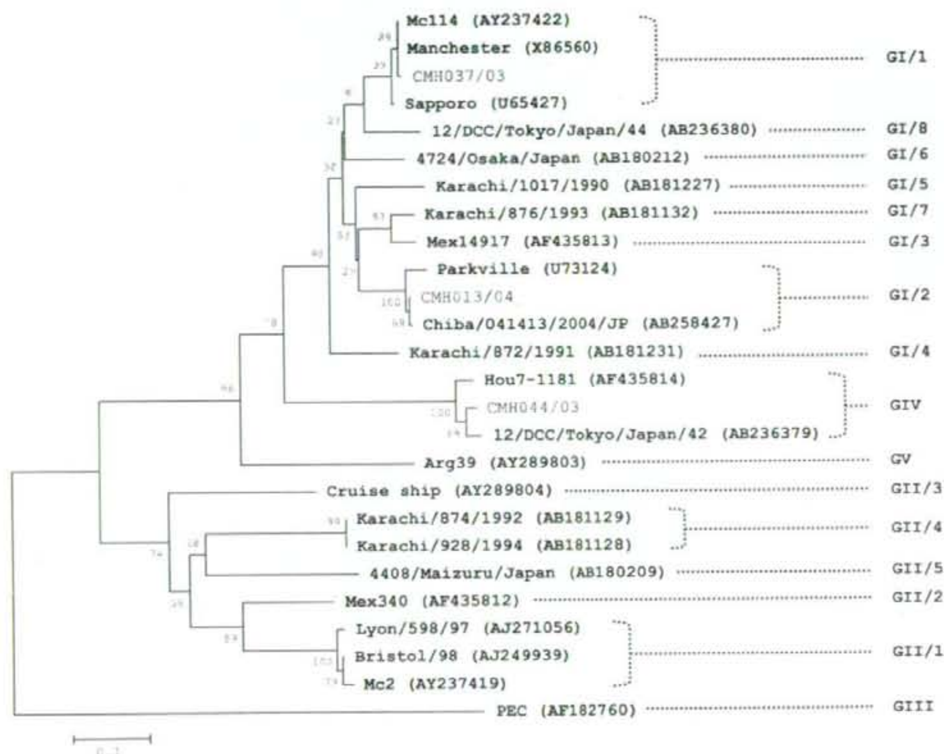

Fig. 2. Phylogenetic analysis of partial capsid sequences of SaV detected in pediatric patients hospitalized with acute gastroenteritis in Chiang Mai, Thailand from 2002 to 2004. The tree was constructed on the basis of the neighbor-joining method and the numbers on each branch indicate the bootstrap 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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Humoral Immune Responses Against Norovirus Infections of Children

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In 2 infants with gastroenteritis associated with Norovirus (NoV), serum immunoglobulin (Ig) G, IgM, IgA, and fecal IgA antibody responses against NoV were examined by enzyme-linked immunosorbent assay using 11 different antigenic and genetic types of NoV virus-like particles expressed in insect cells. These two cases were putative primary single NoV infections, because antibodies against NoVs were not detected in acute-phase serums. In one of two cases, long-term excretion of virus RNA for 33 days was observed. Serum IgG responses demonstrated strong seroresponse to the homologous type, and weak seroresponse to the heterologous types within the genogroup. After more than 2 years, the IgG antibody titer remained high to the homologous type and low to the heterologous type within the genogroup. IgM and IgA were specific to the homologous type. IgM was short lived and the serum IgA antibody titer remained low to the homologous type for a long period. These results improve our understanding of the humoral immune response to NoV infection. ***J. Med. Virol. 79:1187–1193, 2007.***

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KEY WORDS: norovirus; primary infection; humoral immune response; ELISA; virus-like particle

INTRODUCTION

Noroviruses (NoVs) in the family of *Caliciviridae* are the major cause of acute nonbacterial gastroenteritis in all age groups, and NoV infections have occurred worldwide as outbreaks and sporadic cases [Green et al., 2001].

NoVs are a genetically and antigenically diverse group. Genetic analysis of the RNA polymerase and capsid region revealed that human NoVs can be divided into two genogroups, genogroup I (GI) and genogroup II (GII) [Green et al., 1994; Wang et al., 1994]. Recent studies of genotyping on the basis of capsid *N*-terminal/shell (N/S) domain classified NoVs into at least 31 genotypes (14 genotypes in GI and 17 genotypes in GII) [Katayama et al., 2002; Kageyama et al., 2004]. These viruses cannot be grown in cell culture, but the expression of the major capsid protein (VP1) in insect cells resulted in the formation of virus-like particles (VLPs) that are morphologically and antigenically similar to native NoV [Jiang et al., 1992; Lew et al., 1994]. Antigenic analysis using enzyme-linked immunosorbent assay (ELISA) with VLPs and hyperimmune antisera showed that the genetic and antigenic relationship corresponded well [Kobayashi et al., 2000a,b,c; Katayama et al., 2002; Kamata et al., 2005; Hansman et al., 2006], but there were unusual cross-reactivities between certain genogroups and/or genotypes based on the antibody ELISA [Hansman et al., 2006]. Serological studies using these recombinant VLP (rVLPs) have shown a high prevalence and broad responses of NoV-specific antibodies both in children and adults [Parker et al., 1994a, 1995; Noel et al., 1997; Farkas et al., 2003]. IgM, IgA, and IgG serologic responses in adult volunteers and patients also reported [Treanor et al., 1993;

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Gray et al., 1994; Rockx et al., 2005a]. But almost all sera used in previous studies had pre-existing antibodies against many types of NoV. Therefore, it was not clear whether the production of cross-reactivity antibodies emerged after primary infection or by multiple infections during their lifetime. In addition, there is little information about the immune response to NoV infections in infants, including primary infection. To interpret the cross-reactivity of antibodies to NoVs, it is necessary to investigate the immune response to primary NoV infection.

In the present study, serum and fecal samples from infants infected with NoV were examined for specific IgM, IgA, and IgG using ELISA with 11 different genotypes of VLPs. This study describes the cross-reactivity and specific antibody responses in putative primary NoV infection.

MATERIALS AND METHODS

Patients

Fecal or serum samples were collected from 2 infants suffering from nonbacterial gastroenteritis at their medical examination and follow-up in the hospital. After the ethical discussion in this working group, we concluded that this study was ethically acceptable. Informed consent for this study was obtained from their parents. The histo-blood group antigen (HBGA) type of the two infants was unknown. Their development was normal. After recovery from diarrhea, their prognosis was favorable. These two cases were sporadic and not related to each other.

Patient A (male): He had acute gastroenteritis at 2 years in December. The major symptoms were diarrhea and vomiting. The diarrhea was sometimes accompanied by benign afebrile convulsions. He had mild diarrhea for about 6 weeks. During the diarrhea, five fecal and serum samples on the 5th, 12th, 18th, 33rd, and 40th days post-onset were collected. After recovery from diarrhea, one fecal and one serum sample on the 60th day post-onset, and one serum sample at 2 years and 10 months post-onset were collected. Re-infection of NoVs during this long period was unknown.

Patient B (male): He had acute gastroenteritis at 1 year in January. The major symptom was diarrhea. The diarrhea was sometimes accompanied by benign afebrile convulsions. He had mild diarrhea for about 1 week. During the diarrhea, one fecal and one serum sample on the 2nd day post-onset were collected. After recovery from diarrhea, one serum sample on the 105th day post-onset was collected. He did not have gastroenteritis again during the period studied.

Detection of NoVs and Other Viruses in Fecal Specimens

NoVs were detected using RT-PCR with primer pairs, Ando's G1 (SR33, SR48, SR50, and SR52) and G2 (SR33 and SR46) primer sets [Ando et al., 1995] amplifying a 123-bp RNA polymerase region, mon381/mon383 [Noel et al., 1997] amplifying a 322-bp capsid region, and SK primers [Kojima et al., 2002] amplifying a 344-bp capsid N/S region as previously described [Iritani et al., 2000; Seto et al., 2005]. Other gastroenteritis viruses were detected with our laboratory method [Iritani et al., 2003]. Briefly, antigens of group A rotavirus and enteric adenoviruses (serotypes 40 and 41) were tested using commercially available antigen ELISA kits, ROTACLONE, and ADENOCLONE-E, respectively, according to the instructions (Meridian Bioscience, Inc., Cincinnati, OH). Enteroviruses and adenoviruses were tested using cell cultures with Vero and RD-18S cells. The virus-negative samples for group A rotavirus, adenoviruses, enteroviruses, and NoVs were tested using electron microscopy (EM) [Iritani et al., 2000], to directly detect virus particles with a negative stain.

Genetic Analysis of NoVs

Sequencing of RT-PCR products and phylogenetic analysis were performed as previously described [Iritani et al., 2000; Seto et al., 2005]. Genotyping based on the Capsid N/S domain was performed as described by Katayama et al. [2002] and Kageyama et al. [2004].

Expression of VLPs

Eleven VLPs (four genotypes in GI and seven genotypes in GII), expressed in insect cells infected with recombinant baculoviruses carrying the capsid gene, were used for ELISA (Table I). These VLPs were produced in the Department of Virology II, National Institute of Infectious Disease. The expressed capsid antigens were purified by a sucrose gradient followed by CsCl gradient centrifugation and confirmed by EM, as previously described [Kobayashi et al., 2000a,b,c; Tamura et al., 2000; Kamata et al., 2005].

Antibody ELISA for Serum Samples

The wells of 96-well flat-bottom microtiter plates (IMMULON2 HB, Dynex Technologies, Inc., Chantilly, VA) were coated with 100 μl of each VLP (0.5 μg/ml in 0.05 M carbonate–bicarbonate buffer (pH 9.6)), and incubated at 4°C overnight. The wells were then washed twice with 10 mM phosphate-buffered saline containing 0.05% Tween 20 (PBS-T; pH 7.2) using a micro plate washer (S8/12J model, Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) and were blocked at room temperature for 1 hr with 200 μl of Block Ace (Dainippon Pharmaceutical Co., Ltd.). The wells were washed twice and twofold serial dilutions in PBS-T containing 25% Block Ace (25% BA/PBS-T) of serum samples, starting at a 1:50 dilution, were added to antigen-coated plates. After incubation for 1 hr at 37°C, the wells were washed five times, 100 μl of horseradish peroxidase (HRP)-conjugated goat anti-human IgG (heavy and light chains), IgM (μ-chain), or IgA (α-chain) -specific antibodies (Zymed Laboratories, Inc., South San Francisco, CA) at a dilution of 1:4,000 in 25% BA/PBS-T were added, and the plates were incubated for 1 hr at 37°C. After washing the wells five times, 100 μl of substrate, 0.4 mg/ml of *O*-phenylenediamine (Sigma Chemical Co.

TABLE I. Description of 11 VLPs Used in This Study

Genogroup	Genotype	VLP	Strain	Accession no.	References
GI	GI/1	rSeto	Aichi124/1989/JP (Seto)	AB031013	Kobayashi et al. [2000b]
	GI/2	rFUV	Funabashi 258/1996/JP	AB078335	Tamura et al. [2000]
	GI/3	r645	Kashiwa645/1999/JP	BD011871	Kamata et al. [2005]
	GI/4	rChiba	Chiba407/1987/JP	AB022679	Kobayashi et al. [2000a]
GII	GII/3	r809	Sanbu809/1998/JP	BD011876	Kamata et al. [2005]
	GII/4	rNAV	Narita104/1997/JP	AB078336	Kitamoto et al. [2002]
	GII/5	r745	Ichikawa745/1998/JP	BD011877	Kamata et al. [2005]
	GII/6	rUEV	Ueno7k/1994/JP	AB078337	Tamura et al. [2000]
	GII/7	r10–25	Osaka10-25/1999/JP	BD011881	Kamata et al. [2005]
	GII/12	rChitta	Chitta76/1996/JP	AB032758	Kobayashi et al. [2000c]
	GII/14	rKAV	Kashiwa47/2000/JP	AB078334	Kitamoto et al. [2002]

Ltd., St. Louis, MO) was added and the plates were incubated for 30 min at room temperature. The reaction was stopped with 50 µl of 4N H_2SO_4. Absorbance at 492 nm (A492) was measured with a microplate reader (Multickan MS-UV model, Labsystems OY, Helsinki, Finland). IgG, IgM, or IgA titers were defined as the highest dilution of serum given an A492 that was threefold higher than the A492 of the corresponding antigen control well.

Antibody ELISA for Fecal IgA

Five fecal samples on the 5th, 12th, 18th, 40th, and 60th days from Patient A were used for ELISA to detect VLP-specific IgA. There was not sufficient volume of the other samples for ELISA. Fecal samples were prepared as a 10% (wt/vol) suspension in PBS containing 10% fetal bovine serum (FBS). Each fecal suspension was homogenized, and centrifuged at 12,000g for 10 min. The supernatant was used for ELISA to detect VLP-specific IgA.

The plates were coated with VLPs as described above. Twofold serial dilutions of fecal suspensions were made in PBS containing 10% FBS. The diluted suspensions were added to antigen-coated plates; thereafter, the ELISA protocol was performed as described above. Virus-negative fecal samples were included on each plate as a negative control. The sample was considered positive for VLP-specific IgA when the absorbance of the well containing fecal samples from patient A was threefold higher than that of the negative control well.

Nucleotide Sequence Accession Numbers

The nucleotide sequences determined in this study were deposited in DDBJ with the following accession numbers: AB089871, OCS980730 (patient A); AB262773, OCS000564 (patient B).

RESULTS

Detection of the Viruses in Stool Specimens From Two Infants With Gastroenteritis

NoVs were detected in two infants by RT-PCR (Table II). In patient A, who had mild diarrhea for about 6 weeks, four fecal samples on the 5th, 12th, 18th, and 33rd day were NoV-positive, although the sample on the 33rd day produced a thin band (data not shown). Those four RT-PCR products had identical nucleotide sequences. In patients B, who had mild diarrhea, fecal samples on the 2nd were NoV-positive, respectively. These stool specimens were negative to other etiological agents of gastroenteritis. NoV strains from the two infants were classified as GII/4 genotype in the capsid N/S region (Fig. 1). These two NoV strains were closely related, with 100% amino acid identity and 98.8% nucleotide identity in the RNA polymerase region, and 100% amino acid identity and 98.5% nucleotide identities in the capsid region. Pairwise comparison of the capsid N/S region showed that these two NoV strains had 100% amino acid identity and 98.9% nucleotide identities to NAV (GII/4), 73.4–78.7% amino acid identities to the other 6 GII VLP strains, and 60.2% amino acid identities to the 4 GI VLP strains.

Detection of Specific Antibodies to 11 VLPs in the Two Infants

The specific IgG antibody titer to the 11 VLPs is shown in Table II. In two acute-phase serum samples from patient A on the 5th day and patient B on the 2nd day, none of the specific IgG antibodies to all VLPs were detected (<1:50). IgG antibodies to rNAV in patient A showed that the first detection was the 12th day post-onset, and the highest titer was observed on the 33rd to 60th day (1:25,600). IgG antibodies to other five GII VLPs excluding r809 appeared from the 18th to 40th day post-onset and their appearance had a time lag. In a serum sample from patient A at 2 years and 10 months, high IgG titers (1:6,400) to rNAV have persisted, and those IgG titers to the other six GII VLPs were the same or higher than other convalescent-phase serum. In patient B, IgG antibodies to rNAV and other six GII VLPs including r809 were detected with high titer (1:51,200) and low (1:100 – 1:200), respectively. There were no specific IgG antibodies to the four GI VLPs in any serum samples.

Specific IgM antibodies to rNAV were detected in four serum samples on the 12th, 18th, 33rd, and 40th days from patient A (Table III). Two serum samples on the 12th and 18th days had a high titer (1:1,600), and later

TABLE II. Detection of IgG to the 11 Kinds of VLPs in Serum Samples Collected From Infantile Patients by ELISA

Patient (age)	Time post-onset of illness	Symptoms[a]	RT-PCR[b]	Reciprocal of serum dilution										
				Genogroup I				Genogroup II						
				rSeto (GI/1)	rFUV (GI/2)	r645 (GI/3)	rChiba (GI/4)	r809 (GII/3)	rNAV (GII/4)	r745 (GII/5)	rUEV (GII/6)	r10-25 (GII/7)	rChitta (GII/12)	rKAV (GII14)
A (2 years)	5th day	D, V, AC	+	<50	<50	<50	<50	<50	**<50**	<50	<50	<50	<50	<50
	12th day	D	+	<50	<50	<50	<50	<50	**800**	<50	<50	<50	<50	<50
	18th day	D, AC	+	<50	<50	<50	<50	<50	**12,800**	50	<50	50	<50	<50
	33rd day	D, AC	+w	<50	<50	<50	<50	<50	**25,600**	200	<50	100	50	100
	40th day	D, AC	−	<50	<50	<50	<50	<50	**25,600**	200	50	100	50	100
	60th day	Recovered	−	<50	<50	<50	<50	<50	**25,600**	200	50	100	100	100
	2 years 10 months	Normal	NT	<50	<50	<50	<50	400	**6,400**	400	200	200	200	400
B (1 year)	2nd day	D, AC	+	<50	<50	<50	<50	<50	**<50**	<50	<50	<50	<50	<50
	105th day	Recovered	NT	<50	<50	<50	<50	100	**51,200**	100	100	200	100	100

Bold: homologous type.
[a]D, Diarrhea; V, vomiting; AC, afebrile convulsions.
[b]+, Positive; +w, positive but weak band; −, negative; NT, not tested.

samples had a reduction in the IgM titer. There were no specific IgM antibodies to rNAV in serum samples from patient B. IgM antibodies to the other 10 VLPs were not detected in any serum samples.

Specific IgA antibodies to rNAV were detected in all serum samples excluding two acute-phase sera on the 5th day of patient A and the 2nd day of patient B (Table III). For patient A, three serum samples on the 18th, 33rd, and 40th days had a high IgA antibody titer (1:800–1:1,600) and a serum sample at 2 years and 10 months had a low titer (1:100) to rNAV. IgA antibodies to the other 10 VLPs were not detected in any serum samples excluding a serum sample from patient A at 2 years and 10 months to r809 (Table III).

In the five fecal samples of patient A, specific IgA antibodies to rNAV were detected in three samples collected after the 18th day post-onset (Table III). IgA antibodies to the other 10 VLPs were not detected in any fecal samples.

DISCUSSION

From the detection of NoV-specific antibodies in the two patient sera, it appeared that these two patients were putative primary single infections of NoV because antibodies against NoVs were not detected in acute-phase sera. These two cases showed three distinct features compared to the usual NoV-associated gastroenteritis; obstinate mild diarrhea for 1 and 6 weeks, long-term excretion of virus RNAs from patient A for 33 days, and benign afebrile convulsions. The detection of NoVs in patient A indicated that his diarrhea was related to NoV infection up to 33 days, but the direct relation between diarrhea and NoV infection is then unclear, because NoV was not detected from fecal samples of diarrhea on the 40th day post-onset. In a recent study, Rockx et al. [2002] showed that children under 1 year had a tendency toward long-term duration of diarrhea up to the 28th day and excretion of the virus gene up to the 22nd day. Some reports also showed the long-term duration of diarrhea in NoV infections in patients under 2 years [Sakai et al., 2001; Tsugawa et al., 2006]. Our previous study showed that benign afebrile convulsion-associated diarrhea in NoV infections was observed in 6.7% of children under 2 years [Iritani et al., 2003]. Primary NoV infection or age under 2 years may be related to those three distinct features, persistent diarrhea, viral excretion, and afebrile convulsion. Therefore, these two cases in this study were considered general infection cases with NoVs.

Our data showed that the kinetics of serum antibody responses for NoV infection as follows, the peak of IgM in 5th to 12th day, decrease in 18th to 33rd day, and disappearance in 40th to 60th day; the peak of IgA in 12th to 18th day, decrease in 40th to 60th day, and persistence for a long period with low titer; the peak of IgG in >18th day, and persistence for a long period with high titer. This kinetics of serum antibody responses is similar to those seen in previous studies [Erdman et al., 1989; Gray et al., 1994; Brinker et al., 1998, 1999]. Fecal

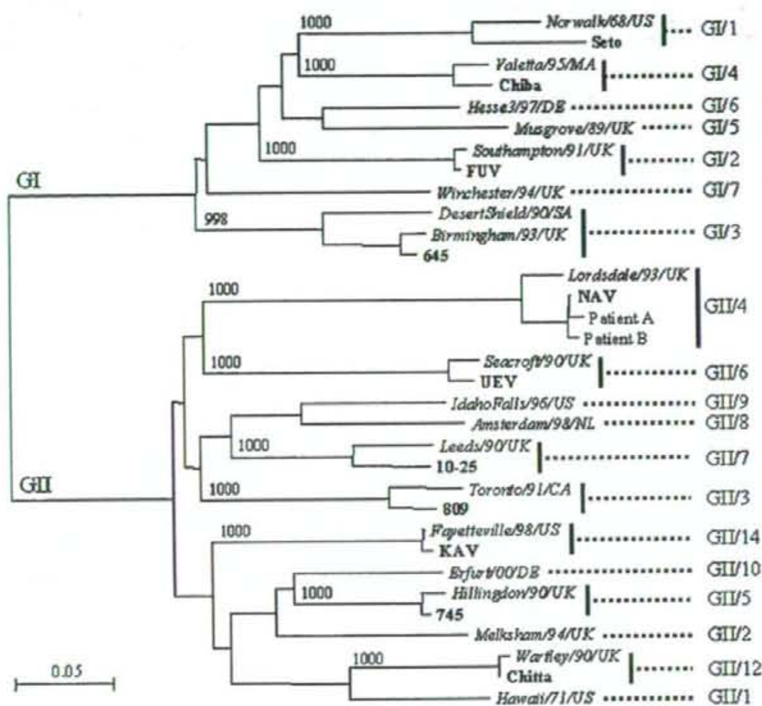

Fig. 1. Phylogenetic tree based on the capsid N/S domain region (GI, 294 nt; GII, 282 nt) constructed using neighbor-joining method. NoV strains for VLP and reference strains are represented in boldface and italics, respectively. Bar indicates the number of substitutions per site. The numbers on each branch indicate the bootstrap value of clusters including NoV strains for VLP. GenBank accession numbers for reference strains used in this analysis are as follows: Amsterdam/98/NL, AF195848; Birmingham/93/UK, AJ277612; DesertShield/90/SA, U04469; Erfurt/00/DE, AF427118; Fayetteville/98/US, AY113106; Hawaii/71/US, U07611; Hesse3/97/DE, AF093797; Hillingdon/90/UK, AJ277607; IdahoFalls/96/US, AY054299; Leeds/90/UK, AJ277608; Lordsdale/93/UK, X86557; Melksham/94/UK, X81879; Musgrove/89/UK, AJ277614; Norwalk/68/US, M87661; Seacroft/90/UK, AJ277620; Southampton/91/UK, L07418; Toronto/91/CA, U02030; Valetta/95/MA, AJ277616; Wartley/90/UK, AJ277618; Winchester/94/UK, AJ277809.

IgA antibodies specific to rNAV were detected from the 18th day post-onset. This specific fecal IgA seems to be followed by the reduction of PCR product or virus excretion, suggesting that fecal IgA was effective in the excretion of NoV.

The cross-reactivity of IgG in this study showed lower reaction to the heterologous type within the genogroup. Many serological studies in adults or children also demonstrated cross-reactivity within and between the genogroups [Treanor et al., 1993; Noel et al., 1997; Hale et al., 1998; Smit et al., 1999; Farkas et al., 2003; Rockx et al., 2005a]. Some reports showed that cross-reactivity was stronger or limited to the genogroups [Noel et al., 1997; Hale et al., 1998; Farkas et al., 2003]. In antibody ELISA using VLPs and their hyperimmune serums, low levels of cross-reaction were observed [Kamata et al., 2005; Hansman et al., 2006]. These findings indicated that NoV strains had a common epitope to stimulate the production of IgG. The common epitopes within and between the genogroups were previously described [Kitamoto et al., 2002; Yoda et al., 2003; Parker et al., 2005]. Yoda et al. [2003] described that the common epitope between genogroups may be located in the *N*-terminus of capsid protein. This region is highly conserved and located in the inner part of capsid protein surrounding the RNA genome [Prasad et al., 1999]. From the above results, cross-reactivity between genogroups may be difficult to produce.

As described in previous reports [Parker et al., 1994a, 1995; Smit et al., 1999], antibodies to NoVs were acquired at a young age and had cross-reactivity; therefore, it is unclear whether cross-reactive antibodies occurred after single infection, or after multiple infections with different genotypes of NoV. Our study demonstrated that cross-reactive antibodies to heterologous types were produced after primary single infection. We could observe that the production of IgG to the heterologous type was later and lower than the homologous type, and had a time lag in patient A. The time lag of IgG production may be related to antigenic differences among these GII NoV strains. The seroconverted against r809 of IgG and IgA in a serum of patient A at 2 years and 10 months are uncertain because the serum was obtained too long after the last serum sample was negative.

TABLE III. Detection of Serum IgM, IgA, and Fecal IgA to the 11 VLPs in Infantile Patients by ELISA

Patient (age)	Time post-onset of illness	RT-PCR[a]	Reciprocal of serum dilution: IgM rNAV (GII/4)	IgM Other VLPs	IgA r809 (GII/3)	IgA rNAV (GII/4)	IgA Other VLPs	Existence of specific fecal IgA[a]: rNAV (GII/4)	Other VLPs
A (2 years)	5th day	+	**<50**	<50	<50	**<50**	<50	–	–
	12th day	+	**1,600**	<50	<50	**50**	<50	–	–
	18th day	+	**1,600**	<50	<50	**1,600**	<50	+	–
	33rd day	+w	**200**	<50	<50	**800**	<50	**NT**	NT
	40th day	–	**200**	<50	<50	**1,600**	<50	+	–
	60th day	–	**<50**	<50	<50	**50**	<50	+	–
	2 years 10 months	NT	**<50**	<50	50	**100**	<50	**NT**	NT
B (1 year)	2nd day	+	**<50**	<50	<50	**<50**	<50	**NT**	NT
	105th day	NT	**<50**	<50	<50	**400**	<50	**NT**	NT

Bold: homologous type.
[a]+, Positive; +w, positive but weak band; –, negative; NT, not tested.

A serum sample from patient A at 2 years and 10 months had IgG antibodies to the homologous type with a high titer and the heterologous type within the genogroup with a low titer. The existence of IgG antibodies to VLPs may have been maintained without re-infection or emerged from re-infection. Patient A might have been re-infected with GII/4 NoV or other GII NoV excluding these seven genotypes in GII, because the IgG titer to the heterologous type was ≥twofold higher and IgM responses were not observed. The data of IgG analysis suggested that the cross-reactivity of IgG is produced in the primary infection and then the level of IgG against NoVs will be raised each time NoV infection occurs throughout life.

IgM and IgA antibody responses were specific to the homologous genotype. Previous studies using adult sera also suggested that serum IgM and IgA antibodies might be more specific for the homologous type than the heterologous type [Parker and Cubitt, 1994b; Brinker et al., 1998, 1999; Hale et al., 1998; Rockx et al., 2005a; Tsugawa et al., 2006]. To interpret the cross-reactivity of IgM and IgA, further investigations are needed based on cases of NoV primary infection.

Recent studies have shown that HBGA may function as receptors of NoV through outbreak investigation [Hennessy et al., 2003; Rockx et al., 2005b] and volunteer challenge studies [Hutson et al., 2002; Lindesmith et al., 2003]. The relation between HBGA and immune response to NoV infection was unknown, because the HBGA type of these two patients was unknown, but these are cases, which were naturally infected with NoV and the typical symptoms appeared, and will represent general immune response to primary NoV infection. This is the first study on humoral immune response in putative primary NoV infection using 11 different antigenic and genetic types of VLPs. Our data will improve understanding of the humoral immune response to NoV infection. However, only two cases were examined in this study. To interpret the immunity for NoV infection, further investigations at the human level are needed.

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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_{630} 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-

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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 **CCA** 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 T**AA** **G**CC CAT GGG AAA TGG AGC	1557–1598	Sense
A(GGC)527K(CTT)	GCT CCA TTT CCC ATG GG**C** **TT**A AGT GTG TAG AAC TGG TTG ACC	1557–1598	Antisense
P(CCC)528A(GCC)	CCA GTT CTA CAC ACT TGC C**G**C 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

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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.

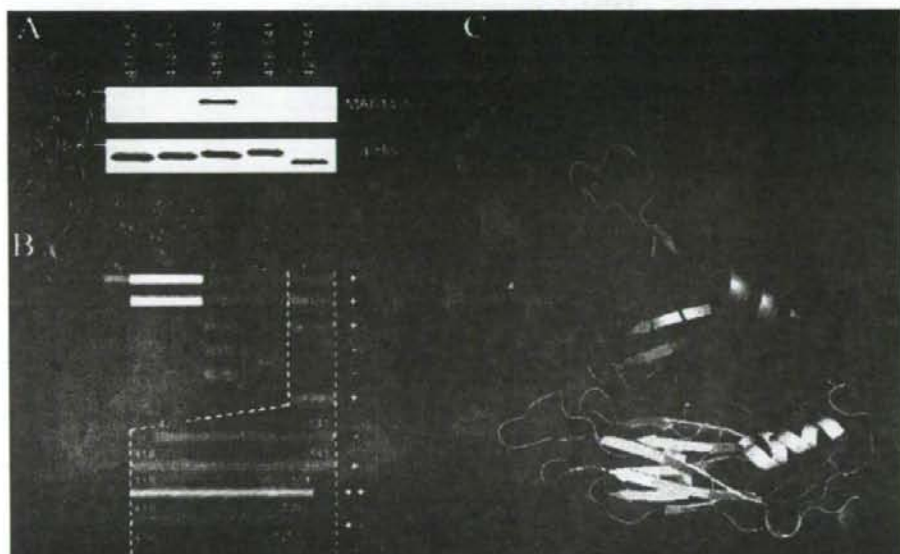

FIG. 1. (A) Reactivities of several capsid fragments for MAb14-1 and anti-His$_6$ antibody (a-His) by Western blotting. 20 K, 20,000. (B) Process of mapping the minimal binding region shown in silver on the map of VP1. WB, Western blotting. Symbols: ++, increase in antigenicity; +, same antigenicity 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

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