博士論文

A study of norovirus in Japanese pediatric patients: epidemiology, genetic variation, and structural analysis of histo-blood group antigen binding specificity

（日本の小児患者におけるノロウイルスの研究：疫学、遺伝的多様性、組織血液型抗原との結合特異性の構造解析）

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ABSTRACT

Acute gastroenteritis continues to be a major public health problem worldwide. In this study, I investigated the epidemiological situation of diarrheal virus infections and the change of their genotypes in 2,908 Japanese pediatric outpatients from 2009-2014. The surveillance identified diarrheal viruses currently circulating in 72.1% of the patients. Norovirus was detected in 43.0% of patients whereas the prevalence of rotavirus, parechovirus, enterovirus, adenovirus, sapovirus, astrovirus, and Aichivirus were 17.2%, 7.3%, 7.0%, 5.8%, 5.3%, 2.9%, and 0.1%, respectively. Most of children with viral diarrhea were younger than 3 years of age. Norovirus and rotavirus were detected throughout the year with a peak in cold and dry seasons, while other common diarrheal viruses were detected without any specific season.

During the epidemiologic study of norovirus, a GII.4 new variant 2012 was found in Japanese population. Norovirus was detected in 43.0% with several genotypes, GII.4 dominated over other genotypes (64.5%). The Den_Haag_2006b (36.9%) was detected as the predominant variant with co-circulation of New_Orleans_2009 (14.9%) until March 2012, and subsequently, displaced by Sydney_2012. Analysis of P2 subdomain showed a high level of diversity compared with other variants in the antigenic sites and HBGA binding sites. The Sydney_2012 showed strong binding to H-active α1,2-fucosylated structures in human saliva and gastric mucosa preparations from secretor individuals irrespective of thier ABO and Lewis phenotypes, but absence of binding specificity to Le^a-active α1,4-fucosylated structure. The expression of α1,2-fucosylated glycans seem to be crucial for susceptibility to infection of Sydney_2012 strain.

ABBRIVIATIONS

%	: Percent
^{o}C	: Degree Celsius
α1,2fuc	: α1,2-fucosyl residues/Fucα1,2Gal
α1,4fuc	: α1,4-fucosyl residues/Fucα1,4GlcNAc
µl	: Microliter
µM	: Micromolar
2-ME	: 2-Mercaptoethanol
AdV	: Adenovirus
AiV	: Aichi virus
bp	: Base pair
BSA	: Bovine serum albumin
cDNA	: Complementary deoxyribonucleic acid
CPE	: Cytopathic effect
CsCl	: Cesium chloride
DMSO	: Dimethyl sulfoxide
DNA	: Deoxyribonucleic acid
dNTP	: Deoxyrinonucleotide triphosphate
dsRNA	: Double stranded RNA
DTT	: Dithiothreitol
ELISA	: Enzyme-linked immunosorbent assays
EM	: Electron microscope
EV	: Enterovirus
i.e.	: *id est*
g	: Gravity
Gal	: Galactose
Glc	: Glucose
GlcNAc	: N-Acetylglucosamine
HAstV	: Human astrovirus
HBGA	: Human blood group antigen
HGM	: Human gastric mucosa
HPeV	: Human parechovirus

hr	: Hour
HRP	: Horseradish peroxidase
kb	: Kilobase
kDa	: Kilodalton
Le	: Lewis
mA	: Millamps
$MgSO_4$	: Magnesium sulfate
min	: Minute
ml	: Milliliter
mM	: Millimolar
NESID	: National Epidemiological Surveillance of Infectious Disease
nm	: Nanometer
NoV	: Norovirus
NSP	: Nonstructural protein
nt	: Nucleotide
ORF	: Open reading frame
PBS	: Phosphate buffered saline
PCR	: Polymerase chain reaction
RdRp	: RNA-dependent RNA polymerase
RNA	: Rinonucleic acid
RNase	: Ribonuclease
RT	: Reverse transcription
RV	: Rotavirus
SDS-PAGE	: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sec	: Second
SV	: Sapovirus
TAE	: Tris-Acetate ethylenediamine tetra-acetic acid
TMB	: Tetramethylbenzidine
UTR	: Untranslated region
VLP	: Virus-like particle
VP	: Viral protein

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LIST OF PUBLICATIONS

This thesis manuscript was written based on the following publications:

1. **Thongprachum A**, Chan-it W, Khamrin P, Okitsu S, Nishimura S, Kikuta H, Yamamoto A, Sugita K, Baba T, Mizuguchi M, Maneekarn N, Hayakawa S, Ushijima H. Reemergence of new variant G3 rotavirus in Japanese pediatric patients, 2009-2011. Infect Genet Evol. 2013 Jan;13:168-74.
2. **Thongprachum A**, Chan-it W, Khamrin P, Saparpakorn P, Okitsu S, Takanashi S, Mizuguchi M, Hayakawa S, Maneekarn N, Ushijima H. Molecular epidemiology of norovirus associated with gastroenteritis and emergence of norovirus GII.4 variant 2012 in Japanese pediatric patients. Infect Genet Evol. 2014 Apr;23:65-73.
3. **Thongprachum A**, Takanashi S, Kalesaran AF, Okitsu S, Mizuguchi M, Hayakawa S, Ushijima H. A Four-Year Study of Viruses that Cause Diarrhea in Japanese Pediatric Outpatients. J Med Virol. (In Press)

CHAPTER I

GENERAL INTRODUCTION

Acute gastroenteritis is a major cause of morbidity and mortality worldwide, especially in children and the elderly. It is estimated that 800,000 infants and young children die worldwide from diarrhea every year (1). Although the number of deaths due to viral gastroenteritis with diarrhea has been steadily declining in developed countries, it remains a leading cause of deaths among children under five years of age in developing world (1). Furthermore, in both developed and developing countries, diarrhea is often associated with substantial medical and healthcare costs and thus has a high economic impact on society.

Surveillances of diarrheal viruses

A gastroenteritis surveillance program has been implemented to monitor outbreak and potential causative agents. Several gastroenteritis viruses have been identified, among which, rotavirus (RV), norovirus (NoV), sapovirus (SV), human astrovirus (HAstV), and adenovirus (AdV) are the most common etiological agents of gastroenteritis in children under 5 years of age.

RV is a major cause of severe gastroenteritis in children <5 years of age worldwide. The consequences of RV infection are particularly different depending on the economic status of each country. RV causes an estimated 453,000 deaths annually among children aged <5 years in developing countries where the access to appropriate medical care is limited (2). RVs are classified into seven groups (A to G) on the basis of their distinct antigenic and genetic properties. Human infection has been reported with group A, B, and C, with group A rotavirus (RAV) is acknowledged as a major cause of severe diarrhea in

infants and children. Based on neutralizing antigen, VP7 and VP4, allowing RAV to be classified into 27 G and 37 P genotypes in human and animal species (3, 4). The knowledge of the prevalence of the G and P genotypes in each country has become more relevant, since two types of human rotavirus vaccines; RotaTeq (Merck&Co.,Inc.,NJ, USA) and Rotarix (GlaxoSmithKline Biological, Belgium), were developed.

NoV and SV are members of the *Caliciviridae* family. Recently, NoV has emerged as an important etiological agent and is responsible for more than half of all associated with outbreaks and sporadic cases in children (5). Based on the variations in capsid sequence, NoV can be genetically classified into five genogroups; most of NoV infections are caused by NoV GI and GII (6). SV infects both children and adults and tends to cause mild illness compared to that caused by NoV (7). SV can be divided into five genogroups (GI to GV), of which GI, GII, GIV and GV are identified in humans.

HAstV infections have been reported in 2 to 9% among children with diarrhea in most areas and the rate up to 30% in some developing counties (8). Although HAstV was detected less frequently than RV or NoV in surveillance studies, HAstV still requires attention from public health viewpoint because of large and frequent outbreaks. Enteric AdV causes acute diarrhea sporadically as well as in outbreaks of 1 to 20% of children with diarrhea in Japan (9).

Many viruses in *Picornaviridae* family, including Aichi virus (AiV), human parechovirus (HPeV), and enterovirus (EV) have also been considered to be associated with diarrhea diseases in different parts of the world (10, 11). The multiplex RT-PCR for detection of AiV, HPeV, and EV was developed (12). However their association with gastroenteritis has not been established, and most of data has been reported in only symptomatic individuals.

Molecular epidemiology of diarrheal viruses in Japan

In Japan, surveys of the trends of infectious gastroenteritis have been conducted by the National Epidemiological Surveillance of Infectious Diseases (NESID). The infectious gastroenteritis cases have been reported from approximately 3,000 pediatric sentinel clinics nationwide (13). Each year, the number of infectious gastroenteritis cases dramatically increases towards the end of the year. Among the reported pathogens, NoV is the most common pathogen causing diarrhea, followed by RV and SV and prevailed in all age groups. NoV accounts for about one third of the pathogens detected from children 3 years old or younger.

Surveillance of diarrheal viruses has also been conducted by my research team. The identification of diarrheal virus infections in 2003-2007 revealed that RAV was the most prevalent pathogen accounting for 16.7% (14-16). NoV as the second prevailing virus was detected in 12.5% (17-19), whereas SV was much less common and represented in 3.7% (20, 21). The epidemiological trend of diarrheal viruses changed between 2007 and 2009. NoV was recognized as the main causative agent of the illnesses accounting for 26.6%, followed by RAV (15.5%) and SV (3.4%) (22-24).

In previous epidemiological studies (14, 15, 17, 19, 22, 24), almost half of the etiologic agents causing acute gastroenteritis remained unknown. It is important to identify specific etiologies of acute gastroenteritis as targets of potential preventive interventions. Epidemiological surveillance of viral agents is critical for the development of effective preventive measures. The accumulated surveillance data have shown that the predominant diarrheal viruses in each epidemic season change over time. Moreover, literature on epidemiology in Japan has shown only a few diarrheal viruses; mostly case numbers of NoV and RV were reported from pediatric sentinel clinic nationwide. The overall picture

of several diarrheal viruses has not been compiled yet. Therefore, the molecular surveillance of diarrheal viruses circulating in Japanese population is needed.

Norovirus structure and classification

NoV is a clinically important diarrheal virus which is considered to be predominant etiological agent of gastroenteritis throughout the world, including Japan. NoV is spread rapidly and associated with sporadic cases and outbreaks of acute gastroenteritis in different settings including schools, daycare facilities, restaurants, nursing homes, and hospitals, resulting in significant economic damage. Control strategies are largely preventive, since there is currently a lack of effective treatments or vaccine available.

NoV is a single-stranded, positive sense RNA virus that belongs to the *Caliciviridae* family. The NoV genome is ~7.5 kb and contains three open reading frames - ORF1 encodes the non-structural proteins, ORF2 encodes the VP1 major capsid protein, and ORF3 encodes the VP2 minor capsid protein (25). VP1 consists of three domains, namely the highly conserved shell (S) domain which is connected by a moderately conserved flexible P1 domain that acts as a hinge to the protruding P2 domain (25), the protruding P2 subdomain possesses several motifs that are involved in binding to the host cell, and the P2 subdomain is therefore responsible for the antigenicity of the virus (26, 27), as shown in **Figure 1**.

Based on the variation in the capsid gene sequences, NoV can be genetically classified into five genogroups (GI-V), of which three have been found in humans; GI, GII, and GIV (6, 28). Genotypes are defined on the basis of either the RdRp sequence or the capsid sequence. NoV genogroups have been further subdivided into at least eight genotypes in GI, 23 genotypes in GII, and only one genotype in GIV (6, 28).

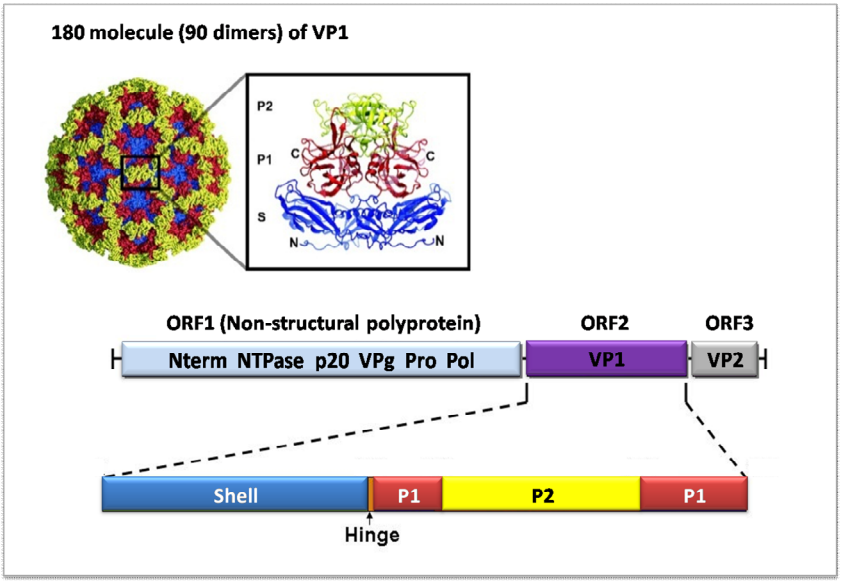


Figure 1. Structure of norovirus capsid. The top left panel shows the icosahedral capsid structure (VP1), color-coded to show the three domains illustrated in the ribbon representation of the VP1 dimer extracted from the capsid structure (top right panel). The genomic codes for three open reading frames (ORFs). The figure was modified from Glass et al. (29)

Among NoV GII, genotype II.4 (GII.4) NoV has been shown to spread rapidly and is the most commonly detected strain worldwide, particularly in association with outbreaks. Recent molecular surveillances indicated that GII.4 has evolved a series of genetic variants over the last 20 years, some of which persisted and replaced the previous circulating variants (30). NoV pattern in human populations include epidemic outbreaks of disease every 2-3 years, punctuated by the emergence of an antigenically distinct GII.4 strain that appears to escape human herd immunity to the previous circulating strains (31, 32). The continuous evolution of the P2 subdomain of the surface exposed major capsid protein (VP1) has been proposed as a key mechanism by which new antigenic variants are generated.

Molecular epidemiology of norovirus

Although more than 35 different human NoV genotypes have been identified, NoV GII.4 is the only genotype associated with global pandemics of gastroenteritis accounting

for more than 80% of all human NoV infection. Since the mid-90s, NoV GII.4 variants have been shown to be associated with at least six pandemics of acute gastroenteritis, and continue to cause millions of infections across the globe annually. The NoV GII.4 variants reported previously include the US 1995/96 in 1996 (33, 34), Farmington_Hills_2002 in 2002 (35, 36), Hunter_2004 in 2004 (37), Minerva_2006b and Den_Haag_2006b virus in 2006-2008 (38), New_Orleans_2009 in 2009-2012 (39), and most recently Sydney_2012 (40-43).

The periodic increases of NoV outbreak have been shown to be related to the emergence of new variant strains, probably owing to evasion of population immunity (44). The emergence of such variants has been shown to be associated with substantial increases in cases worldwide (45). Since late 2012, surveillance systems in many countries have showed increased levels of NoV activity compared to the previous seasons, associated with the emergence of a new variant GII.4 named Sydney_2012 (40-43). Monitoring the emergence of the new NoV GII.4 variant strain, which could display a different pathogenesis and virulence, is important for public health planning. Therefore, rapid evolution and antigenic variation are important evolutionary forces that shape epidemiological success and persistence of the GII.4 viruses in the population.

Genetic variation of norovirus

The lack of culture systems by standard cell culture or animal model has been a significant obstacle to NoV studies. Genetic analysis becomes the principal method to classify, monitor the transmission pathways, and investigate the NoV evolution. NoV continuously undergo the process of genetic diversity and occasionally generate a novel NoV strain by accumulation of mutations or homologous recombination. Both mechanisms have been proposed to drive evolution in the pandemic GII.4 lineage and emerge as the

new variants (46, 47). Antigenic variation is an important factor contributing to the emergence of novel NoVs with most variation observed between different GII.4 variants localized to five evolving antigenic blockade epitopes (A-E) within the capsid protruding P2 subdomain (46). The P2 subdomain is considered to be the extremely variable part of the genome and the most surface-exposed region of the virus capsid. Further, it has been suggested that the P2 subdomain shares the receptor-like site both recognizing blood group-related glycans such as ABO(H) and Lewis-active determinants and involving in the strain-specific antibody recognition.

Even though the majority of acute gastroenteritis outbreaks due to NoV infection are mostly caused by GII.4, NoV other genotypes, such as GII.3 and GII.6, are common genotypes associated with sporadic NoV infections, particularly in children (48). It has been proposed that the incidence of GII.3 increased in the 1990s following GII.4 pandemic periods (49); however few studies have addressed the genetic basis of the epidemiological differences between these GII.3 and GII.6 circulating in Japanese population.

Norovirus binding sites on histo-blood group antigen (HBGA)

Histo-blood group antigen (HBGA) is one of the molecules present in host cells which is predicted to be associated with NoV infection through a series of glycans expressed as the antigenic determinant on the human tissues (50, 51).

Glycans including such determinants have been reported to be present in ABO and Lewis blood group and their related antigens. All these antigens distribute in individuals possessing corresponding blood types and are prepared from not only their red blood cells but also digestive tissues and secretors including saliva and other fluids. Importantly, the presence of ABO(H) blood group antigens in the secretor system is regulated with the action of the secretor gene (*Se* gene). Therefore, no such antigens are present in saliva,

mucosa of digestive organs, milk and other fluids from individuals who lack the *Se* gene (*se/se*) but present only from individuals (*Se*/-), typed non-secretor (non-sec.) and secretor (sec.), accordingly (52).

Together with evidences supporting the association of infectious profiles with ABO blood types of hosts obtained from volunteer challenge studies, with the aid of establishment of recombinant virus-like particles (VLPs), which are morphologically similar to those of the respective viruses, recognition of blood group-related glycans by NoV has been determined with studies on binding specificities of NoV VLPs using a panel of human saliva with different ABO and Lewis blood group phenotypes and secretor status (53). NoV revealed HBGA binding interfaces on the top of the P dimer near the common boundary, and the surrounding regions interact with ABH and Lewis blood group active determinants through binding mode of α-fucosyl residues. Different NoV genotypes are known to recognize HBGAs in a strain specific manner (51, 54).

The previous studies described eight different binding patterns of NoVs based on their binding profiles obtained from binding assays with a series of blood group antigens with different ABO and Lewis blood group phenotypes and secretor status (51, 55). Accordingly, the strains involved in the same binding pattern have been shown to be genetically related and, in particular, VLPs belonging to the same cluster have been shown to share a high sequence identity in the capsid genes as well as their similar binding specificity (56). Further, the same binding patterns depending on the glycan structures have also been demonstrated using chemically synthesized glycans in place of blood group substances from saliva (51).

However, it was of particular interest that some discrepancies of binding patterns were found between human saliva samples and preparations of human gastric mucosa even though they possessed the same blood types and secretor status, as demonstrated in the

previous study (51). NoVs possess genetically high diverse and their binding regions to blood group-related glycans are extremely sensitive against occurrence of mutation resulting in an acquisition of different binding pattern only with a single amino acid mutation around the region (57).

Previous studies on molecular mechanisms of NoV infection indicated the emergence of new variants with substitutions of amino acid in the P2 subdomain of the NoV capsid, containing in the predicted sequences corresponding to not only their antigenicities but also their binding specificities. Most of the data so far reported have been focused mainly on a variety of binding specificities in each NoV genotype including GII.4, but little information has been obtained about detailed changes of binding specificities in NoV genotypes, in particular, in the predominant variant as the prevalent NoV genotype. To understand mechanisms of emergence of new variants in each genotype, analysis on binding specificity of NoV variants should be useful and applicable to extend further molecular analysis of their variants.

CHAPTER II

THE 5-YEAR EPIDEMIOLOGICAL STUDY OF DIARRHEAL VIRUSES IN JAPANESE PEDIATRIC PATIENTS WITH ACUTE GASTROENTERITIS

INTRODUCTION

Acute gastroenteritis is one of the common diseases in infants and children, and continues to be the leading cause of morbidity and mortality worldwide. Several gastroenteritis viruses have been implicated as a cause of deaths in infants and children worldwide. RV is not only the most common cause of diarrhea in children worldwide, but also detectable in any age group (58). Recently, NoV and SV have emerged as important etiological agents of epidemic diarrhea, and are associated frequently with outbreaks and sporadic cases in children (5). NoVs are highly diverse and can be divided into five genogroups, of which NoV GI and GII cause the most infections (6). HAstV and enteric AdV cause gastroenteritis, primarily in children younger than 4 years of age (59). Most of studies reported a prevalence rate of HAstV ranged from 2 to 9% among children with diarrhea worldwide (8). AdVs are responsible for a wide range of symptoms associated with outbreaks of acute gastroenteritis in 1-20% of children with diarrhea in Japan (9).

The literature on epidemiological studies conducted in Japan showed only a few diarrheal viruses, which were mostly NoV and RV cases reported from pediatric sentinel clinics nationwide. In addition, many viruses in the *Picornaviridae* family, such as Aichi virus (AiV), human parechovirus (HPeV), and enterovirus (EV) also have been thought to associate with diarrhea diseases in different parts of the world (10, 11). However, their association with gastroenteritis has not been well established. Therefore, molecular surveillance of viruses that cause diarrhea in the Japanese population is needed.

This study investigated the prevalence of eight viruses that cause diarrhea and described their seasonal distribution pattern in Japanese pediatric patients from 2009 to 2014.

MATERIALS AND METHODS

Patients and samples

A total of 2,908 fecal specimens were collected from Japanese pediatric outpatients with acute gastroenteritis in pediatric clinics in six prefectures from northern to southern Japan including Sapporo city in Hokkaido, Koto-ku in Tokyo, Fujieda city in Shizuoka, Maizuru city in Kyoto, Ibaraki city in Osaka, and Saga city in Saga from July 2009 to June 2014. Among these, 515, 535, 599, 732, and 527 samples were obtained during the period of 2009-2010, 2010-2011, 2011-2012, 2012-2013, and 2013-2014 respectively. In this study, the annual observation period for viral gastroenteritis began in July and ended in June of the following year.

The spectrum of clinical signs exhibited by patients with acute gastroenteritis included acute watery diarrhea, vomiting, abdominal cramps, and fever of various severities and in various combinations. Age of the patients ranged from neonate to 15 years (0-178 months; mean age of 24.3 months). For subsequent data analysis, seven different age-groups were established: <6 months (263 samples), 6-11 months (822 samples), 12-23 months (1005 samples), 24-35 months (399 samples), 36-47 months (145 samples), 48-59 months (86 samples), and >60 months (188 samples). The fecal specimens were stored at minus 20^{o}C until use. This study was approved by ethical committee of the University of Tokyo and Nihon University School of Medicine.

Extraction of viral genome

The fecal specimens were prepared as 10% suspension in distilled water. Viral genome was extracted from the 10% suspension using the QIAamp Viral RNA Mini Kit, according to the manufacturer's instructions (QIAgen, Hilden, Germany). One hundred and

forty µl of the 10% fecal supernatant was mixed with 560 µl AVL viral lysis buffer containing carrier RNA by pulse-vortex for 15 seconds. The mixture was incubated at room temperature for 10 min, and 560 µl of ethanol was added. The mixture was then applied onto the spin column and centrifuged at 6,000 x *g* for 1 min. The column was placed into a new 2 ml collecting tube and 500 µl of AW1 buffer was added. The column was centrifuged at 6,000 x *g* for 1 min to remove unbound materials and washed by adding of 500 µl of AW2 buffer. Then, the column was centrifuged at full-speed (20,000 x *g*) for 3 min, and placed into a new 1.5 ml-microcentrifuge tube. Finally, 60 µl of AVE buffer was added directly onto the column to elute RNA. After incubating at room temperature for 1 min, the column was centrifuged at 6,000 x *g* for 1 min. The viral RNA was spun down into the collecting tube and stored at –20^{o}C until used.

Reverse transcription

Reverse transcription (RT) was performed with Superscript reverse transcriptase III (Invitrogen, Carlsbad, CA). Briefly, 5 µl of viral genome extract were first denatured by heating at 95ºC for 5 min in the presence of 1 µl of 50% dimethyl sulfoxide (DMSO) and then immediately chilled on ice. Five µl of the heated genome were added to a reaction mixture (final, 15 µl) containing 3 µl of 5x first strand buffer (Invitrogen, Carlsbad, CA), 0.8 µl of 10 mM dNTPs (Roche, Mannheim, Germany), 0.8 µl of 10 mM DTT (Invitrogen, Carlsbad, CA), 0.8 µl of random primer (hexa-deoxyribonucleotide mixture) (Takara, Shiga, Japan), 0.8 µl of Superscript reverse transcriptase III (Invitrogen, Carlsbad, CA), 0.5 µl of RNase inhibitor (Toyobo, Osaka, Japan) and 3.3 µl RNase-free distilled water. The RT step was carried out at 50^{o}C for 1 h, followed by 95°C for 5 min and then chilled on ice for 5 min. The cDNA was kept at –20°C.

Diarrheal viruses detection by multiplex RT-PCR

The presence of diarrheal viruses was detected by multiplex PCR, with 3 sets of specific primers as summarized in **Table 1**. The first primer set contained primers Beg9 and VP7-1' for RAV (60), ADG9-1F and ADG9-1R for RBV (60), NG8S1 and NG8S2 for RCV (60), and Ad1 and Ad2 for AdV (60). The second primer set included mixes of PreCAP1 and 82b for HAstV (61), G1SKF and G1SKR for NoV GI (61), COG2F and G2SKR for NoV GII (61), and SLV5317 and SLV5749 for SV (61). The third primer set contained mixes of 6261 and 6779 for AiV (62), ev22(+) and ev22(-) for HPeV (63), and F1 and R1 for EV (64). These three primer sets were utilized in separate reaction. In order to confirm the co-infection, the presence of each diarrheal virus was examined using specific primers.

The PCR components contained 2.5 µl of 5x Taq DNA polymerase buffer (Promega, Madison, WI), 1 µl of 2.5 mM dNTPs (Roche, Mannheim, Germany), 0.25 µl of each specific primer (20 µM) and 0.05 µl of Taq DNA polymerase (Promega, Madison, WI). RNase-free distilled water was added to make up a total volume of 12.5 µl. The PCR was performed at 94^{o}C for 3 min, followed by 30 cycles of 94°C 1 min, 50°C 1 min, 72°C 1 min, and a final extension at 72°C for 7 min, and then held at 4°C.

For NoV GII, semi-nested PCR was performed using G2SKF (5'-CNTGGGAGGGCGATCGCAA-3') and G2SKR primers to detect NoV GII in samples that were found negative by the second primer set. In this step, to avoid a high risk of cross-contamination, NoV positive control was not included in the semi-nested PCR, whereas a negative control was included in the assays throughout the experiment.

Table 1. Specific primers used in multiplex PCR to detect diarrheal viruses in Japanese pediatric patients

Group	Target virus	Primer	Polarity[a]	Sequence (5' to 3')[b]	Position[c]	Length (bp)
I	Group A rotavirus	Beg9	+	GGCTTTAAAAGAGAGAATTTCCGTCTGG	1-28	395
		VP7-1'	-	ACTGATCCTGTTGGCCATCCTTT	395-373	
	Group B rotavirus	ADG9-1F	+	GGCAATAAAATGGCTTCATTGC	1-22	814
		ADG9-1R	-	GGGTTTTTACAGCTTCGGCT	814-795	
	Group C rotavirus	NG8S1	+	ATTATGCTCAGACTATCGCCAC	353-374	351
		NG8S2	-	GTTTCTGTACTAGCTGGTGAAC	704-683	
	Adenovirus	Ad1	+	TTCCCCATGGCICAYAACAC	1834-1853	482
		Ad2	-	CCCTGGTAKCCRATRTTGTA	2296-2315	
II	Astrovirus	PreCAP1	+	GGACTGCAAAGCAGCTTCGTG	4235-4255	719
		82b	-	GTGAGCCACCAGCCATCCCT	4953-4934	
	Norovirus GI	G1SKF	+	CTGCCCGAATTYGTAAATGA	5342-5361	330
		G1SKR	-	CCAACCCARCCATTRTACA	5671-5653	
	Norovirus GII	COG2F	+	CARGARBCNATGTTYAGRTGGATGAG	5002-5028	387
		G2SKR	-	CCRCCNGCATRHCCRTTRTACAT	5389-5367	
	Sapovirus	SLV5317	+	CTCGCCACCTACRAWGCBTGGTT	5083-5105	434
		SLV5749	-	CGGRCYTCAAAVSTACCBCCCCA	5516-5494	
III	Aichi virus	6261	+	ACACTCCCACCTCCCGCCAGT	6261-6282	519
		6779	-	GGAAGAGCTGGGTGTCAAGA	6779-6760	
	Human parechovirus	ev22(+)	+	CYCACACAGCCATCCTC	312-328	270
		ev22(-)	-	TRCGGGTACCTTCTGGG	581-565	
	Enterovirus	F1	+	CAAGCACTTCTGTTTCCCCGG	160-180	440
		R1	-	ATTGTCACCATAAGCAGCCA	599-580	

a: +, Forward primer; -, Reverse primer.
b: Within nucleotide sequence of primers, B=C, G or T; H=A, C or T; N: Any base; R= A or G; S= G or C; V=A, C or G; W= A or T; Y= C or T.
c: The position numbers of primer pairs in the first group correspond to representative strains Wa (K02033), CAL (AF184083), KW408 (AB086968) and Tak (DQ315364) for group A, B and C rotaviruses and adenorovirusirus, respectively. The position numbers of primer pairs in the second group correspond to representative strains Oxford (L23513), Norwalk/68 (M87661), Lordsdale (X86557) and Manchester (X86560) for astrovirus, norovirus (GI, GII) and sapovirus, respectively.

Electrophoresis

The PCR products were detected by electrophoresis on 1.5% agarose gel, stained with SYBR® Safe DNA Stain (Invitrogen, Carlsbad, CA, USA), and visualized under a blue-light transilluminator.

RESULTS

Detection of diarrheal viruses

A total of 2,908 fecal specimens were investigated for the presence of several diarrheal viruses, including RAV, RBV, RCV, AdV, NoV GI, NoV GII, SV, HAstV, AiV, HPeV, and EV. Overall, diarrheal viruses were detected in 72.1% (2,097 out of 2,908) of investigated patients. The existence of aforementioned diarrheal viruses circulating in Japanese pediatric outpatients with acute gastroenteritis is shown in **Table 2**. Among the diarrheal viruses detected, NoV GII was the most predominant viral agent, with an overall detection rate of 42.8% (1,244/2,908). RAV was the second most common viral agent, accounting for 17.1% followed by HPeV (7.3%), EV (7.0%), AdV (5.8%), SV (5.3%), HAstV (2.9%), NoV GI (0.3%), RCV and AiV (0.1% each). No RBV was found in these specimens.

Epidemiology of diarrheal viruses in individual regions in Japan

The pathogen surveillance was conducted in infants and children with diarrhea in six different prefectures (Hokkaido, Tokyo, Shizuoka, Kyoto, Osaka, and Saga) in Japan during July 2009 to June 2014. The presence of diarrheal viruses was found variously in each geographic region with a detection rate ranging from 56.9-87.6% (**Table 3**). The highest detection rate was found in Hokkaido (87.6%), followed by Kyoto (86.5%), Shizuoka (70.1%), Osaka (68.8%), Saga (67.6%), and Tokyo (56.9%). NoV GII was found most frequently in all regions, whereas RAV was detected as the second common pathogen in each area. However, negative results for all diarrheal viruses were shown in 10-39% of the investigated patients included in this study.

Co-infections of diarrheal viruses

Co-infection was detected in 15.5% (450/2,908). Of these, the co-infections between two kinds of target viruses were found in 419 samples (14.4%), in which co-infection between NoV GII and RAV was dominant (3.9%, 113/2,908). Triple-infection was also found in 1.1% (31/2,908), mostly found with NoV GII and RAV. Interestingly, two fecal patients were co-infected for both of NoV GI and GII, as shown in **Table 4**.

It was noted that approximately one third of NoV GII (362/1,244) were mixed infections with other viruses; RAV (113/2,908), HPeV (74/2,908), AdV 55/2,908), EV (49/2,908), SV (20/2,908), HAstV (22/2,908) and other two kinds of target virus (27/2,908). Approximately one-fourth of RAV (190/497) were infected with other diarrheal viruses, especially with NoV GII, HPeV, and EV. The co-infections between NoV GII and RAV occurred predominantly in children younger than 2 years of age, with most commonly found between NoV GII.4, either Sydney_2012 or Dan_Haag_2006b variants, with RAV G1P[8] (27.8%; 35/126) and with G3P[8] (21.4%; 27/126).

Seasonal pattern of diarrheal virus infections

The seasonal pattern of common diarrheal virus infections detected in this study is shown in **Figure 2**. NoV was detected throughout the year. The highest prevalence of NoV was found in December (23.9%; 299/1,251) followed by January (17.9%; 224/1,251), November (11.4%; 142/1,251), and February (9.4%; 117/1,251). On the other hand, RAV was more frequently detected continuously in the 6-month period during January to June with a peak in April (31.6%; 157/497), followed by March (27.0%; 134/497), February (13.5%; 67/497), and May (11.5%; 57/497). Interestingly, a seasonal peak of RAV was absent in 2013-2014. The predominant co-infections between NoV and RAV had a peak from January to March when the detection of RAV and NoV overlapped. A peak of HAstV

infection was most frequently detected in April and May. Furthermore, the seasonal patterns of less common viral pathogens were observed. The positive samples for SV, AdV, HPeV, and EV were detected sporadically throughout the year without any observable peak.

Age distribution of diarrheal virus infections

The age of patients with acute gastroenteritis ranged from new born to 14 years. The mean age of the patients was 24.3 months. The age distribution of the patients by etiologic agents is shown in **Table 5**. The children in the age group of 12-23 months were most commonly infected with any diarrheal viruses. NoV infection was detected in all age groups. The highest prevalence of NoV was found in infants and children aged 12-23 months (41.5%, n=516) followed by 6-11 months (25.5%, n=317), 24-35 months (14.2%, n=177), younger than 6 months (6.8%, n=85), 36-47 months (4.7%, n=59), older than 60 months (52%, n=52), and 48-59 months (3.1%, n=38). Among RAV infection, the highest prevalence was found in children aged 12-23 months (41.2%; 205/497), followed by 6-11 months (26.0%, 129/497), 24-35 months (17.9%, 89/497), and the lowest in infants aged 48-59 months (2.2%; 11/497). The SV and AdV infections were mostly detected in children aged 12-23 months group accounting for 46.4% (71/153) and 35.5% (60/169), respectively. Additionally, HPeV and EV infection cases were detected in all age groups with a high peak in children aged 12-23 month group at 32.9% (70/213) and 38.4% (78/203), respectively.

Table 2. The prevalence of diarrheal virus infections in Japanese pediatric patients with acute gastroenteritis during 2009-2014

Year	No. of specimens	No. of positive cases (%)	No. of positive cases for diarrheal virus (%)										
			RAV	RBV	RCV	NoV GI	NoV GII	SV	AdV	HAstV	EV	HPeV	AiV
2009-2010	515	375(72.8)	95	-	-	-	210	16	31	-	43	43	1
2010-2011	535	356(66.5)	112	-	-	1	215	26	14	3	37	32	1
2011-2012	599	406(67.8)	107	-	1	-	190	29	48	29	39	46	-
2012-2013	732	539(73.6)	162	-	1	7	315	43	41	22	27	35	-
2013-2014	527	421(79.9)	21	-	2	1	314	39	35	32	57	57	-
Total	2,908	2,097(72.1)	497(17.1)	-	4(0.1)	9(0.3)	1,244(42.8)	153(5.3)	169(5.8)	86(2.9)	203(7.0)	213(7.3)	2(0.1)

Table 3. Distribution of diarrheal viral infections in Japanese pediatric patients with acute gastroenteritis in Hokkaido, Tokyo, Shizuoka, Kyoto, Osaka, and Saga from 2009 to 2014

Year	No. of specimens	No. of positive cases (%)	No. of positive cases for diarrheal virus (%)										
			RAV	RBV	RCV	NoV GI	NoV GII	SV	AdV	HAstV	EV	HPeV	AiV
Hokkaido	105	92(87.6)	22	-	-	2	65	6	8	3	2	7	-
Tokyo	327	186(56.9)	59	-	-	-	106	6	19	2	9	22	-
Shizuoka	896	629(70.2)	166	-	2	2	340	54	49	34	88	79	2
Kyoto	601	520(86.5)	137	-		1	342	38	41	14	17	36	-
Osaka	680	468(68.8)	74	-	2	4	275	36	43	22	52	51	-
Saga	299	202(67.6)	39	-	-	-	116	13	9	11	35	18	-
Total	2,908	2,097(72.1)	497(17.1)	-	4(0.1)	9(0.3)	1,244(42.8)	153(5.3)	169(5.8)	86(2.9)	203(7.0)	213(7.3)	2(0.1)

Table 4. Distribution of mix infection of diarrheal virus infections in Japanese pediatric patients with acute gastroenteritis during 2009-2014

Diarrhea viruses	No. of positive case (%)					Total
	2009-2010	2010-2011	2011-2012	2012-2013	2013-2014	
Single infection						
RAV	60	68	76	108	7	319 (11.0)
RCV	-	-	1	1	2	4 (0.1)
NoV GI	-	-	-	6	1	7 (0.2)
NoV GII	162	141	138	228	211	880 (30.3)
SV	13	23	22	36	19	113 (3.9)
AdV	24	8	25	15	15	87 (3.0)
HAstV	-	-	24	17	8	49 (1.7)
EV	33	21	25	10	22	111 (3.8)
HPeV	18	10	18	10	19	75 (2.6)
AiV	1	1	-	-	-	2 (0.1)
Double infection						
RAV + NoV GII	23	36	11	36	7	113 (3.9)
RAV + SV	1	-	-	1	-	2 (0.1)
RAV + AdV	1	-	4	2	-	7 (0.2)
RAV + HAstV	-	1	1	1	-	3 (0.1)
RAV + EV	6	-	2	6	-	14 (0.5)
RAV + HPeV	4	6	9	4	1	24 (0.8)
RCV + EV	-	-	-	-	1	1 (0.03)
NoV GI + NoV GII	-	1	-	1	-	2 (0.1)
NoV GII + SV	-	2	3	1	14	20 (0.7)
NoV GII + AdV	4	4	16	21	10	55 (1.9)
NoV GII + HAstV	-	2	-	1	19	22 (0.8)
NoV GII + EV	4	14	7	8	16	49 (1.7)
NoV GII + HPeV	17	14	10	14	19	74 (2.5)
SV + AdV	-	-	-	1	-	1 (0.03)
SV + EV	-	1	1	2	3	7 (0.2)
SV + HPeV	2	-	2	1	-	5 (0.2)
AdV + HAstV	-	-	-	1	-	1 (0.03)
AdV + EV	-	1	-	-	2	3 (0.1)
AdV + HPeV	2	1	1	1	3	8 (0.3)
HAstV + EV	-	-	1	-	1	2 (0.1)
HAstV + HPeV	-	-	3	-	-	3 (0.1)
HPeV + EV	-	-	-	-	3	3 (0.1)
Triple infection						
RAV + NoV GII + HPeV	-	1	2	2	2	7 (0.2)
RAV + NoV GII + EV	-	-	1	-	3	4 (0.1)
RAV + NoV GII + HAstV	-	-	-	1	1	2 (0.1)
RAV + AdV + EV	-	-	1	-	-	1 (0.03)
RAV + SV + HPeV	-	-	-	1	-	1 (0.03)
NoV GII + SV + AdV	-	-	1	-	-	1 (0.03)
NoV GII + SV + EV	-	-	-	-	1	1 (0.03)
NoV GII + SV + HPeV	-	-	-	-	1	1 (0.03)
NoV GII + AdV + HPeV	-	-	-	-	4	4 (0.1)
NoV GII + HPeV + EV	-	-	1	1	2	4 (0.1)
NoV GII + HAstV + HPeV	-	-	-	1	2	3 (0.1)
NoV GII + HAstV + EV	-	-	-	-	1	1 (0.03)
AdV + HPeV + EV	-	-	-	-	1	1 (0.03)
Total	375	356	406	539	421	2097 (72.1)

No., Number; RAV, Group A rotavirus; RBV, Group B rotavirus; RCV, Group C rotavirus; NoV GI, Norovirus GI; NoV GII, Norovirus GII; SV, Sapovirus; AdV, Adenovirus; HAstV, Human astrovirus; AiV, Aichi virus; HPeV, human parechovirus; EV, enterovirus; %, Refer to total number of specimens tested

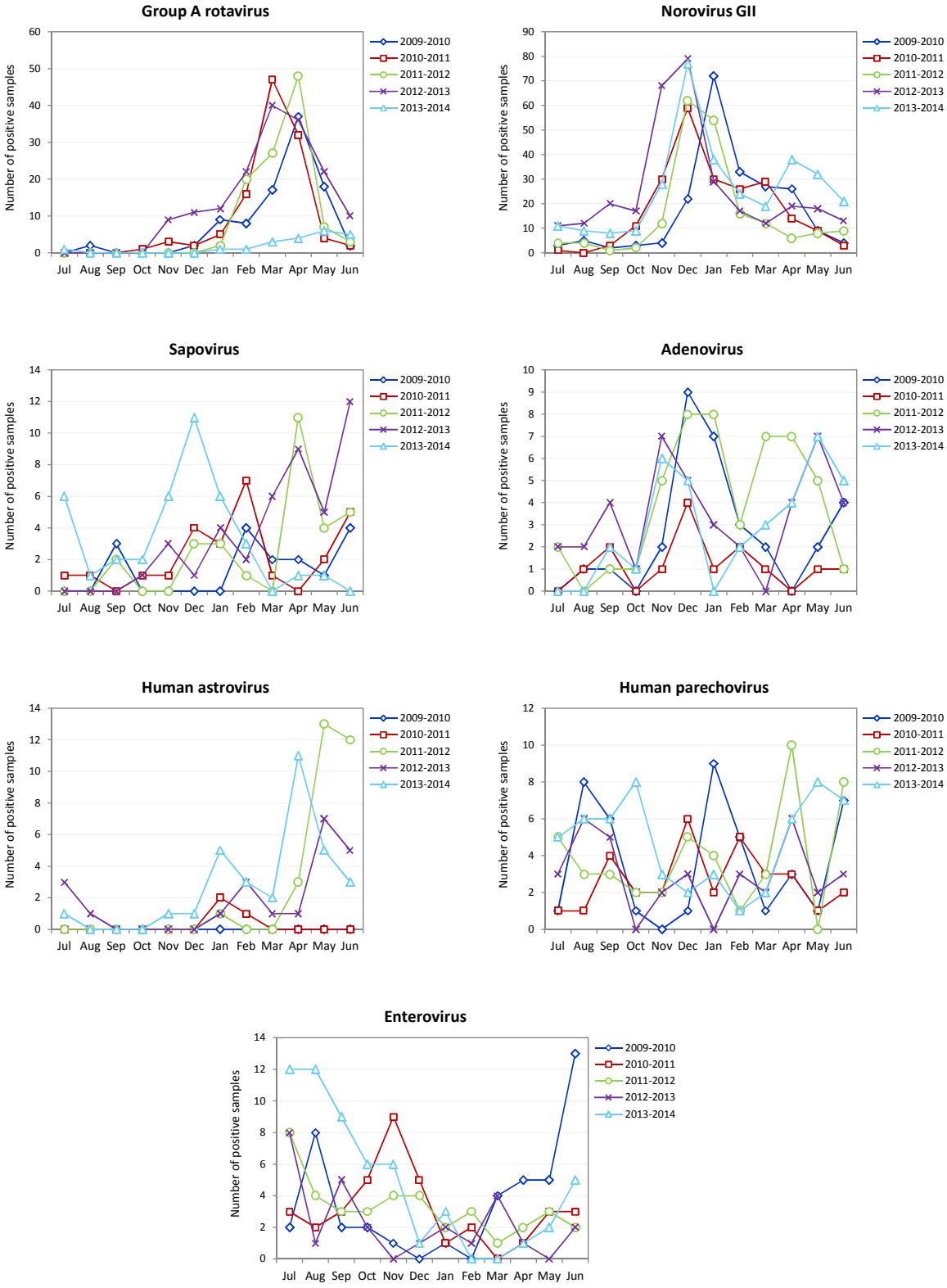


Figure 2. Monthly distribution of diarrheal virus infections among Japanese pediatric patients with acute gastroenteritis in 2009-2014

Table 5. Age-related distribution of diarrheal virus infections among Japanese pediatric patients with acute gastroenteritis during 2009-2014

Age group (months)	No. of samples	No. of diarrhea virus (%)										
		RAV	RBV	RCV	NoV GI	NoV GII	SV	AdV	HAstV	AiV	HPeV	EV
<6	263	25(5.0)	-	-	1(11.1)	85(6.8)	3(2.0)	13(7.7)	4(4.7)	-	22(10.3)	16(7.9)
6-11	822	129(26.0)	-	2(50.0)	3(33.3)	317(25.5)	40(26.1)	56(33.1)	30(34.9)	-	64(30.0)	71(35.0)
12-23	1,005	205(41.2)	-	-	-	516(41.5)	71(46.4)	60(35.5)	32(37.2)	1(50.0)	70(32.9)	78(38.4)
24-35	399	89(17.9)	-	1(25.0)	1(11.1)	177(14.2)	18(11.8)	24(14.2)	12(14.0)	1(50.0)	31(14.6)	18(8.9)
36-47	145	25(5.0)	-	1(25.0)	-	59(4.7)	7(4.6)	8(4.7)	1(1.2)	-	10(4.7)	9(4.4)
48-59	86	11(2.2)	-	-	2(22.2)	38(3.1)	5(3.3)	4(2.4)	2(2.3)	-	6(2.8)	5(2.5)
>60	188	13(2.6)	-	-	2(22.2)	52(4.2)	9(5.9)	4(2.4)	5(5.8)	-	10(4.7)	6(3.0)
Total	2,908	497	-	4	9	1,244	153	169	86	2	213	203

DISCUSSION

This study shows findings from a surveillance of diarrheal virus distributions among Japanese pediatric outpatients with gastroenteritis in six prefectures in Japan during July 2009 to June 2014. The study found several kinds of diarrheal viruses and showed a high proportion (72.1%) of positive samples for many target viruses. NoV and RV remained the most predominant viruses that cause diarrhea in Japanese children, while many other diarrheal viruses such as HPeV, EV, AdV, SV, HAstV, and AiV were also detected but in a much smaller proportion. This pathogen surveillance showed the higher detection rates of diarrheal virus infections in comparison with other studies conducted in Japan [unpublished data] and other countries (65-68). The high detection rates in this study may partly be explained by the inclusion of a larger number of target pathogens.

A comprehensive survey on various viral pathogens was carried out in six prefectures from northern to southern, including Hokkaido, Tokyo, Shizuoka, Kyoto, Osaka, and Saga; the detection rate of diarrheal viruses varies in individual regions. NoV GII was generally more prevalent than RAV. The most common circulating diarrheal viruses and their co-infections were consistently observed in all regions. The different diarrheal viruses circulating in each target area and the number of samples may relate with the difference of pathogen detection rate.

This study demonstrates the contribution of NoV as an important etiologic agent in Japanese outpatients. NoV has been recognized as the most prevalent causative agent of diarrhea (43.0%); most of these NoV were belonged to NoV GII (99.4%). The prevalence of NoV was much higher than that found in the preceding studies conducted in the same geographical areas during 2004 to 2009 showing the detection rates of NoV ranging from 8.5% to 27.0% (17, 19, 24). Taken together, the results in the present and preceding studies

confirmed that NoV was the most dominant agent causing acute gastroenteritis in Japan, and that the detection rate showed a dramatic increase year by year. Interestingly, the increase of NoV infections in pediatric patients was shown to be associated with the emergence of a new variant termed "Sydney_2012" since mid-2012 (69).

RAV was found to be the second most common causative agent, variously detected in each year ranging from 17.9 to 22.1% in 2009-2014, with an average of 17.1% in the 5-year surveillance survey. These figures are comparable with the results from previous studies in the same populations showing a range between 15.5-20.6% (14, 16, 22, 70), whereas they are lower compared with those in other Asian countries accounting for 26.9-37.5% (67, 68, 71).

The predominant genotype RAV in each epidemic season changed over time. The G1 was the most predominant genotype among Japanese population (14, 22). Interestingly, the emergence of G3 was identified, and these strains became the most prevalent genotypes in 2010-2011 (70). The epidemiological trend of RAV genotype is similar to the emergence of G3 in 2002-2004 (72, 73). The change of rotavirus genotype in Japanese populations may be due to an increase of herd immunity against G1 and less immunity to G3, which provides an opportunity for G3 to become more prevalent. Furthermore, the number of RAV increased again in 2012-2013 which was related to the emergence of G1P[8]DS-1 like strains. This strain may represent mutants that shifted, escaped and spread in Japanese population since the introduction of RV vaccines in Japan in 2011 (74). The incidence of RAV-associated acute gastroenteritis dropped in 2013-2014 with the detection rate only 3.9%.

This study shows that SV was the cause of acute gastroenteritis in almost all age groups of the children with the detection rate of 5.3% (153/2,908). The result was more or less in line with findings from other SV epidemiological studies worldwide, in which SV

prevalence ranges from 0.3-9.3% (21, 23, 75, 76). The accumulated data from the present and previous studies indicate that the overall prevalence of SV infection varies from time to time. Even though SV was originally discovered in an outbreak of gastroenteritis in Japan, SV usually tends to cause small-scale epidemics of relatively mild illness.

Previous studies reported that the AdV detection rate in Japan decreased year by year with an average of 7.9% (77). In this study, AdV was detected in 5.8% and the number of AdV positive cases decreased every other year. While no HAstV was found in 2009-2010; thereafter the detection rate increased. However, this rate is higher compared to findings in many other studies conducted in other Asian countries where the infection rate ranged from 2.4-3.9% (78-81).

Although Pham et al. (82) detected HPeV at a rate of 8.1% in samples negative for other common diarrheal viruses in Japanese patients, this figure might not be representative for patients with acute gastroenteritis. Several members of the genus *Picornavirus* were detected in this study; 7.3% of HPeV, 7.0% of EV, and 0.1% of AiV detection rate from all Japanese patients. The data support the presence of HPeV and EV in a significant proportion of patients with gastroenteritis, and also in the case of absence of other enteric viruses. However, these HPeV and EV infections can result in a wide variety of symptoms, and the specific viruses and genotypes should be determined for a clear interpretation of the cause of diseases.

One of the important findings in this study was a high percentage of mixed infections (15.5%), including double infection (14.4%) and triple infection (1.1%), commonly involving RAV and NoV GII (4.3%). Similar findings have been found in studies in other countries showing dual RAV and NoV infections in 0.2-11.0% of children with gastroenteritis (83-85). Most of HPeV and EV positive samples were co-circulated with other enteric viruses accounting for 61.5% and 44.8% of all positive cases. Previous

studies reported that the clinical symptoms of HPeV samples of single infections are not different from those of other diarrheal diseases (82). Therefore, mixed infections detected in this study may be responsible for more severe diarrhea. However, analysis of clinical symptoms and signs, especially in double and triple infection cases, are needed. The frequency of mixed infections was not significant different between children's age groups.

Of the patients with acute gastroenteritis, diarrheal virus infection cases were detected in all age groups. Several studies have found that diarrheal viral infections occur frequently in young children aged younger than 3 years. In agreement with this, these results showed a marked trend toward higher rates of diarrheal virus infection in children younger than 3 years of age, with children between 12 to 23 months being the most frequently affected. NoV (88.0%) and RAV (90.1%) infections were detected in children aged under three years, and were commonly detected in children aged between 12 and 23 months accounting for 41.5% and 41.2%, respectively. No significant difference between NoV and RV infections was found in this study in relation to the age of the patients. Other diarrheal pathogens including HAstV, AdV, HPeV, and EV infections were also observed in children particularly in those aged younger than 3 years. This finding showed that diarrheal virus infections usually occurred in early childhood, possibly because many children aged younger than 3 years may not have adequate antibody protection. The finding supports the RV vaccination schedule at 2, 4, and 6 months of age as recommended by the Centers for Disease Control and Prevention.

Regarding seasonality, several gastroenteritis viruses were detected throughout the year, with higher detection rates in dry and cool seasons. Previous reports have demonstrated a peak of NoV surveillance of viral gastroenteritis during winter in Japan (86, 87). Similar to this study, the seasonal distribution of NoV infection exhibits a peak in winter, with the highest peak of incidence in December, except NoV GII that showed a

peak in January in 2009-2010 survey (**Figure 2**). The low temperature and low humidity had highly consistent effects driving NoV epidemic patterns. RAV appeared to be more prevalent in early spring, peaking in April. This data are in good agreement with the data from other surveillances on pediatric cases that demonstrated the peak of RV from March to April (14, 16, 22, 88). Interestingly, the co-infections by overlapping RAV and NoV detected in this study resulted in the second peak of RAV in January and NoV in March. Epidemiologically, HAstV infections have its peak in spring, while SV, AdV, HPeV, and EV could be detected throughout the year without a specific seasonal peak.

This study confirmed the diversity of viral pathogens and the burden of viral diarrhea disease. One of the strengths of this study is that it was the first report of detection of eight diarrheal viruses in Japan. However, this study also has limitation. The detection rate of gastroenteritis viruses described in the present study is probably underestimated since several new gastroenteritis viruses have recently been reported, for example, human cosavirus (89), Saffold virus (90), salivirus (91), Klassevirus (92), Novel MLB-clade, VA-clade astrovirus (93), and bufavirus (94). Some of the new diarrheal viruses have been examined in Japanese samples, and the prevalence rate of 1.5% was reported for Saffold virus (95). Further surveillance should include identification of other viral agents in the screening protocol. The second limitation of this study is that clinical data was not available for the interpretation of these viruses related with the diarrhea disease. Having been considered as causative agents and their severity of diarrhea illness, the clinical symptom data is needed. Data analysis and clinical interpretation will be further conducted in the next stage.

CONCLUSION

Acute gastroenteritis continues to be a major public health problem worldwide. A wide variety of viruses associated with diarrhea disease is being reported continually. The epidemiological situation of viruses that cause diarrhea in Japanese pediatric patients was investigated. This study provides the first findings on the molecular background of several types of diarrheal viruses among children with gastroenteritis infections in Japan. In this study, 72.1% of the Japanese pediatric outpatients were infected with at least one viral agent. NoV and RAV were the most common pathogens detected in these Japanese pediatric populations. With the increase in the use of RV vaccine in Japan, understanding the etiology of viral gastroenteritis, especially NoV, becomes increasingly important. Moreover, these findings indicate that single detection of minor diarrheal virus including HPeV, EV, and AiV may reflect the true infection. Information from this study are valuable for compiling the overall picture of several viruses that cause diarrhea and for treatment and vaccination programs for the Japanese population and it will contribute to the growing database on the molecular diversity of these diarrheal viruses circulating worldwide. This study also provides useful illustration of epidemiology data as well as guide future research.

CHAPTER III

MOLECULAR EPIDEMIOLOGICAL CHARACTERISTICS AND GENETIC DIVERSITY OF NOROVIRUS IN JAPANESE PEDIATRIC PATIENTS

INTRODUCTION

NoV is a major cause of diarrheal disease in human of children worldwide. Recently, numerous studies have described the molecular epidemiology of NoV causing diarrhea. Although various genotypes of NoV circulate in human population, some particular genotypes have dominant over time. Most of acute gastroenteritis outbreaks due to NoV infections are caused by NoV GII.4. The emergence of novel NoV GII.4 variants has been responsible for the increase of NoV activity globally. Over the last two decades, Many NoV GII.4 variants including the most recently Sydney_2012 have caused pandemics of NoV associated acute gastroenteritis, which were initiated by the emergence of novel variant in every two or three years.

The epidemiological success of GII.4 variants has been contributed to the number of factors (96), including both accumulation of mutation and homologous recombination (97). Antigenic diversity of NoV is generated through genetic variation in the protruding P2 domain, which leads virus escape from herd immunity (31, 46). The changes mostly occur in epitope A-E within P2 subdomain that have been shown to be the antigenic blockade epitopes (98). NoV can also greatly extend its genetic repertoire through recombination, which commonly occur both within and between genotypes (99). Recombination mainly occurs between ORF1 and ORF2, although recombinant hotspots have also been identified within ORF2 and ORF2/3 boundary (97).

Rapid antigenic variations are important evolutionary forces that shape epidemiological success and persistence of the NoV GII.4 in the population. The objectives

of this study were: (1) to describe a surveillance of NoV infections that are associated with gastroenteritis in Japanese pediatric patients; (2) to investigate the adaptive changes of novel GII.4 Sydney_2012 variant in antigenic blockade epitopes within P2 subdomain which may affect antigenic property and contribute to the emergence widespread variant in Japan; and (3) to investigate the genetic diversity of NoV circulating in Japanese population.

MATERIALS AND METHODS

Patients and samples

A total of 2,908 fecal specimens were collected from Japanese pediatric outpatients with acute gastroenteritis in pediatric clinics in six prefectures from northern to southern Japan including Hokkaido, Tokyo, Shizuoka, Kyoto, Osaka, and Saga from July 2009 to June 2014. The ages of the subjects ranged from neonate up to 15 years old. The fecal specimens were kept at -20^{o}C until use.

Detection of Norovirus by RT-PCR

The viral RNA genome was first extracted from 10% of fecal supernatant using the QIAamp viral RNA Mini Kit (Qiagen, Germany). The presence of the NoV in fecal specimens was detected by RT-PCR using the protocol described previously (61). The specimens were tested for the presence of NoV GI and GII using a combination of forward primer G1-SKF (nt 5342-5361) 5'-CTGCCCGAATTYGTAAATGA-3' and the reverse primer G1-SKR (nt 5653-5671) 5'-CCAACCCARCCATTRTACA-3', for NoV GI detection. For NoV GII identification, a forward primer COG2F (nt 5003-5028; 5'-CARGARBCNATGTTYAGRTGGATGAG-3') was used in combination with the reverse primer G2-SKR (nt 5367–5389; 5'-CCRCCNGCATRHCCRTTRTACAT-3'), for the amplification of partial capsid gene of NoV GII.

Electrophoresis

The PCR products were detected by electrophoresis on 1.5% agarose gel, stained with SYBR® Safe DNA Stain (Invitrogen, Carlsbad, CA, USA), and visualized under a blue-light transilluminator.

Phylogenetic analysis for norovirus genotyping

The genotypes of the NoV positive samples were characterized by direct nucleotide sequencing of partial capsid gene and phylogenetic analysis as described previously (28). The PCR products were purified using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and sequenced by using the BigDye Terminator Cycle Sequencing kit (Perkin Elmer-Applied Biosystems, Inc., Foster City, CA) on an automated DNA sequencer (ABI 3100; Perkin Elmer-Applied Biosystems, Inc., Foster City, CA). The nucleotide sequences were analysed with those of NoV strains deposited in GenBank database. Phylogenetic relationship of NoV was examined by aligning sequences with the ClustalX program. A phylogenetic tree was conducted according to the maximum likelihood (ML) method using MEGA version 5 (100).

Amplification of polymerase and capsid genes of norovirus

NoV polymerase gene was amplified to identify recombinant using three pairs of specific primers: GV21/Hep171 (37), P290/P289 (101), or Yuri22F/CB2 (34, 101) (**Table 6**). To confirm the recombinant strain, a long genomic fragment that included the junction between polymerase and capsid genes was generated using forward primers GV21, P290, or Yuri22F and reverse primer G2SKR. Amplification of P2 domain, NoV was performed using primers P2 GII-4 F/P2 GII-4 R. The PCR was carried out at 94°C for 3 min, followed by 35 cycles of 94°C 30 sec, 55°C 30 sec, 72°C 2 min, and a final extension at 72°C for 7 min, and then held at 4°C.

The partial polymerase and full-length capsid region of a new variant was amplified using two pairs of primers, GV21/G2SKF and COG2F/TX30SXN. Long-range PCR was amplified with High Fidelity DNA polymerase KOD-Plus (Toyobo, Osaka, Japan). The PCR was carried out with 2 µl of cDNA, which has been reverse transcribed using

TX30SXN primer, in the reagent mixture containing 2.5 µl of 10x buffer for KOD-Plus, 1 µl of 2 mM dNTPs, 1 µl of 25 mM $MgSO_4$, 0.75 µl of each specific primer (20 µM) and 0.5 µl of KOD Plus enzyme. RNase-free distilled water was added to make up a total volume of 25 µl. The PCR was performed at 94°C for 2 min, followed by 35 cycles of 98°C 20 sec, 50°C or 55°C 30 sec, 68°C 2 min, and a final extension at 68°C for 10 min, and then held at 4°C.

Statistical analysis

The statistical analysis was performed by using the chi-squared test (χ^2) to determine the significance of prevalence, age group, and seasonality of diarrheal virus detection. P-value <0.05 was considered statistically significant.

Table 6. Specific primers used in PCR to amplify polymerase and capsid genes of norovirus detected in children with acute gastroenteritis in Japan

Target gene	Primer	Polarity[a]	Sequence (5' to 3')[b]	Position[c]
Partial	GV21	+	GTBGGNGGYCARATGGGNATG	3368-3388
polymerase	Hep171	-	CGACGCCATCTTCATTCAC	5099-5081
	P290	+	GATTACTCCAAGTGGGACTCCAC	4295-4317
	P289	-	TGACAATGTAATCATCACCATA	4613-4592
	Yuri22F	+	ATGAATGAGGATGGACCCAT	4232-4251
	CB2	-	GTTTYARCCCGTATTCCTTG	4676-4657
P2 domain	P2 GII-4 F	+	GANGATGTCTTCACAGTCTCTT	5661-5682
	P2 GII-4 R	-	CARTCCTGGGGGAGTAGCCA	6432-6451
Full capsid	GV21	+	GTBGGNGGYCARATGGGNATG	3368-3388
genome	G2SKR	-	CCRCCNGCATRHCCRTTRTACAT	5389-5367
	COG2F	+	CARGARBCNATGTTYAGRTGGATGAG	5002-5028
	TX30SXN	-	GACTAGTTCTAGATCGCGAGCGGCCGCCC(T)30	3'end

a: +, Forward primer; -, Reverse primer.
b: Within nucleotide sequence of primers, B=C, G or T; H=A, C or T; N: Any base; R= A or G; K=T or G; W= A or T; Y= C or T
c: The position numbers of primer pairs correspond to representative strains Lordsdale (X86557).

Three-dimensional structure of norovirus

The three-dimensional structures of NoV were constructed by homology modeling technique using Geno3D web server (102), and the structures were checked for the quality of geometry by PROCHECK (103). For the model structures, all sequences were based on the structure of NoV GII.4 2004 using PDB code 3SEJ (104).

RESULTS

Detection of norovirus

NoV was detected in 1,251 out of 2,908 (43.0%) pediatric patients during 2009-2014, comprising 0.3% of GI (7/2,908), 39.0% of GII (1,242/2,908), and 0.1% mixed GI/GII (2/2,908). As shown in **Table 7**, the detection rate of NoV GII infections were 40.8% (210/515) in 2009-2010, 40.2% (215/535) in 2010-2011, 31.7% (190/599) in 2011-2012, 43.0% (315/732) in 2012-2013, and 59.6% (314/527) in 2013-2014.

Out of total samples, 30.3% (880/2,908) of the patients were infected by NoV GII alone, while 12.5% (364/2,908) were co-circulated with other viruses. Mix-infection between NoV and RAV was dominant (3.9%; 113/2,908), followed by HPeV (2.5%), AdV (1.9%), EV (1.7%), HAstV (0.8%), SV (0.7%), and NoV GI (0.1%). Moreover, NoV GII caused mixed infections with other two kinds of diarrheal viruses in 1.0% (28/2,908), mostly with RAV.

Distribution of norovirus genotypes

Based on the analysis of partial sequence of capsid gene of NoV GII, nine distinct genotypes were identified. Of these, GII.4 was the most predominant genotype for five year surveillance (64.5%; 803/1,244), followed by GII.3 (16.3%; 203/1,244), GII.6 (6.4%; 80/1,244), GII.2 (5.0%; 62/1,244), GII.14 (3.3%; 42/1,244), GII.13 (2.6%; 32/1,244), GII.7 (1.0%; 13/1,244), GII.12 (1.0%; 7/1,244), and GII.17 (0.2%; 2/1,244) as shown in **Table 7**. Interestingly, a high frequency of NoV GII.4 was found at a rate of 69.5% (132/190) in 2011-2012 and 88.6% (279/315) in 2012-2013. In contrast, NoV GII.3 decreased rapidly to 0.1% (2/315) in 2012-2013. However, GII.3 reemerged in 2013-2014, accounting for

25.8% (81/314). The NoV GI was detected in 0.3% (9/2,908), consisting of GI.1 (n=2), GI.4 (n=1), and GI.6 (n=6).

Among NoV GII.4 identified in this study, three separate NoV GII.4 variants were mainly identified as the etiologic agents, including Den_Haag_2006b (36.9%; 296/803), New_Orleans_2009 (14.9%; 120/803), and Sydney_2012 (46.0%; 369/803) variants. A number of less common GII.4 variants have been found, such as Hunter 2004, Yerseke_2006a, Apeldoorn_2007, and Hokkaido1_2008 (**Table 8**).

Geographic distribution of norovirus genotype

The distribution of NoV genotypes among 6 regions in Japan was shown in **Table 9**. The detection rates of NoV GII were varied in each region ranging from 32.8% to 61.9% (Hokkaido 61.9%, Kyoto 56.9%, Osaka 40.4%, Saga 38.8%, Shizuoka 37.9%, and Tokyo 32.4%). NoV GII.4 was the most predominant genotype among those of NoV GII detected in each regions; 75.4% in Hokkaido, 72.6% in Tokyo, 62.6% in Shizuoka, 62.6% in Kyoto, 66.9% in Osaka, and 56.9% in Saga. The distribution of NoV GII.3 ranged from 9.5% (Osaka) to 21.6% (Saga). Interestingly, NoV GII.6 was detected in a high prevalence in 2013-2014 in Saga (14.7% of all NoV GII detected in this area), while GII.6 was detected in other regions ranging 0.9-7.0%.

Focusing on NoV GII.4 predominant genotype, NoV GII.4 Den_Haag_2006b, New_Orleans_2009, and Sydney_2012 variants were detected in all investigated regions (**Table 10**). NoV GII.4 Den_Haag_2006b was common found in Shizuoka (n=95), while most of Sydney_2012 was detected in a large number in Kyoto (n=117) and Osaka (n=109).

Table 7. Genotype distributions of norovirus detected in Japanese pediatric patients with diarrhea from 2009-2014

Year	No. of samples tested	No. of norovirus positive (%)	No. of genogroup (%)		No. of genotype cases (%)								
			GI	GII	GII.2	GII.3	GII.4	GII.6	GII.7	GII.12	GII.13	GII.14	GII.17
2009-2010	515	210(40.8)	-	210(40.8)	24	30	135	1	6	5	1	7	1
2010-2011	535	215(40.2)	1(0.2)	215(40.2)	17	60	117	2	1	1	2	15	-
2011-2012	599	190(31.7)	-	190(31.7)	7	30	135	3	-	-	5	10	-
2012-2013	732	321(43.5)	7(0.9)	315(43.0)	12	2	277	6	6	1	2	9	-
2013-2014	527	315(59.8)	1(0.2)	314(59.6)	2	81	139	68	-	-	22	1	1
Total	**2,908**	**1,251(43.0)**	**9(0.3)**	**1,244(42.8)**	**62(2.1)**	**203(7.0)**	**803(27.6)**	**80(2.8)**	**13(0.4)**	**7(0.2)**	**32(1.1)**	**42(1.4)**	**2(0.1)**

Table 8. Distributions of norovirus GII.4 variant detected in Japanese pediatric patients with diarrhea from 2009-2014

Year	No. of samples tested	No. of norovirus GII.4 (%)	No. of positive cases (%)							
			GII.4(2004)	GII.4(2006a)	GII.4(2006b)	GII.4(2007)	GII.4(2008a)	GII.4(2008b)	GII.4(2009)	GII.4(2012)
2009-2010	515	135(26.2)	1	1	89	-	1	-	43	-
2010-2011	535	117(21.9)	-	6	90	-	-	-	21	-
2011-2012	599	135(22.5)	1	-	75	-	3	-	53	3
2012-2013	732	277(37.8)	-	-	33	2	-	2	1	239
2013-2014	527	139(26.4)	-	-	9	-	-	1	2	127
Total	**2,908**	**803(27.6)**	**2(0.2)**	**7(0.9)**	**296(36.9)**	**2(0.2)**	**4(0.5)**	**3(0.4)**	**120(14.9)**	**369(46.0)**

Table 9. Distribution of norovirus GII genotypes in Japanese pediatric patients in 6 regions of Japan during 2009-2014

Place	No. of samples	No. of NoV GII	No. of positive cases								
			GII.2	GII.3	GII.4	GII.6	GII.7	GII.12	GII.13	GII.14	GII.17
Hokkaido	105	65	3	8	49	3	-	1	-	1	-
Tokyo	327	106	6	16	77	1	-	2	-	4	-
Shizuoka	896	340	21	68	213	19	5		3	10	1
Kyoto	601	342	8	60	214	24	7	2	12	15	-
Osaka	680	275	22	26	184	16	1	1	16	8	1
Saga	299	116	2	25	66	17	-	1	1	4	-
Total	2,908	1,244	62	203	803	80	13	7	32	42	2

Table 10. Distribution of norovirus GII.4 variants in Japanese pediatric patients in 6 regions of Japan during 2009-2014

Place	No. of samples	No. of NoV GII.4	No. of positive cases							
			GII.4 (2004)	GII.4 (2006a)	GII.4 (2006b)	GII.4 (2007)	GII.4 (2008a)	GII.4 (2008b)	GII.4 (2009)	GII.4 (2012)
Hokkaido	105	49	-	-	39	1	-	-	1	8
Tokyo	327	77	-	-	37	-	-	-	25	15
Shizuoka	896	213	-	6	95	-	1	1	16	94
Kyoto	601	214	-	1	51	-	1	2	42	117
Osaka	680	184	1	-	43	1	2	-	28	109
Saga	299	66	1	-	31	-	-	-	8	26
Total	2,908	803	2	7	296	2	4	3	120	369

Identification of epidemic norovirus GII.4 variants

The phylogenetic analysis of nucleotide sequences of the partial capsid region of NoV GII isolates was constructed in comparison with the reference strains available in GenBank database (**Figure 3**). The NoV GII.4 consisted of three major variants strains detected in pediatric patients in this study, as shown in **Table 8**. The Den_Haag_2006b strain that appeared during the study period comprised 296 of 803 NoV GII.4 strains (36.9%). The sequences of these NoV strains exhibited 98.0-100% nucleotide identity and 98.7-100% amino acid identity with Hu/GII.4/DenHaag89/2006/NL (GenBank accession number EF126965). The second variant New_Orleans_2009 strain was detected in 14.9% (120/803) and displayed 96.0-100% nucleotide sequence identity and 97.5-100% amino acid identity with the capsid region of Hu/GII.4/NewOrleans1805/2009/USA (GU445325). Interestingly, the new Sydney_2012 variant was detected in 46.0% (369/803) and clustered within the same cluster as the Sydney/NSW0514/2012/AU (JX459908) and Hong Kong/CUHK3655/2012/CHN (JX629456) reference strains and showed 98.7-100% nucleotide sequence identity and 99.2-100% amino acid sequence identity with reference strains, as shown in **Table 11**. However, a number of additional GII.4 variants have also been identified, including, Hunter 2004, Yerseke_2006a, Apeldoorn_2007, and Hokkaido1_2008. These variants were less commonly found in this study.

Seasonal pattern of norovirus infection

The seasonal pattern of NoV infection is shown in **Figure 4**. NoV was detected all year round with a highest peak in December. From this study, it is clearly observed that NoV infection in many genotypes is common in winter season in Japan. The highest prevalence of NoV was found in December (23.9%; 299/1,251), followed by January (17.9%; 224/1,251), and November (11.4%; 142/1,251), respectively.

Focusing on NoV GII.4 variants, most of NoV GII.4 occurred between November and January, with a highest peak in December (28.9% of all NoV GII.4 detected in this study). The Den_Haag_2006b variant was detected as the most predominant variant until March 2012 in co-circulation with New_Orleans_2009 variant. Subsequently, they were replaced by variant Sydney_2012 which was described in June 2012 (**Figure 5**).

Regional distribution of norovirus GII.4 variants

NoV GII.4 Sydney_2012 was first detected in a Japanese patient in Tokyo since November 2011 and Hokkaido in January 2012, as shown in **Figure 6**. Then, these 2012 variant strains spread throughout Japan from mid-2012 with the high detection rate, especially in Shizuoka, Kyoto, and Osaka. In an analysis of P2 subdomain, the Japanese NoV GII.4 variant 2012 strains detected in early 2012 were clustered into two sub-lineages, 2012a and 2012b, within the same cluster of Woonona_2012 (JX459907) and Sydney_2012 (JX459908), respectively (**Figure 7**). All variant 2012 detected belonged to norovirus GII.4 2012b variant. It exhibited a high identity among the strains detected in different regions and showed 98.9% nucleotide and 99.2% amino acid sequence identities with the Sydney/NSW0514/2012 (JX459908), accept only one strain detected in January 2012 (JP10909, Hokkaido) belonged to GII.4 2012a and showed a single amino acid difference in antigenic blockade epitope at the position 393, S in 2012a and G in 2012b.

Age-related distribution of norovirus

The average of infants and children with NoV infection was 22.9 months (range 0 to 452 months). Of the patients with acute gastroenteritis, the highest prevalence of NoV was found in infants and children aged 12-23 months (41.5%, n=516), and the lowest in children aged 48-59 months (3.1%, n=38). For the most predominant genotype, NoV GII.4 was detected in all aged group, being most prevalent in children of age 12-23 months at 39.1% of NoV GII.4 detected in this study (314/803), as shown in **Table 12**. Of these, NoV GII.4 Den_Haag_2006b, New_Orleans_2009, and Sydney_2012 variants were detected more frequently in 12-23 month-old group with frequencies 50.0%, 43.3%, and 36.6% of NoV GII.4, respectively (**Table 13**).

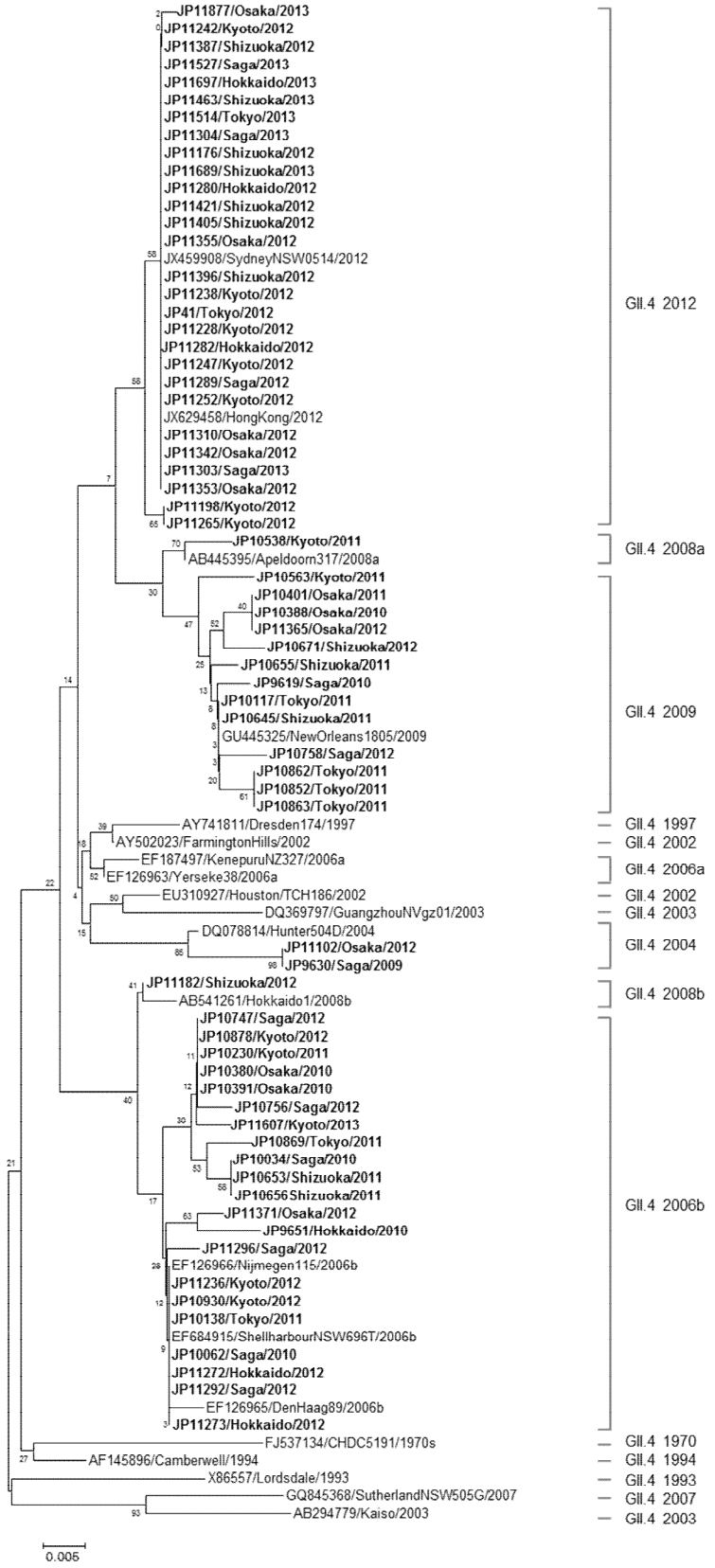


Figure 3. Phylogenetic analysis of the partial capsid sequence of norovirus GII.4 strains. The phylogenetic tree was constructed using the maximum likelihood method. Representative strains detected in this study are presented in boldface.

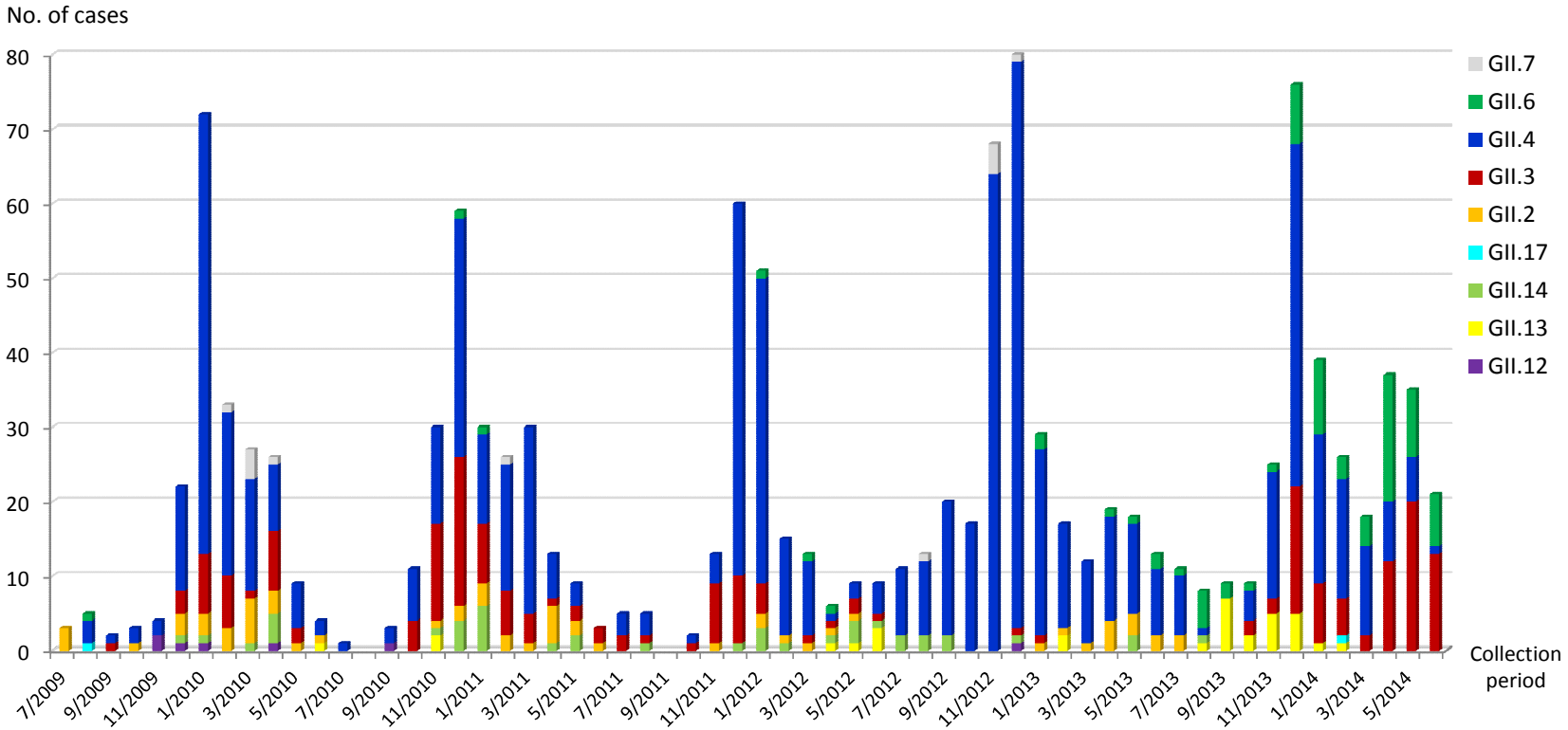


Figure 4. Monthly distribution of norovirus GII genotypes in Japanese pediatric patients during 2009-2014.

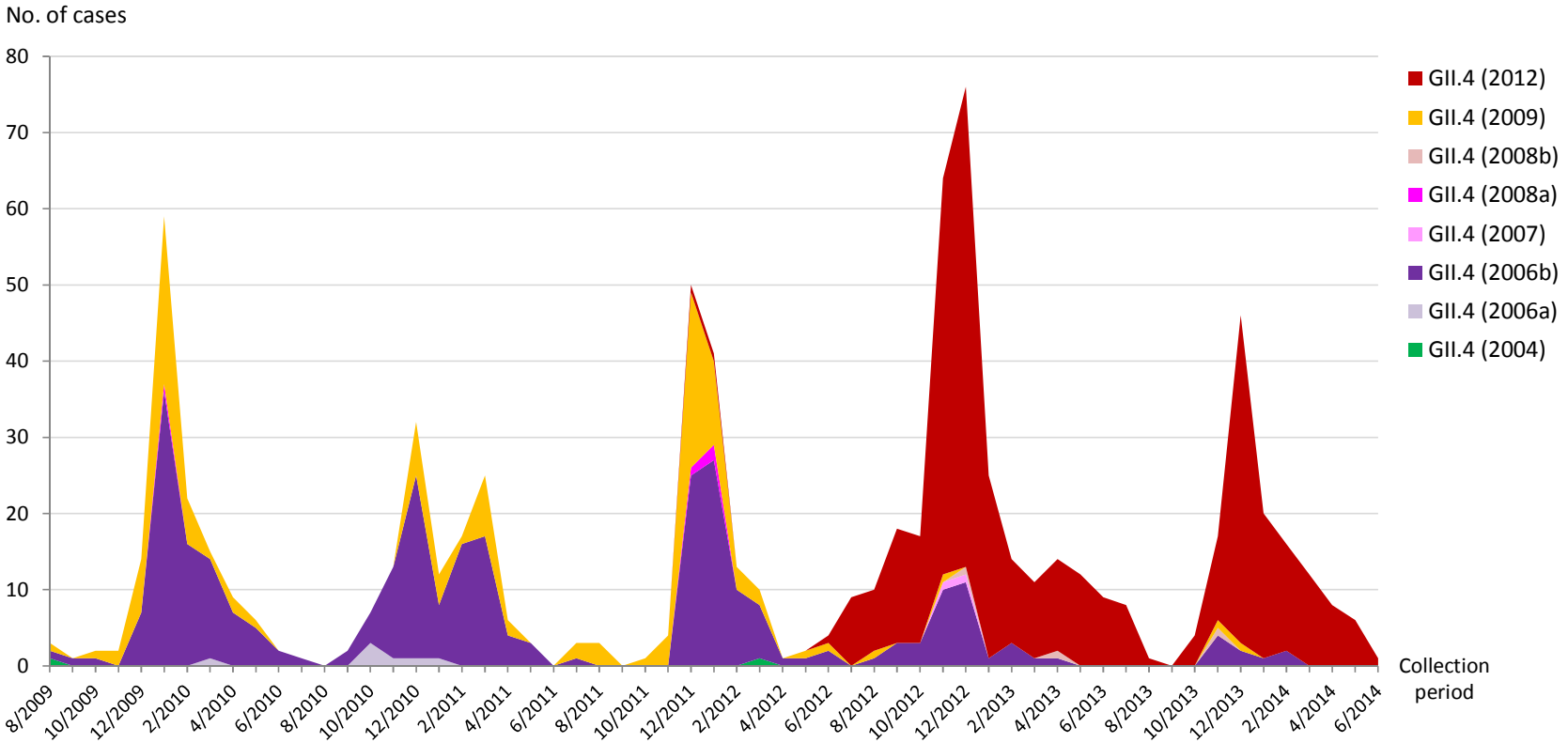


Figure 5. Monthly distribution of norovirus GII.4 variants in Japanese pediatric patients during 2009-2014.

Table 11. Nucleotide and amino acid identities among norovirus GII.4 variants. Representative norovirus GII.4 Sydney_2012 detected in this study are presented in boldface

Amino acid identity

Strains	2006b	2008b	2006a	2004	2007	2012	***JP41***	2009	2002	1997	1994	2003	1970
EF126965/DenHaag89/2006b		99.4	95.0	94.6	95.5	95.3	95.5	96.1	95.5	93.8	92.5	95.1	91.6
AB541261/Hokkaido1/2008b	98.9		95.1	94.4	95.3	95.1	95.3	95.9	95.5	93.8	92.7	95.1	91.8
EF126963/Yerseke38/2006a	91.9	91.7		97.7	94.8	94.2	94.4	95.0	96.1	94.2	92.9	94.6	92.0
DQ078814/Hunter504D/2004	92.2	91.8	96.4		93.7	93.1	93.3	93.8	95.7	94.0	92.0	94.6	91.1
AB445395/Apeldoorn317/2007	92.2	91.9	93.4	92.4		97.4	97.4	97.5	93.7	92.9	92.2	94.0	91.1
JX459908/SydneyNSW0514/2012	91.4	91.2	92.2	91.6	95.8		99.2	97.2	93.1	92.4	91.6	92.9	90.7
JP41	91.3	91.1	92.2	91.3	95.8	98.9		97.2	93.3	92.5	91.8	93.1	90.9
GU445325/NewOrleans/2009	91.3	91.1	92.9	91.9	96.3	94.7	94.5		93.7	92.9	91.8	94.4	91.1
AY502023/FarmingtonHills/2002	93.0	93.0	94.3	94.4	92.6	91.8	91.5	91.9		95.3	93.3	94.4	92.4
AY741811/Dresden174/1997	91.6	91.3	92.5	92.9	90.7	90.1	90.1	90.3	94.1		96.6	94.0	95.1
AF145896/Camberwell/1994	89.3	89.1	89.8	89.8	89.0	88.6	89.0	89.0	90.6	92.7		92.0	97.7
DQ369797/Guangzhou2003	92.6	92.2	92.7	92.9	92.3	91.0	91.1	91.8	94.1	92.7	89.4		90.7
FJ537134/CHDC5191/1970s	88.0	88.2	89.0	88.9	87.5	86.9	87.1	87.7	88.9	91.1	93.3	88.2	

Nucleotide identity

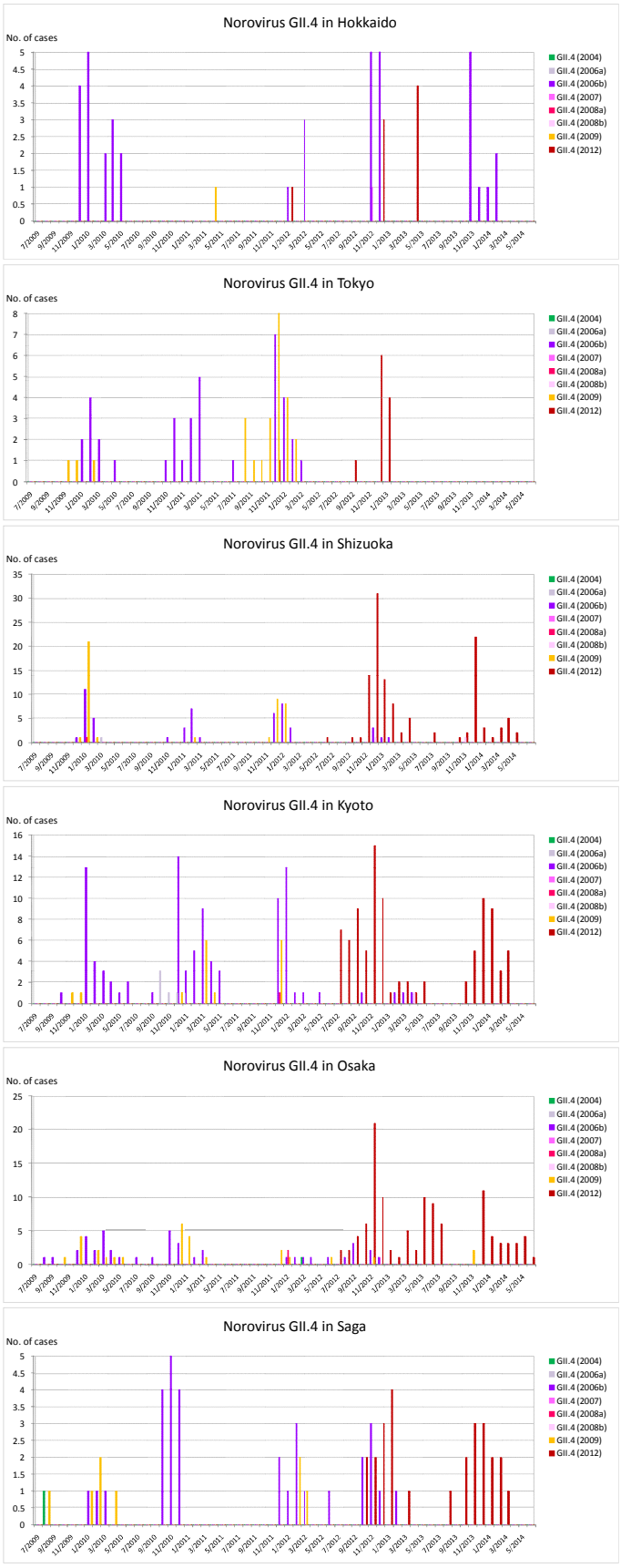


Figure 6. Monthly distribution of norovirus GII.4 variants in 6 prefectures from 2009-2014

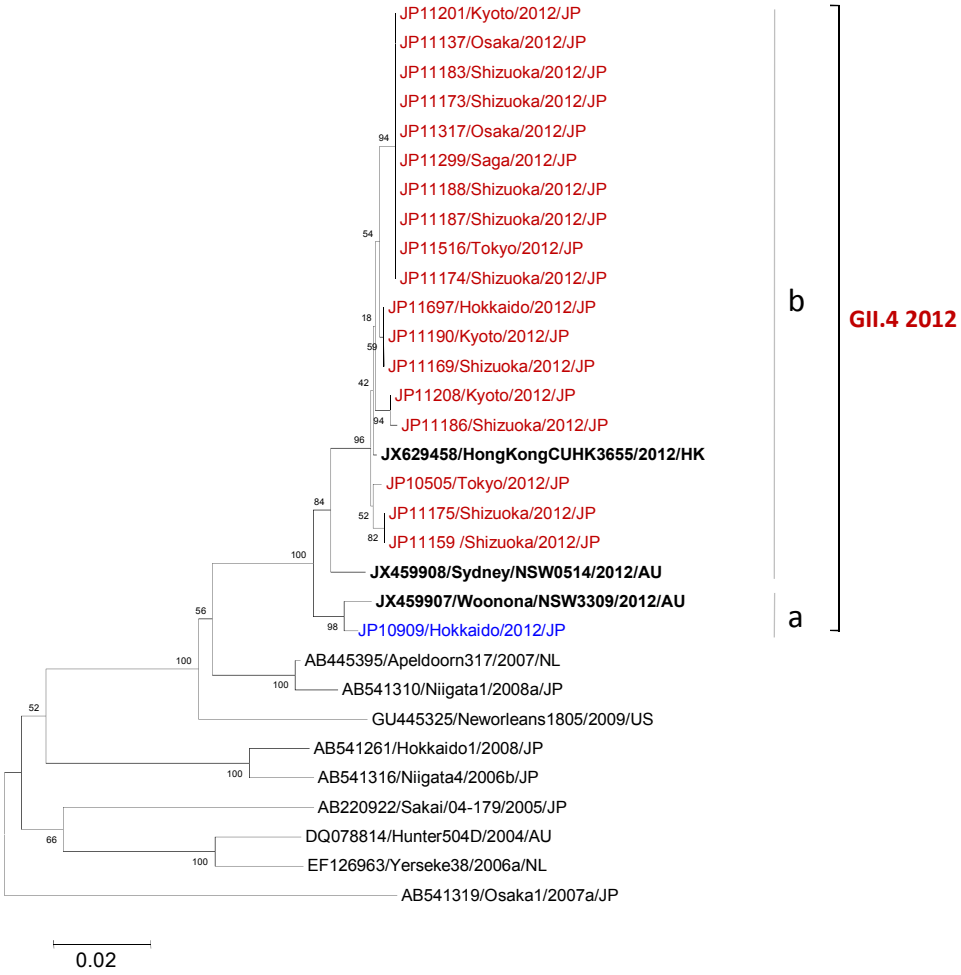


Figure 7. Phylogenetic analysis of the P2 subdomain sequence of norovirus GII.4 strains. The phylogenetic tree was constructed using the maximum likelihood method. Representative strains detected in this study are presented in red and blue.

Table 12. Age-related distribution of norovirus GII genotypes in Japanese pediatric patients during 2009-2014

Age group	No.of samples	No.of norovirus	No. of positive cases								
			GII.2	GII.3	GII.4	GII.6	GII.7	GII.12	GII.13	GII.14	GII.17
<6m	263	85	4	10	61	4	-	-	6	-	-
6-11m	822	317	18	56	219	12	2	-	8	2	-
12-23m	1005	516	18	83	341	34	6	4	6	23	1
24-35m	399	177	11	28	108	10	3	-	6	10	1
36-47m	145	59	4	12	35	5	-	-	1	2	-
48-59m	86	38	5	5	12	7	1	1	4	3	-
>60m	188	52	2	9	27	8	1	2	1	2	-
Total	2,908	1,244	62	203	803	80	13	7	32	42	2

Table 13. Age-related distribution of norovirus GII.4 variants in Japanese pediatric patients during 2009-2014

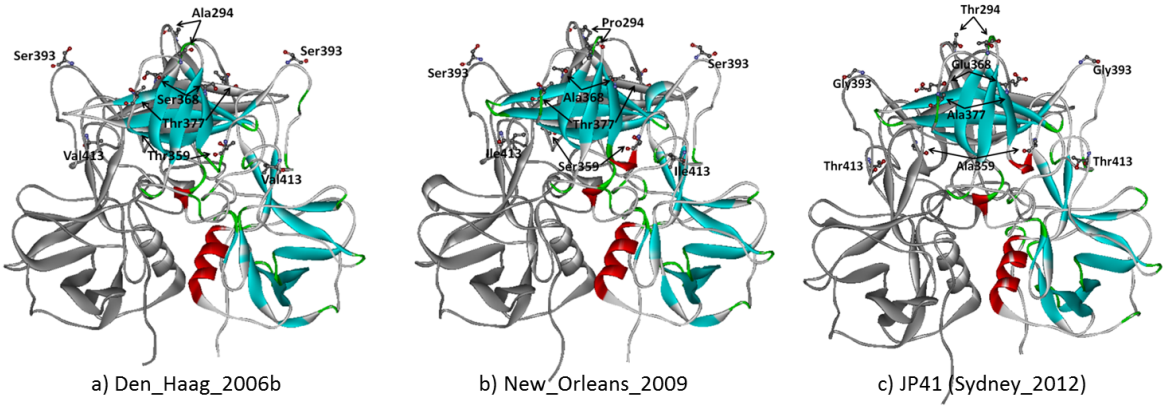
Age group	No.of samples	No.of norovirus	No. of positive cases							
			GII.4 (2004)	GII.4 (2006a)	GII.4 (2006b)	GII.4 (2007)	GII.4 (2008a)	GII.4 (2008b)	GII.4 (2009)	GII.4 (2012)
<6m	263	61	-	2	19	-	-	-	12	28
6-11m	822	219	1	-	69	1	1	1	38	108
12-23m	1005	341	1	3	148	-	1	1	52	135
24-35m	399	108	-	2	35	1	2	-	9	59
36-47m	145	35	-	-	11	-	-	-	4	20
48-59m	86	12	-	-	2	-	-	1	3	6
>60m	188	27	-	-	12	-	-	-	2	13
Total	2,908	803	2	7	296	2	4	3	120	369

Analysis of polymerase and capsid of norovirus GII.4 Sydney_2012

NoV GII.4 Sydney_2012 strain was emerged as the most predominant variant since 2012. To determine antigenic differences between GII.4 Sydney_2012 and the recent circulating ancestral strains Den_Haag_2006b and New_Orleans_2009 strains. VP1 and P2 subdomain especially in blockade epitopes of Sydney_2012 was analysed and compared with other variant strains. The phylogenetic analysis of NoV GII.4 is shown in **Figure 7**. The representative new variant exhibited a high identity in capsid gene. These strains were clustered in the same lineage with Sydney/NSW0514/2012/AU (JX459908) and Hong Kong/CUHK3655/2012/CHN (JX629458). The analysis of partial VP1 capsid of GII.4 variant 2012 representative strains revealed a high diversity with 6 positions of amino acid substitutions compared to Den_Haag_2006b and New_Orleans_2009 variants: positions 294($A_{2006b}\rightarrow P_{2009}\rightarrow T_{2012}$), 359($T_{2006b}\rightarrow S_{2009}\rightarrow A_{2012}$), 368($S_{2006b}\rightarrow A_{2009}\rightarrow E_{2012}$), 377($T_{2006b}\rightarrow T_{2009}\rightarrow A_{2012}$), 393($S_{2006b}\rightarrow S_{2009}\rightarrow G_{2012}$), and 413($V_{2006b}\rightarrow I_{2009}\rightarrow T_{2012}$), as shown in **Table 14**. The positions of amino acid changes in 3D structure of NoV variants were shown in **Figure 8**. In the analysis of P2 subdomain, the Japanese NoV GII.4 variant 2012 strains showed ≥98.9% nucleotide and ≥99.2% amino acid sequence identities with the Sydney/NSW0514/2012/AU (JX459908). Moreover, the Sydney_2012 variant was found to be similar to Apeldoorn_2007 and distinct in both the phylogenetic analysis and accounted for three amino acid substitutions in polymerase region different from other variants.

Table 14. Amino acid variation over time in blockade-epitope regions. Twelve representative sequences of norovirus GII.4 variants and the representative of variant 2012 detected in this study were selected from each major phylogenetic cluster. Each color represents amino acid changes that occurred within the subcluster

Strain	Accession NO.	Variant	Epitope A						Epitope D			Epitope E		
			294	296	297	298	368	372	393	394	395	407	412	413
CHDC5191	FJ537134	1974	G	S	H	D	T	N	D		T	N	S	G
Camberwell	AF145896	1994	V	S	H	D	T	N	D			N	T	G
Dresden	AY741811	1997	A	S	H	D	T	N	N			N	T	G
Farmington_Hills	AY502023	2002	A	T	H	N	N	N	N	G	T	S	T	G
Guangzhou	DQ369797	2003	P	T	R	I	A	D	S	S	A	D	T	V
Hunter	DQ078814	2004	A	T	Q	N	S	S	S	T	T	D	D	S
Yerseke	EF126963	2006a	A	T	Q	E	S	S	S	T	T	D	D	S
Den_Haag	EF126965	2006b	A	S	R	N	S	E	S	T	T	S	N	V
Apeldoorn	AB445395	2007	T	S	R	N	A	D	D	T	A	S	N	N
Hokkaido1	AB541261	2008	A	S	R	N	S	E	S	T	T	S	D	V
New_Orleans	GU445325	2009	P	S	R	N	A	D	S	T	T	S	N	I
Sydney	JX459908	2012	T	S	R	N	E	D	G	T	T	S	N	T
JP41	KF145149	2012	T	S	R	N	E	D	G	T	T	S	N	T



a) Den_Haag_2006b b) New_Orleans_2009 c) JP41 (Sydney_2012)

Figure 8. The three-dimensional structural model of norovirus GII.4 variants showing locations of amino acid changes in blockade-epitope regions of the dominant strains; a) Den_Haag_2006b, b) New_Orleans_2009, c) JP41 [Sydney_2012] variants

Genetic variation of norovirus GII.3

Phylogenetic analysis of partial capsid gene of NoV GII revealed a variety of NoV genotypes circulating in Japanese patients. Among these, NoV GII.3 was detected as the second predominant genotype in these 5 years period. However, NoV GII.3 was detected in only 2 cases in 2012-2013 before showing a high detection rate in 2013-2014, accounting for 25.8%. Therefore, genetic variation of GII.3 was investigated. The sequences of capsid regions of representative GII.3 detected in this study were analysed with GII.3 reference strains. A phylogenetic tree revealed that GII.3 strains detected in late-2013 belonged to the GII.3 Cluster III, which was grouped together other reference strains detected between 2000 and 2006 (**Figure 9**). The representative strains detected around mid-2014 belonged to the same cluster as GII.3 detected in Japan from 2008, however these strains belonged to the new sub-lineage of NoV GII.3 Cluster V.

Emergence of norovirus GII.6 outbreak

A high frequency of emerging NoV GII.6 was found at a rate of 21.6% (68 out of 314) in 2013-2014. Since late 2013, NoV GII.6 emerged dominate over other genotype but under NoV GII.4. NoV GII.6 was detected continuously in a 7-month period from November 2013 to June 2014 and reached its peak in March 2014. This strain was found firstly in Shizuoka and Kyoto before detected in Saga and Osaka. In an attempt to understand the molecular basis of genetic diversity, NoV GII.6 strains were randomly chosen and analysed with other reference strains. At least three distinct GII.6 clusters (A, B, and C) circulated in different part of the world. The phylogenetic analysis of NoV GII.6 was shown in **Figure 10**. The representative GII.6 exhibited high identities among group and clustered in the new sub-lineage GII.6 B-II of lineage B, which distinct from other strains. Analysis on partial capsid region, it showed 7 nucleotide different from other

reference strains which clustered in sub-lineage GII.6 B-I. Only the strain detected in Hokkaido showed some nucleotide different from other GII.6 detected in this study (**Figure 11**). By sequence comparison, the GII.6 detected in 2013-2014 shared 94.5-94.9% and 91.5-92.5% similarities with those in subcluster A and C, indicating 5.1-5.5% and 7.5-8.5% divergence between these clusters. On the other hand, other representative detected in 2009-2013 were clustered into sub-cluster A and C, which were similar to the reference strains detected and reported as the emergence of GII.6 in 2008-2009.

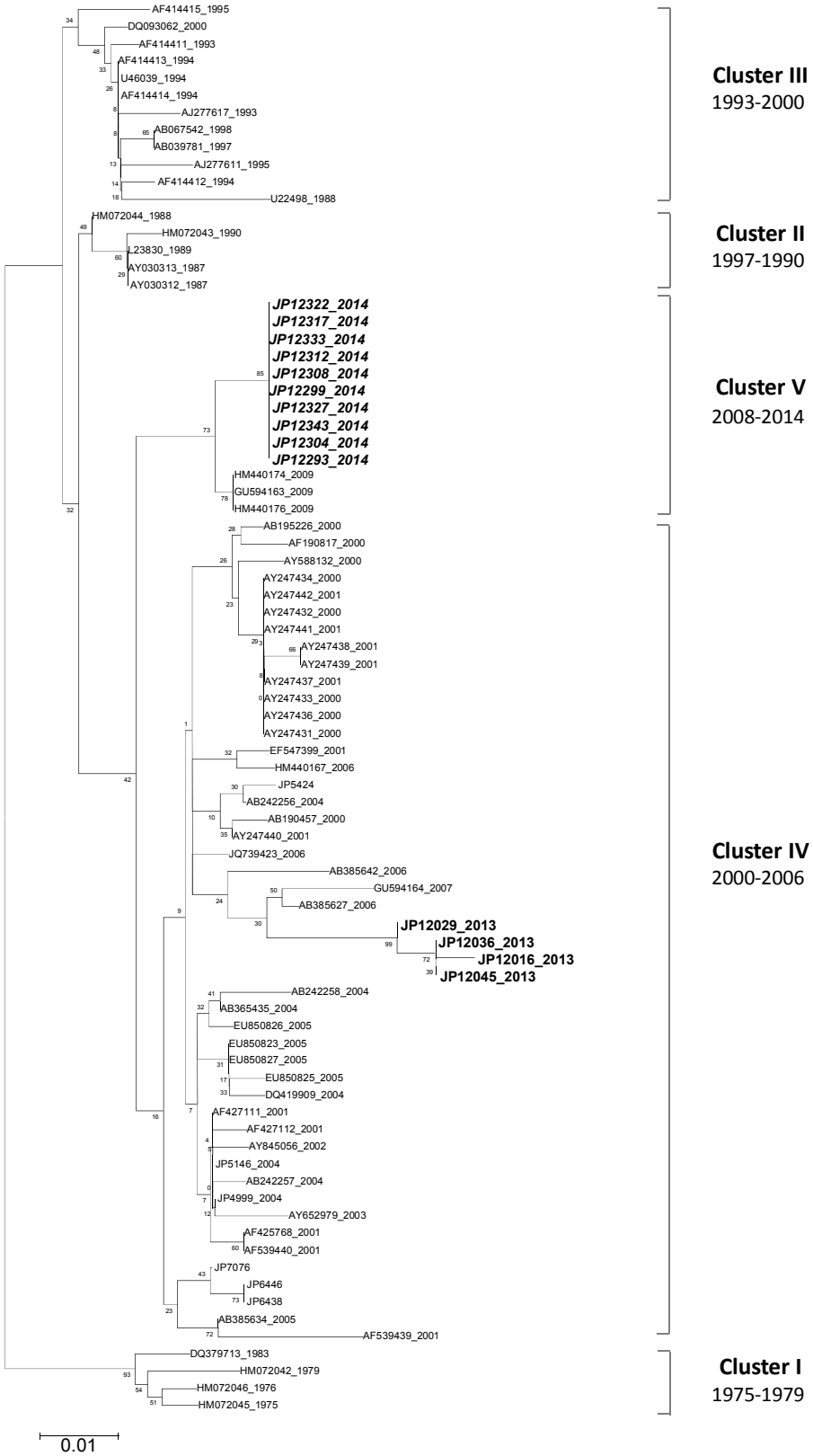


Figure 9. Phylogenetic analysis of the partial capsid sequence of norovirus GII.3 strains. The phylogenetic tree was constructed using the maximum likelihood method. Representative strains detected in this study are presented in boldface

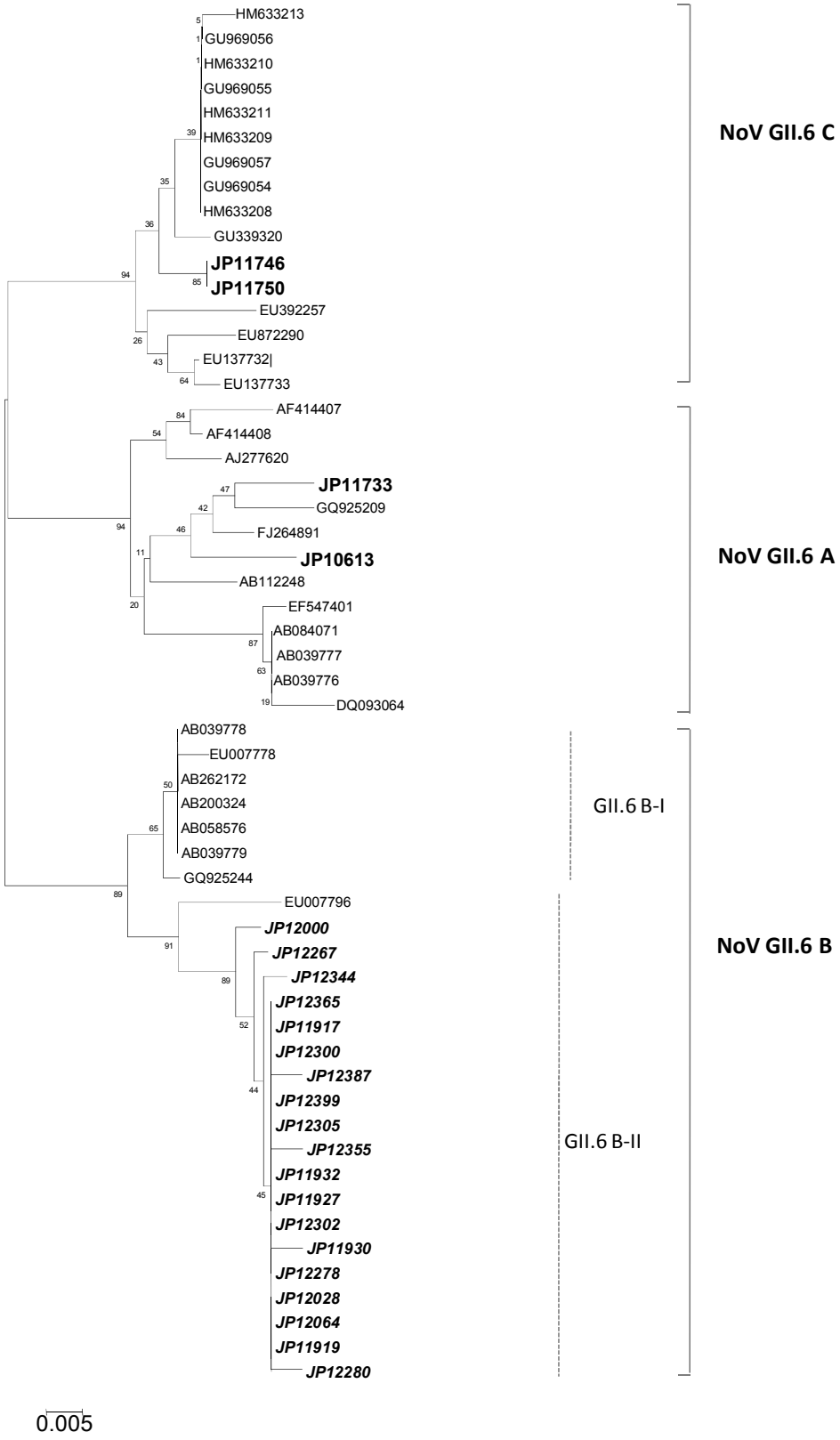


Figure 10. Phylogenetic analysis of the partial capsid sequence of norovirus GII.6 strains. The phylogenetic tree was constructed using the maximum likelihood method. Representative strains detected in this study are presented in boldface

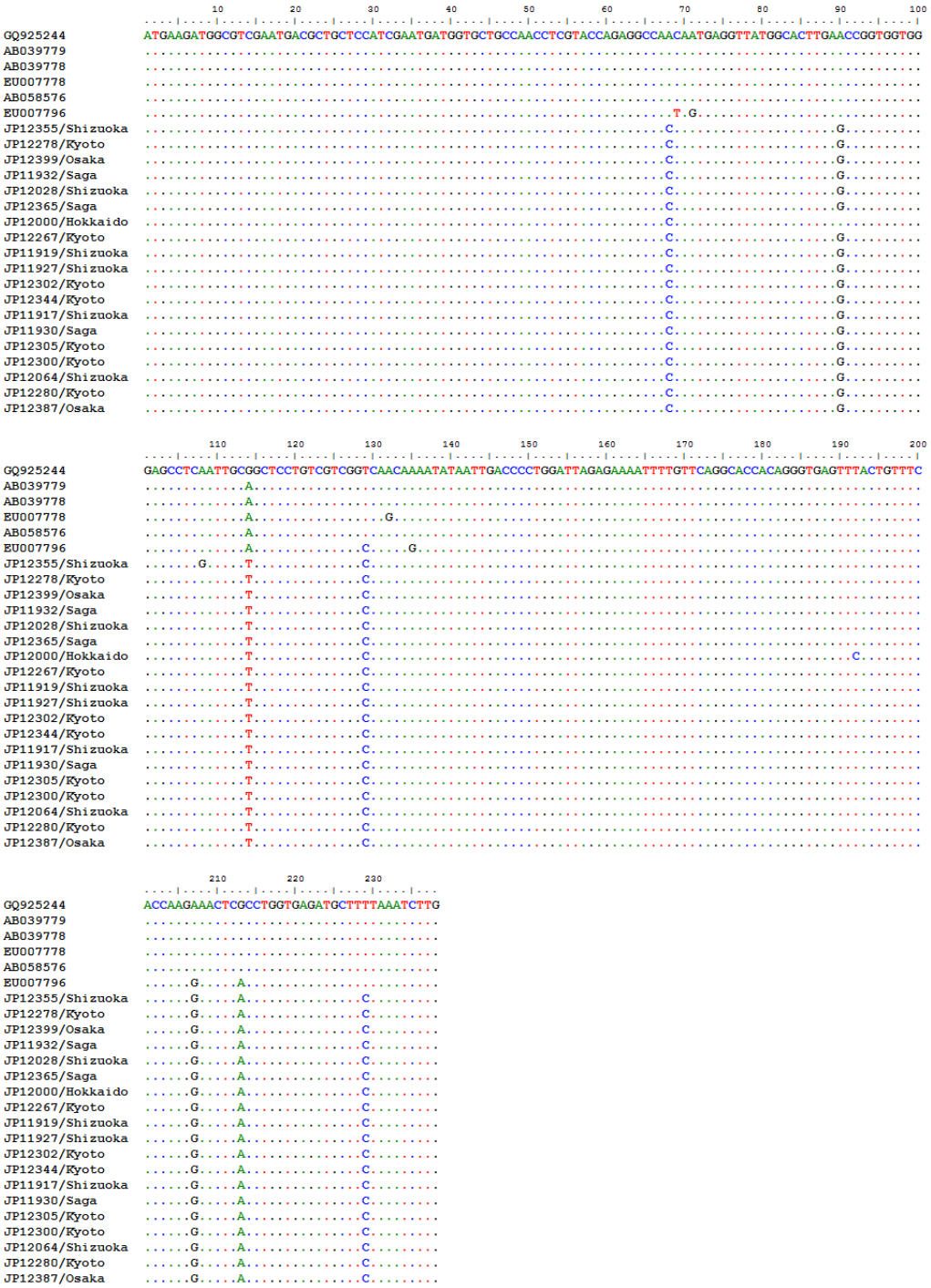


Figure 11. Nucleotide alignment of the partial capsid region of norovirus GII.6 strains showing nucleotide difference across the region.

Norovirus recombination

To investigate NoV recombination, partial polymerase and capsid regions of NoV GII.3, GII.7, and GII.14 strains were randomly sequenced and analyzed. The polymerase-based grouping showed that 3 strains of GII.3, 1 strain of GII.7, and 2 strains of GII.14 belonged to NoV GII.4 when the (**Table 15**). This kind of phenomenon was recognized as the intergenotype recombination. For example, the recombinant JP10009 strain was classified by polymerase into Sakai/04-179/2005 (AB220922), GuangzhouNVgz01/2003 (DQ369797, and Hiroshima/32-754/2003 (AB354294), as clustered into GII.4, while the capsid belonged to the same cluster with NoV GII.3 Toronto (U02030) and Maxico (U22498) In order to localize the potential recombination site, SimPlot analysis using Sakai/04-179/2005 (AB220922), NewTaipei/CGMH68/2012 (KC517378), Hiroshima/32-754/2003 (AB354294) and Toronto (U02030), as the parental strain. There was a high percent similarity in 83-98% in polymerase region with parental GII.4 strains and 90-96% in capsid region with parental GII.3 strain. The JP10009 was indicated to become a recombinant NoV containing GII.4 polymerase and GII.3 capsid. The recombination breakpoints of all recombinant NoVs detected in this study located in the nucleotide position labeled by red vertical lines (**Figure 12**).

Table 15. The recombination norovirus strains detected in Japanese pediatric patients during 2009-2014

Strain	Polymerase	Capsid
JP10009	Norovirus GII.4	Norovirus GII.3
JP10020	Norovirus GII.4	Norovirus GII.3
JP10383	Norovirus GII.4	Norovirus GII.3
JP11167	Norovirus GII.4	Norovirus GII.7
JP11020	Norovirus GII.4	Norovirus GII.14
JP11205	Norovirus GII.4	Norovirus GII.14

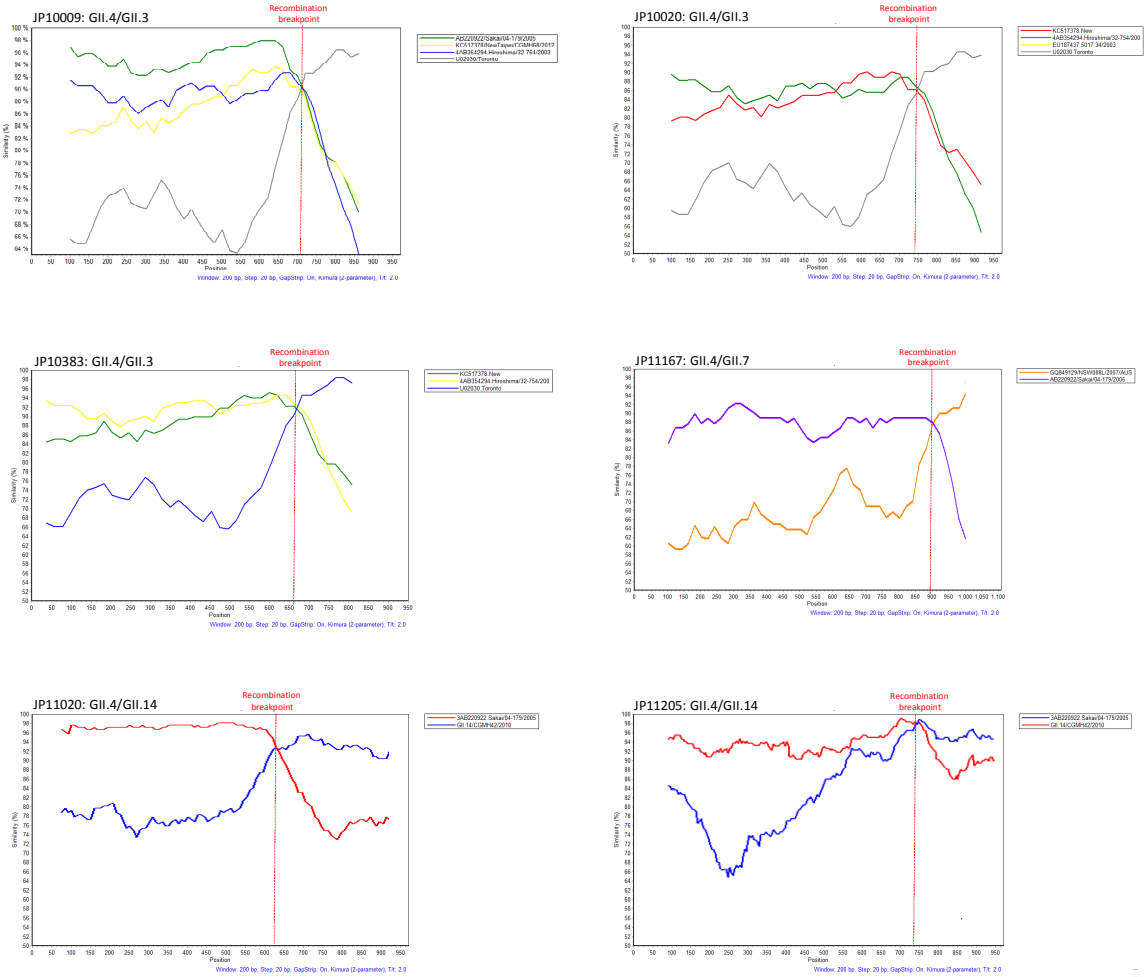


Figure 12. Simplot analysis of recombinant noroviruses. The putative recombination breakpoints are defined by the interception of the parental strains similarity curves in nucleotide labeled by a red vertical lines.

DISCUSSION

Viral gastroenteritis is still a health burden and one of the most frequently encountered problems in developed and developing countries. Global outbreaks of acute gastroenteritis by NoV have frequently been reported. My group study also previously reported the epidemic occurrence of NoV in a Japanese population (17, 19, 24). The current study extended these findings by investigating different epidemic patterns. A high prevalence (43.0%) of NoV infection was found in these Japanese pediatric patients with acute gastroenteritis in Japan during July 2009 to June 2014; 99.4% of all NoV cases were infected by NoV GII. The prevalence of NoV was higher than that found in the preceding studies conducted in the same geographical area during 2004 to 2009 showing the prevalence rates of NoV ranging from 8.5% to 27.0% (17, 19, 24). Taken together, results in the present and preceding studies confirmed that the detection rate increased year by year and that NoV was the most dominant agent in causing acute gastroenteritis in Japan (17, 19, 24, 28). This study documented that 30.3% of all investigated patients were infected by NoV GII alone, and 12.5% by mixed infection between NoV and other diarrheal viruses. The 3.9% mixed infections between NoV GII and RAV was dominant, in accordance with the previous reports showing dual RAV and NoV infections in 0.2-11.0% of children with gastroenteritis (84, 85, 105). The differences in RAV and NoV seasonality may account for the relatively low detection rate of mixed infection. The mixed infections were not related with children's age group.

Regarding NoV seasonality, previous reports have demonstrated a peak of NoV surveillance on pediatric cases of viral gastroenteritis during the winter in Japan (86, 87). Similar to this study, the seasonal distribution of NoV GII infection exhibits a peak in winter seasons, a highest peak of incidence in December. The temperature and humidity

had highly consistent effects driving NoV epidemic patterns (106). A high number of NoV cases detected in every winter season in this study might be associated with cold and dry temperature. Several studies had found that NoV infection occurred frequently in young children under 3 year-old (17, 24). In agreement with this, my results showed a marked trend toward higher rates of infection in children under-3-years of age (88.0%), with children between 12 to 23 months being the most frequently affected (41.5%). It is possible that many children aged younger than 2 years might lack antibody protection.

Based on antigenic analyses and more recent extensive sequence analyses, the circulation of NoV in nature have been shown to be highly variable. In this study, genetic analysis among NoV GII demonstrated that GII.4 genotype was the most predominant genotype accounting for 64.5%, followed by GII.3, GII.6, GII.2, GII.14, GII.13, GII.7, GII.12, and GII.17. However, the number of NoV GI detected in this surveillance study was small, accounting for 0.3%. The highest prevalence of GII.4 is consistent with those of earlier studies conducted in the same settings, as well as other studies conducted elsewhere in Japan (17, 19, 24, 107, 108). NoV GII.4 strains evolved rapidly and spread globally with the emerging variant. On the other hand, the increase of NoV GII.3 was identified and this strain was the second leading genotype in Japan after dropping during 2006 to 2009. A sharp decrease in the number of patients infected by GII.3 was observed when the new emerging variant was spread in 2012-2013 (14.3% in 2009-2010; 27.9% in 2010-2011; 16.0% in 2012-2013; 0.6% in 2012-2013). Interestingly, the emergence of GII.3 was found in 2013-2014 together with the spreading of GII.6, while the detection rate of GII.4 decreased (**Figure 4**).

Focusing on NoV GII.4 variants, analysis of NoV circulating in the past 5 years in Japanese pediatric patients revealed a change of the predominant variant of NoV GII.4 in each epidemic season over time. Among NoV GII.4 identified in this study, three separate

NoV GII.4 variants were mainly identified as the etiologic agents of NoV-associated acute gastroenteritis, including Den_Haag_2006b (36.9%; 296/803), New_Orleans_2009 (14.9%; 120/803), and Sydney_2012 (46.0%; 369/803) variants. A number of additional GII.4 variants have been identified, including Hunter 2004, Yerseke_2006a, Apeldoorn_2007, and Hokkaido1_2008. However, these variants were less common in Japanese population compared to the three major variants found in this study.

The Sydney_2012 new variant was shown to emerge since late 2011 and this emergence has been shown to be associated with the increase of NoV infections in Japanese pediatric patients from May 2012. Interestingly, the previous dominant Den_Haag_2006b and New_Orleans_2009 variants were displaced by new emergence variant Sydney_2012. The Sydney_2012 variant has been shown to be responsible for the majority of NoV infection in 2012-2013, accounting for 85.7% of all NoV GII.4 detected in 2012-2013. Even though many studies reported the detection of Sydney_2012 variant from late 2012 (40, 41, 43), the present study described the detection of Sydney_2012 variant among pediatric patients in Tokyo since November 2011 before spreading throughout Japan from mid-2012. The strain detected in early 2012 showed a single amino acid at the position 393 located in antigenic blockade epitope and HBGA binding site similar to the previous circulating New_Orleans_2009 variant, but different from the emergence variant 2012. This observation suggested that several changes in pre-epidemic were required before reached pandemic potential. The novel GII.4 variants may circulate at the low levels in the population before acquiring the P2 mutations to escape herd immunity and emerged as the pandemic viruses (109).

This study clearly indicated that NoV variant Sydney_2012 was the predominant group causing acute gastroenteritis among Japanese pediatric populations since mid-2012. Interestingly, the Sydney_2012 strains detected in the current study clustered in the same

lineage and showed a high nucleotide sequence (≥98.7%) and amino acid sequence (100%) similar to NoV GII found previously in Australia, the United States, Europe, Canada, and China (40-43). Therefore, the Sydney_2012 emerged as the etiologic agent in acute gastroenteritis across the United States, Europe and Asia, including Japan in the same period.

Although the Sydney_2012 variant has common ancestor with the dominant New_Orleans_2009 variants, analysis of partial VP1 capsid of GII.4 variant 2012 representative strains revealed a high diversity with 6 positions of amino acid substitutions compared to Den_Haag_2006b and New_Orleans_2009 variants which previously emerged as predominant variants cause outbreaks; positions 294, 359, 368, 377, 393, and 413. The P2 subdomain of VP1 contains potential neutralizing antibody epitopes and interacts with histo-blood group antigens (HBGAs), which are a diverse family of carbohydrates and serve as putative receptors for NoV attachment (104). The analysis of P2 subdomain of NoV GII.4 variant Sydney_2012 detected in this study compared with the reference strains revealed four amino acid changes at the antigenic sites in epitope A (positions 294 and 368), epitope D (position 393), and epitope E (position 413).

Epitope A, conformation dependent which likely includes varying amino acid residues 294, 296-298, 368, and 372 has been mapped as a blockade epitope (98). This residue is targeted by a significant portion of isolated monoclonal antibodies and substantially recognized by polyclonal antibody responses elicited in both immunized mice (31) and human (32). Although it is unclear how many and which amino acid in epitope A are needed to mediate an escape mutant phenotype, a previous study demonstrated that epitope A has a complex reactivity pattern between strains (98). Using MAbs mapping, they showed that the blockade of epitope A resulted in significant reduction of reactivity and blockade of Sydney_2012 (110). Thus, the antigenic differences in epitope A positions

294 and 368 between strains may represent the site for driving GII.4 escape from herd immunity, as they undergo evolution in neutralizing epitopes.

Epitope D contains varying amino acids 393 to 395 and other nearby residues that are less clearly defined. Residue 393-395 consistently identified as a putative epitope that commonly alters NoV binding affinity and specificity to HBGAs as well as antibody binding and blockade among epidemic GII.4 strains (98). Based on the evolutionary analysis of GII.4, residues 296 to 298 and 393 to 395 have been identified as "hot spots" for the evolution of GII.4 variants (111). Both sites remained unchanged from 1974 until 1997, when the Grimsby cluster became predominant, indicating that these hot spots were not involved in earlier cluster replacements (112). In the current study, epitope D in amino position 393 changed in Sydney_2012 strain in comparison with Den_haag_2006b and New_Orleans_2009 strains. The change of the amino acid residue at the position 393 in Sydney_2012 may change the HBGA recognition and represent the selective force that drives antigenic variation within around the receptor binding pocket. This may play a key role, particularly in the emergence of widespread Sydney_2012 strain.

Additional surface residues at 333, 340, 356, 368, 372, 407, 412, and 413 were also predicted as potential antibody epitopes (98). Modulation of several residues in close proximity to the HBGA binding pocket influences HBGA binding (31, 46). The other adjacent residues may alter NoV strain binding affinity (110). Diversification of the P2 subdomain through accumulated mutations in antigenic blockade epitopes and several residues close to the HBGAs binding pocket may affect HBGA binding, and has been linked to antigenic escape from host immune responses directed to previous infection that permits the emergence of a new epidemic NoV variant.

The RNA recombination is one of the major driving forces of viral evolution by switching template facilities of RNA polymerase (99). In this study, several intergenotype

recombinants were found (**Figure 12**). In agreement with other studies, the recombinant strains identified in my study had a crossover recombinant point either within or close to the overlap of ORF1/ORF2. This junction located the transcription start site of viral RNA. It was suggested that this recombination occurs when the polymerase switches templates mid-transcription due to complex secondary structure at the start of ORF2. This ORF2 encodes the capsid VP1 protein which contains the antigenic region. The ability of polymerases to switch templates at the start of ORF2 is advantageous because it can possibly help viruses escape immune responses.

The current study characterized the NoV infection among the Japanese pediatric patient with gastroenteritis. The limitation concerns the generalizability of the findings to the entire Japanese populations. Although data were collected from six prefectures from northern to southern part of Japan, they may not represent the entire national situation of NoV outbreak activity.

CONCLUSION

In late 2012, an outbreak of acute gastroenteritis due to NoV variant Sydney_2012 occurred and have been reported from many counties. In this study, I described surveillance study of the incidence of NoV infections among Japanese pediatric patients in association with gastroenteritis and investigated the antigenic change of the new variant Sydney_2012 circulated in Japanese populations. A total of 2,381 fecal specimens collected from children with acute gastroenteritis in 6 prefectures in Japan from 2009 to 2014 were examined for NoV and further analyzed molecularly. This study indicates a high prevalence (43.0%) of NoV infection in Japanese pediatric patients with diarrhea. Analysis of NoVs circulating in the past 5 years revealed a change of the predominant variant of NoV GII.4 in each epidemic season over time. The new variant "Sydney_2012" was shown to emerge in Japanese populations and this emergence has been shown to be associated with the increase of NoV infections in pediatric patients throughout Japan from Mid-2012. Interestingly, the Sydney_2012 appears to replace the previous predominant strains, Den_Haag_2006b and New_Orleans_2009, and shows a high level of diversity across P2 subdomain comparison with those of previous dominant variant strains. The changes of four amino acids in antibody-blockade epitopes may have led to the virus escape from the existing herd immunity and explain the increased outbreak of the virus. These observations suggest that the two existing variants, Den_Haag_2006b and New_Orleans_2009, are currently competing for persistence in human populations by evolving a novel variant 2012 strain. The data from this study may be helpful for the evaluation and implementation of appropriate measures for monitoring NoV infection disease and outbreak in Japan.

CHAPTER IV

ANALYSES OF BLOOD GROUP-RELATED GLYCANS DETERMINING BINDING SPECIFICITIES OF NOROVIRUS GII.4 VARIANTS

INTRODUCTION

Histo-blood group antigen (HBGA) is one of the host factors involved in NoV infection. HBGAs are complex of carbohydrates determinants of ABO, Lewis, and secretor blood types expressed on mucosa surfaces and also present as free oligosaccharides in biologic fluids, such as saliva, intestinal content, milk and blood. Several studies reported that HBGAs is the one of host factors involving in NoV infection (113). Different NoV genotypes are known to recognize HBGAs in a strain specific manner (51, 54).

The interaction between NoV and HBGA is a typical protein carbohydrate interaction. NoVs are genetically highly diverse and the binding region in NoV capsid is extremely sensitive even to point mutations. Studies have shown that a single amino acid changes in the P domain of capsid protein can result in a different HBGA binding pattern (57). The molecular basis of the binding specificities based on in vitro studies has been discussed elsewhere (50).

Molecular surveillance of NoV indicated the emergence of the new variant with a change in capsid protein including the HBGA binding site. To understand on the emergence of the new variant, analysis on binding pattern of NoV variant strains will be explained and extended the previous finding on the same molecular grounds focusing on NoV GII.4 strain.

MATERIALS AND METHODS

Expression of Virus-like particle (VLP)

VLPs of 1207 strain (GII.4 variant 1997), 8174 strain (GII.4 variant 2006b), and JP41 strain (GII.4 variant 2012) were cloned according to Gateway Technology and Bac-to-Bac Baculovirus Expression protocol (Invitrogen Corp., Carlsbad, CA). Primer TX30SXN was used to synthesize cDNA, and PCR was conducted using specific primer pairs and KOD plus polymerase with a high fidelity (Toyobo, Osaka, Japan). A sense primer Mex20attB1S (5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CGA AGG AGA TAG AAC CAT GAA GAT GGC GTC GAA TGA-3') and antisense primer attB2TX30SXN (5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC CTA GAC TAG TTC TAG ATC GCG AGC GGC CGC CC $CC(T)_{30}$-3') was used for amplification of full capsid of NoV. These capsid genes were cut and purified from 1.0% crystal violet agarose gel. These capsid were cloned into a donor vector, pDONR221 (Invitrogen Corp., Carlsbad, CA), and recombinant pDONR221 was purified before transferring into a baculovirus transfer vector pDEST8 (Invitrogen Corp., Carlsbad, CA). The recombinant pDEST8 was purified and used to transform DH10Bac competent cells (Invitrogen Corp., Carlsbad, CA), producing recombinant bacmids containing the capsid gene.

Expression in insect cells

Recombinant bacmids were transfected into Sf9 insect cells using Effectene (QIAgen, Hilden, Germany), incubated for 5-7 days at 27^{o}C. After the culture medium was clarified by centrifugation for 10 min at 3,000 rpm 27^{o}C, the supernatant was stored as seed baculovirus. Tn5 cells were infected with the seed baculovirus at 27^{o}C and harvested at 5-7 days after infection.

After a cytopathic effect (CPE) was observed, VLPs that secreted into the cell medium were separated from the cells by centrifugation for 10 min at 3,000 x *g*, and they were separated from seed baculovirus by further centrifugation for 30 min at 10,000 x *g*. The supernatant was concentrated by ultracentrifugation at 35,000 x *g* for 2 hrs (himac CS100GX II, angle rotor 550A), and suspended in 100 ml of phosphate-buffered saline (PBS). VLPs were purified by CsCl equilibrium gradient ultracentrifugation at 35,000 x *g* at 10^{o}C for 20 hrs (himac CS100GX II, swing rotor). The fraction of VLPs was washed with PBS and concentrated by ultracentrifugation at 35,000 x *g* for 2 hrs (himac CS100GX II, swing rotor). VLP formation was suspended in PBS and kept at 4^{o}C.

Sodium dodecyl sulfate polyacylamide gel electrophoresis (SDS-PAGE)

The VLPs of NoV GII.4 were subjected to detect viral capsid by SDS-PAGE. The PAGE was performed in 12% polyacrylamide separating gel with a 5% stacking gel under the following conditions; each well of the gel was carefully loaded with 7.5 µl of VLP mixed with 7.5 µl of loading dye with 2-mercaptoethanol (2ME). Electrophoresis was carried out for 1 hr at a constant current of 25 mA per gel. The capsid fragment migrated in the gel according to size was visualized by Commassie brilliant blue staining. Then, the acrylamide gel was destained overnight with solution containing methanol and acetic acid.

Examination of binding specificity of norovirus to HBGA samples

Binding specificity of each NoV GII.4 variant was examined by means of an ELISA using saliva and human gastric mucosa (HGM) samples as described previously (51). One hundred µl of saliva samples, diluted at 1:500 with 0.1 M carbohydrate buffer (pH 9.5) or one hundred µl of HGM prepared at 0.01% in saline was added into a 96-well-plate and incubated at 37°C overnight. After three times washing with 250 µl of 0.02 M

phosphate-buffered saline (pH 7.0, PBS) containing 0.05% Tween 20 (PBS-T), the plate was blocked with PBS containing 5% skim milk by an incubation for 1 h at 37°C and washed with three times of PBS-T. Fifty µl of each VLP at 1 µg/ml in 1% BSA/PBS was added to each well of the plate and incubated for 1 h at 37°C. After washing the plate with PBS-T, 100 µl of anti-VLP antibody corresponding to each VLP diluted at 1:500 to 1:1,000 in 5% skim milk/PBS was added to each well and the plate was incubated for 1 h at 37°C. HRP-conjugated anti-mouse or rabbit IgG antibody was then added (100 µl/well) to the wells following by incubation for 1 h at 37°C. Color was developed by adding 200 µl of TMB substrate kit for peroxidase (Vector) and incubating for 5 min at room temperature following by adding 50 µl of 1N sulfuric acid. Reactions of each well were read at 450 nm.

Histochemical analysis of VLP binding with human tissues

Human jejunal tissue sections from an individuals with blood type AB secretor were used in this study. The preparation was described previously (51). After fixing the tissue in 4% paraformaldehyde solution (Sigma-Aldrich, USA), the sections were treated with 100% methanol containing 0.3% hydrogen peroxide and pre-incubated in 2% BSA in PBS overnight. After PBS washing, sections were incubated with VLP of NoV GII.4 (5µg/ml) in 1% BSA in PBS at 37^{o}C for 1 hr. After three times washing with PBS, the sections were incubated with rabbit antibody against NoV GII.4 VLP in dilution of 1:1,000 for 1 hr at room temperature. The sections were covered with labeled anti-rabiit IgG antibodies for 1 hr at room temperature after washing. The signal was determined by treatment with DAB solution (Nichirei Biosciences Inc., Tokyo, Japan). The color was developed using 0.02% 3-3'-diaminobenzidine tetrahydrochloride and 0.01% hydrogen

peroxide in 0.05M of ammonium acetate-citrate buffer, pH 6.0. Then, the section was counterstained with hematoxylin, washed, and mounted.

RESULTS

The structure of the purified virus-like particles (VLPs)

VLPs of NoV GII.4 were prepared from JP1207 (Densden_1997), JP8174 (Den_Haag_2006b) and JP41 (Sydney_2012) strains. The JP1207 strain was isolated and its VLP was prepared as described previously (114).

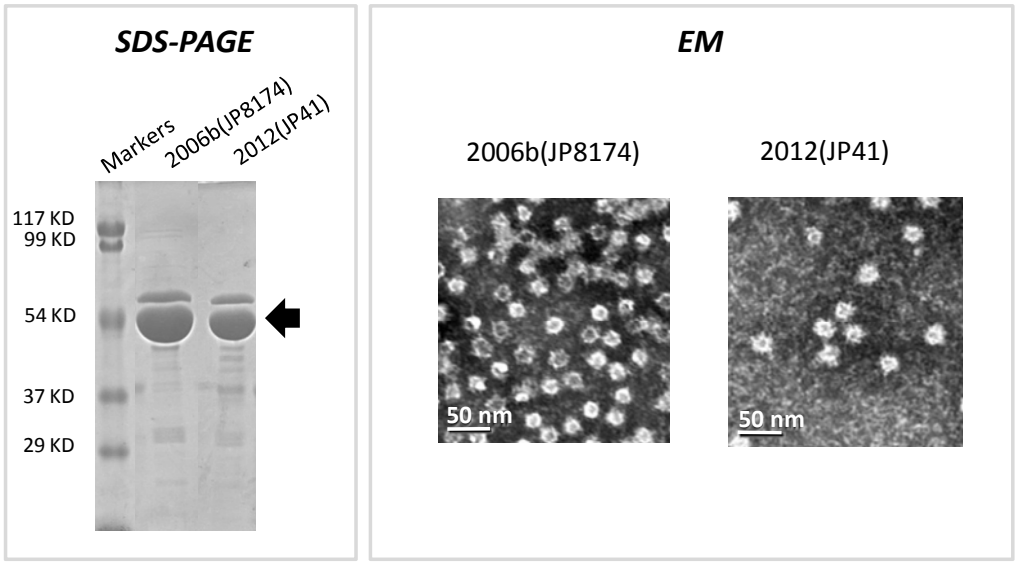


Figure 13. The structure of purified VLPs of norovirus GII.4 JP8174 and JP41 strains. **Left;** SDS-PAGE analysis of the purified VLPs expressed in insect cells, and the arrow indicates the VP1 capsid protein. **Right;** Electron Microscope images of norovirus GII.4 VLPs with scale bars in 50 nm.

The JP8174 strain collected at Saga Baba Clinic, Japan, December 2007 was isolated from a 2.3 year-old boy. The JP41 strain collected at Horinouchi hospital, Saitama, Japan, December 2012 was isolated from a 1-year-old boy. VLPs of JP1207, JP8174 and JP41 strains were purified by CsCl density gradient centrifugation and examined by a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A major and strongly stained band with 58 kDa which corresponds to the major capsid protein VP1 and several minor bands were observed in both the JP8174 and the JP41 VLPs (**Figure 13**) as well as in the JP1207 variant (51). Expressions of each VLP in insect cells resulted in yield

of 2.3 and 1.2 mg/ml, respectively. Electron microscope images of VLPs revealed the presence of homogeneous particles with the expected size in 38 nm diameters.

Sequence variations on HBGA binding site of norovirus GII.4 variants

Phylogenetic analysis of nucleotide and amino acid sequences of the VP1 region confirmed that JP8174 strain (GenBank accession number AY741811) and JP41 strain (GenBank accession number KF145149) belonged to the NoV GII.4 Den_Haag_2006b and Sydney_2012 variants, respectively. The phylogenetic tree revealed JP41 was clustered in the same lineage with Sydney/NSW0514/2012/AU (JX459908). By analysis on the P2 hypervariable region, this JP41 strain shared a high nucleotide and amino acid homologies with Sydney/NSW0514/2012/AU, in 98.9% and 99.2% similarity, respectively.

Since evidence supporting the association of NoV infection with ABO and Lewis blood group phenotypes of hosts was obtained from both volunteer challenge studies and *in vitro* binding assays with ELISA (51), sequence analyses of NoV GII.4 VLPs were conducted in these three variants, Dresden_1997, Den_Haag_2006b and Sydney_2012 focusing on the vicinity of binding sites reported in the VP1 region.

The sequence alterations were found in the P2 subdomain regions corresponding to the HBGA binding domain; substitution of amino acid at positions 346 (in the HBGA binding site I) and 393 (in the HBGA binding site II) occurred from Ala (Dresden_1997) to Gly (Den_Haag_2006b and Sydney_2012) and Asn (Dresden_1997) to Ser (Den_Haag_2006b) and Gly (Sydney_2012), respectively, as shown in **Table 16.**

Effect of amino acid substitutions in the HBGA binding site of norovirus GII.4 variants

In order to investigate the effects of amino acid substitutions in the HBGA binding site on the HBGA binding specificity of NoV GII.4 variants, the superimposition of notable amino acids selected from HBGA binding site and its downstream was conducted between the crystal structure of the GII.4 variants and Le^b-active hexasaccharide (Fucα1,2Galβ1,3[Fucα1,4]GlcNAcβ1,3Galβ1,4Glc) which referred to the data obtained from the Hunter_2004 (PDB code 3SEJ) (**Figure 14**).

The three-dimensional structure of NoV strain revealed the hydrogen bond interaction between Le^b-active hexasaccharide (Fucα1,2Galβ1,3[Fucα1,4]GlcNAcβ1,3 Galβ1,4Glc) and some amino acid residues in both dimers of NoV GII.4 model such as Gln390, Asp391, Gly392, Ser393, Thr394, Thr395, Ser442, and Gly443 (Chain A) and Ser343, Thr344, Arg345, and Asp374 (Chain C). It was of particular interest that the amino acid position 393 changed from Ser (Den_Haag_2006b) to Gly (Sydney_2012).

From the crystal structure, the hydroxyl group of Ser393 seems to form the hydrogen bond interaction to α1,4-fucosyl residue in Le^b-active hexasaccharide, while Gly393 in the Sydney_2012 and JP41_2012 strains could not form the hydrogen bond interaction to this fucosyl residue. Therefore, substitution of the amino acid at position 393 from Ser to Gly may involve occurrence of change in recognition for the Fucα1,4GlcNAc residues resulting in the poor binding of the VLP to the Le^a-active blood group determinant.

Table 16. Substitutions of amino acid found in the HBGA binding sites I and II of GII.4 noroviruses. Twelve representative sequences of norovirus GII.4 variants and sequences of norovirus GII.4 variant VLPs used in this study

Strain	SITE I									SITE II					
	342	343	344	345	346	347	374	440	444	390	391	392	393	394	395
FJ537134/CHDC5191/1970s	G	S	T	R	A	H	D	G	Y	Q	D	G	D	-	T
AF145896/Camberwell/1994	G	S	T	R	A	H	D	G	Y	Q	D	G	D	-	-
AY741811/Dresden174/1997	G	S	T	R	**A**	H	D	G	Y	Q	D	G	**N**	-	-
JP1207/1997	G	S	T	R	**A**	H	D	G	Y	Q	D	G	**N**	-	-
AY502023/FarmingtonHills/2002	G	S	T	R	G	H	D	G	Y	Q	D	G	N	G	T
DQ369797/GuangzhouNVgz01/2003	G	S	T	R	G	H	D	G	Y	Q	D	G	S	S	A
AB294779/Kaiso/2003	G	S	T	R	A	H	D	G	Y	Q	D	G	D	-	A
DQ078814/Hunter504D/2004	G	S	T	R	G	H	D	G	Y	Q	D	G	S	T	T
EF126963/Yerseke38/2006a	G	S	T	R	G	H	D	G	Y	Q	D	G	S	T	T
EF187497/KenepuruNZ327/2006a	G	S	T	R	G	H	D	G	Y	Q	D	G	S	T	T
EF126965/DenHaag89/2006b	G	S	T	R	G	H	D	G	Y	Q	D	G	**S**	T	T
JP8174/2006b	G	S	T	R	G	H	D	G	Y	Q	D	G	**S**	T	T
AB541319/Osaka1/2007	G	S	T	R	G	H	D	G	Y	Q	D	G	S	T	T
AB445395/Apeldoorn317/2008a	G	S	T	R	G	H	D	G	Y	Q	D	G	D	T	A
AB541261/Hokkaido1/2008b	G	S	T	R	G	H	D	G	Y	Q	D	G	S	T	T
GQ845367/OrangeNSW001P/2009	G	S	T	R	G	H	D	G	Y	Q	D	G	N	T	A
GU445325/NewOrlean/2009	G	S	T	R	G	H	D	G	Y	Q	D	G	S	T	T
JX459908/SydneyNSW0514/2012	G	S	T	R	G	H	D	G	Y	Q	D	G	**G**	T	T
JP41/2012	G	S	T	R	G	H	D	G	Y	Q	D	G	**G**	T	T

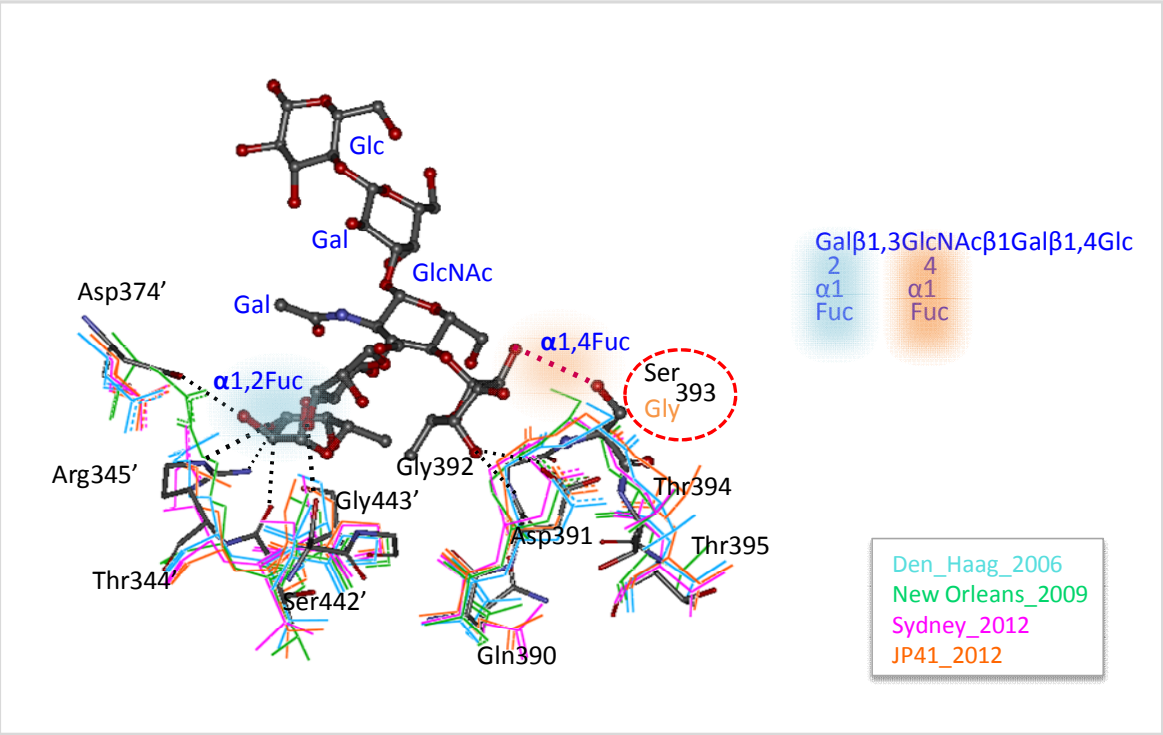


Figure 14. The three-dimensional structure model of norovirus GII.4 variants and representative variant 2012 detected in this study. Hydrogen-bond interactions are indicated in the HBGA binding site between Le^b-active hexasaccharide and norovirus GII.4 (Grey). Structures of Den_Haag_2006 (Blue), New Orleans_2009 (Green), Sydney_2012 (Pink) and JP41 (Orange) are also shown.

*α1,2fuc (α1,2-fucosyl residue), α1,4fuc (α1,4-fucosyl residue), Gal (galactose), GlcNAc (N-acetylglucosamine), and Glc (glucose)

Binding patterns of norovirus VLPs to human saliva and preparations of gastric mucosa from different ABO(H) and Lewis blood group phenotypes and secretor status

The VLPs prepared from Dresden_1997, Den_Haag_2006b and Sydney_2012 strains were examined their binding specificities to HBGA using a panel of human saliva and preparations of human gastric mucosa (HGM) with various ABO and Lewis blood groups phenotypes and secretor status. ABO and Lewis blood group phenotypes were determined with the aid of conventional serological tests. Results from the binding assay of VLPs with human saliva and HGM preparations were shown in **Table 17**, **Table 18**, and **Figure 15**.

Saliva samples from Lewis positive and negative individuals with ABO types were tested in three different samples and a single sample, respectively, and each average level was determined from duplicated experiments. Whereas, preparations of HGM with different blood types and secretor status were obtained from a single individual and each average level was determined as well. As demonstrated previously (51), the VLP from Dresden_1997 showed strong binding levels to all the saliva samples from secretor individuals but a BLe(a-b-)secretor irrespective of their ABO and Lewis phenotypes. Binding levels with this VLP also showed to be influenced by the Le^a determinant since some degrees of positive binding were detected with saliva samples from Le(a+b-)non-secretor individuals. But no significant binding was detected with Le(a-b-)non-secretor. Binding patterns determined with preparations of HGM showed almost the same with those with saliva samples. Therefore, both α1,2- and α1,4-fucosyl residues consisting of the H, Le^a and Le^b determinants and containing in these blood group substances must participate in the binding of the VLP from Dresden_1997.

Most of the binding activities determined with Den_Haag_2006b showed similar binding patterns but under the lower levels in comparison with those Densden_1997, in particular with preparations of HGM from ABO Le^b-positive individuals. However, it remains to be investigated whether some other receptor-like molecules for Den_Haag_2006b VLP are present in these blood group substances in place of reported glycans such as ABO(H) and Lewis blood group active. Surprisingly, strong binding levels were found in the VLP from Sydney_2012 tested with the same saliva and HGM samples and it seemed to be possible for the VLP to distinguish Le(a-b+) and Le(a-b-) secretor individuals from Le(a+b-) and Le(a-b-) non-secretor individuals, irrespective of their ABO(H) phenotypes. Further, binding levels of the VLP with different blood group phenotypes and secretor status from saliva and HGM samples suggested that enhanced binding activities of the VLP from Sydney_2012 to α1,2-fucosyl residues and suppressed binding activities to α1,4-fucosyl residues, in comparison with those of the VLP from Densden_1997. Therefore, α1,2-fucosyl residues responsible for the H determinant must be essential for binding of Sydney_2012 variant but α1,4-fucosyl residues responsible for the Le^a determinant seems to be free from binding of this VLP. As described above, occurrence of the deleted binding activity of the Sydney_2012 variant to the α1,4-fucosyl residues was also predicted in the three-dimensional analysis of the crystal structure of the HBGA-binding interfaces of this VLP using the Le^b-active hexasaccharide (**Figure 14**).

Immunohistochemical detection of norovirus GII.4 VLPs binding to human jejunal mucosa sections

VLPs from NoV GII.4 variants examined in this study were found to bind strongly to ABH and Le^b-active blood group substances by an ELISA using not only a panel of human saliva samples but preparations of HGM with different blood types and secretor

status. Whereas, *in vivo*, human jejunum as well as the proximal small intestine is believed to be susceptible to the NoV infection. Resected human jejunal mucosa from a blood type AB secretor individual was examined immunohistochemically using the VLPs from three NoV GII.4 variants and their corresponding antibodies. A, B and H active antigens expressed on the mucosa were detected by anti-A, anti-B and *Ulex* lectin, respectively **(Figure 16**). Strong positive staining with anti-A, anti-B antibodies and anti-H lectin was observed at the luminal surface of the epithelia from the villi to the crypts. When the same tissues were incubated with VLPs from three GII.4 variants followed with their corresponding antibodies, clear positive staining was also observed with the Dresden_1997 and Sydney_2012 variants which coincided with that anti-A, B and H, but no such positive staining was detected with the VLP from Den_Haag_2006b. No positive staining was also detected in the absence of VLPs (*control* in **Figure 16**). These results from immunohistochemical analyses, therefore, coincided with experiments *in vitro* with ELISA conducted with saliva samples and HGM preparations with blood type ABO secretor individuals.

Table 17. Binding activity of norovirus GII.4 VLPs to saliva samples with different blood group ABO, Lewis phenotypes and secretor status.

Saliva No.	Blood group phenotype & secretor status	JP1207 (Dresden_1997)		JP8174 (Den (Haag_2006b)		JP41 (Sydney_2012)	
		OD450[1]	Reaction[2]	OD450[1]	Reaction[2]	OD450[1]	Reaction[2]
1	A Le(a-b+)sec.	3.482	3+	0.3125	±	2.746	3+
2	A Le(a-b+)sec.	3.217	3+	0.116	(-)	1.799	3+
3	A Le(a-b+)sec.	0.776	1+	0.0205	(-)	0.236	±
4	B Le(a-b+)sec.	1.919	3+	0.092	(-)	2.5095	3+
5	B Le(a-b+)sec.	3.369	3+	0.374	±	2.7665	3+
6	B Le(a-b+)sec.	3.384	3+	0.16	(-)	2.767	3+
7	O Le(a-b+)sec.	0.965	1+	0.381	±	2.76	3+
8	O Le(a-b+)sec.	0.546	1+	0.321	±	2.6775	3+
9	O Le(a-b+)sec.	0.635	1+	0.2095	±	2.318	3+
10	AB Le(a-b+)sec.	2.852	3+	0.1515	(-)	2.649	3+
11	AB Le(a-b+)sec.	3.337	3+	0.302	±	2.7175	3+
12	AB Le(a-b+)sec.	2.535	3+	0.2805	±	2.767	3+
13	A Le(a+b-)non-sec.	0.329	±	0	(-)	0.068	(-)
14	A Le(a+b-)non-sec.	2.452	3+	0	(-)	0.049	(-)
15	A Le(a+b-)non-sec.	0.75	1+	0.0255	(-)	0.165	(-)
16	B Le(a+b-)non-sec.	0.391	±	0.0235	(-)	0.106	(-)
17	B Le(a+b-)non-sec.	0.529	1+	0.053	(-)	0.237	±
18	B Le(a+b-)non-sec.	0.581	1+	0.029	(-)	0.073	(-)
19	O Le(a+b-)non-sec.	0.841	1+	0.038	(-)	0.008	(-)
20	O Le(a+b-)non-sec.	0.207	±	0	(-)	0.014	(-)
21	O Le(a+b-)non-sec.	0.324	±	0	(-)	0	(-)
22	AB Le(a+b-)non-sec.	0.249	±	0.0055	(-)	0.015	(-)
23	AB Le(a+b-)non-sec.	0.55	1+	0.027	(-)	0.069	(-)
24	AB Le(a+b-)non-sec.	1.592	3+	0	(-)	0	(-)
25	A Le(a-b-)sec.	2.983	3+	0.2365	±	2.2175	3+
26	A Le(a-b-)non-sec.	0.084	(-)	0	(-)	0	(-)
27	B Le(a-b-)sec.	0.066	(-)	0.0565	(-)	1.6735	3+
28	B Le(a-b-)non-sec.	0.281	±	0.0055	(-)	0	(-)
29	O Le(a-b-)sec.	0.8	1+	0.1115	(-)	1.445	2+
30	O Le(a-b-)non-sec.	0.104	(-)	0.017	(-)	0.0575	(-)
31	AB Le(a-b-)sec.	2.968	3+	0.333	±	2.7945	3+
32	AB Le(a-b-)non-sec.	0.146	(-)	0.121	(-)	0.006	(-)

[1]Each value indicates the average optical density at 450nm when ELISA of each VLP was conducted with saliva samples from 3 different (Lewis-positive samples) individuals and a single (Lewis-negative samples) individual after duplicated experiments. [2]Reactions were classified into 5 groups based on the OD values; 3+, OD>1.5; 2+, OD1.0 – 1.5; 1+, OD0.5-1.0; ±, OD0.2-0.5; (-), <0.2

Table 18. Binding activity of norovirus GII.4 VLPs variants to human gastric mucosa samples with different ABO, Lewis blood group phenotypes and secretor status

HGM No.	Blood group phenotype & secretor status	JP1207 (Dresden_1997)		JP8174 (Den (Haag_2006b)		JP41 (Sydney_2012)	
		OD450[1]	Reaction[2]	OD450[1]	Reaction[2]	OD450[1]	Reaction[2]
1	A Le(a-b+)sec.	3.053	3+	0.549	1+	2.3415	3+
2	B Le(a-b+)sec.	3.795	3+	0.4745	±	1.9265	3+
3	O Le(a-b+)sec.	2.547	3+	0.506	1+	2.939	3+
4	AB Le(a-b+)sec.	3.818	3+	0.546	1+	2.5965	3+
5	B Le(a+b-)non-sec.	0.206	±	0	(-)	0.0345	(-)
6	O Le(a+b-)non-sec.	0.192	(-)	0.01	(-)	0.013	(-)
7	AB Le(a+b-)non-sec.	0.314	±	0.0525	(-)	0.166	(-)
8	A Le(a-b-)sec.	3.089	3+	0.467	±	2.609	3+
9	B Le(a-b-)sec.	0.813	1+	0.069	(-)	0.188	(-)
10	B Le(a-b-)non-sec.	0.172	(-)	0.0175	(-)	0.009	(-)
11	O Le(a-b-)non-sec.	0.123	(-)	0.114	(-)	0.021	(-)
12	AB Le(a-b-)sec.	0.352	±	0.1665	(-)	0.4185	±

[1]Each value indicates the average optical density at 450nm when ELISA of each VLP was conducted with HGM samples from different (Lewis-positive samples) individuals after duplicated experiments. [2]Reactions were classified into 5 groups based on the OD values; 3+, OD>1.5; 2+, OD1.0 – 1.5; 1+, OD0.5-1.0; ±, OD0.2-0.5; (-), <0.2

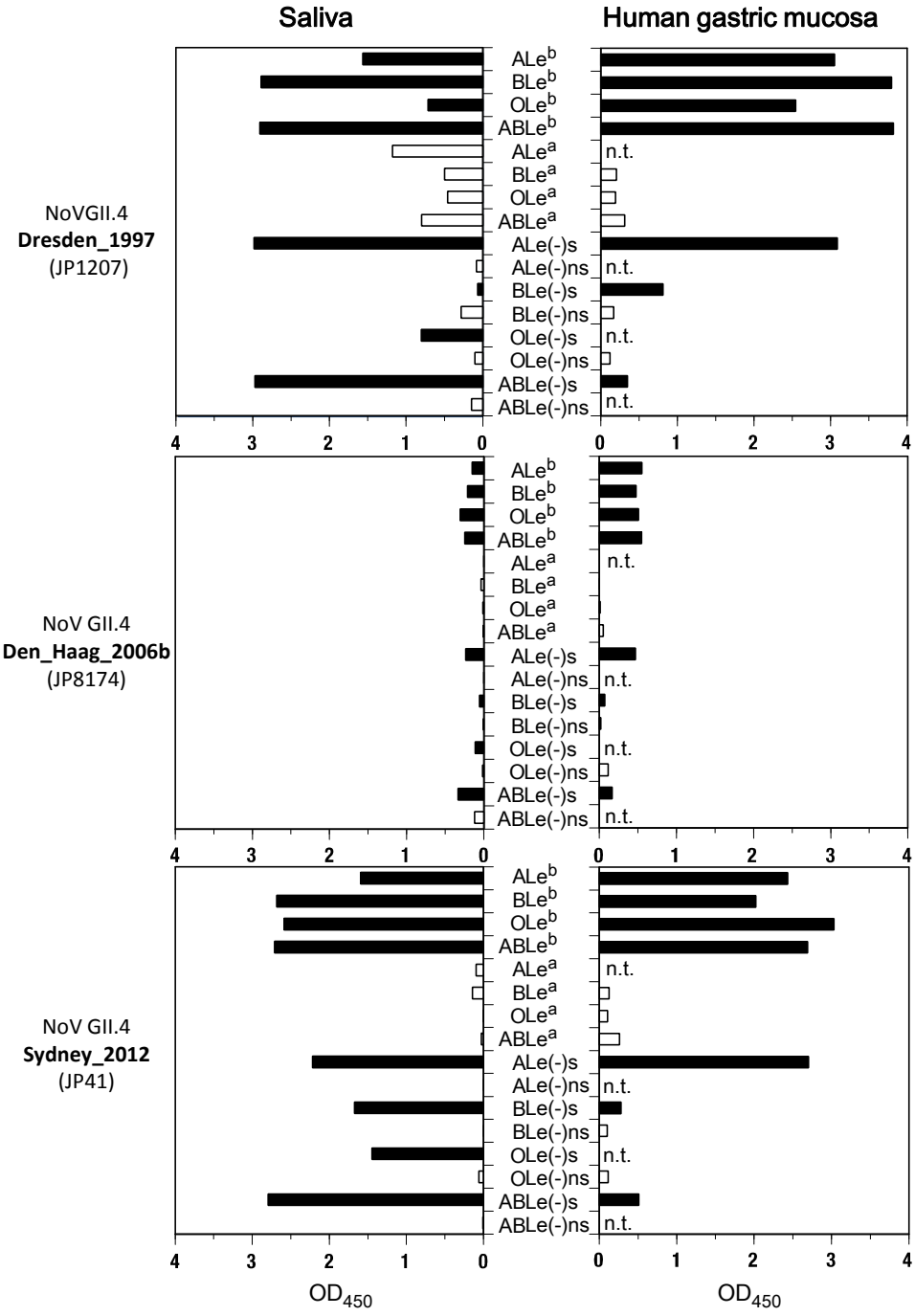


Figure 15. Binding patterns of VLPs from norovirus GII.4 variants determined using human saliva samples and preparations of gastric mucosa with different ABO and Lewis blood group phenotypes and secretor status.

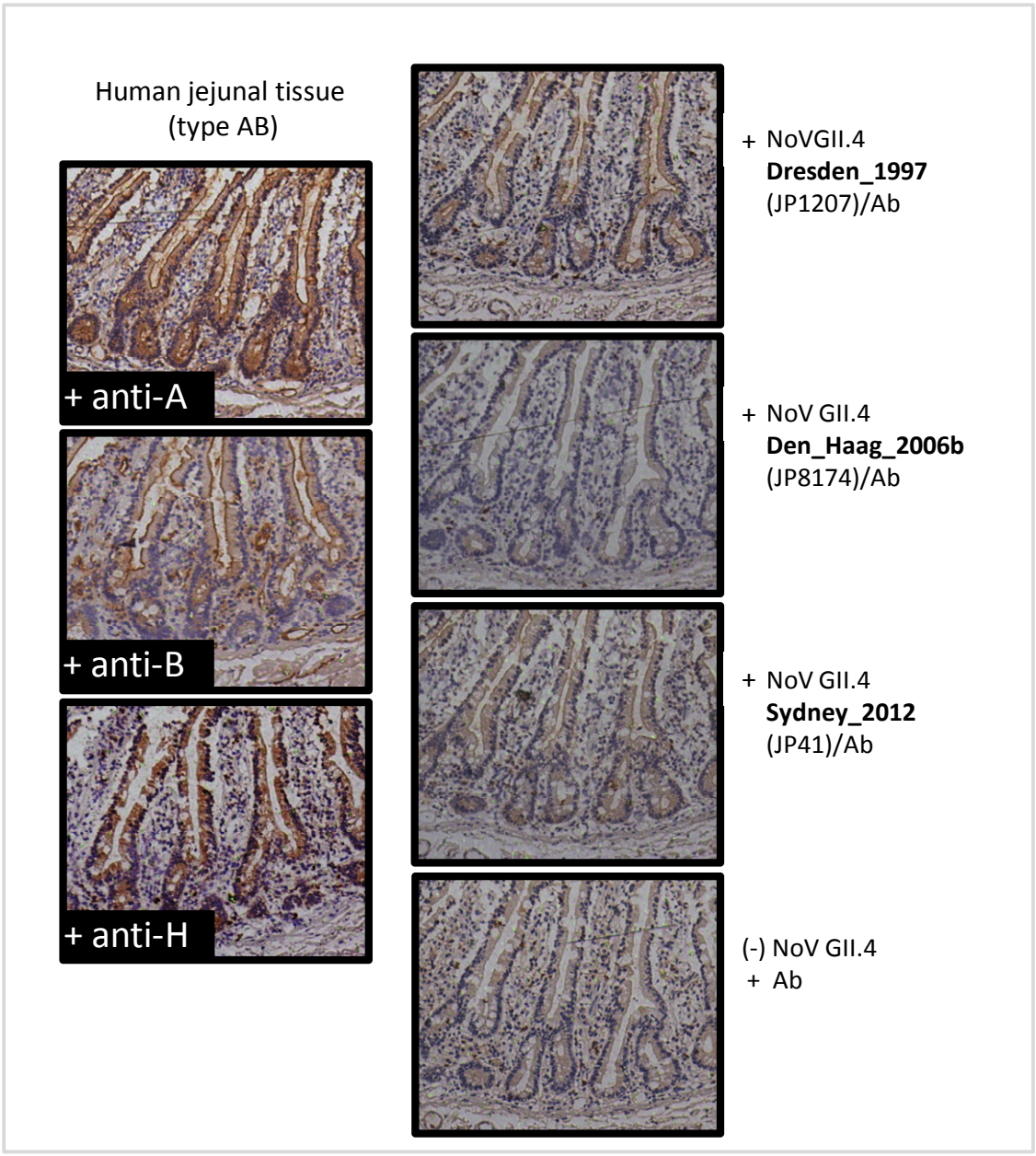


Figure 16. Binding assays of three VLPs of norovirus GII.4 variants, Dresden_1997, Den_Haag_2006b, and Sydney_2012 to human jejunal mucosa sections from a blood type AB secretor individual. Each section was stained with anti-A, anti-B and biotinylated anti-H lectin. Sections incubated with each VLP were then stained with corresponding anti-VLP antibodies. Sections stained with anti-VLP antibodies without incubation with respective VLPs were indicated as control.

DISCUSSION

Evidences supporting a strong association of human NoV infection with blood types of host have been accumulated from not only volunteer challenge studies with different blood types and secretor status individuals but also systematic binding assays using the recombinant virus-like particles (VLPs) and a panel of human blood group substances or blood group-related glycans. Accordingly, it is widely recognized that human NoV binds to human blood group-related glycans that are expressed on the mucosal epithelial cells and secreted as glycoproteins in biologic fluids such as saliva and milk (50, 51).

It has been recently demonstrated that glycans related to the antigenic determinants of blood group ABO(H) and Lewis phenotypes involve mainly in the NoV binding, and that individual binding specificities of VLPs are highly conserved in the two major human-related GI and GII genogroups. Further, numbers of genotypes in these genogroups have been identified and their binding specificities have also been determined respectively in previous studies including this study (51).

Although different genotypes that had caused non-bacterial acute gastroenteritis were found to cocirculate in the same geographical area frequently, it has been observed that the GII.4 has been a predominant genotype causing the disease in many countries during last two decays (17, 24, 28, 30). Whereas, molecular analyses of GII.4 viruses also indicate that they are continuously changing with new variants emerging within a few years (46, 47). It is therefore conceivable that binding specificities of viruses to blood group-related glycans as well as their immunogenetical antigenicities change within a short period.

The binding specificities of GII.4 variants isolated and identified during last two decades in Japan were elucidated in this study. The VLPs prepared from each NoV genome are likely to be indistinguishable from wild-type NoVs, so that they have been used as alterative molecules for NoVs to conduct determination of their binding specificities. In this study, VLPs from three GII.4 strains such as Dresden_1997, Den_Haag_2006b and Sydney_2012 were prepared and investigated since they were either predominant or continual strains during the period. Molecular-based structural analyses of GII.4 VLPs have been suggested that highly diverse changes were present in the P domain of the capsid sequences and amino acid substitutions which locate in the vicinity of the binding sites to blood group-related glycans occur frequently resulting in the modification of binding specificity of the VLPs (110).

Accordingly, to elucidate the binding specificities of three NoV GII.4 variants, sequence analysis with focusing on the predicted HBGA recognition sites was conducted along with determination of their binding specificities using a panel of blood group substances with different ABO and Lewis phenotypes and secretor status. When occurrence of amino acid substitutions was analyzed on the HBGA binding Sites I of the P domain, highly conserved amino acids were found in these three variants which are predicted to involve close interactions with fucosyl (Fuc) as well as *N*-acetylgalactosaminyl (GalNAc) and galactosyl (Gal) residues including as the terminal sugars of blood group ABO(H) active glycans. Interestingly, no substitution of amino acids except position at 346 which was predicted for interactions with GalNAc/Gal residues was detected in this HBGA binding Site I among the three variants **(Table 16).** Importantly, there is no change of amino acid which was predicted for interaction with Fuc residues (postion343 to 345 and 374) in the same variants. The hydrogen-bond interaction predicted between Asp374 and α1,2-fucosyl residues in the synthetic hexasaccharide was also

demonstrated in three-dimensional structure model of GII.4 variants **(Figure 14)**, suggesting that no change for binding specificity to the H-active α1,2-fucosylated structure among these variants. Whereas, amino acid substitution at position 393 in the HBGA binding Site II of the P domain was found in these variants; Asn (Dresden_1997) to Ser (Den_Haag_2006b) and Gly (Sydney_2012). It was of particular interest that substitution of Gly393 found in the Sydney_2012 was predicted to cause the diminished binding level to α1,4-fucosyl residues. In fact, absence of binding to Le^a blood group substance from saliva and preparations of HGM was demonstrated in ELISA binding assays **(Figure 15)**. Such a reduced binding activity was also observed when chemically synthesized Le^a-active trisaccahride attached to BSA was used with VLP from Sydney_2012 in the preliminary study of determination of binding specificity of the VLP. Therefore, in this study, substitution of a few amino acids was found in the HBGA binding Sites I and II of the P domain among three VLPs, and two typical changes of binding specificity were determined with VLPs from Dresden_1997 and Sydney_2012 variants. It still remains to be investigated how an extreme reduction of binding activity occurred in the VLP from Den_Haag_2006b although no substitution of essential amino acids interacting with blood group-related glycans in the same sequence of the VLP was observed, and further, this strain had been a dominant strain as one of the epidemic GII.4 variants.

In my previous (51) and present studies, blood group substances have been used for determination of binding specificity of NoV VLPs and binding patterns associated with presence of corresponding blood group related glycans were found in VLPs from 16 different genotypes of NoV including GII.4. The same blood type-specific binding patterns were also demonstrated with resected human jejunal mucosa sections as well as HGM preparations. The data were confirmed with the aid of treatment with type-specific glycosidases and binding inhibition assays with ABH-active blood groups substances to

block their binding, where blood group ABH active determinants were specifically cleaved and pre-treatment of VLPs with blood group substances inhibited the type specific binding of VLPs, respectively.

Chemically synthesized oligosaccharides consisting of blood group active glycan structures are a powerful and convenient tool for determination of binding specificity of VLPs, in particular, for molecular analysis of the crystal structure of the P subdomain in complexes with such glycans possessing structures. However, as demonstrated in my studies, binding assays of VLP variants seem to show different binding patterns with human intestinal mucosa as well as HGM preparations from others determined using a series of synthetic glycans. This could be due to either by presence of aberrant binding affinity to active glycans in their tissues or by the co-existence of different glycans or some other molecules present in the same samples which are associated with individual VLP binding. It should be noted that occurrence of a genetic drift and evolution of NoV genomes that has been induced by population immunity probably regulated partly with glycans-protein interactions. It was suggested that continual emergence of new variants in GII.4 strains was not accompanied by significant changes of their binding specificities. However, analysis of glycans determining binding specificity of NoV variants both *in vitro* and *in vivo* must still have a fundamental part for further understanding about molecular mechanisms of NoV infections.

CONCLUSION

HBGAs have been implicated in the initial binding of NoV. Sequence analyses of three variants revealed that a high diversity of amino acid substitution was present in the P subdomain, including HBGA binding site. In this study, VLPs of GII.4 variants were investigated not only the molecular sequences but also binding patterns to a series of blood group substances. The present results support the observations that considerable differences were present when binding profiles were characterized by blood group substances from saliva and HGM (51). The binding profiles with saliva samples suggest that GII.4 Sydney_2012 variant acquired elevated and suppressed binding levels of α1,2- and α1,4-fucosylated glycans, respectively. ABO determinants give an increase in binding strength, though the single strongest interacting residue α1,2-linked fucose. However, it remains to be investigated whether molecular changes of GII.4 variants inducing alteration of their antigenicities are accompanied by sequence variations around the binding sites resulting in the occurrence of changes in binding specificities, and further in the emergence of widespread NoV GII.4 variant.

CHAPTER V
SUMMARY

Acute gastroenteritis is still a health burden in both developed and developing countries including Japan. A wide variety of viruses associated with the disease are continually being reported. The accumulated surveillance data have shown that the predominant genotypes of diarrheal virses in each epidemic season change over time. Therefore, the molecular surveillance of diarrheal viruses circulating in Japanese population is needed. A total of 2,908 fecal specimens collected from children with acute gastroenteritis in six prefectures in Japan from 2009 to 2014 were enrolled. The surveillance identified diarrheal viruses currently circulating in Japanese pediatric outpatients in 72.1% of the patients. NoV was detected in 43.0%, followed by RAV (17.2%), HPeV (7.3%), EV (7.0%), AdV (5.8%), SV (5.3%), HAstV (2.9%), and AiV (0.1%). Co-infections of diarrheal viruses were observed, with 13.4% of double infection and 0.5% of triple infection. Mixed viral infections were commonly found in Japanese outpatients, and NoV seems to play a major role in co-infections. Viral diarrhea cases were detected mostly in children younger than 3 years of age and accounting for 72.2%. The NoV and RAV can be detected throughout the year, with a peak during the cold and dry seasons, while other common viruses are found during no specific season.

Surveillance data revealed that a wide variety of viruses has caused diarrhea to circulate currently in Japanese pediatric outpatients, with very high detection rates; and NoV and RAV are the most important pathogens. The results indicated that NoV and RAV infections are needed to pay attention to development of effective vaccine. Furthermore, 30% of etiologic agents caused acute gastroenteritis detected in this study remains unknown. It is also important to identify specific etiologies of acute gastroenteritis in order

to target potential preventive interventions. Since, several novel diarrheal viruses (i.e., cosavirus, Saffold virus, bufavirus, rosavirus, astrovirus novel MLB-clade and VA-clade, and etc.) have been recently reported, the surveillance needs to be conducted continually in order to monitor and keep up with the emergences of new variants and new viruses associated with diarrhea in human. Furthermore, in order to gain an overview picture and relationships of viruses associated gastroenteritis, evolutional dynamic and comprehensive molecular characterization of these viruses are needed to be further elucidated in more detail in the future plan.

Interestingly, NoV GII.4 new variant 2012 was found in Japanese population during the epidemiology study of NoV. From the literature search, GII.4 has been responsible for the majority of outbreaks. It has been reported that epidemic outbreaks occurred every 2 to 3 years, punctuated by the emergence of new variants with different antigenicity which is resulting in an escape from human herd immunity against the circulating strain. From a total of 2,908 specimens, 43.0% of NoV positive sample with several genotypes were detected in this study. GII.4 dominated over other genotypes (64.5%). The Den_Haag_2006b (36.9%) was detected as the predominant variant in co-circulation with New_Orleans_2009 (14.9%) until March 2012, subsequently, was displaced by Sydney_2012. The Sydney_2012 variant has been responsible for the majority of NoV infections since 2012. Although Sydney_2012 variant has a common ancestor with New_Orleans_2009 variant, analysis of P2 subdomain showed a high level of diversity compared with other variants in four amino acid changes at the antigenic sites.

Analysis of the P2 subdomain showed a high level of diversity compared with other variants in the antigenic sites and HBGA binding sites. The Sydney_2012 showed strong binding to human saliva samples and preparations of gastric mucosa from secretor individuals irrespective of thier ABO and Lewis phenotypes, but absence of clear binding

to the same samples from Le^a and Le-negative non-secretor individuals. Along with previous studies on changes of binding specifities occured in GII.4 variants, it was suggested that Sydney_2012 acquired elevalted levels of binding to blood groups substances in secretor individuals but deleted levels of binding to those in non-secretor individuals, indicating the occurence of sequence substitutions in the HBGA binding site which was predicted to participate in the recognition of both α1,2- and α1,4-fucosylated glycan structures. The expression of α1,2-fucosylated glycan is crucial for susceptibility to infect with GII.4 Sydney_2012. However, it still remain to be investigated whether such amino acid substitutions in the HBGA binding sites of GII.4 variants which have been predominant with a high prevalence will occur in close association with emergence of genetically novel variants in the same NoV genotype, and further, whether the outbreak of new genotypes with different antigenicities will result in the change of binding specificity in NoV variants to escape from the present human immune system against NoV infection.

The observation from this study showed that the new variant Sydney_2012 strain showed a high diversity in both antigenic blockade epitope and HBGA binding site. It was suggested that NoV GII.4 persist in the human population by two different mechanisms. First, the changes in P2 subdomain correlated with altered antigenicity may lead virus escape from the existing herd immunity. Second, the changes located in the HBGA receptor binding domain may result in virus adapt to utilize a receptor switching mechanism to persist in human population. These two mechanisms likely occur in overlap, the changes may alter both receptor usage and antigenicity simultaneously. The Sydney_2012 strains detected in Japan from my current study will be further characterized, in particularly, amino acid at the putative antigenic determinant and HBGA binding site in comparison with other circulating variants. I also will investigate NoV binding to host cell using other receptor-like molecules. On the other hand, with regarding to blood group

substances prepared from easily accessible natural sources and medical herbs might have potential for antiviral against NoV, the inhibition effects of medical herbs and food stuffs against NoV binding to HBGAs will be investigated.

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APPENDIX

Properties of amino acids

Abbreviation and molecular weight for the amino acids

Name of amino acid	Three-letter code	One-letter code	Residue mass (Daltons)
Alanine	Ala	A	71.08
Arginine	Arg	R	156.20
Asparagine	Asn	N	114.11
Aspatic acid	Asp	D	115.09
Cysteine	Cys	C	103.14
Glutamine	Gln	Q	128.14
Glutamic acid	Glu	E	129.12
Glycine	Gly	G	57.06
Histidine	His	H	137.15
Isoleucine	Ile	I	113.17
Leucine	Leu	L	113.17
Lysine	Lys	K	128.18
Methionine	Met	M	131.21
Phenylalanine	Phe	F	147.18
Proline	Pro	P	97.12
Serine	Ser	S	87.08
Threonine	Thr	T	101.11
Tryptophan	Trp	W	186.21
Tyrosine	Tyr	Y	163.18
Valine	Val	V	99.14