

**Norovirus Infection in Children:  
Studies on Rapid Detection Method and  
Molecular Epidemiological Characters**

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## ABSTRACT

Norovirus (NoV) is a major cause of non-bacterial acute gastroenteritis in infants and young children all over the world. It is reported that NoV causes up to 200,000 deaths of children in developing countries, and 64,000 episodes of diarrhea requiring hospitalization among children in industrialized countries. Although NoV is such a common infectious disease and more than 30 years have passed since its discovery, the diagnosis of this viral infection has remained difficult in the clinical setting. This is mainly due to the unavailability of a cell culture system to cultivate this virus, which has hampered the development of immunological assays. In consequence, the natural course and pathogenesis of NoV infection have not been fully understood to date.

This research is divided into four chapters. In the first chapter, I describe the development of immunochromatography (IC) for NoV as an alternative rapid detection method. Polyclonal antibodies were raised in rabbits against recombinant virus-like particles (rVLPs) of the prevalent NoV genotypes, namely GII/3 and GII/4, and were applied on to the IC kit as the capture and detection antibodies. This kit was evaluated for its reactivity to rVLPs and for the detection of natural viruses in stool samples collected from diarrheal children compared to the results by RT-PCR. In the prospective assessment, the kit showed agreement rate of 84.1%, sensitivity of 69.8% and specificity of 93.7%. Genotyping of the RT-PCR positive samples by sequence analysis revealed that some heterogeneous genotypes were also detected, while some in homogenous genotypes occasionally showed false negative records resulting in lower sensitivity. No cross reactivity with other common viral pathogens was observed. In

addition, the detection limit of viral load was as small as approximately  $10^{6-7}$  copies/g of stool. Taken together, using the current IC in the screening process for NoV with simple laboratory support is justified .

In the second chapter, I describe detection of NoV RNA in samples other than stool from children with acute gastroenteritis to elucidate potential extraintestinal involvement of NoV gastroenteritis as an unseen feature of its pathogenesis. NoV RNA was detected by seminested RT-PCR in serum samples from 6 out of 39 (15.4%) of the NoV gastroenteritis patients. Neither of the two cerebral spinal fluid samples was positive for NoV RNA. The serum-positive patients showed neurological complications more frequently than the serum-negative patients ( $p=0.028$ ). The viral load in the stools of the serum-positive patients was not significantly greater than that of the serum-negative patients (median:  $9.8 \times 10^9$  copies/g versus  $1.1 \times 10^9$  copies/g ( $p=0.117$ )), and the viral load in the serum and stools of the serum-positive patients did not correlate. However, genotypes of the NoV in stools and serum from the same patient matched completely (GII/3:1, GII/4: 5) with high homology ranging from 99.2% to 100% between the paired samples, demonstrating that RNA in sera originated from the intestines.

In the third chapter, I describe the prevalence and changing distribution of diarrheal viruses in children attending a day care center (DCC) in Tokyo, Japan. NoV emerged as the most predominant diarrheal virus in children attending a DCC ( $p<0.01$ ), while the overall detection rate of viruses decreased ( $p<0.001$ ) in the 6 years since the first study was conducted in the same DCC using the same detection method. Eighteen out of 20 children experienced NoV GII infection during one year, including multiple genotypes infection and re-infection of the same genotype. There was no significant

difference in norovirus load between symptomatic and asymptomatic children (median:  $1.5 \times 10^6$  copies/g versus  $2.6 \times 10^4$  copies/g ( $p=0.6$ )). Sequencing and quantitative analysis revealed that an asymptomatic child excreting an insignificant amount of virus ( $4.7 \times 10^4$  copies per gram of stool) may cause a major outbreak of NoV in a semiclosed setting such as a DCC.

In the final chapter, I describe the prevalence and molecular epidemiological characters of NoV in children hospitalized with acute gastroenteritis in Kandy, Sri Lanka. NoVs were first detected in samples from Sri Lankan children, and their nucleotide data were deposited in the GenBank. NoV was ranked as the second most common causative virus accounting for 10.5% of the cases after group A rotavirus. Among 33 patients showing mono-infection with NoV, the main clinical manifestations observed were diarrhea (100%), vomiting (72.7%), and body temperature  $\geq 37.5^\circ\text{C}$  (51.5%). Regarding the severity of infected patients, NoV showed a high severity score comparable to group A rotavirus and mixed infection group. Each clinical symptom showed no statistically significant difference between NoV infection and other diarrheal viruses. A distinct peak of NoV was observed in the dry and cool season of the year. Great genetic diversity of NoV was recognized, encompassing five genotypes including rare GII/9 and GII/16. GII/3 was the most predominant genotype (22 of 38, 57.9%), showing high homology with globally distributed strains.

In conclusion, a simple and easily performable detection kit with an immunochromatographic system was successfully developed to improve notification of NoV infection in clinical practice. It was suggested that NoV spreads from the intestines to the blood stream, possibly accounting for extraintestinal complications of NoV gastroenteritis. Asymptomatic children were suspected to be an important source of

NoV outbreak, emphasizing the importance of standard precautions. A significant disease burden of NoV infection was recognized in Sri Lanka, where no report of NoV had been made before this study. Although the numbers of molecular epidemiological studies have been increased dramatically over the years, continuous monitoring and detailed genetic analysis are necessary to assess the full implication of the global evolution of this virus.

## ABBREVIATIONS

%	: Percent
°C	: Degree Celsius
$\mu$ g	: Microgram
$\mu$ l	: Microliter
bp	: Base pair
BSA	: Bovine serum albumin
cDNA	: Complementary deoxyribonucleic acid
CSF	: Cerebral spinal fluid
DCC	: Day care center
DDBJ	: DNA Databank of Japan
DNA	: Deoxyribonucleic acid
dNTP	: Deoxyribonucleotide triphosphate
ELISA	: Enzyme-linked immunosorbent assay
G	: Genogroup
g	: Gravity
HEL	: Helicase
IC	: Immunochromatography
IEM	: Immune electron microscopy
IgG	: Immunoglobulin
M	: Molar
min	: Minute
ml	: Milliliter



nm	: Nanometer
NoV	: Norovirus
OD	: Optic density
ORF	: Open reading frame
PBS	: Phosphate-buffered saline
PCR	: Polymerase chain reaction
Pmol	: Picomole
POL	: Polymerase
Poly Ab	: Polyclonal antibody
PRO	: Proteinase
RNA	: Ribonucleic acid
RNase	: Ribonuclease
RT	: Reverse transcription
RT-PCR	: Reverse transcription-polymerase chain reaction
rVLP	: Recombinant virus-like particle
sec	: Second
TAE	: Tris-Acetate-EDTA
TCID <sub>50</sub>	: 50% tissue culture infective dose
VPg	: Virion protein genome

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## INTRODUCTION

### **Overview of norovirus infection and its disease burden**

Despite improved sanitation, water supplies, and food safety, diarrhea remains a common cause of illness worldwide. It is reported to account for approximately 1.8 million annual deaths in children <5 years of age [Bryce et al., 2005], making it quite necessary to explore etiological agents for diarrheal diseases.

Norovirus (NoV) is a major cause of non-bacterial acute gastroenteritis in infants and young children all over the world [Estes et al., 2006; Lopman et al., 2003; Okitsu-Negishi et al., 2004]. NoV was first described as a human pathogen in 1972 by Kapikian and his colleagues by using immune electron microscopy (IEM) [Kapikian et al., 1972]. They observed small and round viruses with a cup-like surface in stools from the adult volunteers who were administered bacteria-free filtrate serially passaged from other volunteers who ingested stool material obtained from an ill individual involved in a gastroenteritis outbreak at an elementary school in Norwalk, Ohio, in October 1968 [Adler and Zickl, 1969].

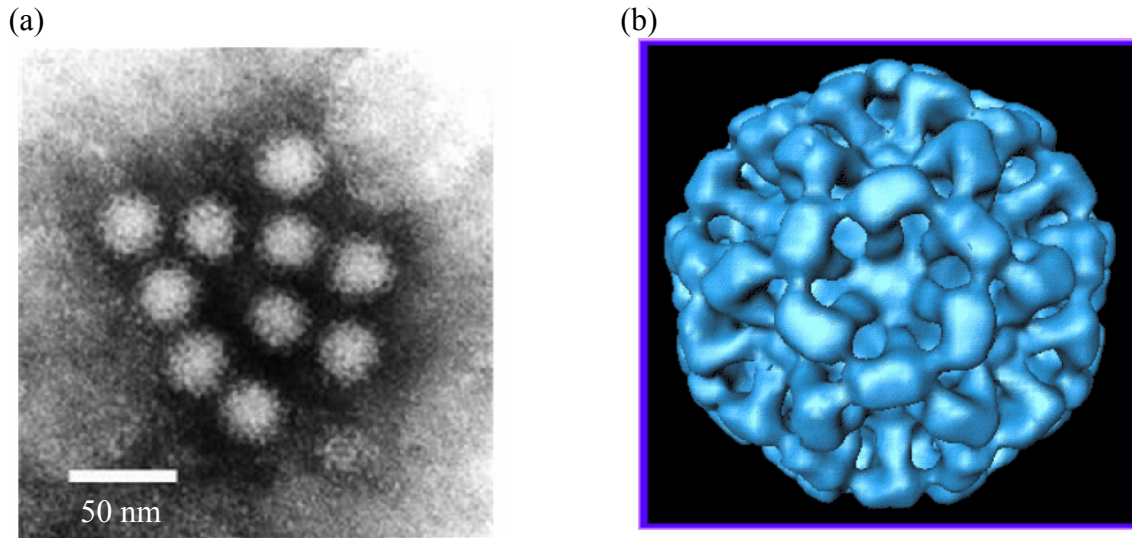
After its discovery, NoV was first recognized as a cause of outbreaks of acute gastroenteritis nicknamed ‘winter vomiting disease.’ In the late 1990s, population-based studies of community-acquired gastroenteritis were undertaken to reveal its incidence, with estimates of around one norovirus episode per 30 people per year [de Wit et al., 2001; Wheeler et al., 1999]. Since the application of sensitive molecular assays for screening diarrheal patients, NoV has been well-documented as the leading cause of sporadic gastroenteritis. According to the systematic review which included studies using reverse-transcription polymerase chain reaction (RT-PCR) for diagnosis, NoV

was estimated to account for 12% of hospitalized gastroenteritis cases among children < 5 years of age and 12% of mild and moderate diarrhea cases among people of all ages who were treated as outpatients [Patel et al., 2008]. It is reported that NoV causes up to 200,000 deaths of children in developing countries, and it causes 64,000 episodes of diarrhea requiring hospitalization and 900,000 clinic visits among children in industrialized countries [Koopmans. 2008; Patel et al., 2008].

### **Virology of norovirus**

NoV is classified as a genus in the *Caliciviridae* family together with three other genera, namely Sapovirus, Lagovirus and Vesivirus. The term “Norwalk-like virus” was tentatively used to mention noroviruses other than the original strain which was described in 1972 (Hu/NoV/GI.1/Norwalk/1968/US). In 2002, however, the International Committee on Taxonomy of Viruses officially announced the genus name as Norovirus, and Norwalk virus was defined as a unique species within the genus. The original strain has been recognized as a prototype strain of the species [Green, 2007].

NoV virions exhibit T=3 icosahedral symmetry with a diameter approximately as small as 30 nm (Figure 1). The capsid contains 90 dimers of the capsid protein that form a shell from which 90 arch-like capsomers protrude. These arches are arranged in such a way that 32 large hollows are present on the surface of the virion [Chen et al., 2004; Prasad et al., 1994, 1999, 2001]. These hollows are seen as cup-like structures of viruses belonging to the family *Caliciviridae*, hence their virological name (*calici* is derived from the Latin word *calyx*, or cup).



**Figure 1.** Pictures of NoV particles. (a) NoV in stool filtrate from an individual with gastroenteritis visualized by negative-stain transmission electron microscopy [Homepage of United States Environmental Protection Agency]. (b) Three-dimensional structure, as viewed along the icosahedral threefold axis, of the recombinant NoV capsid determined by cryo-electron microscopy [Prasad et al., 1999].

Despite intensive efforts spanning over three decades, no fully permissive cell culture system presently exists for NoV [Duizer et al., 2004]. One recent study reported that NoV had infected and replicated in a three-dimensional, organoid model of human small intestinal epithelium. However, the level of virus replication achieved in this system was questionable, indicating that NoV replication in a cell line still remains a difficult challenge [Chen et al., 2007; Straub et al., 2007]. This fastidious nature has hindered the establishment of an animal model for analysis of NoV pathogenesis, as well as the development of antibody-based diagnosis.

Genetic engineering progress has enabled researchers to produce recombinant virus-like particles (rVLPs) by a baculovirus expression system [Jiang et al., 1992]. They are morphologically and antigenically similar to native virion and are very useful

in serving as a surrogate without viral infectivity [Jiang et al., 2000; Kamata et al., 2005; Okitsu-Negishi et al., 2006].

### **Diagnosis of norovirus infection**

Traditionally, electron microscopy has been used to screen stool samples taken from suspected viral gastroenteritis patients [Atmar et al., 2001]. Direct examination of unconcentrated stool material, however, is of limited value for NoV detection because virus particles are usually present in a low concentration and may be difficult to distinguish from other small, round objects present in the stool. For this reason, putative NoV should be identified in stools by the IEM technique, which requires skilled personnel and a well-equipped laboratory [Green, 2007].

Since the successful sequencing of the full gene of NoV [Jiang et al., 1993], RT-PCR with modified primers has been widely used as a gold standard in many reference laboratories [Atmar et al., 2001; Vinjé et al. 2003]. The application of real time PCR has gained widespread use because, when compared with conventional PCR, it allows faster detection as well as comparison of viral RNA levels [Kageyama et al., 2003].

The establishment of rVLPs production has had a major impact on the development of immunoassays such as enzyme-linked immunosorbent assays (ELISA) employing monoclonal or polyclonal antibodies (Poly Ab) against NoV rVLPs [de Bruin et al., 2006; Dimitriadis et al., 2006; Gonzalez et al., 2006; Okitsu-Negishi et al., 2006]. Although ELISA is relatively easy to perform compared to electron microscopy or PCR, it still requires at least 4 hours to obtain the result, which is inadequate for managing outbreaks in hospitals or on-the-spot diagnosis in a clinical setting.



### **Clinical features of norovirus infection**

NoV infection is considered mild and self-limiting, but the patient occasionally needs intravenous rehydration for acute loss of fluid and electrolytes due to watery diarrhea and vomiting [Green, 2007]. Certain individuals with underlying illness have been regarded as at increased risk for more severe disease during cases of outbreak [Mattner et al., 2006]. At the same time, hospitalized cases of sporadic NoV infection have often been described in much literature, accounting for 5.4-15.1% cases of diarrheal patients under surveillance [Hansman et al., 2004a, 2004b; Khamrin et al., 2007; Kirkwood et al., 2005; Yoon et al., 2008; Monica et al., 2007; Onishi et al., 2008].

Interestingly, it has recently been reported that NoV is as common an etiological agent for hospitalization as group A rotavirus, which has been long regarded as the leading cause of viral gastroenteritis. These studies show the capability of NoV to cause severe gastroenteritis [Boga et al., 2004; Marshall et al., 2003; Ribeiro et al., 2008].

Studies of the clinical features of gastroenteritis in the outbreak cases revealed that NoV causes clinical symptoms as severe as rotavirus does, although statistical analysis was not performed [Sakai et al., 2001]. A study of the sporadic cases in Brazil also showed similar results [Nakagomi et al., 2008]. Surveillance of hospitalized cases in Taiwan reported that rotavirus and mixed viral infection show a higher severity score than NoV, although the number of cases analyzed was relatively small (total: 257) [Chen et al., 2007].

### **NoV infection in a community**

It is well documented that NoV can lead to large outbreaks all over the world [Centers for Disease Control and Prevention, 2005; Lopman et al., 2003; Seto et al., 2005]. The predominant modes of transmission are person-to-person contact and food-borne spread [Green, 2007]. In the pediatric population, NoV outbreaks mostly occur in the settings of schools and day care centers (DCCs) [Fankhauser et al., 2002; Götz et al., 2002; Gallimore et al., 2004; Isakbaeva et al., 2005].

Intensive surveillances toward detection of NoV in children have been carried out in many Asian countries including Japan [Onishi et al., 2008; Phan et al., 2006a, 2006b]; South Korea [Lee et al., 2007; Yoon et al., 2008]; China [Liu et al., 2006], Thailand [Khamrin et al., 2007; Hansman et al., 2004b; Malasao et al., 2008], Vietnam [Hansman et al., 2004a; Nguyen et al., 2007; Nguyen et al., 2008], Bangladesh [Dey et al., 2007], and India [Rachakonda et al., 2008; Monica et al., 2007; Girish et al., 2002; Chhabra et al., 2008]. These studies identified the significant impact of NoV in pediatric clinical settings in Asian regions, and warranted further investigation in other countries to elucidate the disease burden of this infectious disease.

## OBJECTIVES

The main objectives of this study were the following:

1. To develop a rapid detection method, namely immunochromatography (IC), for NoV with evaluation by clinical stool samples and rVLPs.
2. To elucidate potential extraintestinal involvement of NoV gastroenteritis as an unseen feature of NoV infection by detecting NoV RNA in samples other than stool.
3. To investigate the prevalence and changing distribution of diarrheal viruses in children attending a day care center in Tokyo, Japan, and highlight the impact of asymptomatic NoV infection in children.
4. To investigate the prevalence and molecular epidemiological characters of NoV in children hospitalized with acute gastroenteritis in Kandy, Sri Lanka, and highlight the impact of NoV infection in a country where no previous report is available.

The first aim was to develop easily performable detection method, so that I could contribute to the establishment of the real clinical entity of NoV infection. Secondly, in order to gain further insight to the pathogenesis of NoV, I intended to investigate the possible systemic spread of NoV from the intestines. Finally, I would like to report the NoV infection in a community observed in Japan as well as that observed in Sri Lanka.

# **Chapter I: Development of a rapid immunochromatographic test for noroviruses genogroups I and II**

## **Introduction**

IC is one of the representative methods in rapid diagnosis, and it is widely used to detect various infectious diseases, such as influenza virus, rotavirus, and adenovirus [Bon et al., 2007; Fujimoto et al., 2004; Hara et al., 2006]. The assays are usually completed within 30 minutes, and require only limited equipment such as centrifuge machines and micropipettes. Before the initiation of the current study, only one report of IC for NoV [Okame et al., 2003] was published, which pointed out difficulties in obtaining good sensitivity and specificity due to diverse genotypes with distinct antigenicities of NoV. Thus, further improvements are yet to be made to this technique before it is employed as a clinically applicable detection method for NoV infection.

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

## **Materials and methods**

### **Recombinant virus-like particle**

Fifteen types of rVLP (GII/1 strain 4656, GII/3 strain 3634, GII/4 strain 2876, GII/8 strain 3006, GII/1 strain 3101, GII/2 strain 2840, GII/3 strain 3229, GII/4 strain 1207, GII/5 strain 3611, GII/6 strain 3612, GII/7 strain 419, GII/12 strain 2087, GII/13 strain 3385, GII/14 strain 2468, GII/15 strain 3625), which were generated previously [Okame et al., 2003; Okitsu-Negishi et al., 2006], were used in this study. Protein concentration of each rVLP was measured by BCA Coomassie protein assay (Pierce Biotechnology, Inc., Rockford, IL) and 150 µg/ml was prepared as stock solution.

### **Polyclonal antibody production**

Rabbits were immunized subcutaneously with 100 µg of CsCl-purified rVLP GII/3 or GII/4 in Freund's complete adjuvant 4 times at two-week intervals. The serum was collected one week after the last injection. Rabbit IgG was purified from sera using Hi Trap<sup>TM</sup> rProtein A FF (Amersham Bioscience, Piscataway, NJ) and then used as Poly Ab.

### **Enzyme-linked immunosorbent assay for titration of polyclonal antibody**

96-well plates (Maxisorp, Nunc, Denmark) were each coated with 90 ng of rVLPs/well in 60 µl of 0.1M carbonate buffer (pH 9.6) for 1 hour at 37°C. The wells were blocked with 1% BSA in PBS containing 0.1% Tween 20 (PBS-T). The plates were incubated overnight at 4°C. After the wells were washed three times with PBS-T, 60 µl twofold serially diluted Poly Ab from a starting dilution of 1: 400 in PBS-T

containing 1% BSA was added to each well, and the plate was incubated for 1 hour at 37°C. After washing three times with PBS-T, 60 µl of a 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) was added to each well, and the plate was incubated again for 1 hour at 37°C. After washing three times with PBS-T, 60 µl of substrate *o*-phenylenediamine containing 0.012% H<sub>2</sub>O<sub>2</sub>, 0.2 M citrate-phosphate buffer (pH 5.0) was added to each well and left in the dark for 20 minutes at room temperature. The reaction was stopped by the addition of 60 µl of 2 M H<sub>2</sub>SO<sub>4</sub> to each well and the optical density (OD) at 492 nm (620 nm as reference) was determined with a Labsystems Multiskan MCC microplate reader. For this experiment, a lysate of Tn5 cell, which was used to generate recombinant protein, was included as a negative control. A sample with OD>0.2 and a sample/negative control ratio >2.0 was considered positive.

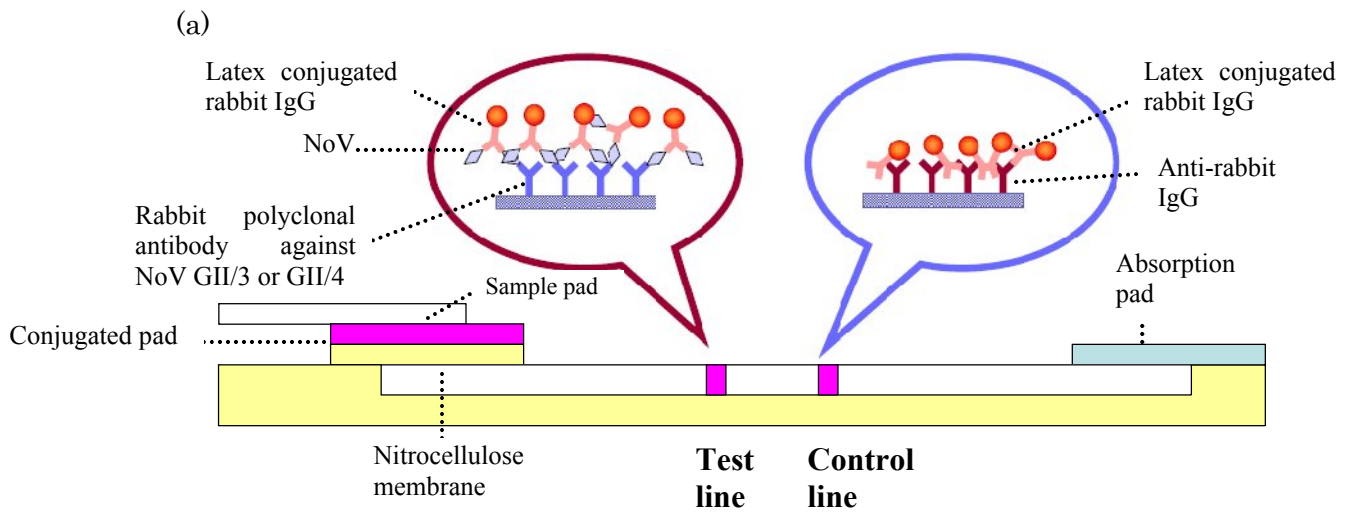
### **Conjugation of polyclonal antibody with colored latex**

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

### **Immunochromatography**

This test kit included two antibodies; one was against rVLP either GII/3 (IC-GII/3) or GII/4 (IC-GII/4), while the other was against general rabbit IgG which was applied on the control line. The former was applied in a conjugated pad with colored latex, and also in the test line as a capture antibody for complex of virus in the sample and latex-conjugated antibody. All the lines were painted onto the Hiflow plus nitrocellulose membrane (Millipore, Billerica, MA). The test strip was assembled in the order shown in Figure 2a: sample pad, conjugated pad, nitrocellulose membrane and absorption pad. All the pads were overlapped to enable migration of the complex of NoV and antibody along the test strip.

Each stool sample was diluted with distilled water to 10% suspension, and clarified by centrifugation at 10,000×g for 10 minutes. Fifty µl of the diluted stool sample and 50 µl of the reaction buffer (0.2 M NH<sub>4</sub>Cl buffer containing 0.15 M NaCl and 0.5% Tween20) were put into the well of Nunc-Immuno<sup>TM</sup> Module (Nunc, Denmark), and mixed well by gentle pipetting. Then, the sample pad of the test strip was inserted in the well to let the reaction mixture come up along the strip by capillary filling. When NoV existed in the sample, it bound with the Poly Ab in the conjugate pad. The complex was captured by the immobilized Poly Ab applied on the test line and a pink band was formed. The immobilized anti-rabbit IgG combined with the latex-conjugated rabbit Poly Ab independently of the NoV antigen, and confirmed that the assay had been performed correctly. The observer waited 15 minutes before determining the result of the assay. Examples are shown in Figure 2b for a positive sample and in Figure 2c for a negative sample.



(b)



(c)



**Figure 2.** (a) Diagram of the IC for norovirus. (b) A representative photograph of a positive sample. (c) A representative photograph of a negative sample.



### **Detection limit of IC**

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

### **Prospective assessment of IC**

In order to evaluate the IC with no interference, which may arise due to repeated freezing and thawing of the samples, as well as the effect of centrifugation performed for other analyses, prospective assessment was conducted using freshly collected samples. A total of 107 stool samples were obtained from children with acute gastroenteritis visiting one pediatric clinic in Kyoto, Japan, during the winter season from 2005 December to 2006 March, during which period NoV infection reaches its peak in Japan [Okame et al., 2006]. Ethical approval was obtained from the ethical committee of the University of Tokyo (Title: Research on molecular epidemiology, diagnosis, treatment, pathogenesis of viral gastroenteritis in children; registration No

1139; dated June 13<sup>th</sup>, 2005) and the patients' guardians gave informed consent.

### **Extraction of viral genome from stool samples**

Detection of diarrheal viruses was conducted using the protocol modified from the method previously described by Yan et al. [2003, 2004]. The viral genome was first extracted from stool supernatant using QIAamp viral RNA Mini Kit (Qiagen, Hildren, Germany). One hundred and forty  $\mu\text{l}$  of 10% stool supernatant was mixed with 560  $\mu\text{l}$  of AVL viral lysis buffer containing carrier RNA by pulse-vortexing for 15 sec. The mixture was incubated at room temperature for 10 min, and 560  $\mu\text{l}$  of ethanol was added. Thereafter, the mixture was applied onto the spin column, and centrifuged at  $4,300 \times g$  for 1 min. The column was placed into a new 2 ml collection tube and 500  $\mu\text{l}$  AW1 buffer was added. The column was centrifuged at  $4,300 \times g$  for 1 min to remove unbound materials, and washed adding 500  $\mu\text{l}$  of AW2 buffer. Then, the column was centrifuged at full-speed (about  $13,000 \times g$ ) for 3 min, and placed into a new 1.5 ml microcentrifuge tube. Finally, 60  $\mu\text{l}$  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  $4,300 \times g$  for 1 min. The viral RNA was spin down into the collection tube, and used as a template for the RT-PCR.

### **RT reaction**

First, 5 $\mu\text{l}$  of the extracted RNA was heated at 95°C for 5 min to denature the viral RNA, and then immediately cooled on ice. The RT reaction components contained 3.0  $\mu\text{l}$  of 5  $\times$  First-Strand Buffer (Invitrogen Japan, Tokyo, Japan), 0.8  $\mu\text{l}$  of 0.1 M DTT (Invitrogen Japan, Tokyo, Japan), 0.8  $\mu\text{l}$  of 10 mM deoxynucleotide triphosphate

(dNTP) Mix (Roche, Mannheim Germany), 0.8  $\mu$ l (200 U/ $\mu$ l) of SuperScript III Reverse Transcriptase (Invitrogen Japan, Tokyo, Japan), 0.8  $\mu$ l (1  $\mu$ g/ $\mu$ l) of random primer (hexa-deoxyribonucleotide mixture) (Takara, Shiga, Japan), 0.5  $\mu$ l (40 units/ $\mu$ l) of RNase Inhibitor (Toyobo, Osaka, Japan), and 5.0  $\mu$ l of heat denatured RNA. Milli-Q water was added to give a total volume of 15.0  $\mu$ l. The RT step was carried out at 50<sup>o</sup> C

**Table 1.** Oligonucleotide primers for detection of diarrheal viruses and sequencing of capsid gene of norovirus

Target virus	Primer	Polarity	Sequence (5'-3') <sup>a</sup>	Position	Amplicon size (bp)	Reference
<b>A set</b>						
RAV	Beg9	+	GGCTTTAAAAGAGAGAATTTCCGTCTG <sup>i</sup>	1-28	395	Yan et al.,2004
	VP7-1'	-	ACTGATCCTGTTGGCCATCCTTT	373-395		Yan et al.,2004
RBV	ADG9-1F	+	GGCAATAAAAATGGCTTCATTGC	1-22	814	Yan et al.,2004
	ADG9-1R	-	GGGTTTTTACAGCTTCGGCT	795-814		Yan et al.,2004
RCV	G8NS1	+	ATTATGCTCAGACTATCGCCAC	353-374	351	Yan et al.,2004
	G8NA2	-	GTTTCTGTACTAGCTGGTGAA	683-704		Yan et al.,2004
AdV	Ad1	+	TTCCCCATGGCICAYAACAC	1834-1853	462	Yan et al.,2004
	Ad2	-	CCCTGGTAKCCRATRTTGTA	2296-2315		Yan et al.,2004
<b>B set</b>						
NoV GI	G1-SKF <sup>b,d</sup>	+	CTGCCGAATTYGTAATGA	5342-5361	330	Yan et al.,2003
	G1-SKR <sup>b,c,d</sup>	-	CCAACCCARCCATRTACA	5653-5671		Yan et al.,2003
	COG1F <sup>c</sup>	+	CGYTGGATGCGNTTYCATGA	5291-5310	381 <sup>e</sup>	Shinohara et al., 2002
NoV GII	G2-SKF <sup>d</sup>	+	CNTGGGAGGGCGATCGCAA	5058-5076	342	Kojima et al., 2002
	G2-SKR <sup>b,c,d</sup>	-	CCRCCNGCATRHCCRTTRTACAT	5367-5389		Yan et al.,2003
	COG2F <sup>b,c</sup>	+	CARGARBCNATGTTYAGRTGGATGAG	5003-5028	387 <sup>f</sup>	Yan et al.,2003
SaV	SLV5317	+	CTCGCCACCTACRAWGCBTGGTT	5317-5339	434	Yan et al.,2003
	SLV5749	-	CGGRCYTCAA AVSTACCBCCCCA	5727-5749		Yan et al.,2003
HAstV	PreCAP1	+	GGACTGCAAAGCAGCTTCGTG	4235-4255	719	Yan et al.,2003
	82b	-	GTGAGCCACCAGCATCCCT	4934-4953		Yan et al.,2003

<sup>a</sup> within nucleotide sequence of primers, B=C,G or T; H=A,C or T; I=inosine; K=G 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.

<sup>b</sup> used in multiplex PCR as set B

<sup>c</sup> used in monoplex PCR

<sup>d</sup> used in seminested PCR

<sup>e</sup> in combination with G1-SKR

<sup>f</sup> in combination with G2-SKR

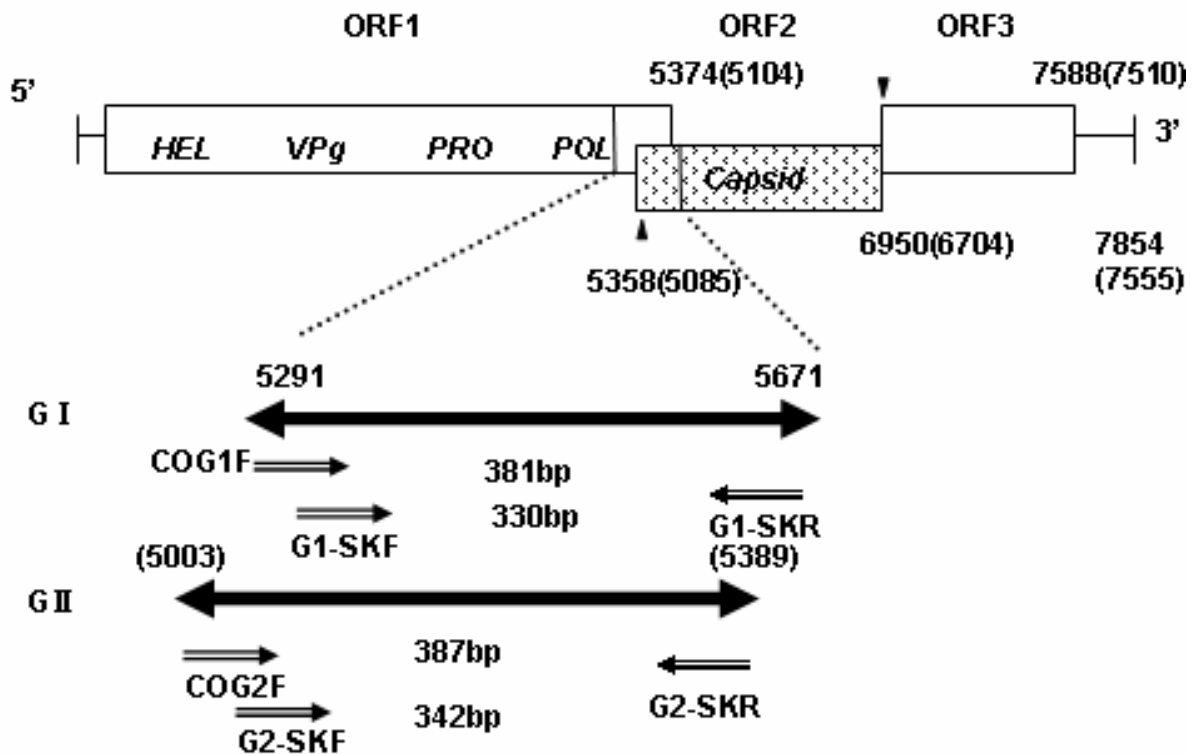
RAV, group A rotavirus; RBV, group B rotavirus; RCV, group C rotavirus; AdV, adenovirus; NoV GI, Norovirus genogroup I; NoV GII, norovirus genogroup II; SaV, sapovirus; HAstV, human astrovirus

for 1 h, followed by heating at 95°C for 5 min, and then the samples were immediately cooled on ice.

## **PCR**

Two sets of primers were used to screen the common diarrheal viruses from stool with the primers shown in Table 1. This process was performed in two assays. The first assay was done using the primer set termed A, containing 4 pairs of primers for rotaviruses group A, B and C, and adenovirus. The second set, termed B, contained 4 pairs of primers for norovirus genogroup I and II, sapovirus and astrovirus. Monoplex PCR and seminested PCR for NoV were performed for some samples negative for the above multiplex PCR, as specified in the Results section. Schematic representation of the genes of norovirus, primer binding sites and the expected PCR products are shown in Figure 3.

The PCR components contained 5.0 µl of 5 × Colorless GoTaq PCR buffer containing MgCl<sub>2</sub> at a concentration of 7.5 mM for a final concentration of 1.5 mM in the 1× reaction (Promega, Madison, WI), 2.0 µl of 2.5 mM of dNTP Mix (Roche, Germany), 0.5 µl of each 33 pmol primer, 0.1 µl of 5 units/µl GoTaq DNA polymerase (Promega, Madison, WI), and 3.0 µl of cDNA template. Milli-Q water was added to give a total volume of 25.0 µl. Amplification was performed by an initial denaturation at 94°C for 3 min and 35 cycles at 94°C for 30 sec, 55°C for 30 sec, 72°C for 60 sec, and an additional extension at 72°C for 7 min. The PCR product was detected by electrophoresis through 1.5% agarose gel in Tris-Acetate-EDTA (TAE) buffer at 100 volts for 30 min. The gel was stained with ethidium bromide (0.5 µg/ml) and then visualized under ultraviolet light.



**Figure 3.** Schematic representation of the genes of norovirus, primer binding sites, and the expected PCR products. Reference strains for GI and GII were Norwalk/68/US (M87661) and Lordsdale/93/UK (X86557), respectively. The location for GII is indicated in parenthesis. ORF, open reading frame; HEL, helicase; VPg, Virion protein, genome; PRO, proteinase; POL, polymerase; G, genogroup.

### Nucleotide sequence analysis

Positive PCR products for NoV were subjected to nucleotide sequencing to determine genotypes of NoV using a Big-Dye terminator cycle sequencing kit and an ABI prism 310 Genetic Analyzer (Applied Biosystemen, Inc., Foster City, CA). Sequence analysis was performed using CLUSTAL X software (Version 1.81). A phylogenetic tree with 100 bootstrap resamples of the nucleotide sequence alignment

data sets was generated using the neighbor-joining method with CLUSTAL X. The genetic distance was calculated using Kimura's two parameter method (PHYLIP). NoV reference strains and accession numbers used in this study were as follows:

Saitama/T56aGII/02/JP (AB112261), SaitamaU3/97/JP (AB039776), Miami/292/94/US (AF414410), Alphontrou/98/NE (AF195847), Saitama/T68aGII/02/JP (AB112284), Arg320/95/AR (AF190817), SaitamaU201/98/J (AB067542), Saitama/T3GII/00/JP (AB112238), Leeds/90/UK (AJ277608), SaitamaU25/98/JP (AB067543), IdahoFalls/378/9 (AY054299), M7/99/US (AY130761), Saitama/T57bGII/02/JP (AB112272), Lordsdale/93/UK (X86557), Bristol/93/UK (X76716), SaitamaKU80aGII (AB058582), SaitamaU1/97/JP (AB039775), Hawaii/71/US (U07611), Melksham/89/UK (X81879), Hillingdon/90/UK (AJ277607), Mc37/99/Thai (AY237415), Kashiwa47/00/JP (AB078334), SaitamaT29GII/0 (AB112221), SaitamaT53GII/0 (AB112260), Manchester\_Sapovirus (X86560).

## **Results**

### **Cross-reactivity of Poly Ab against rVLPs in ELISA**

The cross-reactivity of Poly Ab raised against rVLP GII/3 or GII/4 was checked with an ELISA system (Table 2). Poly Ab raised against rVLP GII/3 reacted broadly with many genotypes including those in genogroup I. The titer, expressed as the reciprocal of the highest dilution of each antibody yielding positive results, was generally high against many genotypes.

**Cross-reactivity of IC against various genotypes of rVLP and NoV in stored clinical samples**

A panel of various genotypes of rVLP and NoV in stored clinical samples was used to test the cross-reactivity of the IC (Table 3). Although highly concentrated rVLP of each genotype was applied, none of the heterogeneous genotypes reacted with IC-GII/4, and only a few did with IC-GII/3. Similar results were obtained using the stored clinical samples where few samples containing GII/1 or GII/6 NoV weakly reacted with IC-GII/3.

**Table 2.** Cross reactivity of polyclonal antibody in enzyme-linked immunosorbent assay

	Genogroup	Genotype	Poly Ab	
			GII/3	GII/4
rVLP (90 ng/well)	I	1	102,400	3,200
		3	25,600	<400
		4	204,800	12,800
		8	204,800	51,200
	II	1	>819,200	51,200
		2	>819,200	25,600
		3	>819,200	6,400
		4	409,600	>819,200
		5	>819,200	25,600
		6	409,600	25,600
		7	>819,200	12,800
		12	204,800	800
		13	409,600	25,600
		14	>819,200	6,400
		15	3,200	25,600

Titers were expressed as reciprocal dilution that gave the positive results. Titers more than 409,600 were painted by .

**Table 3.** Reactivity of IC with recombinant virus like particles and clinical stool samples

Genogroup	Genotype	IC-GII/3		IC-GII/4	
		rVLP (750 ng/ml)	No. of positive stools / No. of tested stools	rVLP (750 ng/ml)	No. of positive stools / No. of tested stools
I	1	-	0/3	-	0/3
	3	-	NT	-	NT
	4	-	0/1	-	0/1
	8	-	NT	-	NT
II	1	-	1/2	-	0/2
	2	±	0/8	-	0/2
	3	+	18/19	-	0/10
	4	-	0/10	+	26/31
	5	-	0/1	-	0/1
	6	±	2/4	-	0/2
	7	±	NT	-	NT
	12	±	NT	-	NT
	13	-	0/2	-	0/2
	14	±	0/1	-	0/1
	15	-	NT	-	NT

**Table 4.** Sensitivity, specificity, and agreement of IC and RT-PCR<sup>a</sup>

IC	Detection (no. of samples) by RT-PCR		
	+	-	Total
+	30 <sup>b</sup>	4 <sup>c</sup>	34
-	13	60	73
Total	43	64	107

<sup>a</sup> Sensitivity=69.8% (30/43); specificity=93.7% (60/64); agreement=84.1% (90/107)

<sup>b</sup> Nineteen were positive by IC-GII/3 and 11 by IC-GII/4

<sup>c</sup> These samples were positive as determined by seminested PCR

+, positive; -, negative



### **Detection limit of IC**

Stock solution of both rVLP GII/3 and GII/4, as well as stool samples with a known viral copy number, were serially diluted to determine the minimal concentration of antigenic protein and virus copy number by the IC. For IC-GII/3, the detection limit of rVLP was  $3.0 \times 10^{-3}$  ng/ $\mu$ l and that of viral load was  $3.5 \times 10^7$  copies/g of stool, whereas IC-GII/4 gave values for rVLP of  $7.5 \times 10^{-3}$  ng/ $\mu$ l and for viral load of  $4.6 \times 10^6$  copies/g of stool. The assays with rVLPs were performed in triplicate and showed the same results, giving a kappa value of 1.0.

### **Sensitivity, specificity and agreement rate based on RT-PCR**

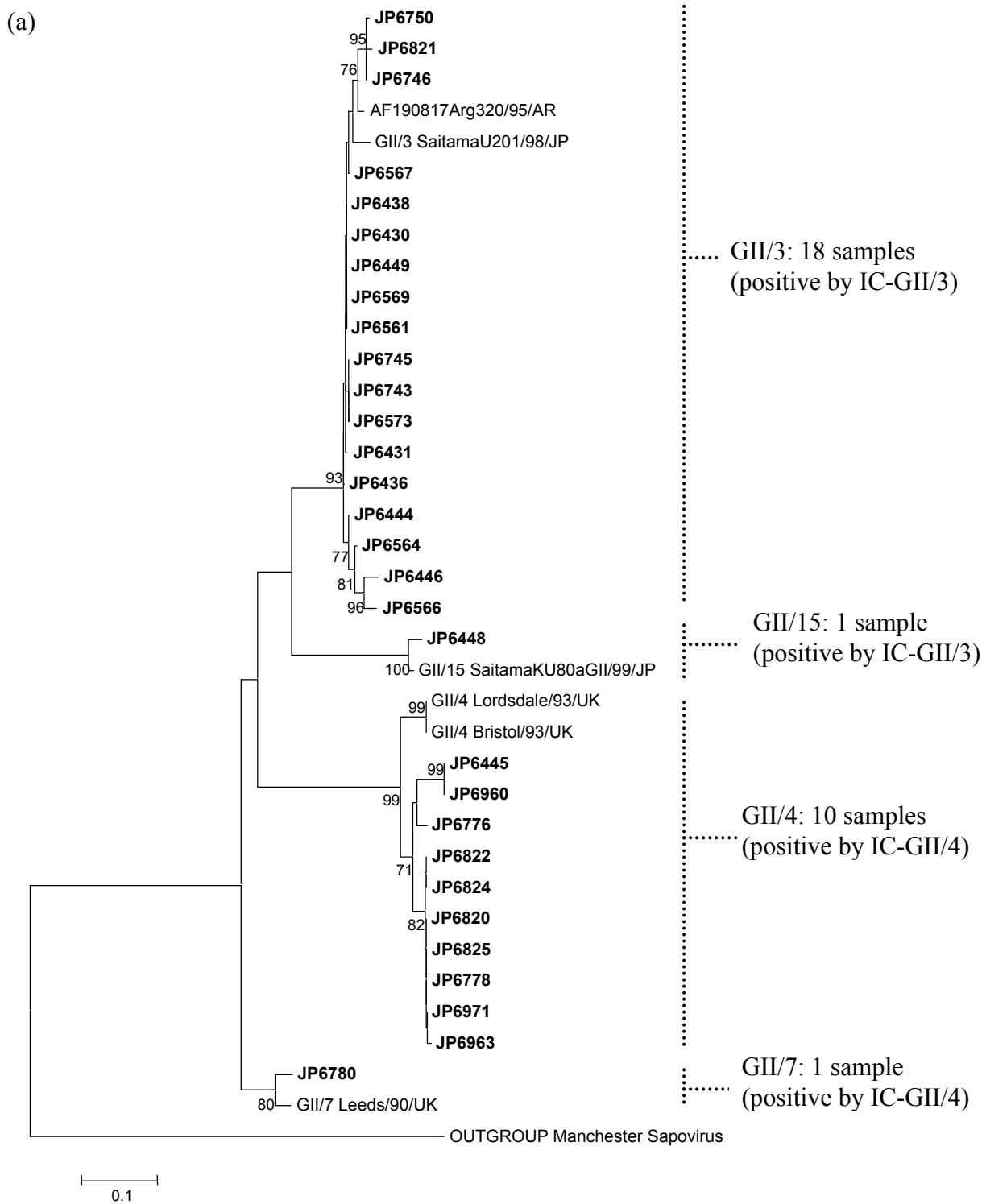
NoV in clinical stool samples collected during the winter season in Japan were examined by both the current IC and RT-PCR, and the kit was evaluated based on RT-PCR (Table 4). Among 107 samples, 43 proved to be positive by RT-PCR. Of these samples, 30 were correctly determined as positive by the IC, and the sensitivity was calculated as 69.8%. Nineteen samples were determined to be positive by IC-GII/3, and 11 samples by IC-GII/4. The former and latter samples were collected mainly in December and in March, respectively, indicating genotype change during the study period. Sixty-four samples were negative by RT-PCR. Of these, 60 were properly determined to be negative by the IC, giving a specificity of 93.7%. The agreement rate between the IC and RT-PCR was calculated as 84.1%. Four samples were determined to be positive by the IC but negative by RT-PCR. Subsequently, these samples were subjected to seminested PCR which gave positive results for all the four samples.

### **Genotyping of positive samples by RT-PCR**

Stool samples found to be positive for NoV by RT-PCR were further analyzed by sequencing to identify their genotypes. The phylogenetic tree for samples positive for both the IC and RT-PCR is shown in Figure 4a, whereas that for negative samples for the IC but positive with RT-PCR is shown in Figure 4b. All samples which were positive for GII/3 and GII/4 were determined by homologous IC. Interestingly, one GII/15 sample became positive with IC-GII/3, and one GII/7 sample with IC-GII/4. Thirteen false negative samples, which were negative with the IC but positive with RT-PCR, included not only heterogeneous genotypes such as GII/2 and GII/6, but also, unexpectedly, two homogeneous genotypes, GII/3 and GII/4. The nucleotide identities of 282 bp which were used for genotyping ranged 98-100% between false negative and true positive samples of GII/3 or GII/4 (data not shown).

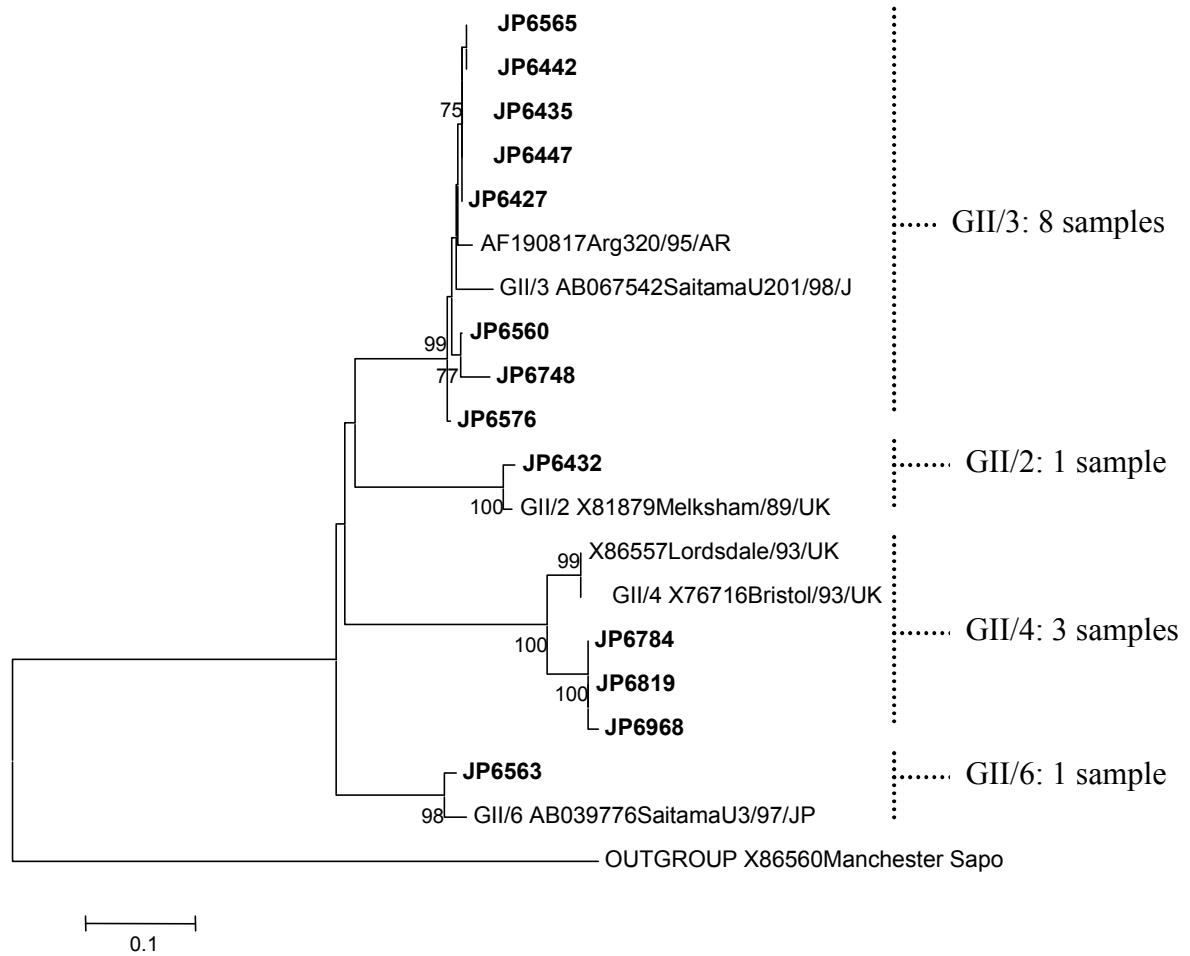
### **Cross-reactivity with other common enteric viruses**

During the study period, several enteric viruses were encountered in pediatric patients. The results of RT-multiplex PCR and the IC are shown in Table 5. Since no sample was positive for astrovirus during this period, two samples known to be positive for astrovirus were tested and the results of these samples are also summarized in Table 5. Of note, the IC succeeded in determining a positive result for a sample of mixed infection with both NoV GII and sapovirus. The IC correctly gave negative results for other single viral infections, such as group A rotavirus, sapovirus, adenovirus and astrovirus, which indicates the high specificity of the IC kit.



**Figure 4.** Phylogenetic tree of nucleotide sequences of norovirus in samples positive by RT-PCR. (a) Samples positive by both IC and RT-PCR. Reference strains selected from the DDBJ/GenBank database under the accession numbers indicated in the text. Study norovirus was highlighted in bold. Manchester strain was used as an out-group strain for phylogenetic analysis. The scale indicates nucleotide substitutions per position. The numbers in the branches indicate the bootstrap values. (b) Samples negative by IC but positive by RT-PCR.

(b)



**Figure 4. (Continued)**

**Table 5.** Cross-reactivity of IC with other enteric viruses

Viruses	Number of positive samples	Results of IC
Group A rotavirus	10	All negative
Sapovirus	5	All negative
Adenovirus	1	Negative
Astrovirus	2	Both negative
Mixed infection <sup>a</sup>	1	Positive

<sup>a</sup> Mixed infection between norovirus GII and sapovirus

## Discussion

With an increasing number of reports on NoV and an estimated increase in the number of patients of NoV infection [Estes et al., 2006], the demand for rapid diagnosis of this infectious disease is dramatically expanding. ELISA has been developed as a rapid diagnostic test, although it still takes more than 4 hours to obtain the results. The results of studies evaluating commercial ELISA kits differed greatly depending on the type of kits and researchers; sensitivity ranged from 36.0% to 76.3% and specificity ranged from 69.0% to 100% [Burton-MacLeod et al., 2004; de Bruin et al., 2006; Dimitriadis et al., 2006; Okitsu-Negishi et al., 2006].

In this study, a simple, easy-to-read, and rapid detection test for NoV using an IC membrane strip was developed. This method took a shorter time; approximately 30 minutes to complete the assay with limited equipment needed, and the results were reproducible with a kappa value of 1.0. Based on RT-PCR using freshly collected samples in winter, the peak season, the agreement rate was 84.1%, sensitivity 69.8%, and specificity 93.7%. The high specificity and the absence of cross-reactivity with other enteric viral pathogens, such as group A rotavirus, sapovirus, and adenovirus, clearly indicated the potential applicability of the IC in screening samples for NoV infection.

The false negative samples, which were negative by IC but positive by RT-PCR, included GII/2, GII/6, GII/3, and GII/4. For the first two, the genotypes were reasonable, since the cross-reactivity of the IC with rVLP had been initially checked and it was found that the broad cross-reactivity observed in the ELISA assessment of Poly Ab raised against rVLP GII/3 did not necessarily appear after applying it to the IC system. However, the latter two genotypes were the ones used as antigens to immunize

rabbits, and were expected to react strongly with the Poly Ab in the IC. A possible reason is that the viral loads in these false negative samples were smaller than those in the true positive samples.

On the other hand, the genotypes of 4 samples, which were positive by IC and seminested PCR, were 3 GII/3 and 1 GII/4. These samples may contain a smaller viral load than monoplex PCR-positive samples. This phenomenon was also reported elsewhere [Okitsu-Negishi et al., 2006], suggesting that factors other than viral load cause the lower sensitivity in an immunological detection test. Recently, Hansman et al. [2006] reported that the helix structure in amino acid residues 219-237 from the start codon of VP1 may play an important role in influencing the reactivity of GII/3 NoV in stool samples and Poly Ab raised against rVLP GII/3. Secondary structural prediction by software PSIPRED [McGuffin et al., 2000] revealed that our strain 3229, belonging to GII/3 and being used as an antigen for Poly Ab, possessed the helix structure in the abovementioned site (data not shown). Hence, there is a possibility to increase the sensitivity against GII/3 if Poly Ab raised against a strain without such a structure is also applied in the IC.

Other possible explanations for lower sensitivity of IC might be inner epitopes of NoV and the existence of inhibitors in mucus in the human stools. Of interest, the genotype-specific sensitivity for GII/3 and GII/4 with stored stool samples were 18/19=94.7% and 26/31=83.9%, respectively, and these were higher than those with freshly collected samples, which were 18/26=69.2% for GII/3 and 10/13=76.9% for GII/4.

Bon et al. [2007] reported the change in OD of ELISA for rotavirus with stocked samples; OD of 14 samples decreased whereas those of 9 samples increased.

They speculated that freezing and thawing as well as repeated centrifugation might have affected the immunological detection system. In our assessment, extra freezing and thawing as well as centrifugation performed for previous analyses might have exposed the inner epitopes or removed inhibitors from the stool suspension in stored samples, resulting in better sensitivity.

Determining the detection limit of IC to rVLP is important, especially because NoV cannot be cultured in any cell line, and therefore cannot give the minimal TCID<sub>50</sub> for positive results, which is usually considered as a meaningful index for quality constituency between different batches of the kit [Okitsu-Negishi et al., 2006]. In this study, the detection limit of the rVLP used as antigen for each type of IC is clearly shown. This value may be useful as a reference point for future attempts using various genotypes. The viral load of NoV GII in the clinical stool samples is reported to be around 10<sup>8</sup> copies/g of stool [Chan et al., 2006].

Based on the results in the current study, the IC can theoretically detect 1/100 to 1/10 of the viral load found in clinical samples, which is almost equivalent to the detection power of electron microscopy [Atmar et al., 2001]. Therefore it may be justified to use IC for screening the stool samples.

Regarding cross-reactivity to heterogeneous genotypes, broad reactivity of IC-GII/3 was expected on the basis of the titration result of Poly Ab raised against GII/3 in ELISA. This cross-reactivity was also confirmed in the sandwich ELISA where Poly Ab was used for both capture and detection antibody just like the combination used in the IC system (data not shown).

In the assessment of IC using the panel of various genotypes of NoV, however, discordant results were found in reactive genotypes from those in ELISA. Although IC

and ELISA are both classified into immunological methods, several factors such as the pH level in reaction buffer and pore size in the immunochromatographic membrane might influence the reactivity of these methods. Lessons learned from this observation would be the necessity of optimizing the reaction buffer or materials of IC after screening a broadly reactive antibody in an ELISA system. Additional attempts for simplifying the process of IC would also be needed by applying the mixture of antibodies raised against several genotypes.

In conclusion, a simple and rapid detection kit with immunochromatographic system was successfully developed using Poly Ab against rVLP for the two most prevailing genotypes of NoV, and a panel of various genotypes of rVLP as well as clinical stool samples were evaluated with this kit. Excellent specificity and detection limit of virus copy number supported the idea of applying the kit in the screening process of samples for NoV infection, whereas the lower sensitivity required further efforts in optimizing many factors, such as establishing broadly reactive mono/polyclonal antibodies, selection of antigen with several types of ideal secondary structure, and pretreatment of stool samples.



## **Chapter II: Detection of norovirus RNA from sera of children with gastroenteritis and analysis of their virological/clinical characteristics**

### **Introduction**

With the increasing interest in NoV in clinical settings, there have been several reports showing various manifestations of NoV other than gastroenteritis. These include NoV-associated encephalopathy with altered consciousness [Ito et al., 2006], potentially triggering convulsions [Abe et al., 2000; Kawano et al., 2007], as well as disseminated intravascular coagulation with obtundation, headache and photophobia [Centers for Disease Control and Prevention, 2002]. These reports are anecdotal but indicate a potential pathogenicity of NoV for organs other than the intestines.

Many studies have been conducted seeking evidence of extraintestinal manifestations of rotavirus infection, which is another leading cause of acute viral gastroenteritis. These attempts included the detection of rotavirus RNA in blood [Chiappini et al., 2005], cerebrospinal fluids (CSF) [Iturriza-Gomara et al., 2002; Lynch et al., 2003], and throat swabs [Ushijima et al., 1994], as well as detection of rotavirus nonstructural proteins in the liver and kidney [Gilger et al., 1992]. Although early works suggested that this was due to unusual rotavirus strains or rare host genetic or immunologic defects in the infected child [Salsburry et al., 1980], recent analyses have revealed that rotavirus antigen is commonly detected in the sera of immunocompetent children with rotavirus diarrhea (43% to 67%) [Blutt et al., 2003; Fischer et al., 2005, Nakagomi et al., 2005].

Human NoV, unlike rotavirus, is not capable of growing in cell lines and has no animal model available, which has hindered study of its systemic spread after intestinal

infection. Detection of NoV RNA from specimens other than stools has been limited to only one case in which NoV was present in the serum and CSF from a previously healthy NoV-infected girl with encephalopathy [Ito et al., 2006]. In this regard, proper assessment is clearly needed to evaluate potential spread in the bloodstream after the NoV invasion of the intestines.

In this study, I sought to detect NoV RNA in blood and CSF from patients with NoV gastroenteritis. Genetic analyses and quantification of NoV RNA were undertaken on positive samples, and clinical manifestations were compared among the patients with or without NoV RNA in serum.

## **Materials and methods**

### **Sample collection**

From the diarrheal patients who attended the Departments of Pediatrics in Teikyo University Hospital, Eijudo Clinic, and Red Cross Society Wakayama Medical Center from December 2005-December 2006, 56 cases who needed venepuncture for examination or infusion as treatment were recruited in this study. The interval between stool and blood sample collection was less than 2 days. Stool samples were collected from the 56 cases only once, but successive blood samples were taken from some patients (total=90). CSF was also obtained from 2 patients who had convulsions.

Soon after collection, blood samples were centrifuged and all the samples were stored at -20°C. Ethical approval was obtained from the ethical committee of the University of Tokyo (Title: Research on molecular epidemiology, diagnosis, treatment, pathogenesis of viral gastroenteritis in children; registration No 1139; dated June 13<sup>th</sup>, 2005) and the patients' guardians gave informed consent.

## **RT-PCR**

This method is described in detail on pages 14 to 17.

## **Nucleotide sequence analysis**

This method is described in detail on pages 17 to 18. The nucleotide sequence data of NoV reported in this chapter have been submitted to the DDBJ/GenBank databases under accession no: FJ152428-FJ152439.

## **Real-time PCR**

Real-time PCR was performed to quantify NoV GII viral cDNA in PCR positive clinical samples as previously described [Kageyama, 2003]. Briefly, a mixture containing 4 µl of cDNA, 17.5 µl of TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA), a 100 pmol/µl concentration of primers COG2F and COG2R, and 4 pmol/µl of a fluorogenic probe [RING2-TP; 5'-TGG GAG GGC GAT CGC AAT CT-3'] was prepared. The following PCR protocol was used: 2 min at 50 °C and 10 min at 95 °C, 40 cycles of 95 °C for 15 s, and 56 °C for 60 s. Data were corrected by using ROX passive reference as an internal standard. Moreover, recovery rate of the NoV genome was tested by highly purified NoV plasmid standards containing PCR products of the ORF1-ORF2 junction of the GII strain (Saitama U201, AB039782).

## **Statistical analysis**

SPSS software version 12.0 J was used for data analysis and graphical representations. Continuous variables were analyzed by Kruskal Wallis test and Mann-Whitney U test, and dichotomous variables by the chi-square test. A *p* value less

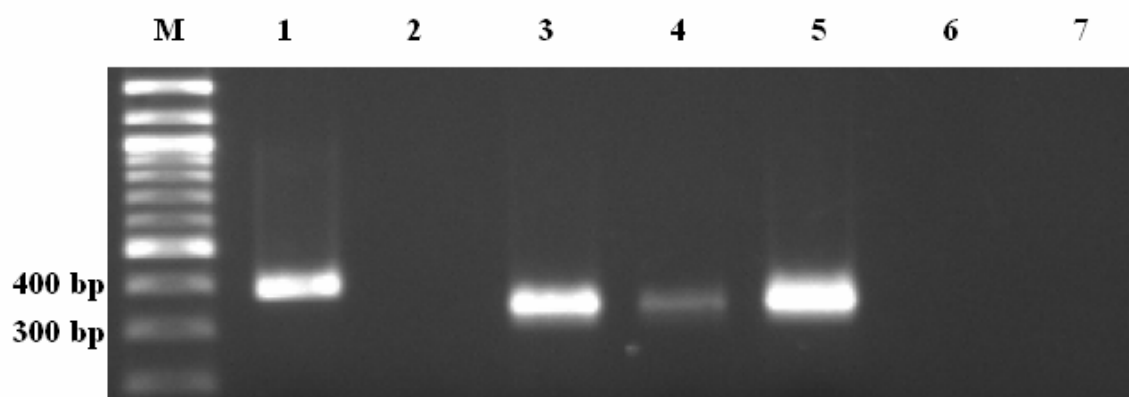
than 0.05 was considered to be statistically significant.

## **Results**

### **RT-PCR and patients' characteristics**

Age of the 56 recruited patients ranged from 1 month to 15 years and 9 months (mean  $\pm$  standard deviation: 49.2 $\pm$ 46.2 months) and their male/female ratio was 33:23. Among the 56 stool samples collected, 26 were positive for NoV GII by the first round PCR and 13 were positive by the second round, making the positive rate 69.6%. Among the 90 serum samples collected from the 56 patients, 6 were positive for NoV GII by the second round PCR, and were shown by sequence analysis to be NoV. These 6 patients showed stools positive for NoV GII by the first round PCR. Neither of the 2 CSF samples collected from patients who had convulsions contained NoV RNA, even by second round PCR, although stool samples from these patients contained NoV GII RNA by the first round PCR. All of the samples tested were negative for NoV GI. A photograph of electrophoresis for samples from Patient A is shown in Figure 5.

Clinical manifestations and timing of sample collection from patients whose serum samples were positive for NoV GII are shown in Table 6. All the patients except for patient D, who had non-insulin-dependent diabetes mellitus, were healthy before this study. Patient A, B, and F had convulsions and Patient C developed cerebellar ataxia with ataxic gait and disorder of tongue movement without any abnormal findings in computed tomography, magnetic resonance imaging or electroencephalogram. Stool samples from the serum-positive cases were screened for common diarrheal viruses (Group A, B and C rotavirus, adenovirus, sapovirus, astrovirus), using RT-multiplex PCR described by Yan et al. [2003 and 2004], which gave negative results.



**Figure 5.** Detection of norovirus GII capsid gene from stool and serum samples. M, marker; lanes 1 and 2, first PCR for stool and serum from Patient A, respectively; lane 3 and 4, second PCR for stool and serum from Patient A, respectively; lane 5, positive control for second PCR; lanes 6 and 7, negative control for second PCR. First PCR product: 386 bp, second PCR product: 342 bp.

**Table 6.** Clinical manifestations and timing of sample collection of serum positive (NoV GII) cases

ID	Sex	Age (months)	Date of stool collection	Date of serum collection	Neurological symptom	Notes
A	Male	19	Dec. 19	Dec. 19 <sup>a</sup>	+ <sup>b</sup>	
B	Female	26	Jan. 18	Jan. 17 <sup>a</sup>	+ <sup>b</sup>	
C	Male	30	Jan. 26	Jan. 24, 25 <sup>a</sup>	+ <sup>c</sup>	
D	Male	73	Nov. 25	Nov. 25 <sup>a</sup>	-	
E	Female	70	Nov. 28	Nov. 28 <sup>a</sup>	-	NIDDM
F	Female	18	Dec. 2	Dec. 2, 3, 4 <sup>a</sup>	+ <sup>b</sup>	

NoV GII, norovirus genogroup II; NIDDM, non-insulin-dependent diabetes mellitus

<sup>a</sup> Sample collected on this date was positive for NoV GII.

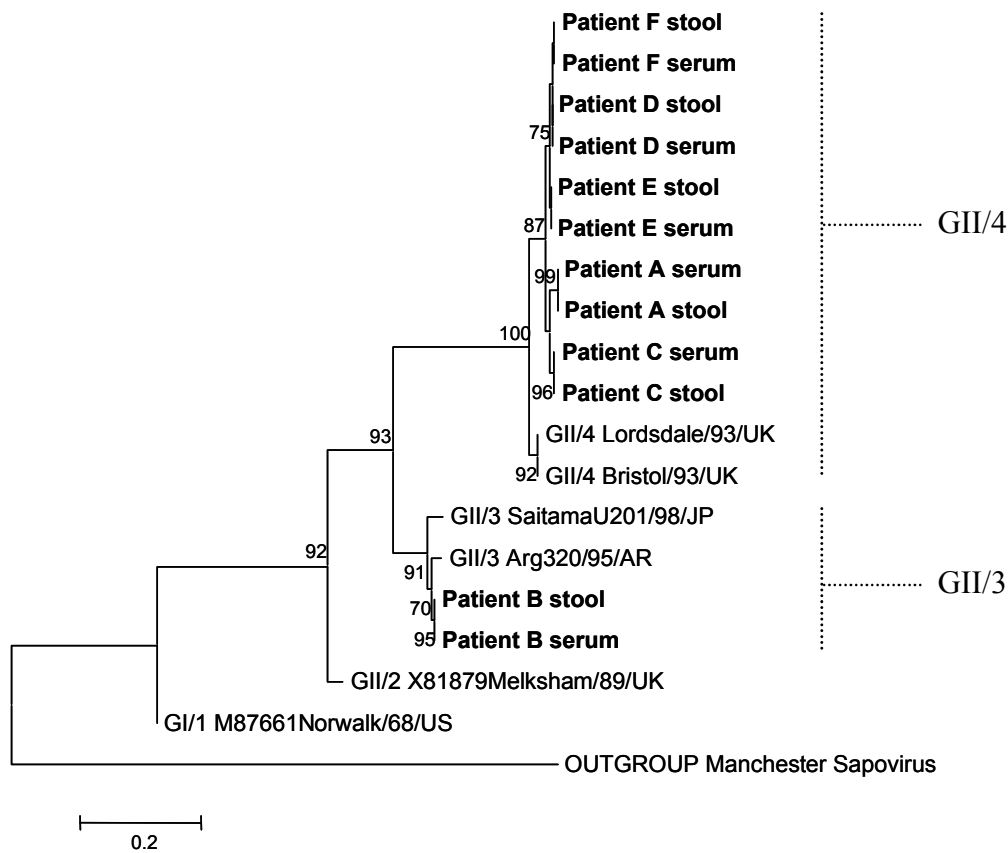
<sup>b</sup> Convulsion. <sup>c</sup> Cerebellar ataxia.

Patient C had fever of unknown origin 17 days before the symptom of cerebellar ataxia. Therefore, we tested CSF for *Mycoplasma pneumoniae* and varicella zoster virus; serum for anti-VCA IgG, anti-VCA IgM, anti-EBNA and EA-DR IgG for Epstein-Barr virus; and HI for influenza A-H1, A-H3 and B-1, all of which were negative.

### **Nucleotide sequence and phylogenetic analysis of NoV GII**

The nucleotide sequences of NoV obtained in this study were compared to each other, as well as to NoV reference strains available in the DDBJ DNA/GenBank database by BLAST. The partial nucleotide sequences (282 bp) of the NoV capsid gene were analyzed by phylogenetic grouping based on the NoV capsid region classification schemes described by Kageyama et al. [2004]. They were grouped into 3 distinct genotypes: 2 stool samples into genogroup II genotype 2 (GII/2), 4 stool samples and 1 serum sample into GII/3, and 33 stool and 5 serum samples into GII/4. The genotypes of stool and serum samples collected from the same patients were all identical, and the identities of nucleotide sequences between these pairs ranged between 99.2% and 100%.

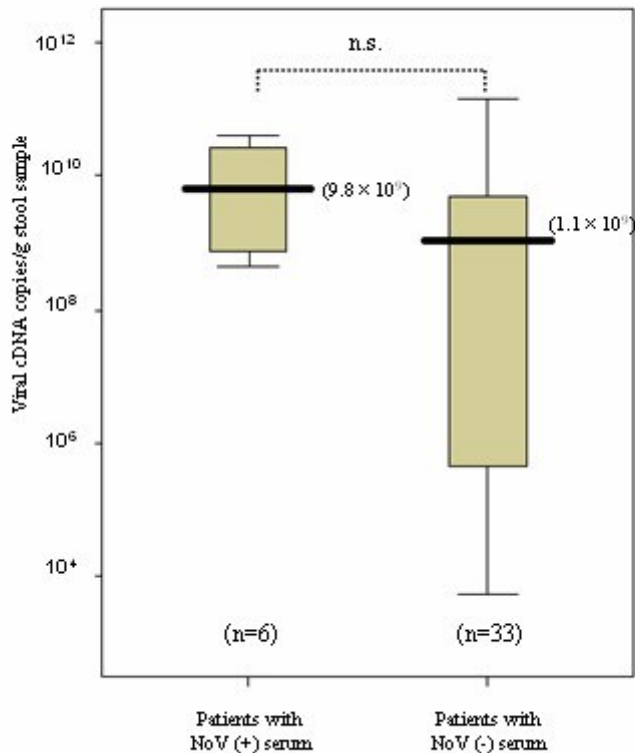
The phylogenetic tree of the 6 pairs of stool and serum samples together with the reference strains is shown in Figure 6. The identities among strains in the same genotypes ranged from 96.8% to 99.6%, and each strain showed high identities (98.1% to 100%) with previously registered Japanese strains in the DDBJ DNA/GenBank database.



**Figure 6.** Phylogenetic tree of norovirus GII nucleotide sequences detected in pairs of stool and serum samples. The tree was constructed from partial nucleotide sequences of the capsid region. Reference strains of NoV were selected from the DDBJ DNA/GenBank database under the accession numbers indicated in the text. Strains detected in this study are highlighted in bold. Percentage bootstrap values above 70% are shown at the branch nodes.

### Quantification of NoV cDNA

The median cDNA viral load detected in the stool samples from patients with serum-positive and serum-negative for NoV GII was  $9.8 \times 10^9$  (range  $4.2 \times 10^8$ - $4.0 \times 10^{10}$ ) and  $1.1 \times 10^9$  (range  $5.6 \times 10^3$ - $1.4 \times 10^{11}$ ) copies per gram of stool, respectively (Figure 7). This difference was not statistically significant ( $p=0.117$ ).



**Figure 7.** Quantification of norovirus GII cDNA in stool and serum samples. Box plot of cDNA in stools for NoV gastroenteritis patients (serum positive and serum negative patients). n.s., not significant.

### **Statistical analysis of patient characteristics in serum-positive and negative groups**

The characteristics of the patients whose stool samples showed positive results for NoV GII are summarized in Table 7. When convulsion and cerebellar ataxia were included, 4 of 6 patients (66.7%) in the serum-positive group had these neurological complications, whereas 6 of 33 (18.2%) had these complications in the serum-negative group ( $p=0.028$ ). Two of these 10 patients had fever at the time of neurological complications, and both were in the serum-negative group. Sex, age, sample collection month, and the NoV genotype did not differ significantly between groups.



**Table 7.** Comparison of clinical characteristics between serum positive and negative cases in stool positive (NoV GII) cases

		Serum (+)	Serum (-)	<i>p</i> value
Sex	Male	3	21	0.421
	Female	3	12	
Age (mean±SD)		39.2±25.4	34.6±26.3	0.701
Neurological complication	+	4	6	0.028*
	-	2	27	
Sample collection month	January	2	1	0.182
	March	0	4	
	July	0	1	
	October	0	1	
	November	2	17	
	December	2	9	
Genotype of NoV	GII/2	0	2	0.723
	GII/3	1	3	
	GII/4	5	28	
Total		6	33	

NoV GII, norovirus genogroup II; SD, standard deviation

\*statistically significant

## Discussion

Extraintestinal involvement is important in understanding the pathogenesis of viral gastroenteritis. For rotavirus research, previous studies have shown that antigenemia is a common event in immunocompetent children [Blutt et al., 2003; Fischer et al., 2005; Nakagomi et al., 2005] and that antigenemia is associated with viremia, i.e. existence of infectious virus in sera [Blutt et al., 2007]. With regard to NoV, however, only a few data are available.

There is a case report showing NoV gene in samples other than stool, such as

the serum and CSF in a girl with encephalopathy [Ito et al., 2006]. In this study, a prospective survey was conducted in order to detect NoV RNA in the serum and CSF, and compared clinical characteristics in children with NoV gastroenteritis. I observed that 15.4% of the cases (6/39) had NoV RNA in the serum and NoV RNA could not be detected in either of the two CSF samples.

The genetic analysis showed a very high homology between strains found in stools and serum, suggesting that the viral RNA in the blood had originated from the intestinal tract. The high homology between the strains in this study and those deposited in GenBank implied that no unique strains were associated with detection in the bloodstream. This finding is comparable to those of a rotavirus study reporting that the presence of viral RNA and antigen is not a property of a specific viral type [Xin et al., 1993; Ushijima et al., 1994; Blutt et al., 2007].

In this study, there was no difference in NoV RNA in the stool of patients with or without NoV RNA in the blood. Several previous studies showed that, in rotavirus diarrhea children, the antigen level in blood samples decreased as diarrhea subsided, implying a relationship between the presence of virus in the intestines and in the blood [Blutt et al., 2003; Fischer et al., 2005]. These studies succeeded in collecting samples in both the acute and subacute phases. Hence, I may be able to observe a similar relationship with a larger sample size or consecutive collection of samples from the same group.

Commercial ELISA kits manufactured for rotavirus diarrhea have been widely used to detect antigenemia in rotavirus infection [Blutt et al., 2003; Fischer et al., 2005]. This prompted us to conduct a small pilot study to apply a commercial NoV ELISA kit (NV-AD, Denka Seiken, Japan) for the remaining 28 serum samples from NoV diarrhea

children in this study. When I followed the manufacturer's protocol in preparing stool samples, none of the serum samples became positive, although three of them had previously shown positive by RT-PCR (data not shown). This was the same in both two-fold dilution and four-fold dilution of serum. Further attempts with optimal modifications of ELISA conditions are needed to conclude whether antigenemia is indeed absent in NoV infection.

When I looked into the clinical characteristics of cases with positive results for RNA in the serum, neurological complications were more frequently encountered with statistical significance ( $p=0.028$ ). Convulsion is a rather common symptom in gastroenteritis, and NoV diarrhea patients are also reported to experience this symptom [Kawano et al., 2007], sometimes more frequently than patients with rotavirus [Abe et al., 2000]. Of note, our study pointed out the existence of NoV genes in the blood circulation, which may provide a clue to the pathogenesis of neurological complications. At the same time, a remarkably high rate of NoV as a cause of gastroenteritis was observed, possibly due to the outbreak in 2006 [Motomura et al., 2008]. In addition, the number of patients enrolled was quite small. Taking these research limitations into account, future studies with more patients for longer periods are warranted to confirm our results.

Recently, experiments with murine NoV in a mouse model have shown that infectious virus was detected from peripheral organs such as the spleen, liver, and lung after oral inoculation, and that histopathological changes were observed not only in the intestine but also in the spleen, even in immunocompetent mice [Mumphrey et al., 2007; Wobus et al., 2006]. These findings are unique, and significant in the light of current comprehension that human NoV is confined to the intestine and does not spread

to other organs. It warrants a consideration of potential extraintestinal spread of human NoV.

In summary, the results of this study demonstrate the spread of NoV from the intestines to the bloodstream, possibly accounting for extraintestinal complications of NoV gastroenteritis. Further research is expected to provide a better picture of this infectious disease.

## **Chapter III: Changing trends of diarrheal viruses with the emergence of norovirus in children attending a day care center in Japan**

### **Introduction**

Akihara et al. [2005], during their study from June 1999 to July 2000, demonstrated multiple outbreaks of viral gastroenteritis due to NoV and other viral agents, such as astrovirus, adenovirus and sapovirus, in a single DCC in Tokyo. Surprisingly, in their study 27.6% of all the samples collected from asymptomatic children showed positive for diarrheal viruses by RT-multiplex PCR. Similarly, several other studies have detected NoV in the stools of asymptomatic people [García et al., 2006; Monica et al., 2007], and a quantitative analysis has revealed that fairly high doses of NoV were excreted by asymptomatic adults during the outbreak [Ozawa et al., 2007]. To date, however, reports on the quantification of NoV in the pediatric population have been limited to patients with special conditions such as immunocompromised children [Primrose et al., 2008].

The objectives of the current study were to detect common diarrheal viruses in children with and without acute gastroenteritis in the same DCC as Akihara's study, to compare the results after a six-year interval, and to describe genetic and quantitative characteristics of norovirus in this population.

### **Materials and methods**

#### **Subjects and sample collection**

Stool samples were collected from children attending a DCC in Tokyo, Japan, from July 2005 to June 2006. This DCC was the same one where Akihara et al.

conducted their survey in 1999, and the number of staff as well as attending children had not changed significantly since their study.

The schedule of sample collection was essentially the same as previously described [Akihara et al., 2005]. Briefly, one stool sample was collected from each child once a week, and when children presented diarrhea (defined as at least three passings of unformed stool per day in comparison with the usual bowel habits of the child), more than one stool was collected from the subject in a week. Caretakers and a nurse at the DCC recorded symptoms such as diarrhea, vomiting, fever, and abdominal pain.

Twenty healthy children, 10 males and 10 females, were included in this study. Their age ranged from 5 months to 19 months (average 12 months) at the time of enrollment for this study.

In total, 479 stool samples were collected during the study period, out of which 36 were from children with diarrhea and 443 were from asymptomatic children. They were prepared as a 10% suspension in distilled water, and clarified by centrifugation at  $10,000 \times g$  for 10 min. The supernatants were collected and stored at  $-20^{\circ}\text{C}$  until testing.

Ethical approval was obtained from the ethical committee of the University of Tokyo (Title: Molecular epidemiological change of diarrheal viruses in a DCC in Japan; registration No 1202; dated September 12<sup>th</sup>, 2005) and the children's guardians gave informed consent.

## **RT-PCR**

This method is described in detail on pages 14 to 17.

### **Nucleotide sequence analysis**

This method is described in detail on pages 17 to 18. The nucleotide sequence data of NoV reported in this chapter have been submitted to the DDBJ/GenBank databases under accession no: FJ152440-FJ152461.

### **Real-time PCR**

This method is described in detail on page 31.

### **Statistical analysis**

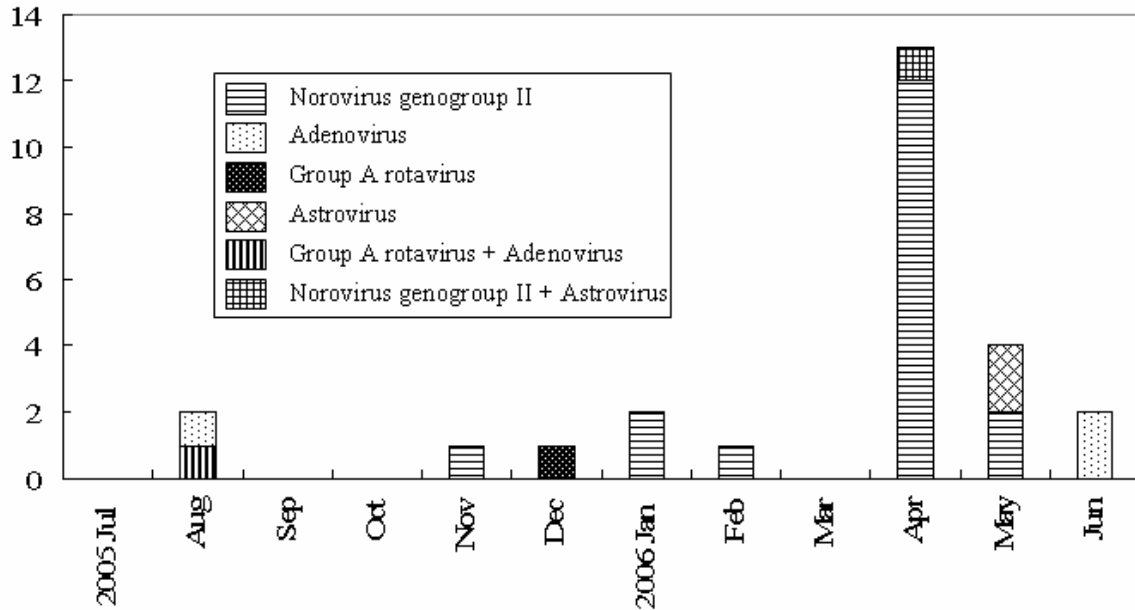
SPSS software version 12.0 J was used for data analysis and graphical representations. Continuous variables were analyzed by Kruskal Wallis test and Mann-Whitney U test, and dichotomous variables by the chi-square test. A *p* value less than 0.05 was considered to be statistically significant.

## **Results**

### **Detection of diarrheal viruses**

Among 36 stool samples collected from children with acute gastroenteritis, 16.7% were positive for diarrheal viruses by RT-multiplex PCR. NoV GII was the most prevalent virus among them (11.1%, 4 of 36), followed by adenovirus (2.8%, 1 of 36) and group A rotavirus (2.8%, 1 of 36). Out of 443 samples of asymptomatic children, I detected diarrheal viruses in 4.5% (20 of 443) by RT-multiplex PCR. They included NoV GII (3.2%, 14 of 443), astrovirus (0.5%, 2 of 443), and adenovirus (0.5%, 2 of 443). Two samples demonstrated mixed infection; one was by group A rotavirus and adenovirus, and the other was by NoV GII and astrovirus.

Number of cases



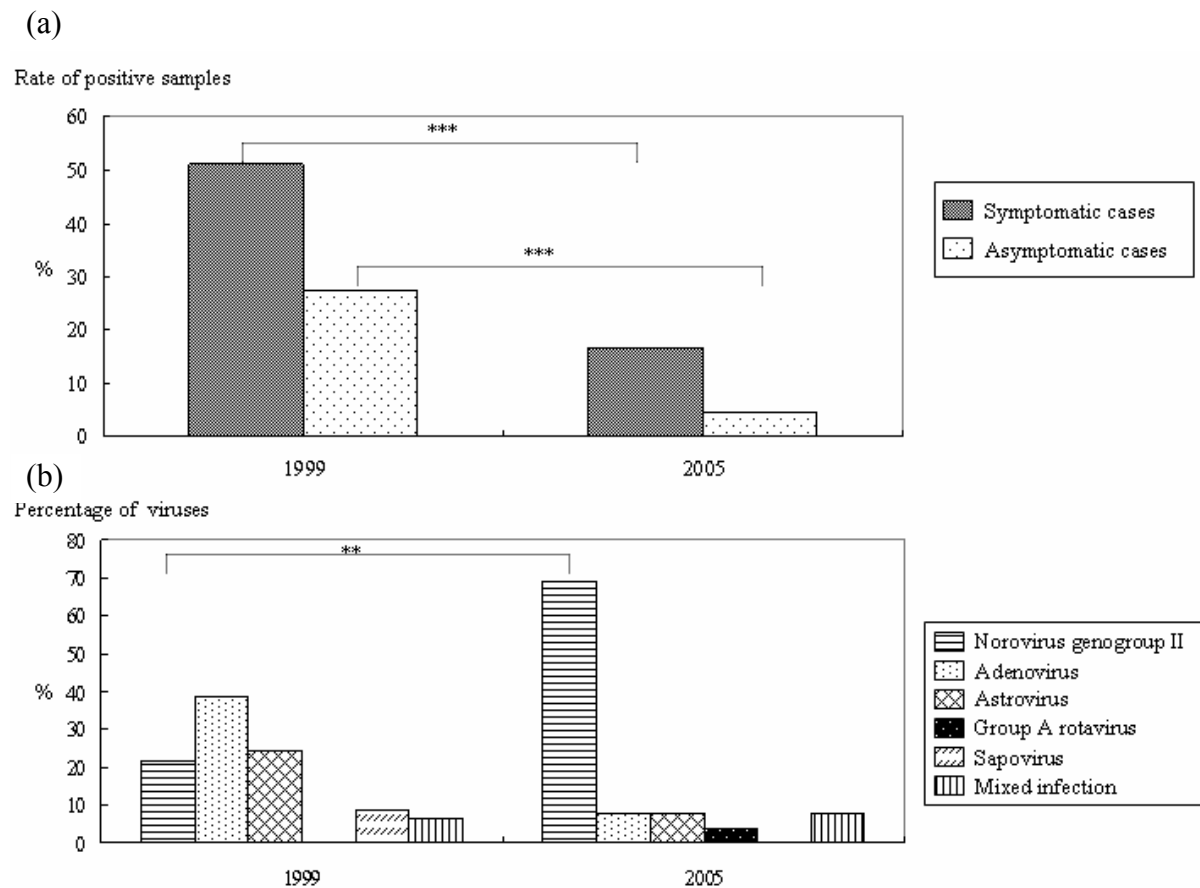
**Figure 8.** Monthly distribution of detected diarrheal viruses in stools collected from children attending a day care center in Tokyo, Japan, during the period from July 2005 to June 2006.

Monthly distribution of detected diarrheal viruses is shown in Figure 8. An outbreak of NoV GII occurred from April 19<sup>th</sup> to 21<sup>st</sup> in which 8 out of 32 attending children and 6 out of 19 staff were affected (overall incidence: 27.5%). In this outbreak, a local health officer reported that NoV was not detected in the food provided by the DCC. Moreover, none of the children in one class were affected, suggesting that the transmission route of this outbreak was not food-borne but person-to-person contact.

Figure 9 shows a comparison of the results between the previous study done by Akihara et al. (1999) and the present study (2005). Figure 9a expresses the positive rate for viral agents in symptomatic and asymptomatic cases, whereas Figure 9b shows the rate of each virus compared to all the viruses detected. Although positive rates for both symptomatic and asymptomatic cases significantly decreased after 6 years ( $p < 0.001$ ),



the NoV rate of all the viruses increased during this period ( $p<0.01$ ). Further analysis by RT-monoplex and seminested PCR for samples negative for RT-multiplex PCR demonstrated positivity for NoV GII in 8, 3, 4, 19, 23, and 1 samples in January, February, March, April, May, and June, respectively. Of these 58 samples, 30 were from symptomatic cases whereas 28 were from asymptomatic cases. All the samples were negative for NoV GI even by RT-seminested PCR.



**Figure 9.** Comparison between the current study (2005) and the previous study in the same DCC [Akihara et al. 1999]. (a) Positive rates among the samples collected from symptomatic cases and asymptomatic cases.  $***p<0.001$ . (b) Rates of each virus among all the viruses detected.  $**p<0.01$ .

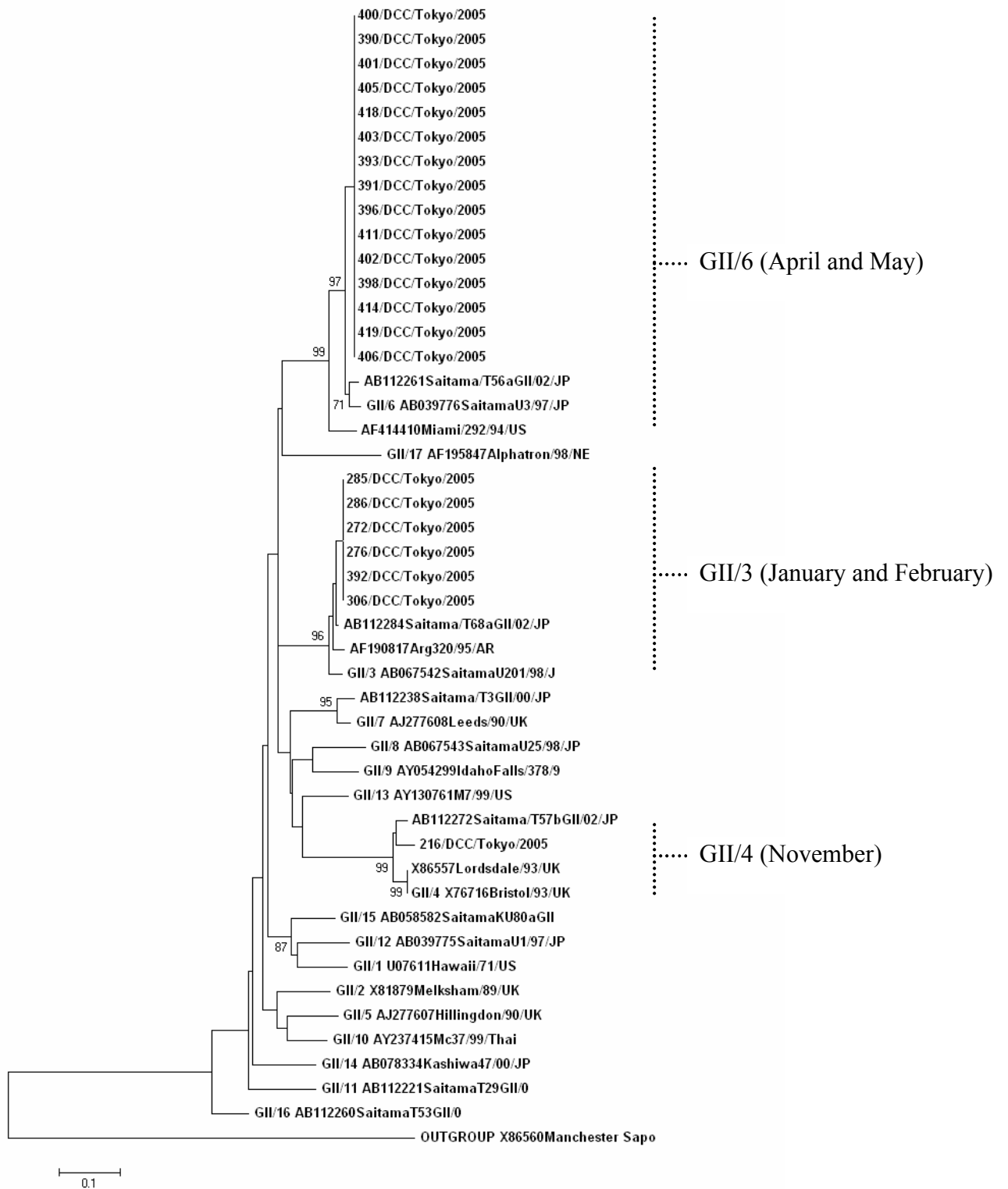
### **Nucleotide sequencing and phylogenetic analysis of norovirus**

All of the samples positive for NoV were subjected to nucleotide sequencing and phylogenetically analyzed by the NoV capsid region classification scheme of Kageyama et al. [2004]. It was found that they were grouped into three distinct genotypes: GII/3, GII/4, and GII/6. Figure 10 shows the phylogenetic tree of the samples positive for NoV GII by RT-multiplex or monoplex PCR, which had a comparatively higher amount of viral load than that of seminested PCR positive samples.

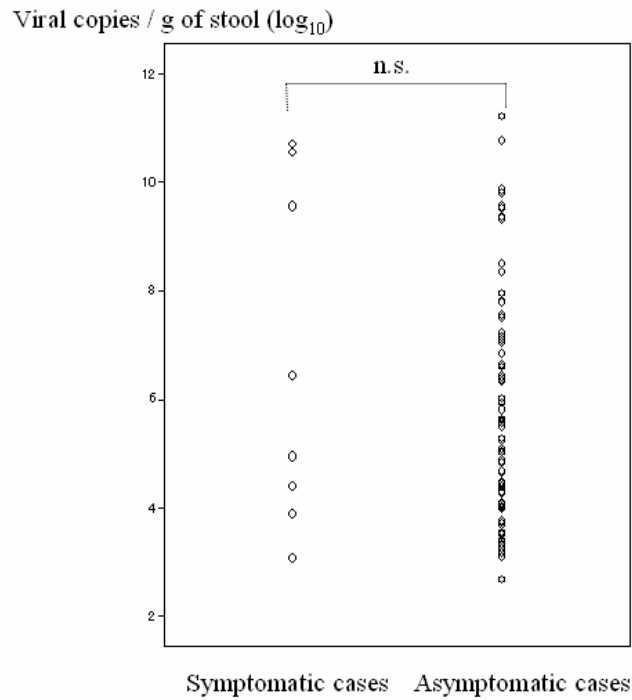
The dominant genotype in this DCC changed from GII/4 in November, to GII/3 in January and February, and to GII/6 in April and May. However, the RT-seminested PCR positive samples contained a small amount of GII/4 in January and GII/3 and GII/4 in April and May, as shown in Figure 12. The identity at the nucleotide as well as the amino acid level among the samples in the same genotypes was 100%. Each sample showed high identities (97.8% to 99.8%) of Japanese strains which were previously registered in the DDBJ DNA/GenBank database.

### **Quantification of norovirus cDNA**

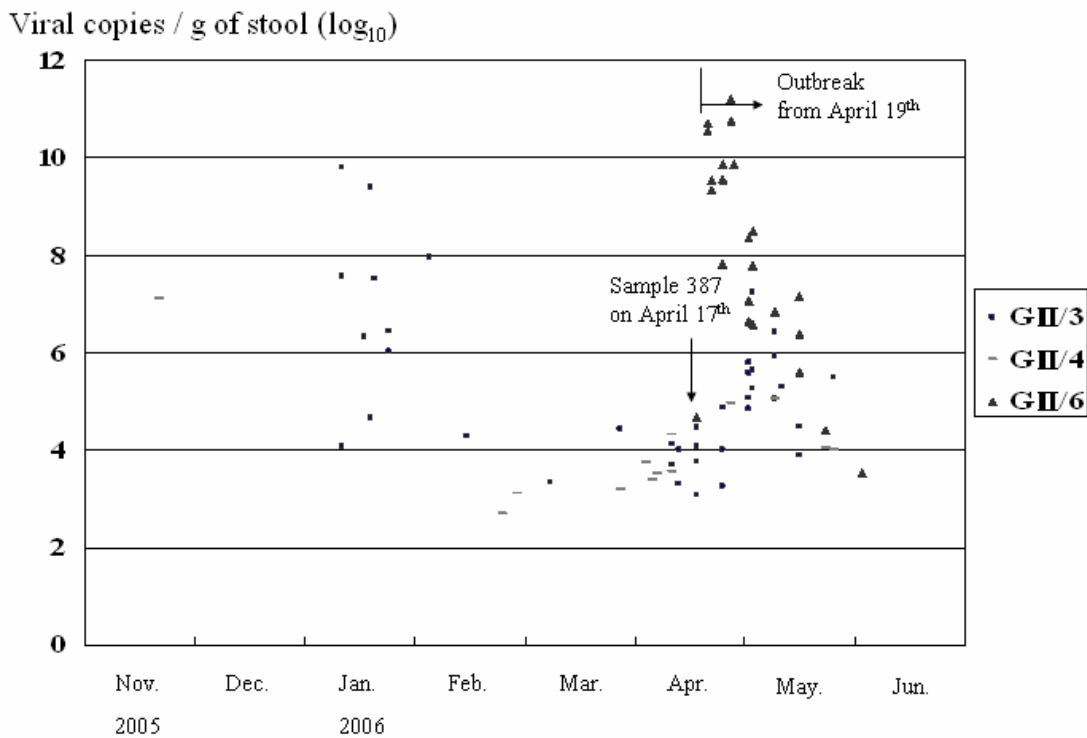
All the positive samples for NoV were subjected to real-time PCR to determine the cDNA viral load in the stool. The average viral load found in samples positive by RT-multiplex, monoplex, and seminested PCR were  $2.0 \times 10^{10}$  copies per gram of stool,  $4.0 \times 10^7$  copies per gram of stool, and  $1.2 \times 10^6$  copies per gram of stool, respectively. There was no statistically significant difference in the viral loads between children with diarrhea and asymptomatic children (median:  $1.5 \times 10^6$  copies/g versus  $2.6 \times 10^4$  copies/g ( $p=0.600$ )) (Figure 11.).



**Figure 10.** Phylogenetic tree of nucleotide sequences of NoV GII detected in RT-multiplex and RT-monoplex PCR in DCC. The tree was constructed from partial nucleotide sequences of the capsid region. Reference strains of NoV were selected from the DDBJ DNA/GenBank database under the accession numbers indicated in the text. Percentage bootstrap values above 70% are shown at the branch nodes.



**Figure 11.** Quantification of NoV GII cDNA in stool from symptomatic and asymptomatic children. n.s.:not significant.



**Figure 12.** Quantification and genotypes of NoV GII in stool from children attending a DCC in Tokyo, Japan, during the period from July 2005 to June 2006.

The viral load and genotype of each sample are shown in Figure 12. Sample 387 was collected two days before the onset of NoV outbreak in April. It was determined to be positive only by RT-nested PCR, and its viral load was as small as  $4.7 \times 10^4$  copies per gram of stool. Nevertheless, the child who provided this sample was considered to be the index case, since the NoV genotype in this sample turned out to be GII/6, which was the same as those of symptomatic cases during the outbreak. This 2-year-old girl came to the DCC as usual on the morning of the 17<sup>th</sup> of April, and passed a normal stool which was collected by our group on a routine basis. Thereafter she developed fever up to 39.0°C in the evening without having diarrhea on that day. She was absent from the DCC from 18<sup>th</sup> to 21<sup>st</sup> of April due to symptoms of acute gastroenteritis.

### **Re-infection of norovirus**

During the one-year study period, I observed that 18 out of 20 children experienced NoV GII infection. There were children who had experienced multiple genotypes of NoV infection (GII/3+GII/4, 3; GII/3+GII/6, 2; GII/4+GII/6, 1; GII/3+GII/4+GII/6, 4). Moreover, 6 children experienced re-infection of the same genotype of NoV (GII/3, 5; GII/4, 1) after more than one NoV-negative sample in the interval.

### **Discussion**

Children attending DCCs are considered to encounter more infectious diseases than those who are taken care of at home [Alexander et al., 1990; Bartlett et al., 1985; Fleming et al., 1987; Hurwitz et al., 2004; Lu et al., 2004; Staat et al., 1991].

Encouraging results from the current study were that hygienic measures, such as avoiding cloth diaper usage and common diaper covers, together with education on infection control, might have contributed to the decreased detection rate of diarrheal viruses by RT-multiplex PCR in both symptomatic and asymptomatic cases in the same DCC after 6 years. Interestingly, the relative NoV percentage of all the viruses increased during this period, suggesting that the norovirus tends to retain infectivity even in improved hygienic circumstances. This finding becomes more important when one considers the recent introduction of effective rotavirus vaccine [Linhares et al., 2008; Vesikari et al., 2006] and expects that the picture of causative diarrheal viruses may change.

It is also noteworthy that, in total, I found 17.5% (84 of 479) of the samples positive for diarrheal viruses by RT-multiplex, monoplex and seminested PCR. Quantitative analysis revealed that there was no statistically significant difference in NoV viral load in stools from symptomatic and asymptomatic children. This finding is consistent with the previous study reporting that asymptomatic individuals had mean viral loads similar to those of symptomatic individuals [Ozawa et al., 2007]. Samples in the present study were not collected from patients coming to clinics or hospitals but from healthy children who regularly come to the DCC. This fact underscores the importance of taking standard precautions in handling feces from all children in DCCs.

Another interesting finding of the current study was the demonstration of genetic variation and re-infection of NoV in children. I observed that the dominant genotype in both RT-multiplex and RT-monoplex PCR positive samples clearly shifted from GII/4 to GII/3 and GII/6 as shown in Figure 10. However, when I included RT-seminested PCR positive samples, there was a mixed distribution of these genotypes

as shown in Figure 12. I also noted that some children experienced re-infection of GII/3 and GII/4 but not GII/6. Many previous studies in Japan have reported that GII/3 and GII/4 were the most prevalent genotypes, whereas GII/6 was reported to a lesser degree [Okame et al., 2006; Onishi et al., 2008; Phan et al., 2006a]. Therefore, our results imply that the common genotypes, GII/3 and GII/4 can circulate even without being excreted much, which may enable re-infection of children. At the same time, the absence of antibody in children against rare genotypes, such as GII/6, may cause a large outbreak in a DCC, although direct evidence is lacking. There is controversy over whether previous infection by heterogeneous genotypes of NoV would prevent further NoV infection in adults [Johnson et al., 1990; Lindesmith et al., 2003; Parrino et al., 1977; Rockx et al., 2005]. In this context, our observation of children who experienced multiple genotypes of NoV infection is noteworthy, since for ethical reasons, it is impossible to transmit experimental NoV infection to volunteers in a pediatric population.

Nested PCR is considered to be useful in detecting asymptomatic infection of NoV. Gallimore et al. reported that all 27 samples from asymptomatic persons in the outbreak of NoV GII in the hospital were positive only by RT-nested PCR [2004]. In our study, the viral loads in RT-nested PCR positive samples were smaller than those in RT-multiplex and RT-monoplex PCR positive samples. The majority of samples from asymptomatic children (51 out of 70) were positive only by RT-nested PCR. Generally, sample collection starts after notification of an outbreak, whereas I collected samples routinely even from asymptomatic children in this study. Therefore, it is plausible to mention that sensitive methods such as nested PCR, as well as laborious sample collection, enabled us to obtain the important sample, sample 387,

which led to the identification of the child excreting a small amount of NoV as the source of the major outbreak in April in this DCC.

In summary, NoV emerged as the most predominant diarrheal virus in children attending the DCC, while the overall detection rate of viruses decreased in the 6 years since the first study. Sequencing and quantitative analysis revealed that a child excreting an insignificant amount of virus may cause a major outbreak of NoV. Further research recruiting more DCCs for longer periods of sample collection is warranted to generalize the observations in this study.



## **Chapter IV: Molecular epidemiology of norovirus in children hospitalized with acute gastroenteritis in Kandy, Sri Lanka**

### **Introduction**

Recently, intensive surveillances toward detection of NoV in children have been carried out in many Asian countries, including Japan [Onishi et al., 2008; Phan et al., 2006a, 2006b], South Korea [Lee et al., 2007; Yoon et al., 2008], China [Liu et al., 2006], Thailand [Khamrin et al., 2007; Hansman et al., 2004b; Malasao et al., 2008], Vietnam [Hansman et al., 2004a; Nguyen et al., 2007; Nguyen et al., 2008], Bangladesh [Dey et al., 2007], and India [Rachakonda et al., 2008; Monica et al., 2007; Girish et al., 2002; Chhabra et al., 2008]. These studies identified the significant impact of NoV in pediatric clinical settings in Asian regions, and showed the interesting feature of NoV seasonal distribution in tropical countries, which showed a peak in the dry season [Hansman et al., 2004a; Nguyen et al., 2007] or cool season, different from winter from a distinct viewpoint [Dey et al., 2007].

For rotavirus research, an international network among Asian countries conducted epidemiological studies using a standardized protocol. The studies promoted policy-making for rotavirus control by enhancing new vaccines [Bresee et al., 2004; Nelson et al., 2008]. This movement would be a model case for NoV, which is the second most common infection after rotavirus. Basic information on NoV from various regions is definitely necessary to build a future strategy for controlling this infectious disease.

Sri Lanka is a small island country located in the Indian Ocean where no cases of NoV have been reported up to now. In this study, I aimed to report the detection of

NoV among pediatric patients with acute gastroenteritis in a Kandy hospital, and describe the clinical manifestations of NoV infection, as well as analyzing the molecular characteristics of the NoV detected.

## **Materials and methods**

### **Patients and sample collection**

According to the WHO generic protocol prepared for hospital-based surveillance for rotavirus gastroenteritis in children [WHO, 2002], all the patients under 5 years of age who were admitted to the department of Paediatrics, Peradeniya University Hospital in Kandy for the treatment of diarrhea were recruited in this study. Kandy is the second largest city after the capital Colombo in Sri Lanka, with a population of approximately 1.5 million.

A total of 362 stool samples were collected from infants and children from the period of September 2005 to August 2006. The symptoms of dehydration were assessed by pediatricians, based on WHO guidelines [World Health Organization, 1995], and the severity of diarrheic patients was evaluated based on the 20-point numerical score [Ruuska and Vesikari, 1990]. Ethical approval was obtained from the Committee on Research and Ethical Review of University of Peradeniya (Title: Molecular epidemiology of viral gastroenteritis in children in Kandy; dated October 10<sup>th</sup>, 2005) and the patients' guardians gave informed consent.

### **RT-PCR**

This method is described in detail on pages 14 to 17.

### **Nucleotide sequence analysis**

This method is described in detail on pages 17 to 18. The nucleotide sequence data of NoV reported in this chapter have been submitted to the DDBJ/GenBank databases under accession no: FJ173238-FJ173275.

### **Statistical analysis**

SPSS software version 12.0 J was used for data analysis and graphical representations. Continuous variables were analyzed by Kruskal Wallis test and Mann-Whitney U test, and dichotomous variables by the chi-square test. A *p* value less than 0.05 was considered to be statistically significant.

## **Results**

### **Detection of target viruses**

Five out of eight target viral agents were detected in 220 out of 362 (60.1%) stool samples (Table 8). Among them, group A rotavirus was the most predominant causative virus, with an overall prevalence of 44.2%. NoV GII was the second most common viral enteropathogen in this study (38/362, 10.5%), followed by adenovirus, sapovirus, and human astrovirus, with overall prevalences of 4.1%, 1.9%, and 0.8%, respectively. Mixed infection between two diarrheal viruses was identified in 12 samples (3.3%), in which coinfection between group A rotavirus and another viral agent was dominant.

**Table 8.** Distribution of target viral agents detected from 362 children with acute gastroenteritis in Kandy, Sri Lanka during 2005 to 2006.

	Monoinfection					Mixed infection						
	RAV	NoV GII	AdV	SaV	HAstV	RAV-NoV GII	RAV-AdV	RAV-SaV	RAV-HAstV	NoV-AdV	AdV-SaV	SaV-HAstV
No. (%) of samples	160 (44.2)	33 (9.7)	11 (3.0)	3 (0.8)	1 (0.3)	4 (1.1)	2 (0.6)	2 (0.6)	1 (0.3)	1 (0.3)	1 (0.3)	1 (0.3)

RAV, group A rotavirus; NoV GII, norovirus genogroup II; AdV, adenovirus; SaV, sapovirus; HAstV, human astrovirus

**Table 9.** Attributes of 38 NoV positive cases and mean severity score of patients in each group

	Age (month)					<i>p</i> value	Gender			<i>p</i> value	Genotypes				
	<6	6-11	12-23	24-35	>35		Male	Female	<i>p</i> value		GII/3	GII/4	GII/6	GII/9	GII/16
No. (%) of cases <sup>a</sup>	3 (7.9)	14 (36.8)	11 (28.9)	4 (10.5)	6 (15.8)		23 (60.5)	15 (39.5)		22 (57.9)	9 (23.7)	2 (5.3)	1 (2.6)	4 (10.5)	
Vesikari's score <sup>b</sup>	8.7±4.0	12.1±2.5	12.0±3.4	14.2±1.0	13.0±2.0	0.247	12.3±3.0	11.8±3.0	0.650	12.2±2.9	12.5±3.8	9.5±3.8	11	12.5±2.7	0.698

<sup>a</sup> Data based on 38 NoV positive cases. <sup>b</sup> Data based on 33 out of 38 cases, which showed mono-infection with NoV.

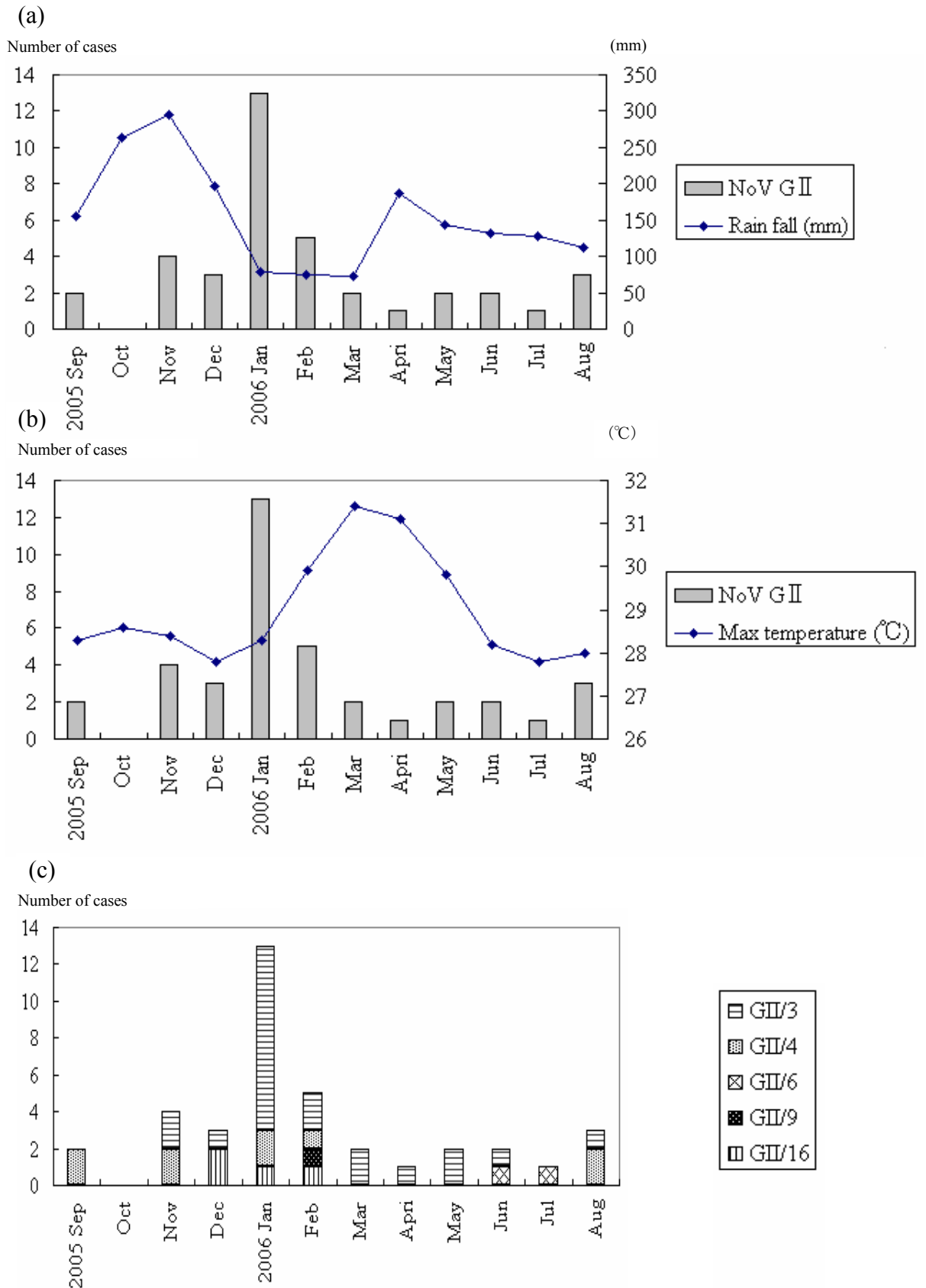
### **Seasonal pattern of norovirus infections**

The central part of Sri Lanka, where Kandy is located, has a tropical climate with two monsoon seasons. The southwest monsoon generally starts from April to July, while the northeast monsoon brings rain from October to December. The maximum temperature throughout the year varies slightly from 27.8 to 31.4°C. The minimum temperature ranges from 18.4 to 21.5°C, with the lowest temperature in January. In this study, although NoV was found throughout the year except for October, the distinct peak was observed in January, which falls at the start of the drier season and the end of the cooler season (Figure 13 a and b). Interestingly, more than half (20 out of 38, 52.6%) of NoV infections were detected in the drier season between the two monsoon periods (January to March).

### **Clinical features and symptoms of norovirus infections**

NoV was detected in 38 (10.5%) of 362 samples analyzed in boys (25/210, 11.9%), who were more frequently infected than girls (13/152, 8.6%), but this difference was not statistically significant ( $p=0.197$ ). Patients who were enrolled in this study were classified into five age groups: <6, 6–11, 12–23, 24–35, and >35 months old. The age groups included 26, 132, 117, 65, and 22 patients, respectively. Diarrhea due to NoV was seen in all five age groups, and NoV infections were more likely found in patients 6–11 and 12–23 months old ( $p<0.001$ ) (Table 9).

Among 33 patients showing mono-infection with NoV, the main clinical manifestations observed were diarrhea (100%), vomiting (72.7%), body temperature  $\geq 37.5^\circ\text{C}$  (51.5%), coughing (33.3%), and coryza (21.2%). According to WHO guidelines [World Health Organization, 1995] for assessing dehydration, the majority of



**Figure 13.** Monthly distribution of NoV in stools collected from children in Kandy, Sri Lanka, during the period from September 2005 to August 2006. (a) Correlation between

cases of NoV diarrhea and rain fall. (b) Correlation between cases of NoV and daily max temperature. (c) Distribution by genotype.

patients (90.6%) presented some dehydration, whereas 9.4% of the patients experienced severe dehydration. Evaluation of severity in patients by using the 20-point numerical score (Vesikari's score) showed that the scores ranged between 5 and 17 points, with an overall mean score of  $12.1 \pm 3.0$ . The severity scores were further analyzed by age groups, gender, and genotypes as shown in Table 9. Although the mean score of patients <6 months of age was lower than that of patients in the other age groups, this difference was not statistically significant ( $p=0.247$ ). Similarly, the mean scores of severity between male and female patients, as well as between patients with various genotypes, were not statistically different ( $p=0.650$  and  $0.698$ , respectively).

### Comparison of clinical features among virus groups

Clinical features in monoinfection of NoV, RAV, AdV and mixed infection are summarized in Table 10. Monoinfection of SaV and HAstV were excluded since the number in each group was small. The mean severity score and vomiting episodes/day in the NoV group were the highest, although there was no statistically significant difference among viral groups.

**Table 10.** Comparison of clinical features among virus groups

	Norovirus	Rotavirus	Adenovirus	Mixed infection	<i>p</i> value
Gender (Male:Female)	20:13	93:67	9:2	6:6	0.415
Age (month) (mean±SD)	17.4±14.5	16.8±12.6	21.1±20.2	22.6±17.1	0.927
Severity score (mean±SD)	12.1±3.0	10.9±3.0	11.4±2.8	10.6±3.0	0.156
Diarrhea episode/day (mean±SD)	7.3±3.9	7.7±5.1	5.9±2.0	7.6±4.9	0.899
Diarrhea period (day) (mean±SD)	3.9±2.3	3.9±2.1	3.6±1.1	4.1±2.1	0.890
Vomiting episode/day (mean±SD)	4.9±4.0	4.4±4.9	2.9±2.6	2.7±2.6	0.320
Vomiting period (day) (mean±SD)	1.9±1.7	1.6±1.5	2.3±2.1	1.9±1.8	0.567
Fever ( $\geq 37.5^{\circ}\text{C}$ )	17/33	67/160	8/11	4/12	0.154
Total number	33	160	11	12	

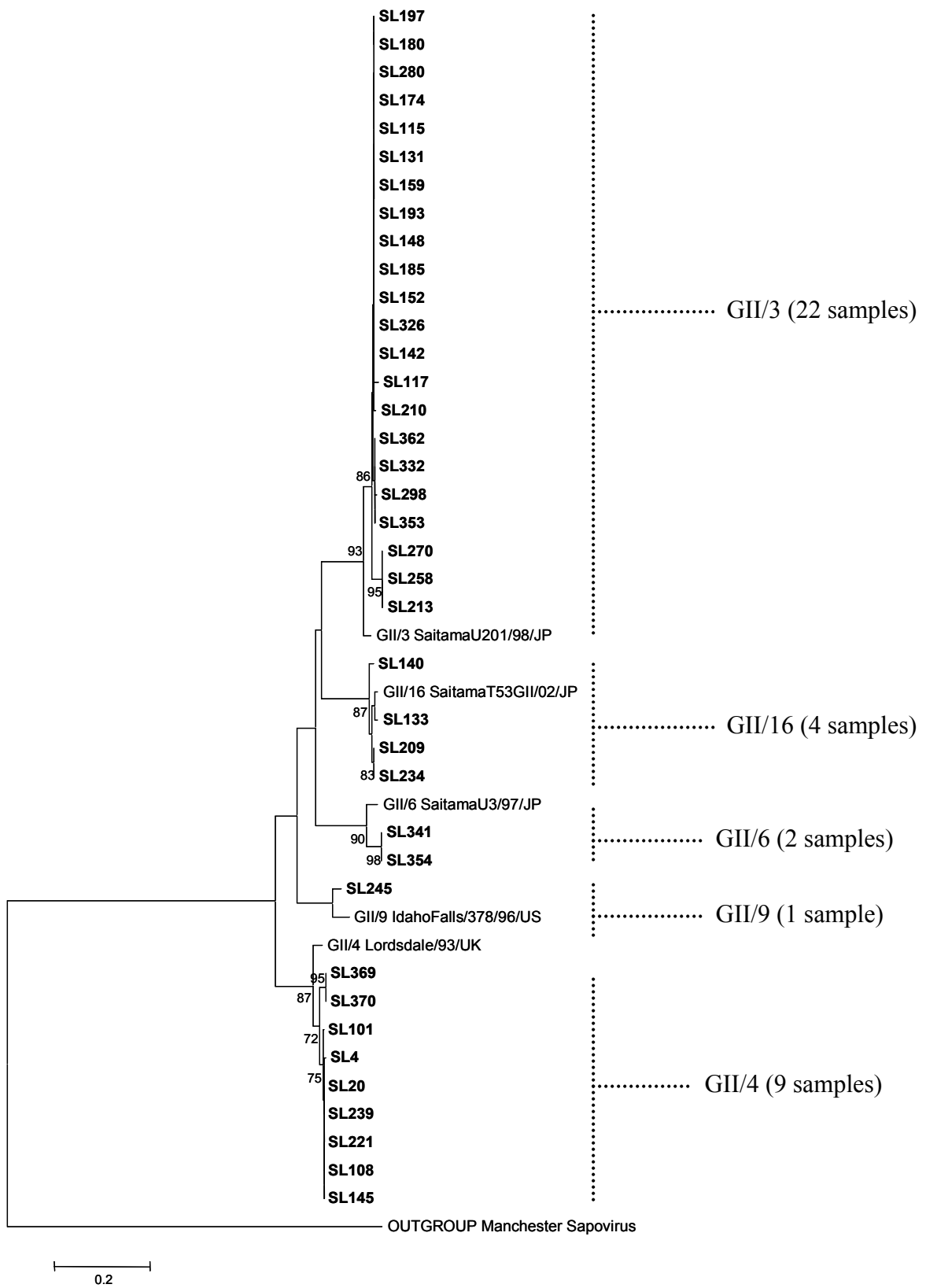


### **Nucleotide sequencing and phylogenetic analysis of norovirus**

All Nov-positive samples were subjected to nucleotide sequencing and phylogenetically analyzed by the NoV capsid region classification scheme of Kageyama et al. [2004]. The phylogenetic tree clearly shows that they were grouped into five distinct genotypes (Figure 14). GII/3 was the most predominant genotype, accounting for 57.9% (22 of 38), followed by GII/4 (23.7%, 9 out of 38), GII/16 (10.5%, 4 of 38), GII/6 (5.3%, 2 of 38), and GII/9 (2.6%, 1 of 38). All the samples except for 4 strains in GII/16 had the high identity over 98% on the amino acid as well as the nucleotide level with other strains found in Asian countries, especially in Japan. Interestingly, the highest homology at 96% of GII/16 strains was found with the strain detected in Iraq in 2003 (Norovirus NLV/IF1998/2003/Iraq, accession number: AY675554).

### **Genotypes and seasonality**

The highest diversity of NoV genotypes was observed in February, when at least four genotypes circulated; these genotypes were GII/3, GII/4, GII/9, and GII/16 (Figure 13c). GII/3 was found throughout most of the year, except for September, October and July, even when the genotype diversity was relatively restricted. This genotype caused the NoV peak in January (76.9%, 10 of 13). GII/4 was also seen for a relatively long period. Notably, the two small clusters of rare genotypes, namely GII/16 and GII/6, were seen from December to February and June to July, respectively.



**Figure 14 (above).** Phylogenetic tree of nucleotide sequences of NoV GII detected in children hospitalized with acute gastroenteritis in Kandy, Sri Lanka. The tree was constructed from partial nucleotide sequences of the capsid region. Reference strains of NoV were selected from the DDBJ DNA/GenBank database under the accession numbers indicated in the text. Percentage bootstrap values above 70% are shown at the branch nodes.

## Discussion

This study describes the incidence and genetic diversity of NoV in pediatric diarrhea in Kandy, Sri Lanka. The incidence found in this study (10.5%) is comparable to that in other reports dealing with hospitalized cases. This finding confirms that infection rates in the studied population are similar to those for other high- and low-income countries [Chen et al., 2007; Hansman et al., 2004a, 2004b; Khamrin et al., 2007; Lee et al., 2007; Onishi et al., 2008; Phan et al., 2004]. Comparisons of this type should be done with caution, since the inclusion criteria used in these studies have been so heterogeneous.

In this study, we strictly followed the WHO generic protocol for hospital-based surveillance of rotavirus gastroenteritis in children. This protocol defines the cases as children under 5 years of age who were admitted to the hospital for treatment of diarrhea. Samples should be taken within 48 hours of admission so as to avoid the detection of nosocomial infection [World Health Organization, 2002]. In this regard, the data obtained in the current study would be significant in the comparison of diarrheal viruses' detection in other countries following the same globally used protocol.

The predominance of group A rotavirus (46.7%) was concordant with previous investigations on the viral etiology of pediatric gastroenteritis [Nguyen et al., 2007; Yan et al., 2004; Chen et al., 2007]. Of interest, mixed infection of diarrheal viruses was found in 12 cases (3.3%), of which 5 were with NoV GII. In other words, 13.2% (5 of

38) of the NoV cases found in this study were discovered with other virological agents. This phenomenon has been reported in other studies [Nguyen et al., 2007; Chen et al., 2007], suggesting that children are vulnerable to several viruses at the same time. It is noteworthy to point out that clinical symptoms and total severity among viral groups did not differ significantly even with rotavirus and mixed infection in this study, suggesting that the most common causative virus for hospitalization for diarrhea and vulnerability to several viruses did not necessarily incur severe symptoms.

When I looked at the clinical symptoms of NoV monoinfected cases, the main features of the patients were diarrhea (100%) and vomiting (72.7%). Some patients presented upper respiratory symptoms such as coughing (33.3%) and coryza (21.2%), which are concordant with the previous study [Dey et al., 2007]. In this study, NoVs were found in samples collected from patients in all five age groups. However, infants aged from 6 to 11 months and children from 12 to 23 months old were found to be infected more frequently than patients in the other age groups. This result was in agreement with other NoV studies from different countries [Dey et al., 2007; Nguyen et al., 2007; Bucardo et al., 2008].

The protection from NoV infection might be explained by passive immunity from mothers to infants <6 months, or active immunity from previously acquired infections in children more than 2 years of age. The mean severity score of patients <6 months was not significantly lower than that of patients in the other age groups. This observation may suggest that passive immunity only helps to protect from infection, but does not influence the severity of disease.

Regarding the severity among different genotypes, the data shows no statistical difference even with rare genotypes such as GII/16. Great caution is needed before

concluding this issue, since the number analyzed in each genotype was very small. Further research recruiting more cases of gastroenteritis is needed.

This study is the first to report NoV infection in Sri Lankan children. The data on nucleotide analysis of this virus was registered in the DDBJ/GenBank databases. Great genetic diversity was observed, encompassing 5 genotypes, namely GII/3, GII/4, GII/6, GII/9, and GII/16. Interestingly, GII/3 was found with the highest prevalence, accounting for 57.9% (22 of 38) in the present study, although many reports on NoV from various countries have reported GII/4 dominance over other genotypes [Onishi et al., 2008; Okame et al., 2006; Dey et al., 2007; Nguyen et al., 2007; Bucardo et al., 2008, Vinje et al., 1997]. In Japan, however, a change in distribution of predominant NoV genotypes, GII/4 to GII/3 in 2003-2004 and GII/3 to again GII/4 in 2004-2005, was observed in the consecutive surveillance [Phan et al., 2006a, 2006b]. Therefore, continuous monitoring of NoV genetic property in Sri Lankan children is important for updating and comparing global trends of NoV infection.

In the current study, samples were collected throughout a year. A distinct peak of NoV was observed in January consisting of the most common genotype, GII/3. This occurs at the start of the drier season and the end of the cooler season, which is concordant with other studies conducted in tropical countries such as Vietnam [Nguyen et al., 2007] showing a peak in the dry season, and Bangladesh [Dey et al., 2007] showing a peak in the cooler season. In rotavirus surveillance, the difference in the season of peak between temperate and tropical climates has been described [Estes and Kapikian, 2007]. Similarly, accumulation of knowledge on the natural history of NoV from countries with tropical climates is warranted.

In summary, the results of this study highlight the impact of NoV in diarrheal diseases among children in Kandy, Sri Lanka, where no report of NoV has previously been made, and are the first to describe the clinical and epidemiological features as well as the genetic properties of this virus. The knowledge provided by the current study will be of use in disease control for this viral infection and future strategy, especially using NoV vaccine in Sri Lanka.

## CONCLUSIONS

NoV infection is a significant cause of acute gastroenteritis with relatively little preparedness for diagnostic option, and with little knowledge of its pathogenesis, as well as its disease burden in the pediatric population. The major findings of this study are the following.

1. A simple and rapid detection kit with an immunochromatographic system was successfully developed using Poly Abs against rVLPs for the two most prevalent genotypes of NoV, namely GII/3 and GII/4. Based on RT-PCR, its agreement rate, sensitivity, and specificity were 84.1%, 69.8%, and 93.7%, respectively. The high specificity, no cross-reactivity with other enteric viral pathogens and excellent detection limit ( $3.5 \times 10^7$  copies/g of stool for GII/3 and  $4.6 \times 10^6$  copies/g of stool for GII/4) clearly indicated the potential applicability of the IC in the screening process for NoV infection.
2. NoV RNA existed in the bloodstream in 15.4% of the NoV gastroenteritis patients analyzed. The serum-positive patients showed neurological complications more frequently than the serum-negative patients ( $p=0.028$ ). Although association of viral load in stools and sera was not observed, genotypes of the NoV in stools and sera from the same patient matched completely, indicating that RNA in sera originated in the intestines.

3. NoV emerged as the most predominant diarrheal virus in children attending a DCC in Tokyo, Japan ( $p < 0.01$ ), while the overall detection rate of viruses decreased ( $p < 0.001$ ) in the 6 years since the first study was conducted in the same DCC using the same detection method. Eighteen out of 20 children experienced NoV genogroup II infection during one year, including multiple genotypes infection and re-infection of the same genotype. There was no significant difference in NoV load between symptomatic and asymptomatic children (median:  $1.5 \times 10^6$  copies/g versus  $2.6 \times 10^4$  copies/g ( $p = 0.6$ )). Sequencing and quantitative analysis revealed that an asymptomatic child excreting an insignificant amount of virus ( $4.7 \times 10^4$  copies per gram of stool) may cause a major outbreak of NoV in a semi-enclosed setting such as a DCC.
  
4. NoV ranked as the second most common causative virus, accounting for 10.5% of the hospitalized children with acute gastroenteritis in Kandy, Sri Lanka. I observed no statistically significant difference in clinical symptoms among viral groups. Among 33 patients showing monoinfection with NoV, the main clinical manifestations observed were diarrhea (100%), vomiting (72.7%), and body temperature  $\geq 37.5^\circ\text{C}$  (51.5%). A distinct peak of NoV was observed in the drier and cooler seasons of the year. Great genetic diversity was recognized, encompassing five genotypes, including rare GII/9 and GII/16. GII/3 was the most predominant genotype (22 of 38, 57.9%).

In summary, a simple and easily performable detection kit with an immunochromatographic system was successfully developed. It is suggested that NoV



spreads from the intestines to the bloodstream, possibly accounting for extraintestinal complications of NoV gastroenteritis. It is also suggested that asymptomatic children may be an important source of NoV outbreak, emphasizing the importance of standard precautions. A significant disease burden of NoV infection was recognized in Sri Lanka for the first time, where no cases of NoV had been reported before this study.

The results of the present study would promote further recognition of NoV infection by physicians so that unknown clinical outcomes might be encountered that would better facilitate interpretation of the natural history of this viral infection. Although the number of molecular epidemiological studies has been increased dramatically over the years, continuous monitoring and detailed genetic analyses are necessary to visualize the whole picture of the global evolution of this virus.

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