Table 2
Comparison of attack rate between oyster-associated outbreaks and food handler-associated outbreaks

Group	Number of cases	Attack rate (%)		
		Median	25th percentile	75th percentile
Oyster-associated outbreaks	95	58.3*	40	75
Food handler-associated outbreaks	195	47.2*	33.3	67.7

*: The difference between the median attack rates were statistically significant (P 0.007) by using the Mann–Whitney U test.

handler-associated outbreaks. The reason may be explained as follows from the viewpoint of the difference in contamination route. First, in the oyster-associated outbreaks, one or more NoV strains to which each person is susceptible might have selectively grown in its intestinal cells after the oyster-consumption because oysters might accumulate various NoV strains during their sea life. In contrast, most of the food handler-associated outbreaks were caused by a single NoV strain that had contaminated the foods after propagation in the food handlers. Some consumers, therefore, are not susceptible to an NoV strain because of the lack of the receptor(s) for it (Huang et al., 2003; Huang et al., 2005; Larsson et al., 2006; Marionneau et al., 2002; Tan and Jiang, 2005). Indeed, a single NoV genotype was responsible for most of the food handler-associated outbreaks, whereas multiple NoV genotype strains were frequently involved in the oyster-associated outbreaks (Table 1). Second, the amount of NoV particles in foods that might have been contaminated from food handlers during the food processing and/or food serving might vary, and some of the implicated food might not have been contaminated with NoV. The number of patients in the food handler-associated outbreaks tended to be more than that in the oyster-associated outbreaks. This may be explained by the difference in involved facilities; most of the facilities associated with oyster-associated outbreaks were small restaurants, while most of the facilities associated with food handler-associated outbreaks were large restaurants such as hotels serving food for parties and schools.

The attack rate in the oyster-associated outbreaks varied between 40% (25th percentile) and 75% (75th percentile), and the median attack rate was 58.3% (Table 2). Therefore, some individuals did not develop any symptoms despite the fact that they had consumed oysters suspected of being the food vehicle. In the oyster-associated outbreaks, susceptibility of the host to NoV infection might have been one of the reasons but was not

Table 3
Comparison of number of patients between oyster-associated outbreaks and food handler-associated outbreaks

Group	Number of cases	Number of patients		
		Median	25th percentile	75th percentile
Oyster-associated outbreaks	95	17	7	39
Food handler-associated outbreaks	195	40	12	108

Table 4
Comparison of attack rate between GII.4-associated outbreaks and other outbreaks

Group	Number of cases	Attack rate (%)		
		Median	25th percentile	75th percentile
GII.4-associated outbreaks	27	41*	29.9	54.5
Other outbreaks	136	56.9*	40	75

*: The difference between the median attack rates were statistically significant (P 0.004) by using the Mann–Whitney U test.

thought to be a major factor because some NoV strains to which the host is susceptible included in oysters could selectively propagate in individuals as stated above. There are three other possible reasons. First, some individuals might have asymptomatic infection. Second, some individuals might have blocked the NoV infection by their immunity. Third, the oysters eaten by individuals who were not affected might have been less or not contaminated with NoVs because it has been reported that oysters packed in the same package for sale or harvested from the same balsa raft in the cultivating sea area have different copy numbers of the NoV genome and that some oysters selected from the same lot in which NoV was detected in other oysters do not include NoV (Nishio et al., 2004; Noda et al., 2004).

Recent studies have suggested that NoVs use a histo-blood group antigen(s) expressed on intestinal epithelial cells as their receptor and that different NoV strains may use different types of histo-blood group antigen (Huang et al., 2005; Tan and Jiang, 2005). These reports suggest that the attack rate may depend on difference of genotype. GII/4 is thought to be able to infect all secretor individuals, suggesting that susceptibility to GII/4 appears to be greater than that to other NoV genotypes. From this viewpoint, we compared the attack rate in outbreaks associated with different genotypes. Unexpectedly, the attack rate in outbreaks associated with GII/4 genotype was lower than that in outbreaks associated with other NoV genotypes, while the attack rate in outbreaks associated with GII/3 genotype was higher than that in outbreaks associated with other NoV genotypes. The low attack rate in the GII/4 cases can not be explained only by the fact that GII/4 was more frequently associated with the food handler-associated outbreaks than were other NoV genotypes (Table 1) because GII/3 has the same tendency as GII/4 and the attack rate in the GII/4 cases was lower than that in the food handler cases (Tables 2, 4). Therefore, it is possible that GII/4 causes asymptomatic infection more frequently than do other NoV genotypes. This

Table 5
Comparison of attack rate between GII.3-associated outbreaks and other outbreaks

Group	Number of cases	Attack rate (%)		
		Median	25th percentile	75th percentile
GII.3-associated outbreaks	20	64.8*	40.2	81.7
Other outbreaks	143	53.2*	37.6	73.8

*: The difference between the median attack rates were statistically significant (P 0.247) by using the Mann–Whitney U test.

hypothesis is supported by the fact that GII/4 was frequently detected from asymptomatic in-hospital patients and staff in a study by Gallimore et al. (2004). This unique characteristic of GII/4 may be responsible for the recent increase in outbreaks caused by GII/4 strains, especially in hospitals, elderly home facilities, residential facilities, and nursing homes, all over the world (Bull et al., 2006; Ike et al., 2006; Lopman et al., 2004; Okada et al., 2006; Siebenga et al., 2007; Wu et al., 2006; Yoshizumi et al., 2005). The high attack rate in the GII/3 cases was also unexpected because binding ability of GII/3 strains to secretor individuals with blood type O and non-secretor individuals was shown to be weak in a saliva binding assay (Huang et al., 2005; Tan and Jiang, 2005). In recent years, GII/3 genotype as well as GII/4 has been frequently associated with gastroenteritis outbreaks (Bull et al., 2006; Gallimore et al., 2004; Wu et al., 2006). GII/3 strains may have high pathogenicity, transmissibility, or infectivity. We may not exclude the influence of mass immunity for difference in attack rate between different genotypes. However, secretory IgA antibodies specific for NoVs, which can block NoV infection, exist in intestinal tracts for a short period after the recovery from illness (Nishio et al., 1988). Mass immunity, therefore, does not considerably affect our results of analysis in attack rate between different genotypes.

In conclusion, we showed in this study that there were differences between attack rates in oyster-associated outbreaks and food handler-associated outbreaks and between attack rates in GII/4- or GII/3-associated outbreaks and other NoV genotype-associated outbreaks. These results show that the attack rate in NoV foodborne outbreaks may be influenced by differences in implicated foods, susceptibility of the host for NoV infection, and pathogenicity of NoVs. To estimate the minimum amount of virus particles in oysters required for infection, it is necessary to investigate foodborne outbreaks epidemiologically in detail and to perform genotyping of NoVs involved for obtaining data on attack rate as well as quantitation of copy numbers of NoV genomes, or titration of infectious NoV particles if possible (Straub et al., 2007), in the foods such as oysters, involved in foodborne outbreaks. Our results should be useful for risk analysis for the prevention and control of NoV in food.

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

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Abstract

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

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

1. Introduction

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

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

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

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

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

2. Materials and methods

2.1. Expression of rVLPs

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

2.2. Polyclonal antibody production

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

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

Ninety-six-well Maxisorp plates (Nunc, Roskilde, Denmark) were coated with 90 ng of each rVLP per well in 60 μl of 0.1 M carbonate buffer for 1 h at 37 °C. The wells were blocked with 1% BSA in PBS containing 0.1% Tween 20 (PBS-T). The plates were incubated overnight at 4 °C. After the wells were washed three times with PBS-T, 60 μl twofold serially diluted Poly Ab from a starting dilution of 1:400 in PBS-T containing 1% BSA was added to each well and the plate was incubated for 1 h at 37 °C. After washing three times with PBS-T, 60 μl of a 1:10 000 dilution of Horse radish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) was added to each well and the plate was incubated again for 1 h at 37 °C. Having washed three times with PBS-T, 60 μl of substrate *o*-phenylenediamine containing 0.012% H_2O_2, 0.2 M citrate–phosphate buffer was added to each well and left in the dark for 20 min at room temperature. The reaction was stopped by the addition of 60 μl of 2 M H_2SO_4 to each well and the optical density (OD) at 492 nm (620 nm as reference) was determined with a Labsystems Mutiskan MCC microplate reader. For this experiment, the lysate of Tn5 cell was included as negative control. A sample of which sample–negative OD > 0.2 and sample/negative ratio >2.0 was considered positive.

2.4. Conjugation of Poly Ab with colored latex

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

2.5. Immunochromatography

2.5.1. Test principles

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

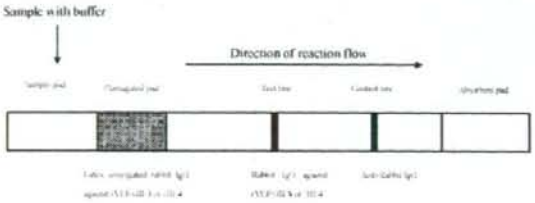

Fig. 1. Diagram of the immunochromatography for NoV.

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

2.5.2. Test procedures

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

2.6. Detection limit of immunochromatography

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

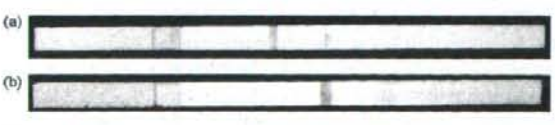

Fig. 2. (a) A representative photograph of a positive sample. (b) A representative photograph of a negative sample.

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

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

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

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

2.9. RT-PCR and sequencing

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

Foster City, CA). Sequence analysis was performed using CLUSTAL X software (Version 1.81). Phylogenetic tree with 100 bootstrap resamples of the nucleotide sequence alignment data sets was generated using neighbor-joining method with CLUSTAL X. The genetic distance was calculated using Kimura's two-parameter method (PHYLIP). Reference NoV strains and accession numbers used in this study were as follows: Manchester (X86560), Melksham/89/UK (X81879), SaitamaU201/98/J (AB067542), Arg320/95/AR (AF190817), Lordsdale/93/UK (X86557), Bristol/93/UK (X76716), SaitamaU3/97/JP (AB039776), Leeds/90/UK (AJ277698), SaitamaKU80aGII (AB058582).

3. Results

3.1. 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 in ELISA system (Table 1). Poly Ab raised against rVLP GII/3 reacted broadly with many genotypes including the ones in genogroup I and the titer, expressed as the reciprocal of the highest dilution of each antibody for positive results, generally ranged high against many genotypes. On the other hand, Poly Ab raised against rVLP GII/4 had the tendency to react only with the homologous genotype, GII/4, showing a very high titer compared with the titers against other genotypes.

3.2. Cross-reactivity of immunochromatography 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 immunochromatography (Table 2). Although highly concentrated rVLP of each genotype was applied, none of heterogeneous genotypes reacted with Immunochromatography-GII/4 and only few did with Immunochromatography-GII/3. This tendency was also observed in the assessment using the stored clinical samples where few samples containing GII/1 or GII/6 NoV were weakly reactive with Immunochromatography-GII/3.

Table 1
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 reprocical dilution that gave the positive results. Titers more than 409 600 were painted by .

3.3. Detection limit of immunochromatography

Stock solution of both rVLP GII/3 and GII/4, as well as stool samples with known viral copy number were serially diluted to determine the minimal concentration of antigenic protein and virus copy number by the immunochromatography. For

Table 2
Reactivity of immunochromatography 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

Note: NT, not tested; +, positive; ±, weakly positive; –, negative.

Immunochromatography-GII/3, the detection limit of rVLP was 3.0×10^{-3} ng/μl and that of viral load was 3.5×10^{7} copies/g of stool, whereas Immunochromatography-GII/4 gave the values for rVLP of 7.5×10^{-3} ng/μl and for viral load of 4.6×10^{6} copies/g of stool. The assays with rVLPs were performed in triplicate and showed the same results, giving a κ-value of 1.0.

3.4. Sensitivity, specificity and agreement rate based on RT-PCR

NoV in clinical stool samples collected during the winter season in Japan was examined by both the current immunochromatography test and RT-PCR and the kit was evaluated based

Table 3
Sensitivity, specificity, and agreement of immunochromatography and RT-PCR[a]

IC	Detection (no. of samples) by RT-PCR		
	+	−	Total
+	30[b]	4[c]	34
−	13	60	73
Total	43	64	107

+, positive; −, negative.

[a] Sensitivity = 69.8% (30/43); specificity = 93.7% (60/64); agreement = 84.1% (90/107).

[b] Nineteen was positive by Immunochromatography-GII/3 and 11 by Immunochromatography-GII/4.

[c] These samples were positive as determined by semi-nested PCR.

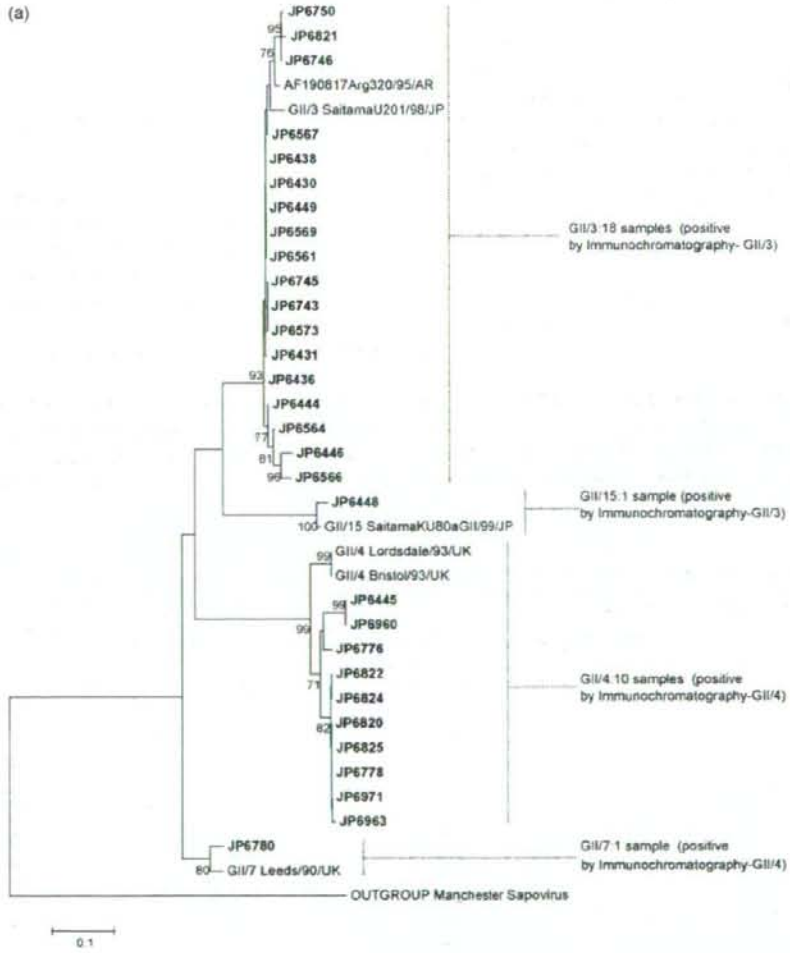

Fig. 3. (a) Phylogenetic tree of nucleotide sequences of NoV in samples positive by both immunochromatography and RT-PCR. Reference strains selected from the DDBJ/GenBank database under the accession numbers indicated in the text. Study NoV was highlighted in bold. Manchester strain was used as an out-group strain for phylogenetic analysis. The scale indicates nucleotide substitutions per position. The numbers in the branches indicate the bootstrap values. (b) Phylogenetic tree of nucleotide sequences of NoV in samples negative by immunochromatography but positive by RT-PCR.

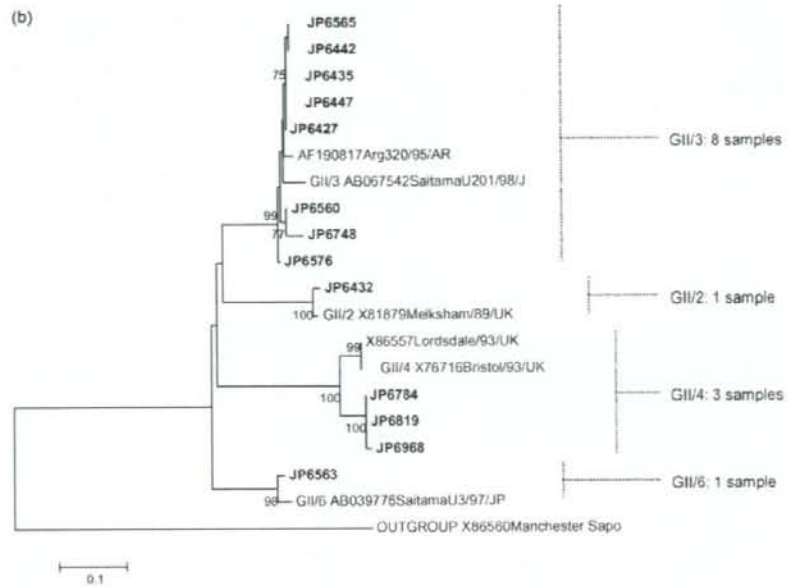

Fig. 3. (*Continued*).

on RT-PCR (Table 3). Among 107 samples, 43 became positive by RT-PCR from which 30 were determined correctly as positive by the immunochromatography and the sensitivity was calculated as 69.8%. Nineteen samples were recorded positive by Immunochromatography-GII/3, whereas 11 samples by Immunochromatography-GII/4 and the former appeared mainly in December, the latter did in March, indicating genotype change during the study period (data not shown). Sixty-four samples were negative by RT-PCR, among which 60 were negative by the immunochromatography, giving the specificity as 93.7%. The agreement rate between the immunochromatography and RT-PCR was calculated as 84.1%. Four samples were determined to be positive by the immunochromatography but negative by RT-PCR. Subsequently, these samples were subjected to semi-nested PCR which gave positive results for all the four samples.

3.5. Genotyping of positive samples by RT-PCR

Stool samples found to be positive for NoV by RT-PCR were analyzed further by sequencing to identify their genotypes. The phylogenetic tree for samples positive for both the immunochromatography and RT-PCR is shown in Fig. 3a, whereas that for negative samples for the immunochromatography but positive with RT-PCR is shown in Fig. 3b. All the GII/3 and GII/4 samples found positive by immunochromatography were done by the homologous immunochromatography. Interestingly, one GII/15 sample became positive with Immunochromatography-GII/3 and one GII/7 sample positive with Immunochromatography-GII/4. Thirteen false negative samples, which were negative by the immunochromatography 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 was used for genotyping ranged 98–100% between false negative and true positive samples of GII/3 or GII/4 (data not shown).

3.6. 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 immunochromatography are shown in Table 4. Since no sample was positive for astrovirus during this period, two samples known to be positive for astrovirus as described in Section 2.7 were tested and the results of these samples are also summarized in Table 4. Of note, the immunochromatography succeeded in determining positive result for a sample of mix infection between NoV GII and sapovirus. The immunochromatography gave correctly negative results for other single viral infections, such as group A rotavirus, sapovirus, adenovirus and astrovirus, which implied the high specificity of the immunochromatography kit.

Table 4
Cross-reactivity of immunochromatography with other enteric viruses

Viruses	Number of positive samples	Results of immunochromatography
Group A rotavirus	10	All negative
Sapovirus	5	All negative
Adenovirus	1	Negative
Astrovirus	2	Both negative
Mix infection[a]	1	Positive

[a] Mix infection between norovirus GII and sapovirus.

4. Discussion

With increasing number of reports on NoV and estimated increase in the number of patients with NoV infection (Estes et al., 2006), the demand of offering rapid diagnosis of this infectious disease is expanding dramatically. The results of studies evaluating commercial ELISAs as rapid diagnostic tests, which still take more than 4 h to obtain the results, 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, simple, easy-to-read, and rapid detection test for NoV using immunochromatography membrane strip was developed. This took a shorter time; approximately 30 min to complete the assay with limited equipment needed, and the results were reproducible with the κ-value of 1.0. Based on RT-PCR using the freshly collected samples in winter when NoV infection reaches its peak, the agreement rate was 84.1%, sensitivity 69.8%, and specificity 93.7%. The high percentage of specificity and no cross-reactivity with other enteric viral pathogens, such as group A rotavirus, sapovirus, and adenovirus clearly indicated the potential applicability of the immunochromatography in screening samples for NoV infection.

The false negative samples, which were negative by immunochromatography but RT-PCR positive included GII/2, GII/6, GII/3, and GII/4. The first two genotypes were reasonable as the cross-reactivity of immunochromatography with rVLP had been checked initially and it was found that the broad cross-reactivity observed by the ELISA assessment of Poly Ab raised against rVLP GII/3 did not necessarily appear after applying the immunochromatography system. However, the latter two genotypes were the ones used as antigens to immunize rabbit and expected to react strongly with the Poly Ab in the immunochromatography. The possible reason was 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 four samples, which were positive by immunochromatography and by the semi-nested PCR, were three GII/3 and one GII/4. Then these samples may contain a smaller viral load than monoplex PCR positive samples. This phenomenon was also described elsewhere (Okitsu-Negishi et al., 2006), suggesting that the factors other than viral load cause the lower sensitivity in the 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 above-mentioned site (data not shown). Hence, there is a possibility of increasing the sensitivity against GII/3 if Poly Ab raised against a strain without such a structure is also applied for the immunochromatography test.

Another possible explanation for lower sensitivity of immunochromatography might be inner epitopes of NoV or the existence of inhibitors in mucous 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 stored samples; OD of 14 samples decreased whereas those of 9 samples increased, and 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 tests might have revealed the inner epitopes or removed inhibitors easily from the stool suspension with stored samples, resulting in better sensitivity.

Determining the detection limit of immunochromatography to rVLP is of importance especially because NoV cannot be cultured in any cell line, and therefore cannot give the minimal $TCID_{50}$ for positive results which is usually considered 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 an antigen for each type of immunochromatography was clearly showed and this might be useful as a reference point for future attempts using various genotypes. The viral load of NoV GII in the clinical stool samples are reported to range mostly around 10^8 copies/g of stool (Chan et al., 2006). Based on the results in the current study, the immunochromatography can theoretically detect 1/100 to 1/10 of viral load found in clinical samples, which is almost equivalent to the detection power of electron microscopy (Atmar and Estes, 2001), and might be justified to use for screening the stool samples.

Regarding the cross-reactivity among heterogeneous genotypes, broad reactivity of Immunochromatography-GII/3 was expected to be observed according to 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 immunochromatography system (data not shown). In the assessment of immunochromatography using the panel of various genotypes of NoV, however, the discordant results were found in reactive genotypes from those in ELISA. Although immunochromatography and ELISA are both classified as immunological methods, several factors such as pH level in the reaction buffer and size of pore in the immunochromatographic membrane might influence the reactivity in these methods. Lessons learnt from this observation would indicate the need for optimizing the reaction buffer or materials of immunochromatography after screening broadly reactive antibody in ELISA system. Additional attempts for simplifying the process of immunochromatography 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 developed using a polyclonal antibody 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 for screening 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.

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Human Sapovirus in Clams, Japan

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Human sapovirus was detected in 4 of 57 clam packages by reverse transcription–PCR and sequence analysis. This represents the first finding of sapovirus contamination in food. Closely matching sequences have been detected in stool specimens from patients with gastroenteritis in Japan, which indicates a possible food-to-human transmission link.

Sapoviruses and noroviruses are etiologic agents of human gastroenteritis. Human noroviruses are the most important cause of outbreaks of gastroenteritis worldwide and can be transmitted by a variety of routes, including food (*1*). Sapovirus infections are mostly associated with sporadic gastroenteritis in young children; however, foodborne transmission routes are yet to be determined. The most widely used method of detection is reverse transcription–PCR (RT-PCR), which has a high sensitivity and can also be used for genetic analysis. Sapovirus strains can be divided into 5 genogroups; GI–GV infect humans; sapovirus GIII infects porcine species. Phylogenetic studies have also designated sapovirus clusters or genotypes to further describe strains.

The Study

The purpose of this study was to detect sapovirus in the clam *Corbicula japonica* (bivalve mollusk) and describe the genetic diversity of the strains. A total of 57 clam packages (30–60 clams per package) were collected from supermarkets or fish markets from 6 different areas in western Japan from December 8, 2005, to September 6, 2006. The samples were shucked, and the digestive diverticulum was removed by dissection and weighed. One gram of digestive diverticulum (10–15 clam/package) was homogenized with an Omini-mixer (Sorvall Inc., Newtown, CT, USA) in 10 mL phosphate-buffered saline. After centrifugation at 10,000× *g* for 30 min at 4°C, the supernatant was centrifuged at 100,000× *g* for 2 h (SW41 Rotor, Beckman Instruments, Inc., Fullerton, CA, USA). The pellet was resuspended in 140 μL distilled water and stored at –80°C until use.

RNA extraction and nested RT-PCR were performed as described (*2*). Briefly, for the first PCR, F13, F14, R13, and R14 primers were used; for the nested PCR, F22 and R2 primers were used. All RT-PCR products were analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. RT-PCR products were excised from the gel and purified by the QIAquick gel extraction kit (QIAGEN, Hilden, Germany). Nucleotide sequences were prepared with the terminator cycle sequence kit (version 3.1, Applied Biosystems, Warrington, England) and determined with the ABI 3130 sequencer (ABI, Boston, MA, USA). Nucleotide sequences were aligned with ClustalX, and the distances were calculated by Kimura's 2-parameter method, as described elsewhere (*2*). Nucleotide sequence data determined in this study have been deposited in GenBank under accession nos. EF104251–EF104254.

Four (7%) of 57 clam packages were contaminated with sapovirus (termed Shijimi1, Shijimi2, Shijimi3, and Shijimi4). Genetic analysis of the partial capsid gene showed that these 4 sequences shared >98% nucleotide similarity and >97% amino acid identity. Phylogenetic analysis grouped these 4 sequences in the same genotype, i.e., GI/1 (Figure). Similar sequences were found on the database (Figure). Strains from this cluster likely represent the dominant genotype worldwide (*3*). Three of 4 sapovirus-positive clam packages were collected from different areas and at different times (Figure). The clam packages that were contaminated with Shijimi1 and Shijimi3 were collected from the same area, but 6 weeks apart, which indicates an ongoing sapovirus contamination or resistance in the natural environment. The seasonality of sapovirus infection in Japan is unknown; however, as with norovirus, sapovirus infections may also peak during winter, although further epidemiologic and environmental studies are needed.

In a recent study, we detected sapovirus strains in 7 of 69 water samples, which included untreated wastewater, treated wastewater, and a river in Japan (*4*). Three of 7 sapovirus sequences detected in the water samples belonged to GI/1 and shared >97% nucleotide similarity with the sapovirus sequences detected in the clam packages. Additionally, sapovirus sequences belonging to GI/1 and sharing >99% nucleotide similarity, for example, Chiba/010598F strain (Figure), have been detected in stool specimens from children with sporadic gastroenteritis in Japan (*5*,*6*). The closely matching sapovirus sequences detected in the water, clams, and patients suggest that sapovirus contamination in the natural environment can

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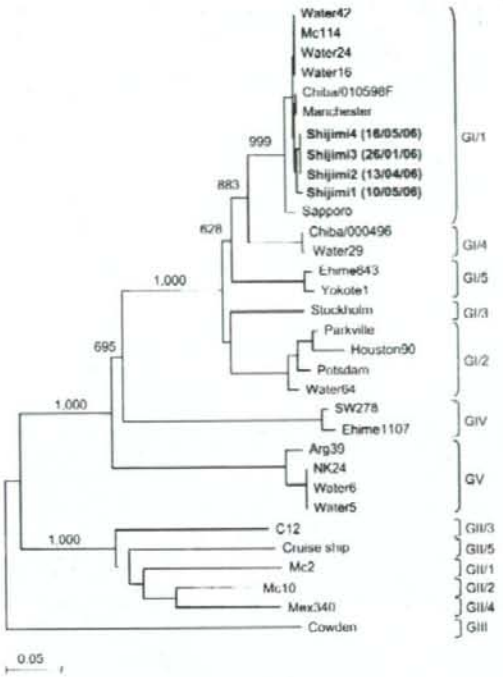

Figure. Phylogenetic analysis of sapovirus capsid sequences (≈300 nt) showing the different genogroups and clusters. Numbers on each branch indicate bootstrap values for the genotype. Bootstrap values of ≥950 were considered statistically significant for the grouping. The scale represents nucleotide substitutions per site. GenBank accession nos.: Mex340, AF435812; Parkville, U73124; Cowden, AF182760; Potsdam, AF294739; Sapporo, U65427; Stockholm, AF194182; SW278, DQ125333; water samples, DQ915088–DQ915094; and Yokote, AB253740. **Boldface** represents sequences detected in this study.

lead to foodborne infections in humans, although direct evidence is lacking. More important, a recent study found animal sapovirus in oysters and suggested that coinfection with human and animal sapovirus strains could result in genomic recombination and the emergence of new strains (*7*). At the same time, we recently described the first human sapovirus intergenogroup recombinant strain (*8*). Phylogenetic analysis of the nonstructural region (i.e., genome start to capsid start) grouped this sapovirus strain in GII, while the structural region (i.e., capsid start to genome end) grouped this strain in GIV.

A large number of studies have detected norovirus in oysters. In 2 recent studies, norovirus was detected in oysters (*Crassostrea gigas*) harvested from geographically isolated areas in Japan (*9,10*). We also screened the same oyster samples for sapovirus; however, all of the samples were negative for sapovirus. That sapovirus was detected in the clam samples, but not in the oyster samples, is of interest. In the past several years, increasing evidence has emerged that human noroviruses bind to histo-blood group antigens (HBGAs) (*11*). These carbohydrate epitopes are present in mucosal secretions and throughout many tissues of the human body, including the small intestine, and in oyster digestive tissues. A number of studies have found that different norovirus strains exhibit different binding patterns to HBGAs and oyster digestive tissues (*12,13*). In a recent study, we found that sapovirus GI and GV strains showed no such binding activity to HBGAs (*14*). These results suggest that human norovirus and sapovirus strains have different binding receptors or that human sapovirus may not concentrate in detectable levels in oysters.

Conclusions

Foodborne diseases are a major problem worldwide. We report what is, to the best of our knowledge, the first account of sapovirus contamination in food destined for human consumption. The report may represent a possible food-to-human transmission link, although direct evidence is lacking. In Japan, clams are usually boiled before they are consumed in soups. However, boiling to open the clam may not inactivate the virus (*15*); in addition, some areas in Japan do not boil clams before eating them. Further studies are needed to determine if boiling inactivates sapovirus and if the contaminated clams are indeed infectious. In conclusion, these novel results highlight the importance of sapovirus, in particular the GI/1 strains. A new awareness of sapovirus transmission routes is necessary and may help reduce sapovirus infections.

Dr Hansman is a scientist at the National Institute of Infectious Diseases, Japan. He studies viruses that cause gastroenteritis in humans, namely sapovirus and norovirus. His research interests include epidemiology, virus expression, and cross-reactivity.

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Short communication

Evaluation of immunochromatography and commercial enzyme-linked immunosorbent assay for rapid detection of norovirus antigen in stool samples

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Abstract

The efficiency of immunochromatography and commercial enzyme-linked immunosorbent assay (ELISA) kit (Denka Seiken Co. Ltd., Tokyo, Japan) were evaluated for rapid detection of norovirus (NoV) from stool specimens. A total of 503 stool specimens collected from infants and young children who suffered from acute gastroenteritis were tested for NoV by the NoV-immunochromatography kit, Denka ELISA kit, and by a monoplex RT-PCR method. The NoV-immunochromatography revealed 78.9% sensitivity, 96.4% specificity, and 92.4% efficiency with the monoplex RT-PCR method. The Denka ELISA kit had a sensitivity of 90.4%, specificity of 96.4%, and an efficiency level of 95%. The findings indicate that the newly developed NoV-immunochromatography kit provides the specificity equal to that of the Denka ELISA kit, even through the sensitivity of detection was lower. However, the advantage of the NoV-immunochromatography kit is less time consuming and simpler. The data show that both the Denka ELISA and the NoV-immunochromatography kits may be used as an alternative method for screening of NoV in stool samples.

Keywords: Norovirus; Immunochromatography; Denka ELISA; RT-PCR; Gastroenteritis

Viral gastroenteritis is one of the most common illnesses in humans worldwide, and different agents such as rotavirus, astrovirus, adenovirus, and calicivirus have been associated with the disease (Clark and McKendrick, 2004). Norovirus (NoV) is one of the four members of the family *Caliciviridae*, which is a nonenveloped, positive-sense, single-stranded RNA virus. NoV is a leading cause of gastroenteritis worldwide and responsible for outbreaks in various epidemiological settings, including restaurants, schools, day-care centers, hospitals, nursing homes, and cruiser (McEvoy et al., 1996; McIntyre et al., 2002; Russo et al., 1997). Currently, based on the diversity of the capsid sequences, NoVs are grouped into five genogroups (G), of which GI, GII, and GIV have been found in humans (Kageyama et al., 2004; Zheng et al., 2006). Human NoV genogroups are subdivided further into at least 15 genotypes in GI, 18 genotypes in GII, and only one genotype in GIV (Kageyama et al., 2004; Okada et al., 2005; Vinje et al., 2004). Several epidemiological studies clearly indicated that NoV GII is the main causative agent among NoVs that cause acute diarrhea in humans (Hansman et al., 2004; Phan et al., 2006a,b; Tseng et al., 2007).

Noroviruses were first discovered by Kapikian et al. (1972) under electron microscopy (EM). More recently, application of reverse transcription-polymerase chain reaction (RT-PCR) and DNA sequencing techniques to detect and characterize NoV became the standard methods for detecting this pathogen. Although RT-PCR is used around the world as a standard tool for routine diagnosis of NoV infection, detection of viral agents with molecular techniques requires well-trained personnel and sophisticated equipments. Thus, a rapid and sensitive diagnostic test for NoV detection is required. Currently, a number

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of enzyme-linked immunosorbent assay (ELISA) kits for the detection of NoV in stool samples have been developed and commercialized (Burton-MacLeod et al., 2004; de Bruin et al., 2006; Richards et al., 2003). In addition to these laboratory diagnostic techniques, the immunochromatography diagnostic test is another alternative choice. The immunochromatography method is easy to perform and less time consuming to conduct the test. In this study, we evaluated the newly developed immunochromatography kit (Immuno-Probe, Co. Ltd., Saitama, Japan) in comparison with a new commercial ELISA kit (NV-AD; Denka Seiken Co. Ltd., Tokyo, Japan) to assess their sensitivities in detecting NoV antigen in stool samples. The monoplex RT-PCR was used as a "gold standard" method for this assessment.

A total of 503 stool samples were collected from infants and children with acute gastroenteritis, encompassing five different geographical settings in Japan (Maizuru, Tokyo, Sapporo, Saga, and Osaka) from July 2004 to March 2005. The presence of NoV GI and GII in fecal specimens was detected by RT-PCR using a protocol described previously (Yan et al., 2003). A forward primer G1-SKF (nt 5342–5261) 5′-CTGCCCGAATTYGTAAATGA-3′ was used in combination with the reverse primer G1-SKR (nt 5653–5671) 5′-CCAACCCARCCATTRTACA-3′, for the amplification of NoV GI. For NoV GII identification, a forward primer COG2F (nt 5003–5028) 5′-CARGARBCNATGTTYAGRTGGATGAG-3′ was used in combination with the reverse primer G2-SKR (nt 5367–5389) 5′-CCRCCNGCATRHCCRTTRTACAT-3′. All of the NoV positive samples were characterized further for their genotypes by direct DNA sequencing using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and analyzed the sequence on an automated sequencer (ABI 3100; Applied Biosystems). The sequences obtained were compared to those of NoV strains deposited in the GenBank using the BLAST program, and the genotypes were classified using the clustering determined previously by Kageyama et al. (2004).

To evaluate the sensitivity and specificity of this NoV-immunochromatography test, all of the 503 samples were tested for NoV antigen using the newly developed NoV-immunochromatography kit, which was kindly provided by the Immuno-Probe Company. The immunochromatography test was performed according to the manufacturer's directions. The NoV-immunochromatography strip used in this study was a nitrocellulose membrane coated with gold colloid conjugated with mouse monoclonal antibody (MAb 14-1) against GII/4 on right hand side of the adsorbent pad as shown in Fig. 1. From the previous study, the MAb 14-1 showed a broad range of cross-reactivity with several genotypes of the virus-like particles (VLPs of NoV) as tested by ELISA (Shiota et al., 2007). Therefore, the MAb 14-1 was selected and used as a capture antibody in this NoV-immunochromatography kit. The test line was coated with NoV polyclonal antibodies against NoV GII/3 and GII/4, while the control line was coated with antibody against mouse immunoglobulin. For a negative immunochromatography reaction, only a band of control was appeared on the immunochromatography strip, while a positive immunochromatography reaction both the control and test bands were appeared on the immunochromatography strip (Fig. 1).

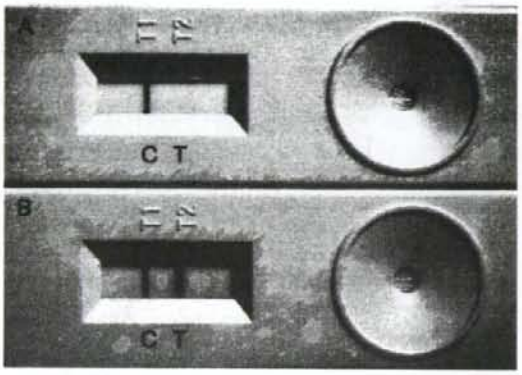

Fig. 1. Detection of NoV in a stool sample using the NoV-immunochromatography kit. The test is negative when only one band appears in the control area (A). The test is positive if two bands appear in the membrane (B). C represents the control band and T represents the test band.

An improved version of commercial ELISA kit, NV-AD (Denka Seiken Co. Ltd.), was evaluated using the same set of stool samples. The detection of NoV using the NV-AD Denka ELISA kit was slightly modified from the manufacturer's instruction. Briefly, 100 μl of a 20% stool suspension was mixed with 120 μl of sample extraction buffer. The reactions for the detection of NoV antigens were performed by mixing 100 μl of the NoV extract with 100 μl of peroxidase conjugated antibodies against NoV GI and GII in the wells which coated with antibodies against NoV GI and GII. After the wells were washed, 3,3′,5,5′-tetramethylbenzidine and hydrogen peroxide were added, and a colorimetric reaction was allowed to develop for 30 min at room temperature. The optical density (OD) of the colorimetric reaction was measured at 450 and 630 nm by an ELISA plate reader (Titertek Multiskan, USA). The sample with OD value greater than cut-off value was regarded as a positive reaction.

From a total of 503 fecal specimens collected from pediatric patients with diarrhea, 114 (22.7%) were found to be positive for NoV using the monoplex RT-PCR screening method (Table 1). Of these, majority of the samples (112; 98.2%) belonged to the NoV subgroup II (GII), while only 2 (1.8%) carried subgroup

Table 1
Comparison of NoV detection in stool samples between NoV-immunochromatography kit and commercial ELISA kit (Denka) with monoplex RT-PCR method

Test kit	Monoplex RT-PCR		
	Positive	Negative	Total (%)
Immunochromatography			
Positive	90	14	104 (20.7)
Negative	24	375	399 (79.3)
Denka ELISA			
Positive	103	14	117 (23.3)
Negative	11	375	386 (76.7)
Total (%)	114 (22.7)	389 (77.3)	503 (100)

Table 2
Comparison of the accuracy of the NoV genotype detection between NoV-immunochromatography and ELISA Denka kit with monoplex RT-PCR

Test kit	NoV genotypes determined by monoplex RT-PCR and sequence analysis				
	GI/1	GII/3	GII/4	GII/6	Total (%)
Immunochromatography					
Positive	1	13	75	1	90 (78.9)
Negative	1	1	20	2	24 (21.1)
Denka ELISA					
Positive	2	12	86	3	103 (90.4)
Negative	–	2	9	–	11 (9.6)
Total (%)	2 (1.8)	14 (12.3)	95 (83.3)	3 (2.6)	114 (100)

I (GI) specificity. All of the NoV positive samples were characterized further for identification of their genotypes by sequence analysis of the capsid regions. It was found that both of the GI strains belonged to the GI/1 genotype (1.8%). Of the total 112 GII specimens detected, 95 (83.3%) were GII/4, 14 (12.3%) were GII/3, and 3 (2.6%) were GII/6 genotypes (Table 2).

To evaluate sensitivity, specificity, and efficiency of the newly developed NoV-immunochromatography kit, the results of 503 stool samples tested by NoV-immunochromatography were compared with those of the monoplex RT-PCR method. Of 114 samples that were positive by RT-PCR, 90 were positive by NoV-immunochromatography test, and 24 were negative. In addition, of 389 samples that negative by RT-PCR, 14 were positive by NoV-immunochromatography kit (Table 1). It was interesting to note that although this NoV-immunochromatography kit was developed for the detection of NoV GII/3 and GII/4, which were the major NoV genotypes circulating in humans, a cross-reactivity with some of GI/1 and GII/6 specimens were also observed (Table 2). The sensitivity and specificity of this NoV-immunochromatography test were 78.9 and 96.4%, respectively, and the overall efficiency compared to the monoplex RT-PCR method was 92.4%.

Based on the monoplex RT-PCR standard method, a total of 114 samples were positive for NoV detection. Of these, 103 samples were also positive by the Denka ELISA kit, while 11 samples showed discrepant results. In addition, from the 389 samples that negative by PCR, 14 were positive by the Denka ELISA kit (Table 1). Moreover, it was found that the Denka kit could detect four NoV genotypes (GI/1, GII/3, GII/4, and GII/6 as determined by sequence analysis) (Table 2). Overall, the sensitivity, specificity, and level of efficiency between the Denka ELISA kit and monoplex RT-PCR conventional method were 90.4, 96.4, and 95%, respectively. In comparison of the Denka ELISA assay and immunochromatography kit, the Denka ELISA showed a higher level of sensitivity than the immunochromatography kit (Denka kit: 90.4%, immunochromatography kit: 78.9%). However, specificity of the two kits was equal (96.4%).

Recently, large outbreaks of NoV occurred in various epidemiological settings in Japan. Although the main causative agent of these outbreaks was the GII/4 genotype, other genotypes were also detected and the predominant genotype was changed from one season to others (Morioka et al., 2006; Okada et al., 2005; Sasaki et al., 2006; Tokutake et al., 2006). When patients are diagnosed as severe diarrhea, rapid virus detection is essential for the intervention of appropriate treatment. For this reason, a new NoV-immunochromatography kit was developed to serve as a rapid method for identification of NoV directly from stool samples. In addition to the NoV-immunochromatography kit, the ELISA assay is another attractive supplementary method for screening of NoV in stool samples.

Previously, the sensitivity and specificity of immunochromatography for the detection of NoV in stool samples has been evaluated in our laboratory with a low sensitivity (72.7%) (Okame et al., 2003). The detection of NoV in stool specimens using the commercial RIDASCREEN ELISA kit (R-Biopharm AG, Darmstadt, Germany) was also reported with the sensitivity and specificity of 76.3 and 94.9%, respectively (Okitsu-Negishi et al., 2006). In the present study, the newly developed NoV-immunochromatography kit was evaluated and the results were compared with that of previous NoV-immunochromatography kit. It seems likely that the newly developed NoV-immunochromatography kit shows the sensitivity (78.9%) and specificity (96.4%) higher than that of the previous NoV-immunochromatography kit.

In this study, the new commercial ELISA kit (NV-AD) developed by Denka Seiken Co. Ltd. was also evaluated for sensitivity and specificity by comparing with the monoplex RT-PCR method using an identical set of stool samples that tested by the NoV-immunochromatography kit. It was clearly observed that the new Denka ELISA kit (NV-AD) showed the higher sensitivity (90.4%) than those of the NoV-immunochromatography test (78.9%) and RIDASCREEN ELISA kit (76.3%). When comparing of NoV-immunochromatography and RIDASCREEN ELISA kit, there was no significant difference in sensitivity and specificity. However, it should be pointed out that the advantage of NoV-immunochromatography kit is that it takes only 20 min which is much less time consuming to perform the test compare to 4 h by Denka ELISA and RIDASCREEN ELISA kits.

The detection limit of the NoV-immunochromatography assay, using the standard NoV strains, was found to be approximately 10^8 and 10^7 copies per gram of stool for NoV GII/3 and GII/4, respectively, without cross reaction with other diarrheal viruses (Immuno-Probe Co. Ltd., unpublished data). By using monoplex RT-PCR as a standard method, there were 24 samples that showed false negative results by the NoV-immunochromatography kit (Table 1). The discrepancy might be due to a low viral load in stool specimens or genetic variation that leads to antigenic change of NoV protein and fails to be recognized by MAb as reported by other commercial kits (Burton-MacLeod et al., 2004; de Bruin et al., 2006; Richards et al., 2003). Although the new NoV-immunochromatography kit was developed for the detection of GII/3 and GII/4 genotypes, which are the major genotypes detected in humans, it seems likely that this NoV-immunochromatography kit shows cross-reactivity with some of GI/1 and GII/6 from clinical samples (Table 2). Additionally, several NoV-VLP genotypes were used for testing this NoV-immunochromatography kit to determine a cross-reactivity with other NoV genotypes. It was found

that this NoV-immunochromatography kit could detect other genotypes of GII VLPs, including GII/1, GII/12, GII/13, and GII/14 genotypes. However, cross-reactivity with GI VLPs was not observed. In order to clarify this point, additional testing with several other NoV genotypes from clinical samples is essential.

In conclusion, the present study demonstrated that the NoV-immunochromatography kit or Denka ELISA kit could be used as an alternative method for detecting of NoV in stool specimens and may be practical for screening of NoV during outbreaks of food-borne and person-to-person transmitted gastroenteritis.

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Short communication

Emergence of intragenotype recombinant sapovirus in Japan

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Abstract

Sapovirus is an important causative agent of sporadic cases as well as of outbreaks of acute gastroenteritis in humans worldwide. A total of 603 fecal specimens collected from July 2005 to June 2006 from children with acute gastroenteritis in five localities in Japan (Maizuru, Tokyo, Sapporo, Saga, and Osaka) were screened for sapovirus by RT-PCR. It was found that 17 specimens were positive for sapovirus and it represented 2.8%. Interestingly, intragenotype recombinant sapovirus GI/1 emerged with 76.4% (13 of 17) and rapidly became the leading cause of acute gastroenteritis in Japan for the first time. The lower frequency of sapovirus GI/2 and GI/4 (each of 11.8%), which were the second prevailing genotypes, was also detected. A novel nomenclature of sapovirus was proposed, in which worldwide sapovirus strains were classified into seven genogroups. Of these, novel sapovirus genogroups VI and VII demonstrated the very low homologies, only 32.8–41.6% at the amino acid level and 43.6–49.9% at the nucleotide level, to those of sapovirus genogroups I–V. Of note, two distinct clusters of sapovirus were co-circulating in porcine. Interestingly, the worldwide sapovirus strains shared the 25 nucleotide-conserved region, covering the polymerase–capsid junction which differed according to each species due to multiple nucleotide substitutions. The finding suggests that the sapovirus recombination between human and animal hardly takes place in nature. This is also the first, to our best knowledge, demonstrating the emergence of the intragenotype recombinant sapovirus with its causing diarrheal illness in Japan.

Keywords: Sapovirus; Emergence; Genogroup; Recombination; Japan

1. The study

Viral gastroenteritis is a common disease with a high morbidity reported worldwide, especially in infants and the elderly. The mortality in children due to gastroenteritis is greater in developing than in developed countries. Acute gastroenteritis ranks consistently as one of the principal six causes of all deaths (Murray and Lopez, 1997; Parashar et al., 2003; Thapar and Sanderson, 2004). Sapovirus is recognized as a significant global enteropathogen, being a common cause of sporadic cases as well as of outbreaks of acute nonbacterial gastroenteritis in humans of all age in various epidemiological settings such as kindergartens, schools, and nursing home for the elderly (Chiba et al., 1979, 2000; Lopman et al., 2002; Akihara et al., 2005; Yan et al., 2005). Sapovirus is the distinct genus within the family Caliciviridae. The sapovirus genome contains two ORFs. The ORF1 encodes non-structural and capsid proteins while ORF2 encodes a small protein. Sapovirus has a typical "Star of David" configuration by electron microscopy. The prototype sapovirus is the Sapporo virus (Hu/SaV/Sapporo virus/1977/JP), which was originally discovered from an outbreak in a home for infants in Sapporo, Japan, in 1977 (Chiba et al., 1979).

A total of 603 fecal specimens were collected from sporadic cases of acute gastroenteritis in pediatric clinics, encompassing five localities (Maizuru, Tokyo, Sapporo, Saga, and Osaka) in Japan from July 2005 to June 2006. The ages of the subjects ranged from 2 months to 15 years, with a median of 26 months. The fecal specimens were diluted with distilled water to 10% suspensions, and clarified by centrifugation at 10,000 × *g* for 10 min. The supernatants were collected and the viral genomes were extracted by using a QIAamp Viral RNA kit (QIAGEN®,

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Hilden, Germany). Using PCR with specific primers SLV5317 and SLV5749 as previously reported resulted in the identification of sapovirus (Phan et al., 2005). The polymerase region was also amplified to detect recombinant sapovirus using primers SR80 and JV33. Products were sequenced directly on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence analysis was performed using CLUSTAL X (Version 1.6). A phylogenetic tree with 100 bootstrap resamples of the nucleotide alignment datasets was generated using the neighbor-joining method with CLUSTAL X. The genetic distance was calculated using Kimura's two-parameter method (PHYLIP). SimPlot software (Version 1.3) was used to compare recombinant sapovirus sequences. Reference sapovirus strains and accession numbers used in this study were as follows: Cowden (AF182760), Manchester (X86560), Hou7-1181 (AF435814), Mex11859/99 (AY157857), Sapporo/82 (U65427), Plymouth (X86559), Dresden (AY694184), Houston/90 (U95644), 6728/05/Maizuru/JP (DQ395300), Bristol/98 (AJ249939), London/92 (U956445), Parkville (U73124), MEC151A (AY144337), C12 (AY603425), SK15 (AY646855), SW278 (DQ125333), 5863/Osaka/JP (DQ401095), NK24 (AY646856), JJ681 (AY974192), LL14 (AY425671), MM280 (AY823308), QW270 (AY826426), Lyon/598 (AJ271056) and JJ259 (AY826423).

Here sapovirus was detected in 17 out of 603 specimens tested, accounting for 2.8%. Fig. 1 reveals that sapovirus was divided into three distinct genotypes 1, 2, and 4 within genogroup I (GI). Of these, GI/1 was the most predominant genotype with 76.4% (13 of 17), followed by GI/2 and GI/4 with 11.8% of each (2 of 17). Thus, there was the changing epidemiology of sapovirus genotypes in Japan with the emergence of sapovirus GI/1 together with the sudden disappearance of predominant sapovirus GI/6 in the previous year (Phan et al., 2007). All sapovirus GI/1 isolates had great homologies (99–100%) each other. Obviously, they came from the same source of infection and very likely represented the same strain, the JP-6732. By BLAST, both capsid and polymerase sequences of the JP-6732 were highly identical (99–100%) to those of the novel intragenotype recombinant sapovirus 6728/Maizuru/JP (the GI/1b polymerase and the GI/1a capsid) (Phan et al., 2006a). Taken together, the results indicated that the JP-6732 was also recognized as a recombinant strain. In contrast, all sapoviruses belonging to the GI/2 and the GI/4, the genotypes remained the same no matter the polymerase or capsid regions were analyzed.

The novel intragenotype recombinant sapovirus was first noted in a 10-month old male child with acute gastroenteritis in Maizuru City in 2005 (Phan et al., 2006a) and no additional case was reported so far. Interestingly, this virus emerged and rapidly became the leading cause of acute gastroenteritis in Japan for the first time in this study. Sapovirus capsid contains the determinants which are important for the immune recognition (Chen et al., 2004, 2006). The emergence of recombinant virus with GI/1 capsid could be explained by the insufficient antibody protection from acquired viral immunity against sapovirus GI/1 due to the lack of a trigger of the previous sapovirus GI/1 infection in the previous year.

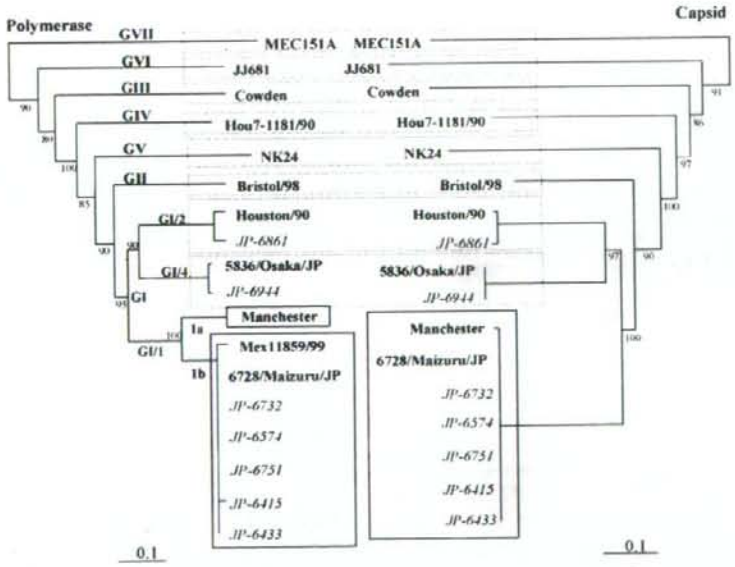

Fig. 1. Observation of changes of sapovirus subgenotypes (GI/1a and GI/1b) on the basis of phylogentic trees. The trees were constructed from nucleotide sequences of the capsid and polymerase regions of sapovirus isolates and reference sapovirus strains available in GenBank. The sapovirus isolates detected in the study are highlighted in italics. The scale indicates nucleotide substitutions per position. The numbers in the branches indicate the bootstrap values. The novel genogroup VI (known as the JJ681 virus cluster) and novel genogroup VII (known as the MEC151A virus cluster) were also shown.