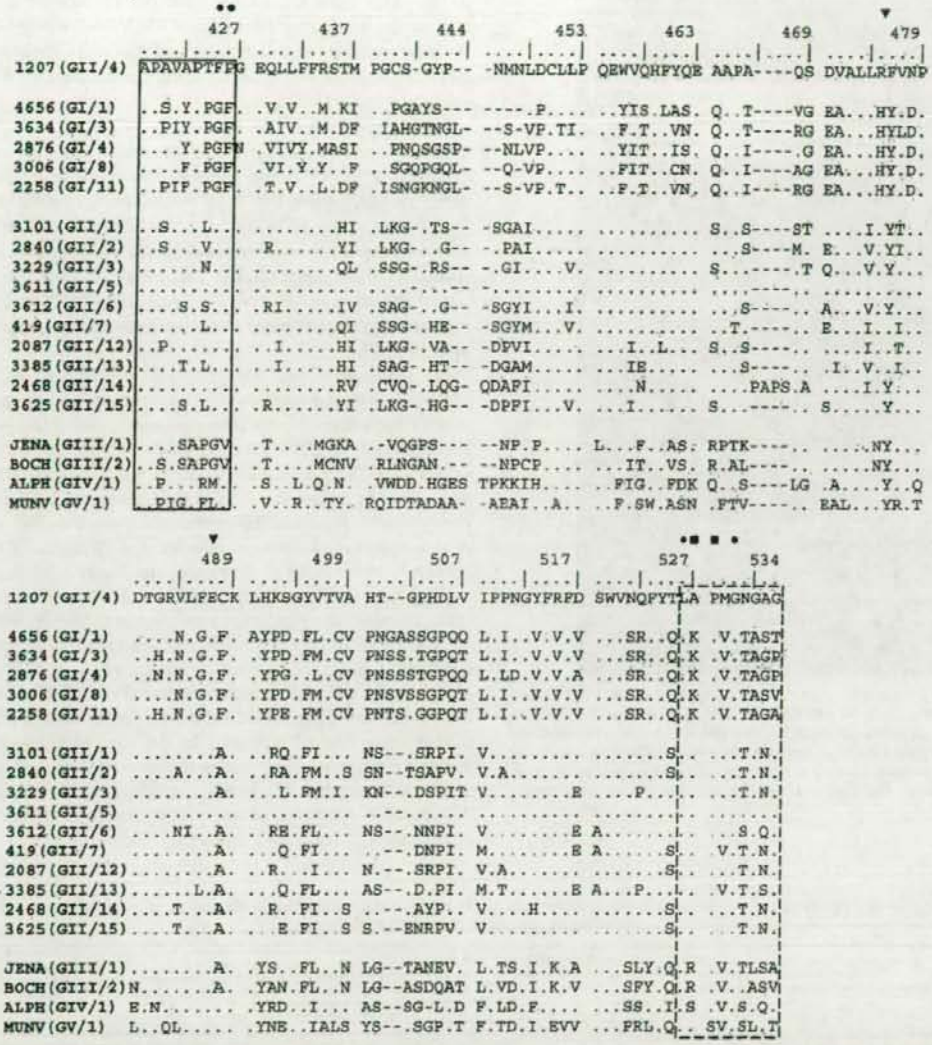

FIG. 2. Alignment of the amino acid sequence of the minimal binding region on noroviruses for MAb14-1. The MAb14-1-specific residues (●), identical components of the previously reported conformational epitope of NV3901 and NV3912 (▼), and the amino acid positions shared between the epitopes of MAb14-1,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

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

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

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

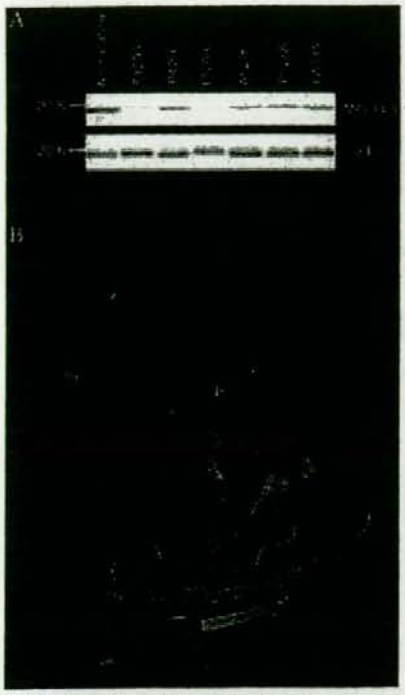

FIG. 3. (A) Reactivities of the six point mutations by Western blotting analysis. 20 K, 20,000; a-His, anti-His_5 antibody. (B) Position of each point mutation on the r1207 prediction structure. The phenylalanine at position 426 (blue), proline at position 427 (red), leucine at position 526 (yellow),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

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

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

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

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

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

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

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

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

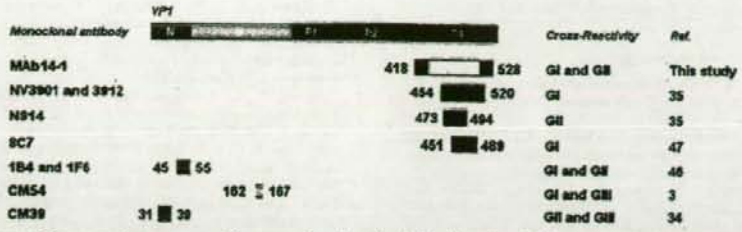

FIG. 4. Location map of the norovirus cross-reactive monoclonal antibody binding sites (being or containing an epitope) on VP1. The blank (amino acids positions 427 to 525) on the binding site for MAb14-1 means that it is not the region for a binding site but is necessary for generating a binding site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

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

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

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

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

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

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

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

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

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

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Abstract

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

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

1. Introduction

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

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

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

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

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

2. Materials and methods

2.1. Expression of rVLPs

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

2.2. Polyclonal antibody production

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

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

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

2.4. Conjugation of Poly Ab with colored latex

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

2.5. Immunochromatography

2.5.1. Test principles

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

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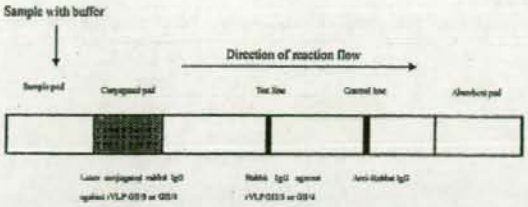

Fig. 1. Diagram of the immunochromatography for NoV.

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.

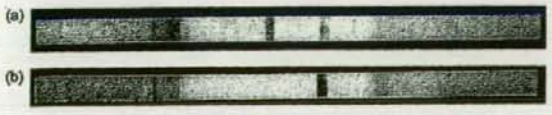

Fig. 2. (a) A representative photograph of a positive sample. (b) A representative photograph of a negative sample.

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,

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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 [illegible].

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.

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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.

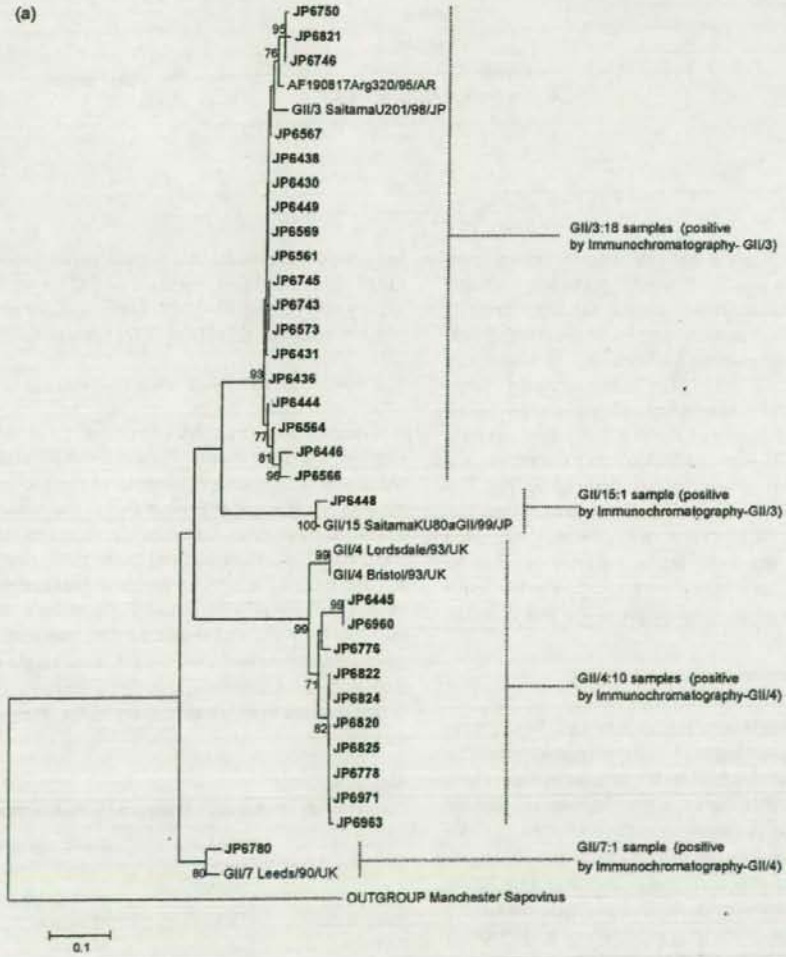

Fig. 3. (a) Phylogenetic tree of nucleotide sequences of NoV in samples positive by both immunochromatography and RT-PCR. Reference strains selected from the DDBJ/GenBank database under the accession numbers indicated in the text. Study NoV was highlighted in bold. Manchester strain was used as an out-group 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.

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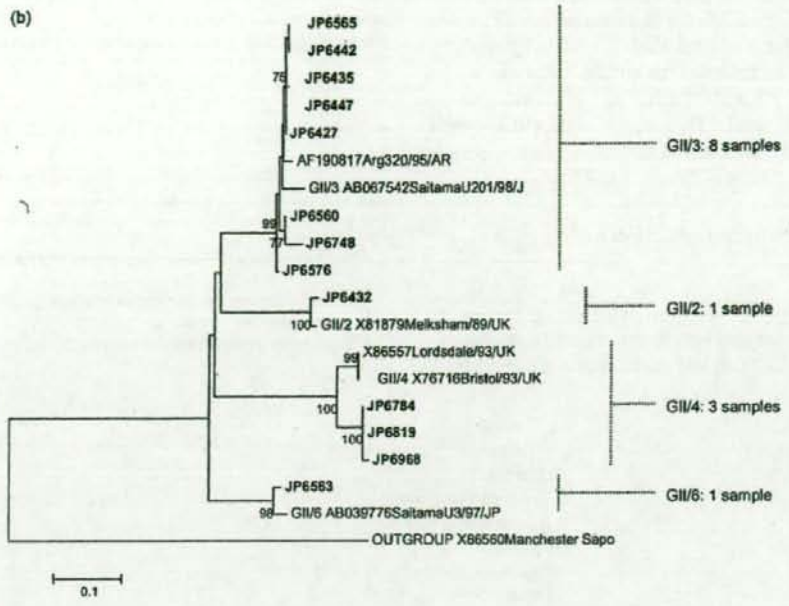

Fig. 3. (*Continued*).

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.

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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

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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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Use of sequence analysis of the VP4 gene to classify recent Vietnamese rotavirus isolates

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ABSTRACT

Twenty-eight strains of P(8), four of P(4) and one of P(19) rotavirus, isolated in Ho Chi Minh City, Vietnam, during 2002–2003, were investigated by sequence analysis of the VP4 gene. Seven of the 28 P(8) rotavirus VP4 sequences clustered in the P(8)-3 lineage, or the rare, so-called OP354-like lineage. Amino-acid sequence comparison revealed that Vietnamese P(8)-3 rotaviruses were generally very similar to Malawian strains, including the prototype OP354 strain. The numerical severity scores of diarrhoeal disease caused by the Vietnamese P(8)-3 rotaviruses were statistically higher than those of diarrhoeal disease caused by rotaviruses in the more common P(8)-2 lineage. Sequence and phylogenetic analysis of the VP4 gene of a Vietnamese G9P(19) rotavirus isolate showed a high degree of homology with the cognate genes of other human and porcine rotaviruses, including the prototype 4F strain.

Keywords Diarrhoeal disease, epidemiology, phylogenetic analysis, rotavirus, sequence analysis, Vietnam

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INTRODUCTION

Group A rotaviruses are members of the family Reoviridae and are a major cause of acute gastroenteritis in infants and young children. Rotaviruses cause *c.* 22% of childhood hospitalisations associated with diarrhoea, and are responsible worldwide for *c.* 611 000 childhood deaths annually [1]. The rotavirus genome consists of 11 segments of double-stranded RNA enclosed in a triple-layered capsid. The outer capsid layer is composed of two proteins: VP7, which defines G types (derived from glycoprotein); and VP4, which defines P types (derived from protease-sensitive protein) [2]. At least 15 different G genotypes and 27 P genotypes have been established, based on sequence analysis of the VP7 and VP4 genes, respectively [2–4]. Epidemiological studies worldwide have revealed that rotaviruses of types G1–G4, P(4) and P(8) are responsible for most infections, and four G–P combinations, G1P(8), G2P(4), G3P(8), and G4P(8), have been linked to 88.5% of the cases of rotavirus diarrhoea among children worldwide. However, these four G–P combinations accounted for <70% of rotavirus infections in South America, Asia and Africa [5].

Since VP7 is one of the two neutralising proteins, extensive studies on the molecular characteristics of the gene encoding this protein have been conducted. However, epidemiological studies of the distribution of P genotypes are also necessary for the development of a rotavirus vaccine, as sequence analyses of the VP4 gene could help to identify novel genotypes or subgenotypes in the human population, including recombinant viruses containing genes of animal origin. Data from epidemiological studies have indicated that P(8) is the predominant type, and that types P(8) and P(4) account for >90% of all cases of rotavirus diarrhoea in most parts of the world [5]. Sequence analysis of available P(8)

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rotavirus strains has revealed that they can be divided into three lineages: the P(8)-1 or Wa-like lineage; the P(8)-2 or F45-like lineage; and the P(8)-3 or OP354-like lineage, with the last being a rare subtype comprising only a few rotavirus strains isolated from Malawi [6]. It has also been reported that, besides the dominance of the P(8) type, unusual P genotypes, e.g., P(19), which are normally predominant in animals, have been found increasingly in humans [7]. Further investigation of these strains could provide important insights into the evolution of rotaviruses, including the emergence of new P serotypes or evidence of human–animal recombinant viruses [8,9].

Vietnam is a developing country with a population of 80 million individuals. There are 1.52 million births annually, and a high mortality rate among children aged <5 years (42.2 deaths/1000/year), with 15.4% of deaths being related to diarrhoeal disease [10]. Several epidemiological studies of viruses causing acute gastroenteritis have been conducted, and these have revealed the significant impact of rotavirus on diarrhoeal disease in Vietnam [11–14]. The burden of rotavirus diarrhoea in Vietnam has also been estimated in order to consider the feasibility of a vaccine trial in the near future [15,16]. However, studies concerning the molecular characteristics of rotaviruses, especially the gene encoding the VP4 protein, have been very limited. The first study of the VP4 gene of Vietnamese rotavirus strains reported the presence of P(6) rotaviruses in Khanh Hoa Province, with these viruses probably originating from porcine rotavirus [17]. The present study determined the characteristics of the VP4 gene of rotaviruses belonging to the P(8), P(4) and P(19) genotypes that were detected during surveillance in Ho Chi Minh City in 2002–2003.

MATERIALS AND METHODS

Viruses and patients

A 1-year surveillance study of common viruses causing acute gastroenteritis among children in Ho Chi Minh City was performed and has been described in detail previously [13]. In brief, 1010 faecal specimens were collected from paediatric patients who were admitted to Children's Hospital 1 with a clinical diagnosis of acute gastroenteritis between October 2002 and September 2003. Disease severity was recorded using the 20-point Vesikari score [18]. Group A rotavirus was the most common cause, accounting for 681 (67.4%) of the 1010 cases. Among the 640 samples in which the P genotype could be determined, P(8) was the most frequent (362/640), followed by P(4), P(6) and P(19) (202, five and one cases, respectively). Infections involving more than one P genotype were found in 70 cases. Representative specimens from among the rotavirus-positive samples belonging to each P type were chosen randomly for investigation in the present study.

RT-PCR and nucleotide sequencing of the VP4 gene

A 687-bp fragment of the gene encoding the VP4 protein, including the majority of the hypervariable region VP8* cleavage and a small part of the more conserved region VP5*, was amplified by primers HumCom5 (sense, nucleotides 200–221, 5′-CTCTCGATGGTCCATATCAACC) [19] and Con2 (antisense, nucleotides 887–868, 5′-ATTTCGGACCATTTATA ACC) [20]. PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and were sequenced using a Big Dye Terminator Cycle Sequencing Kit v.3.1 and an ABI Prism 310 Genetic Analyser (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instruction. The HumCom5 and Con2 primers were also used for sequencing.

RT-PCR and nucleotide sequencing of the VP6 gene

The full length of the gene encoding the VP6 protein of the G9P(19) strain, VN375/2003, was amplified as described previously [21]. Primers VP6R (antisense, nucleotides 1356–1339, 5′-GGTCACATCCTCTCACTA), derived from primer 1 [21], and VP6_694 (sense, nucleotides 694–711, 5′-CCTTAT TACCAGATGCTG), designed for this study, were used for sequencing. Sequencing was performed as described above.

Sequence and phylogenetic analysis

Similarities of the sequenced strains with other strains were assessed by BLAST searches of partial nucleotide sequences using the default options (DNA DataBank of Japan). Multiple sequence alignments were calculated using the CLUSTALW program, and the phylogenetic trees were constructed using the neighbour-joining method with the MEGA 3.1 software package [22], with the different rotavirus sequences available in GenBank being used for comparisons and as outgroups.

RESULTS

The nucleotide sequences of a 687-bp fragment of the P gene were determined for 28 strains of P(8), four strains of P(4), and one strain of P(19) rotavirus. Results from the BLAST searches confirmed the P genotyping results obtained previously by nested type-specific PCR (data not shown). Table 1 summarises the clinical data of the patients studied. The age distribution of the patients ranged from 2 to 31 months. None showed dehydration, except for one patient, VN964, who was moderately dehydrated. Evaluation of diarrhoea severity, using a 20-point numerical score, showed a range between 7 and 15 points, with a mean score of 10.5 (Table 1).

Table 1. Clinical data and P genotypes of selected Vietnamese P(8) and P(4) rotavirus strains isolated from hospitalised children in Ho Chi Minh City, Vietnam, 2002–2003

Isolate	Date of collection	P type	Age (months)	Dehydration	Vesikari's score	Mean Vesikari score ± SE (number)*
VN545	April 2003	P[8]-3	15	None	9	12.6 ± 1.0 (n = 6)
VN546	April 2003	P[8]-3	7	None	12	
VN564	April 2003	P[8]-3	NA	NA	NA	
VN645	May 2003	P[8]-3	11	None	15	
VN827	July 2003	P[8]-3	10	None	15	
VN929	September 2003	P[8]-3	21	None	11	
VN952	September 2003	P[8]-3	9	None	14	
VN6	October 2002	P[8]-2	18	None	7	9.8 ± 0.7 (n = 16)
VN21	October 2002	P[8]-2	14	None	8	
VN23	October 2002	P[8]-2	8	None	8	
VN45	October 2002	P[8]-2	12	NA	NA	
VN318	February 2003	P[8]-2	NA	NA	NA	
VN368	March 2003	P[8]-2	NA	NA	NA	
VN517	April 2003	P[8]-2	15	None	13	
VN532	April 2003	P[8]-2	8	None	12	
VN537	April 2003	P[8]-2	6	None	8	
VN538	April 2003	P[8]-2	4	None	7	
VN543	April 2003	P[8]-2	NA	NA	NA	
VN544	April 2003	P[8]-2	31	None	15	
VN548	April 2003	P[8]-2	NA	NA	NA	
VN550	April 2003	P[8]-2	22	None	12	
VN553	April 2003	P[8]-2	10	None	12	
VN574	April 2003	P[8]-2	24	None	7	
VN621	April 2003	P[8]-2	9	None	8	
VN626	May 2003	P[8]-2	14	None	9	
VN828	July 2003	P[8]-2	30	None	9	
VN851	August 2003	P[8]-2	18	None	8	
VN964	September 2003	P[8]-2	9	Moderate	14	
VN271	February 2003	P[4]	12	None	12	9.5 ± 2.5 (n = 2)
VN322	February 2003	P[4]	NA	NA	NA	
VN580	April 2003	P[4]	4	None	7	
VN594	April 2003	P[4]	NA	NA	NA	

NA, not available or not known.
*Mean severity scores ± standard error of diarrhoeal disease caused by rotaviruses in lineages P[8]-3, P[8]-2, and P[4], respectively. Numbers of patients evaluated for the severity scores are also shown.

Sequence analysis of the VP4 gene of P(8) rotaviruses

The phylogenetic tree of the nucleotide sequences demonstrated that the 28 Vietnamese P(8) rotaviruses clustered into two different lineages, lineage P(8)-2 or F45-like (21/28), and lineage P(8)-3 or OP354-like (7/28) (Fig. 1). The results of amino-acid (aa) comparisons, spanning aa 86–263, for seven Vietnamese P(8)-3 rotavirus strains, revealed that they had a high degree of homology with other strains within lineage P(8)-3 (98.3–98.8%), and a lower homology with other P(8) rotaviruses in lineages 1 and 2 (85.9–88.2%) (data not shown). Amino-acid alignments within the same region are shown in Fig. S1 (see Supplementary material), and revealed that the highly conserved proline molecules at residues 224 and 225 were seen in all strains studied. The seven Vietnamese P(8)-3 strains were, in general, identical with other rotaviruses in the OP354-like lineage, within the studied region, and had several unique residues that were characteristic of OP354-like viruses (aa 140-F, aa 149-S, aa 178-K, aa 182-G, aa 184-V, aa 187-G, SD at position 191–192, aa 211-I, aa 221-Y). However, the Vietnamese P(8) rotavirus strains did not have the same conserved residues as some Malawian strains at two positions (aa 194, I instead of T; and aa 258, A instead of T).

In view of the hypothesis that the rare OP354-like viruses might cause more severe diarrhoeal disease than other viruses in common lineages because they could escape protective immunity in communities with common P(8) rotaviruses, the severity of diarrhoea in the patients was investigated using Vesikari's numerical score. The mean score for disease caused by rotaviruses in lineages P(8)-3, P(8)-2 and P(4) was 12.6 ± 1.0 (n = 6), 9.8 ± 0.7 (n = 16) and 9.5 ± 2.5 (n = 2), respectively. The difference between the mean score for the rare P(8)-3 lineage and that for the other lineages was statistically significant ($p < 0.05$).

Sequence analysis of the VP4 gene of P(4) rotaviruses

All four Vietnamese P(4) rotavirus strains in this study were identical with each other, and clustered into a single lineage with other recent P(4) rotaviruses isolated worldwide (Fig. 2).

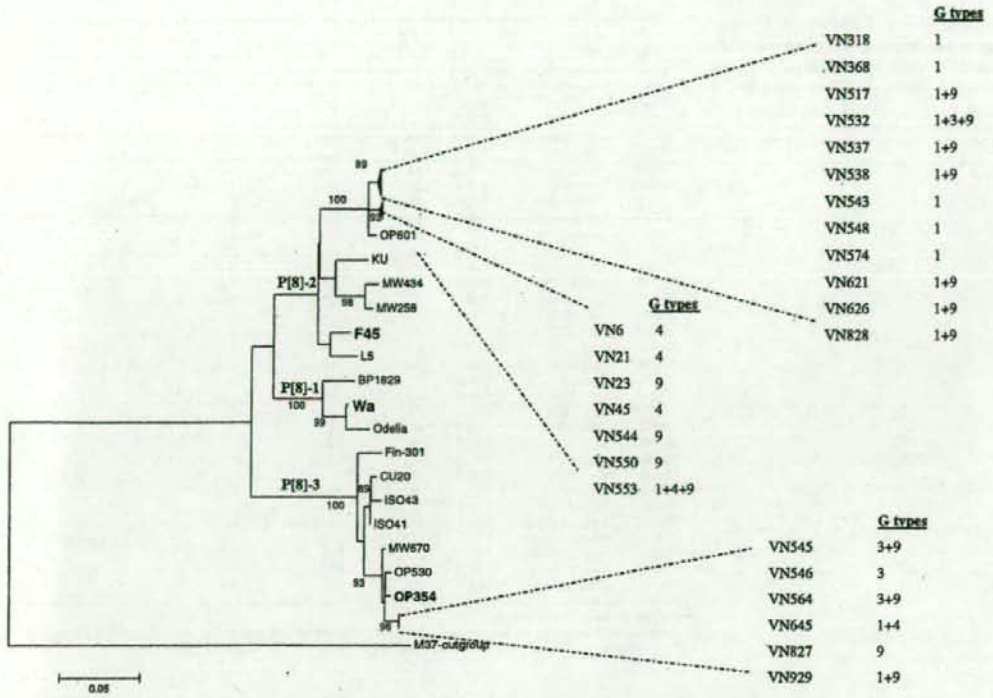

Fig. 1. Phylogenetic tree of the VP8* fragment of 28 Vietnamese P(8) rotavirus strains. Bootstrap values >70% are indicated at the branch nodes. The prototype strains of three lineages are in shown bold type. The associated G types are also shown next to each isolate. Three sub-lineages are indicated. The P(6) rotavirus strain M37 was used as an outgroup.

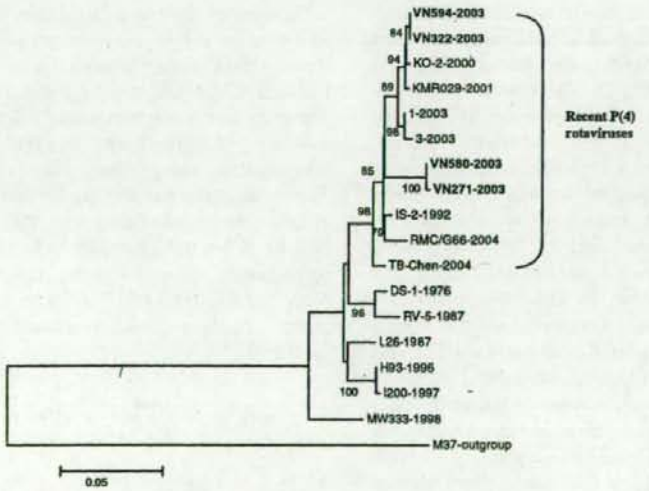

Fig. 2. Phylogenetic tree of the VP8* fragment of four Vietnamese P(4) rotavirus strains. Bootstrap values >70% are indicated at the branch nodes. Year of isolation is also shown. Vietnamese P(4) rotaviruses are shown in bold type. The P(6) rotavirus strain M37 was used as an outgroup.

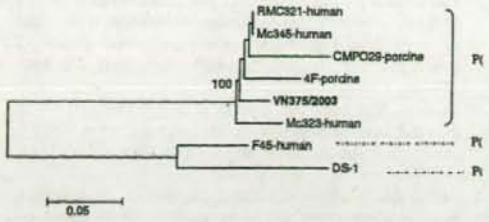

Fig. 3. Phylogenetic tree of the VP8* fragment of Vietnamese and other human and porcine P(19) rotavirus strains. The Vietnamese P(19) isolate is shown in bold type.

Sequence analysis of the VP4 and VP6 genes of a P(19) rotavirus

Nucleotide and amino-acid sequence analysis of the VP4 gene of the VN375/2003 isolate detected during 2002–2003 revealed that this strain had greater identity with P(19) rotaviruses (94.5–96.7% for nucleotides; 94.4–97.2% for amino-acids) than other P genotypes (24.2–68.1% for nucleotides; 44.6–72.2% for amino acids). The phylogenetic tree indicated that the Vietnamese P(19) strain clustered in the same group as other human and several porcine P(19) rotaviruses (Fig. 3). A deduced amino-acid comparison, from aa 88 to aa 273, showed that these P(19) strains were identical. However, the VN375 strain had several unique residues at positions aa 89-I, aa 147-N and aa 195-K. Interestingly, only the Vietnamese strain and the prototype porcine strain 4F had a valine at position 254 (Fig. S2, see Supplementary material).

A 462-bp nucleotide sequence of the VP6 gene of the VN375 strain was also analysed. The results revealed that this gene had closer identity with cognate genes of porcine rotavirus strains (91.1–93.7% for nucleotides; 98.7–99.3% for amino-acids) than with other human strains (82.4–83.7% for nucleotides; 91.5–92.2% for amino-acids).

DISCUSSION

Genotype P(8) has been found to be the most frequent P type among rotaviruses worldwide, with a prevalence of 51% in Africa and 87.8% in Europe [5]. Reports from several surveillance studies in Vietnam have also indicated a high prevalence of P(8) rotavirus (70.7–71.8%) [11,14]. The present study analysed the VP4 gene sequences of these isolates and found that, surprisingly, seven of 28 randomly chosen P(8) strains clustered in the rare P(8)-3, or so-called OP354-like lineage. Since the first report of OP354-like viruses in Malawi during 1998–1999 [6], these rare rotavirus strains have not been isolated elsewhere. A BLAST search of P(8) nucleotide sequences available from GenBank showed that only a few P(8) rotavirus strains had a high identity with the OP354 virus, including some strains from Malawi [6] and India [23], and one each from Thailand and Finland. The detection of these viruses from various countries in different continents suggests that these viruses are widespread around the world. The rather low rate of detection of these rare strains could be explained by large-scale mutation in the nucleotide sequence of the VP4 gene at the primer-binding site of the primer, 1T-1, specifically for P(8), which may make recognition of these rotavirus strains more difficult [6].

Cunliffe *et al.* [6] reported that Malawian P(8) rotaviruses predominantly comprised strains belonging to a distinct G type, in which the G4 type for OP354, the G3 type for MW258, and the G1 type for OP601 were clustered. In the present study, the phylogenetic tree indicated clearly that Vietnamese P(8) rotaviruses belonged to two separate clusters, the OP354 cluster and the OP601 cluster. The strains in the OP601 cluster could be divided further into two variants, in which one variant had a tendency to belong to G types 1 and/or 9, and the second variant had the characteristics of types G9 or G4. However, the concordance between P and G type in the OP354-like viruses was unclear (Fig. 1).

A rotavirus vaccine, which includes a P(8)-1 rotavirus strain as a component, has begun to be used widely in some countries. The finding in the present study that viruses of the P(8)-3 lineage caused, on average, more severe diarrhoeal disease may be important for the development and application of rotavirus vaccines, especially in countries in which OP354-like rotaviruses are prevalent.

The P(19) rotaviruses are known to commonly infect pigs, with the prototype virus being the porcine 4F strain [24]. To our knowledge, there have been only two reports of the isolation of the P(19) rotavirus in humans worldwide, one from Thailand and one from India [7,9]. The detection of this virus in Vietnam emphasises that the P(19)

rotavirus is circulating in Asia. Sequence analysis of the VP6 gene of the Vietnamese G9P(19) strain also showed closest identity with the cognate genes of other porcine rotaviruses, suggesting gene reassortment or a human infection by a virus similar to one that infects animals. Further investigation of this virus might provide interesting data concerning the relationship between human and porcine rotaviruses, especially in Asian countries in which farming is the main activity and close contact between humans and animals could promote inter-species transmission.

ACCESSION NUMBERS

The VP4 nucleotide sequences of the Vietnamese isolates described in this study have been deposited in GenBank under the following accession numbers: EF673752–EF673779 for the P(8) strains; and EF673780–EF673783 for the P(4) strains. The accession numbers of the nucleotide sequences of the VP4 and VP6 genes of the VN375/2003 strain are EF063154 and EU042135, respectively.

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SUPPLEMENTARY MATERIAL

The following supplementary material is available for this article online at http://www.blackwell-synergy.com:

Fig. S1. Partial amino-acid sequence alignment (aa 86-263) for selected P(8) rotaviruses. Lineages and sub-lineages are indicated.

Fig. S2. Partial amino-acid sequence (aa 88-273) alignment within selected P(19) rotaviruses. Human and porcine rotaviruses are indicated.

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