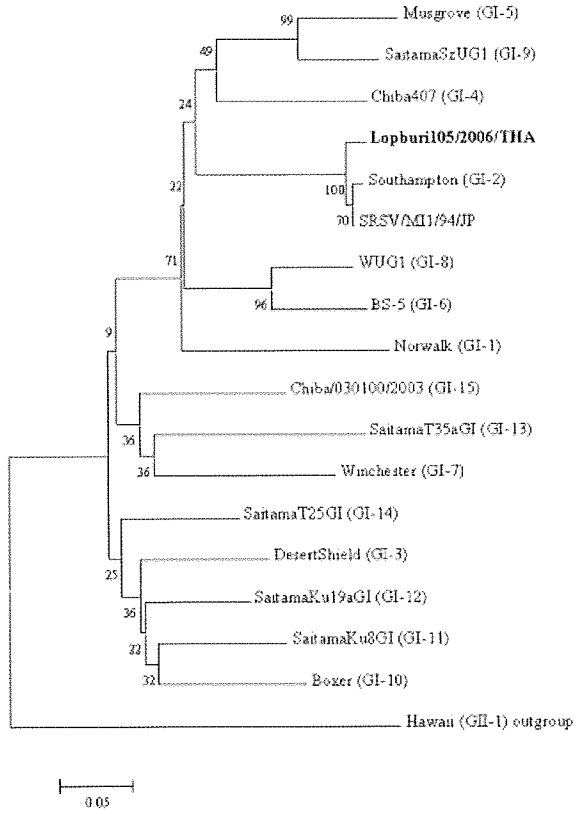

Fig. 3. Phylogenetic analysis of partial capsid sequence of norovirus GI detected in a patient with acute gastroenteritis (Lopburi105/2006/THA). The GenBank accession numbers for 15 known GI strains include: GI-1; Norwalk (M87661), GI-2; Southampton (L07418), GI-3; DesertShield (U04469), GI-4; Chiba407 (AB042808), GI-5; Musgrove (AJ277614), GI-6; BS-5 (AF093797), GI-7; Winchester (AJ277609), GI-8; WUG1 (AB081723), GI-9; Saitama SzUG1 (AB039774), GI-10; Boxer (AF538679), GI-11; Saitama KU8GI (AB058547), GI-12; Saitama KU19aGI (AB058525), GI-13; Saitama T35aGI (AB112132), GI-14; Saitama T25GI (AB112100), GI-15; Chiba/030100/2003 (AJ844469), and for GII-1; Hawaii (U07611) as outgroup. The tree was generated based on the neighbor-joining method and the numbers on each branch indicate the bootstrap values.

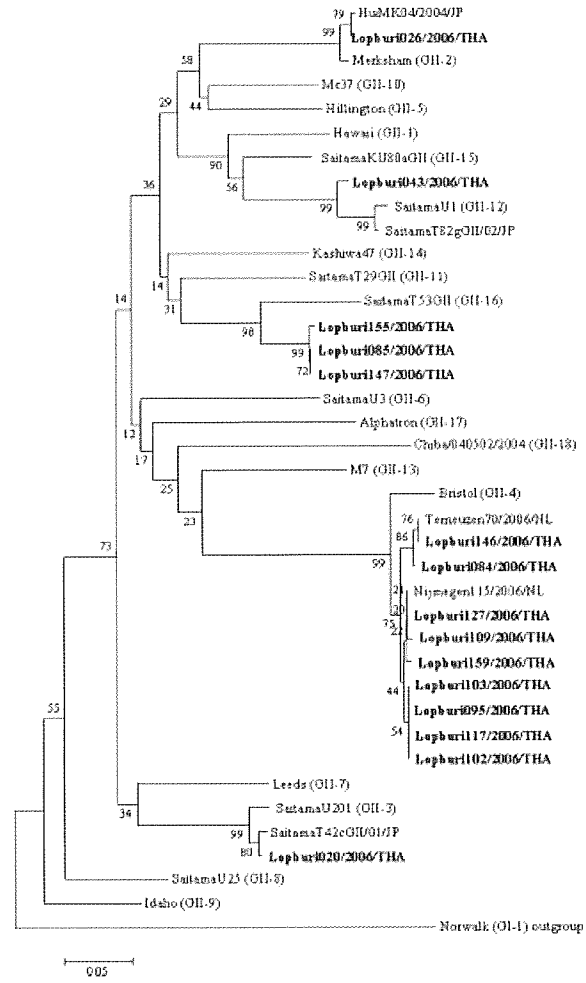

Fig. 4. Phylogenetic analysis of partial capsid sequences of norovirus GII detected in patients with acute gastroenteritis (Lopburi020-159/2006/THA). The GenBank accession numbers for 18 known GII strains include: GII-1; Hawaii (U07611), GII-2; Merksham (X81879), GII-3; Saitama U201 (AB067542), GII-4; Bristol (X76716), GII-5; Hillington (AJ277607), GII-6; Saitama U3 (AB039776), GII-7; Leeds (AJ277608), GII-8; Saitama U3 (AB067543), GII-9; Idaho Fall (AY054299), GII-10; Mc37 (AY237415), GII-11; Saitama T29GII (AB112221), GII-12; Saitama U1 (AB039775), GII-13; M7 (AY130761), GII-14; Kashiwa47 (AB078334), GII-15; Saitama KU80aGII (AB058582), GII-16; Saitama T53GII (AB112260), GII-17; Alphatron (AF195847), GII-18; Chiba/040502/2004 (AJ844470), and for GI-1; Norwalk (M87661) as outgroup. The tree was generated based on the neighbor-joining method and the numbers on each branch indicate the bootstrap values.

have not been used routinely in a hospital setting. In this study, the RT-multiplex PCR method has been used to investigate the role of enteric viruses in acute gastroenteritis in all age groups attending a hospital in Thailand. Identification and genetic analysis were undertaken by molecular technique.

Among 273 stool samples, enteropathogenic bacteria were identified only in 11 cases since most of the patients had been treated with antibiotics prior to attending the hospital. During the 14-month period study, 14.9% (39/262 patients) were detected with enteric viruses, using the RT-multiplex PCR. NoVs are the etiologic agents of acute gastroenteritis in people of all age groups but group A RVs, SaVs, AsVs, and AdVs are the cause of the infection mainly in children or the elderly [Koopmans, 2005]. The most common viruses found in the patients were NoVs as well as RVs, followed by AdVs and SaVs. Among NoV-infected patients, NoV GII was predominant. This finding is consistent with that reported previously [Hansman et al., 2004a,b; Fabiana et al., 2007; Nguyen et al., 2007; Papaventsis et al., 2007; Siebenga et al., 2007] including Thailand [Guntapong et al., 2004; Khamrin et al., 2007a]. However, the difference in NoV and SaV detection rates between this study and previous studies can be explained by the selection criteria for patients enrolled in this study. In this study, the patients enrolled were from all age groups, as opposed to the previous studies which enrolled children. Statistical significance indicates the enteric viruses caused acute gastroenteritis mainly in children less than 15 years old, as opposed to in adults.

The seasonal distribution of RV infection demonstrated a peak in January and occurred continually through March; this finding is consistent with the study

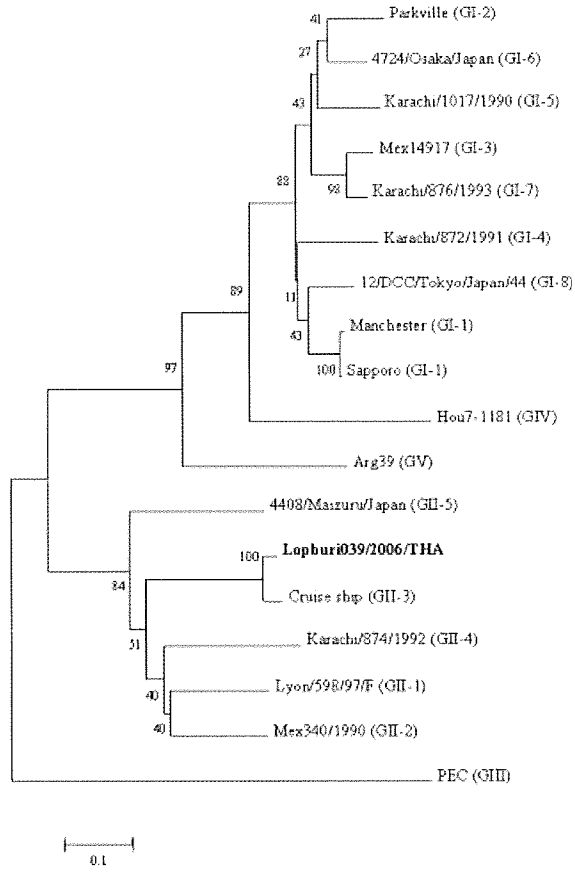

Fig. 5. Phylogenetic analysis of partial capsid sequence of sapovirus detected in a patient with acute gastroenteritis (Lopburi039/2006/THA). The GenBank accession numbers for eight known GI strains include: GI-1; Sapporo (U65427), Manchester (X86560), GI-2; Parkville (U73124), GI-3; Mex 14917 (AF435813), GI-4; Karashi/872/1991 (AB181231), GI-5; Karashi/1017/1990 (AB181132), GI-6; 4724/Osaka/Japan (AB180212), GI-7; Karashi/876/1993 (AB181227), GI-8; 12/DCC/Tokyo/Japan/44 (AB236380), for 5 known GII strains include: GII-1; Lyon/598/97/F (AJ271056), GII-2; Mex 340/1990 (AF435812), GII-3; Cruise ship (AY289804), GII-4; Karashi/874/1992 (AB181129), GII-5; 4408/Maizuru/Japan (AB180209), for GIII; PEC (AF182760), for GIV; Hou 7-1181 (AF435814), and for GV; Arg39 (AY289803). The tree was generated based on the neighbor-joining method and the numbers on each branch indicate the bootstrap values.

by Jiraphongsa et al. [2005]. NoV GI infection took place in October and NoV GII had a peak in August and September, which was earlier than the peak of RV infection, which could be found throughout the year. SaV and AdV infections, however, were only observed in children and in early summer. The seasonal pattern should be interpreted carefully since this study has some limitations regarding the collection of stool samples; the distribution of gastroenteritis patients in age groups and in each month is very different and a number of patients with gastroenteritis who had HIV infection were excluded from the study. Although viral RNA extraction kit (QIAGEN GmbH, Hilden, Germany) was used to extract total RNA from the viruses, identification of AdVs provided the feasibility of co-extracting DNA with total RNA. Using RT-multiplex PCR and DNA sequencing, AdVs found in this study were associated with human AdVs type 41, type 2 or type 38. AdV type 41 is the predominant serotype in Asian countries and associated with acute gastroenteritis in infants and children [Li et al., 2005].

In this study, RV G1 strains were the most prevalent, followed by G3 and G9. RV G9 strains were predominant in Thailand during the year 2001–2003 [Jiraphongsa et al., 2005] and declined with a reemergence of G1 and G2 during 2002–2004 [Khamrin et al., 2007c]. In 2006, RV G1 strains were found to be predominant both in this study and in other countries such as Australia [Kirkwood et al., 2007], Germany [Mas Marques et al., 2007], and Japan [Phan et al., 2007a]. One RV G1 strain showed 98% nucleotide sequence identity with human RV G1 (Chi-87), and belonged to lineage III [Trinh et al., 2007]. Other RV G1 strains showed 98–99% identity with rotavirus A strain (7265/JP) which was found in Japan during 2005–2006 and belonging to lineage II; sublineage IIC [Phan et al., 2007a].

Interestingly, a stool sample was obtained from an infant, with a RV G9 associated with a nucleotide sequence that was a close match to the reference strain GH 3574 (AY 211068). This was in accord with a previous report on the detection of group A RV in raw oysters [Kittigul et al., 2008]. These findings confirmed the presence of human RV genotypes circulating in gastroenteritis patients and bivalve shellfish. Therefore, shellfish is likely to be one of the potential vehicles for rotavirus transmission as well as environmental water, as reported by Kittigul et al. [2005].

The predominance of NoVs GII-4 in this study (60%) is similar to other studies in Thailand [Guntapong et al., 2004; Khamrin et al., 2007a]. NoV GII-4 strains cause 80% of gastroenteritis outbreaks and emergence of the GII-4 variants in The Netherlands has been reported [Siebenga et al., 2007]. In 2006, the emergence of two GII-4 variants (2006a and 2006b) were reported worldwide as having caused outbreaks of gastroenteritis in cruise ships [Koopmans et al., 2006], and healthcare settings [Buesa et al., 2008]. NoV 2006a and 2006b have been implicated as the etiologic agents in acute gastroenteritis in Europe and also in Australia and New Zealand [Tu et al., 2008]. The present study carried out during the year 2006–2007, identified both NoV GII-4 strains; two samples similar to NoV Hu/GII.4/Terneuzen70/2006/NL (2006a variant) and seven samples similar to NoV Hu/GII.4/Nijmegen115/2006/NL (2006b variant). Therefore, these NoV GII-4 strains might be associated with 2006a and 2006b variants, respectively. Further characterization of the viruses would provide information of outbreak strains circulating globally. Using the screening RT-multiplex PCR, NoVs GI were identified in one child (4 years old) and one adult (47 years old). The child infected with NoV GI had the GI-2 genotype. NoVs GI were detected in the year 2000–2001 [Hansman et al., 2004b], but disappeared during 2002–2004 [Khamrin et al., 2007b] and reemerged in this study (year 2006–2007); two NoV GI-positive stool samples were collected in October 2006. NoVs GI in patients with acute gastroenteritis are detected rarely in Japan [Yan et al., 2003] or Vietnam [Hansman et al.,

2004a; Nguyen et al., 2007]. In Thailand, NoVs are circulating in humans, oysters, and environmental water, as with RVs. Of note, NoV GI is predominant genogroup in the environment (manuscript in preparation). Further studies on the ecology of NoVs GI and GII need to be elucidated.

SaVs were found at a lower frequency than NoVs. One SaV strain belonged to GII-3, with an almost 100% nucleotide sequence identity with the cruise ship reference strain. Two previous studies have demonstrated the presence of SaV GI/1, GI/2, GIV [Khamrin et al., 2007a] and GI, GII, GV strains [Hansman et al., 2004b] with genetic diversity. The differences in SaV genotypes circulating depend on the different study sites.

The hospital-based study reported above demonstrate the importance of enteric viruses as causes of gastroenteritis in Thailand. An accurate diagnosis of acute gastroenteritis would facilitate appropriate management of patients and reduce extensive or unnecessary use of antibiotics for treatment. The use of a short conserved sequence is valuable for diagnosis of enteric virus infections, however, genotyping and phylogenetic analyses using the short sequence should be determined with caution. In conclusion, by screening acute gastroenteritis using molecular methods including RT-multiplex PCR and confirmation by DNA sequencing, an etiologic identification and genetic characterization of enteric viruses can be achieved, leading to a comprehensive investigation of the disease burden caused by RVs, NoVs, SaVs, and AdVs.

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G2 Strain of Rotavirus among Infants and Children, Bangladesh

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To determine G and P genotypes, we performed nested PCR on 307 rotavirus specimens collected in Dhaka, Bangladesh, during 2004–2005. G2 (43.3%) was detected at the highest frequency, followed by G4 (19.5%), G9 (13.7%), G1 (12.7%), and G3 (2.6%). P[8] was the most predominant genotype (53.2%), followed by P[4] (42.9%).

Group A rotavirus (RAV) is the leading cause of severe gastroenteritis in infants and young children worldwide and accounts for ≈600,000 deaths in children <5 years of age (*1*). Rotaviruses are members of the *Reoviridae* family (*2*) and are classified into 7 groups (A–G) on the basis of distinct antigenic and genetic properties (*3*). On the basis of neutralization assay and sequence analysis, a total of 15 G and 27 P genotypes of RAV have been documented (*4*). The major human G types are G1, G2, G3, G4, and G9, which, when combined with the P types P[8], P[4], and P[6], account for >80% of rotavirus-associated gastroenteritis (*5*).

The Study

A total of 917 stool specimens were collected from infants and children with acute gastroenteritis in Dhaka Children's Hospital, Bangladesh, during October 2004 to September 2005. Fecal specimens were diluted with distilled water to 10% suspensions and clarified by centrifugation at 10,000 × *g* for 10 min. The supernatant was collected and viral genomes were extracted from fecal specimens by using the QIAamp viral RNA Mini Kit (QIAGEN, Hilden, Germany). Using reverse transcription–PCR (RT-PCR) with specific primers, as previously reported (*6*), resulted in the identification of diarrheal viruses, including group A, B, and C rotaviruses and adenovirus.

RAV-positive samples were then subjected to G and P genotyping by nested PCR with previously published primers (*7–9*).The RAV isolate for which the G and P types could not be determined by RT-PCR method was subjected to nucleotide sequence analysis of PCR products positive for VP7 and VP4 genes with the BigDye Terminator Cycle Sequencing Kit and ABI Prism 3100 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA, USA). Their VP7 and VP4 nucleotide sequences were compared as well as those of reference rotavirus strains available in GenBank by using BLAST (http://blast.ncbi.nlm.nih.,gov/Blast.cgi). Phylogenetic and molecular evolutionary analyses were conducted by using MEGA version 3.2 software (*10*). The sequences of VP4 and VP7 genes of rotaviruses detected in this study had been submitted to GenBank under accession nos. EU855813–EU855822 and EU855823–EU855830, respectively.

Among diarrhea-causing viruses detected, RAV was the most prevalent (33.5%), followed by adenoviruses (1.9%). Rotavirus group B and rotavirus group C could not be detected in this study. Ninety-seven percent of RAV-infected patients were hospitalized. Co-infection between RAV and adenoviruses was identified in 7 of 917 samples. Most of the patients in this study were 1 to 24 months of age; RAV infection was most commonly detected in patients 6 to 23 months of age. Gender distribution of patients with RAV-positive samples was 56% male and 44% female.

We could not initially determine G type for 10 RAV and P type for 8 RAV isolates, even though their VP7 and VP4 genes were successfully amplified by RT-PCR. Sequence analysis showed that all of untypeable RAV were G1 and P[8]. Ten rotavirus G1 sequences were classified into a distinct lineage, lineage1 and sublineage 1a. G1 strains analyzed in this study belonged to the Asian cluster and were most closely related to Dhaka-02, Dhaka-03, and Thai-1602 strains, which had high identities at the nucleotide level with each other (99%–100%). Eight rotavirus P[8] sequences in this study belonged to 1 distinct lineage, lineage P[8]-II, but made a novel sublineage, sublineage P[8]-IIB, which had a high nucleotide sequence identity of 100% within lineage P[8]-II (Figure 1). These untypeable rotavirus P[8] strains contained 2–3 point mutations at the VP4 primer-binding site.

RAV was detected all year round, but 2 peaks in infections occurred: 1 peak apparently lasted 4 months (October 2004 through January 2005) and another peak lasted 3 months (June 2005 through August 2005) (Figure 2). Five different G types, G1–G4 and G9, were detected during the study period. Of these, G2 was the most common (43.3%) followed by G4 (19.5%), G9 (13.7%), G1 (12.7%), and G3 (2.6%). Mixed infections between >1 G genotypes were

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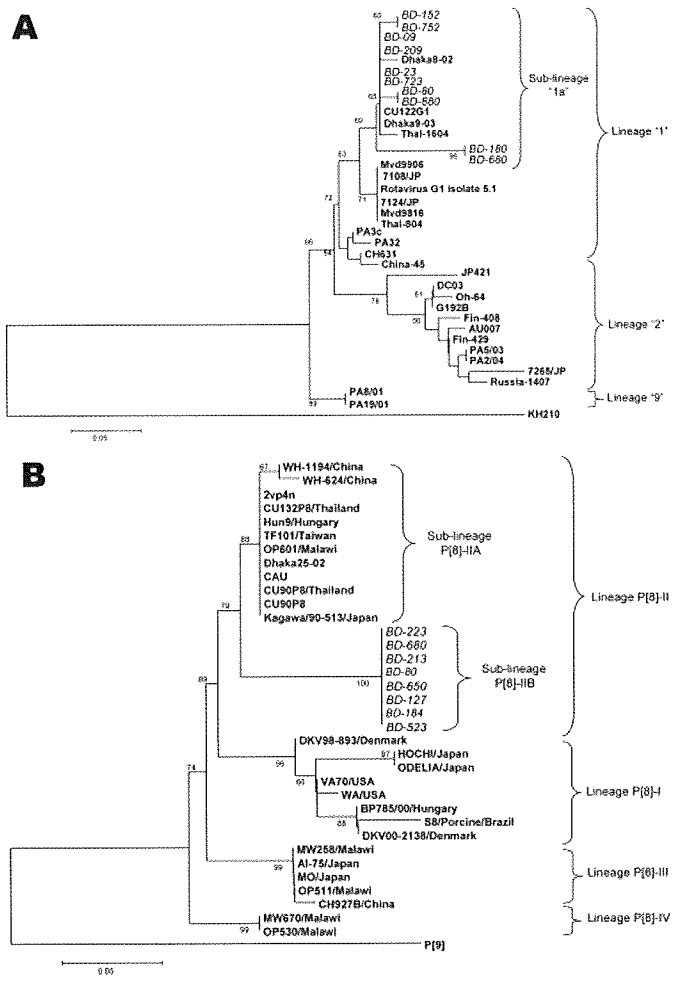

Figure 1. Phylogenetic analysis of the nucleotide sequences of the VP7 and VP4 genes of untypeable group A rotavirus strains (RAV) from Bangladesh. A) Neighbor-joining phylogenetic tree based on nucleotide sequences of the VP7 encoding genes for untypeable RAV strains. B) Neighbor-joining phylogenetic tree based on nucleotide sequences of the VP4 encoding genes for untypeable RAV strains. The numbers in the branches indicate the bootstrap values. Reference strains of RAV G1 and P[8] strains were selected from DNA database of Japan/GenBank under the accession number indicated in **boldface**. G1 strains from Bangladesh are highlighted in *italics*. The scale bars indicate nucleotide substitutions per position. Reference RAV strains used in this study and their accession numbers are as follows: RAV P[8] strains: BP785/00/Hungary (AJ605315), VA70/USA (AJ540229), WA/ USA (L34161), HOCHI/Japan (AB039943), ODELIA/Japan (AB039942), MW670/Malawi (AJ302146), OP530/Malawi (AJ302152), AI-75/Japan (AB008285), MW258/Malawi (AJ302143), OP511/Malawi (AJ302151), CH927B/China (AB008273), MO/Japan (AB008278), Kagawa/90-513/Japan (AB039944), OP601/Malawi (AJ302153), CU132P8/Thailand (DQ235955), DK V98-893/Denmark (AY509908), DK V00- 2138/Denmark (AY509910), S8/Porcine/Brazil (AF052449), CU90P8/Thailand (DQ235978), TF101/Taiwan (AF183870), Hun9/Hungary (AJ605320), WH-1194/China (AY856445), Dhaka25-02 (DQ146652), CU90P8 (DQ235978), 2vp4n (DQ675009), CAU 164 (EU679398) and WH-624/China (AY856444); RAV G1 strains: Dhaka9-03 (DQ482715), CU122G1 (DQ236053), PA5/03 (DQ377596), KH210 (AB303218), 7014/JP (EF079064), rotavirus G1 isolate 5.1(DQ672628), Mvd9906 (AF480278), 7265/JP (EF079066), 7124/JP(EF079069), 7108/JP (EF079068), JP421 (D16326), Fin-408 (Z80303), PA2/04 (DQ377598), Fin-429 (Z80312), AU007 (AB081799), G192B (AF043678), DC03 (AF183859), Oh-64 (U26387), PA3c (DQ377566), PA32 (DQ377574), Thai-1604 (DQ512981), Dhaka8-02 (AY631049), Thai-804 (DQ512979), Mvd9816 (AF480293) CH631 (AF183857), China-45 (U26371), Russia-1407 (S83903), PA8/01 (DQ377592), PA19/01 (DQ377593).

identified in 4% of the specimens (Table). Genotype G2 was detected in every month with a relatively high incidence rate. Among 307 RAV-positive samples, 280 samples were P typed successfully, and P[8] was the most predominant genotype (53.2%), followed by P[4] (42.9%) and mixed infections between different P genotypes (5.0%). G2P[4] combination was the most predominant genotype (39%), followed by G4P[8] (18.2%), G9P[8] (13%), G1P[8] (11.8%), and G3P[8] (2.9%).

Conclusions

Of 917 fecal specimens tested, 307 (33.5%) were positive for RAV. This result was consistent with the previous findings on rotavirus epidemiology in Bangladesh in which its prevalence was ≈29% (*11,12*). Our study demonstrated 2 peaks of rotavirus infection. The winter rotavirus peak is usually observed worldwide, but the monsoon peak is not common in settings with temperate climates. Why there was a relation between rainy season and viral infection in this study is not clear. We identified most of the globally common rotavirus types (G1, G2, G4, and G9) in our study. Even though G3 is one of the most prevalent rotavirus types worldwide, the G3 strain has not been detected in Bangladesh since 1993 (*13*). However, we found that 4% of the rotavirus types identified in this study were G3. Results of rotavirus diversity from this study were compared with results of previous studies in Bangladesh (*13*), and we found that G2 was a predominant rotavirus strain among infants and children in Dhaka, Bangladesh.

Rotavirus G4 genotype was the most common genotype in Dhaka from 1992 through 1997 but became a less common rotavirus strain over time; G9 was the leading genotype followed by G2, G4, and G1 in Dhaka (*12,14*). The prevalence of G9 strains was nearly the same in our study, but G2 strains showed a dramatic increase. From 2001–2004, the most common rotavirus genotype was P[8] (76%); non-P[8] strains constituted ≈20%. We also found that rotavirus P[8] (53.2%) strain was the most prevalent. We found that the 4 most common strains globally, G1P[8], G2P[4], G3P[8], and G4[8], were found in 83.9% of cas-

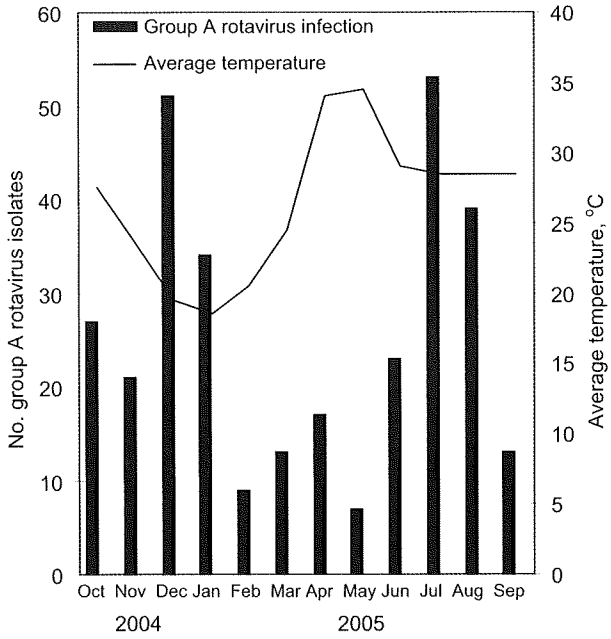

Figure 2. Seasonal pattern of group A rotavirus infection in infants and children with acute gastroenteritis in Dhaka, Bangladesh, October 2004–September 2005.

es. The G1P[8] strains, less common in 2001, became the predominant strains in the following years, but decreased again in 2005–06. Rotaviruses show great genomic diversity, and several studies in different regions of Bangladesh have identified types not targeted by candidate rotavirus vaccines (*11,14*). The frequent genomic reassortment among different rotavirus types was accelerated by mixed infection and generated huge genomic diversity (*13*).

RAV has been associated with gastroenteritis outbreaks in infants and children <5 years of age. However, less is known of the age distribution of rotavirus infection in Bangladesh. In this study, infections were most commonly detected in children <2 years of age.

Common clinical symptoms of RAV-infected patients were dehydration (84%), vomiting (69%), abdominal pain (52%), and fever (31%), which are in agreement with previous published reports (*15*). Number of loose stools per day was increased, with most patients (76%) having loose stools 3–5 times per day. Our study is limited because we could not conduct other tests such as enzyme immunoassay or polyacrylamide gel electrophoresis to confirm rotavirus illness. The incidence of rotavirus gastroenteritis identified by RT-PCR could be an overestimate because healthy controls tested by RT-PCR had a 5%–10% general incidence of rotavirus.

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Mr Dey is a PhD student at The University of Tokyo. His research interest focuses on molecular epidemiology of gastroenteritis viruses in humans.

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Table. Distribution of group A rotavirus G and P genotypes among infants and children with acute gastroenteritis in Dhaka City, Bangladesh, 2004–2005*

Genotype	P[8]	P[4]	P[6]	Mixed*	Nontypeable	Total
G1	33	4	0	1	2	40
G2	11	109	0	9	13	142
G3	8	0	0	1	0	9
G4	51	4	0	2	5	62
G9	36	2	1	2	3	44
Mixed*	10	0	0	0	0	10
Total	149	119	1	15	23	307

*>1 G or P genotype was recognized.

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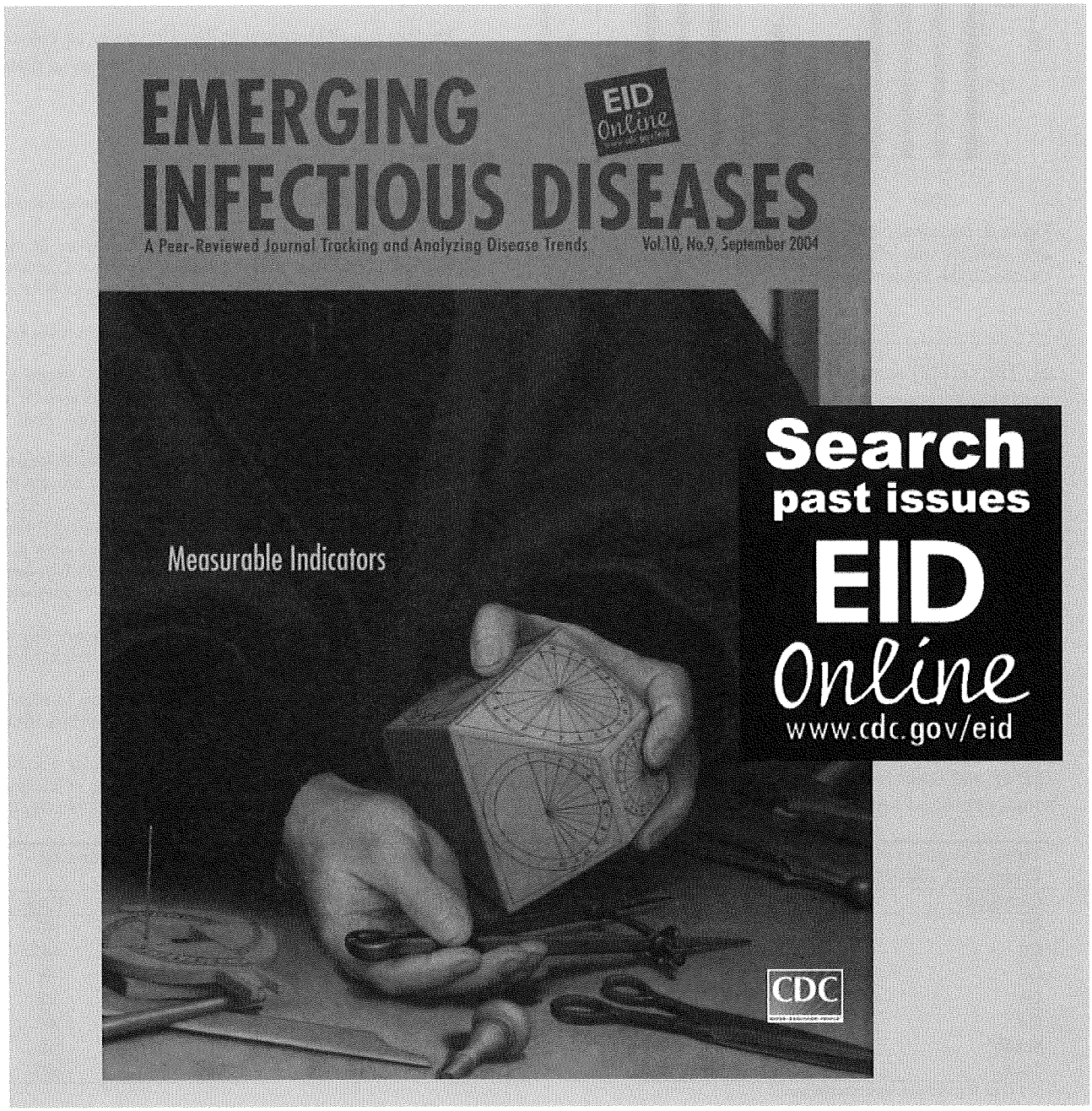

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Short communication

Detection, genetic characterization, and quantification of norovirus RNA from sera of children with gastroenteritis

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ABSTRACT

Background: Norovirus (NoV) infection is thought to be confined to the intestines, whereas many reports suggest antigenemia and viremia occur during rotavirus gastroenteritis.
Objectives: To detect NoV RNA in sera and cerebrospinal fluids (CSF) from NoV-infected children, and to quantify and genetically characterize the NoV found in these compartments.
Study design: Semi-nested PCR was conducted on stool, serum and CSF samples from 56 patients with acute gastroenteritis. Positive samples for NoV were analyzed further by sequencing and real-time PCR.
Results: From 39 patients with NoV RNA in stools, 6 also had NoV RNA in sera and none had NoV RNA in CSF. Genotypes of the NoV in stool and serum from the same patient matched completely. The strains in this study had high homology (98.1–100%) with registered strains in the database. The median viral load in stools of the serum-positive patients was greater than that of the serum-negative patients, but this difference was not statistically significant (9.8×10^9 copies/g versus 1.1×10^9 copies/g ($p = 0.117$)).
Conclusions: NoV RNA appeared in the blood stream in 15% of the patients of NoV gastroenteritis. Although the viral load in stool was not statistically correlated with NoV appearance in serum, genetic analysis indicated that NoV RNA in sera originated from the NoV gastroenteritis.
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1. Introduction

Norovirus (NoV), a member of the family *Caliciviridae* in the genus *Norovirus*, is a major cause of non-bacterial acute gastroenteritis all over the world.[1–3] The main symptoms of NoV infection are diarrhea and vomiting, which are usually mild and self-limiting. However, a recent case report demonstrated that a patient suffered from disseminated intravascular coagulation during a NoV outbreak, in association with obtundation, headache and photophobia[4] and Ito et al.[5] reported NoV-associated encephalopathy with altered consciousness. These reports indicate a potential spread of NoV to organs other than the intestines.

Many studies have been conducted seeking evidence of extra-intestinal manifestations of rotavirus infection. These included detection of rotavirus RNA in blood[6,7], CSF[7–9] and throat swabs.[7] Although early works suggested that this was due to unusual rotavirus strains or rare host genetic or immunologic defects in the infected child,[10] recent analysis revealed that rotavirus antigen is commonly detected in sera of immunocompetent children with rotavirus diarrhea (43–67%).[11–13]

Human NoV, unlike rotavirus, is not capable of growing in cell lines and has no animal model available, thus hindering study of systemic spread after intestinal infection. Detection of NoV RNA from specimens other than stools has been limited to one case in which NoV was present in serum and CSF from a previously healthy NoV-infected girl with encephalopathy.[5]

In this study, we sought to detect NoV RNA in blood and CSF from patients with NoV gastroenteritis. Genetic analyses and quantification of NoV RNA were undertaken on positive samples.

2. Materials and methods

2.1. Sample collection

From the diarrheal patients who attended Department of Pediatrics in Teikyo University Hospital, Eijudo clinic, and Red Cross

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Society Wakayama Medical Center from December 2005–2006, 56 cases who needed venepuncture for examination or infusion were recruited into this study. Stool samples were collected from 56 cases only once, while several blood samples were taken from patients who were required additional venepuncture in later course (total = 90). The interval between stool and the first blood sample collection was less than 2 days. CSF was also obtained from two patients who had convulsions. Ethical approvals were obtained from the Ethical Committees and informed consent was given by the guardians of the patients.

2.2. Reverse transcription (RT)-PCR

RT-PCR was performed according to the previously described protocol[14] with primers COG1F/G1-SKR (first round PCR) and G1-SKF/G1-SKR (second round (semi-nested) PCR) for NoV genogroup I (GI), and COG2F/G2-SK (first round PCR) and G2-SKF/G2-SKR (second round PCR) for NoV genogroup II (GII).[15,16] We used known-positive specimen extracts as positive controls, JP 6146 for NoV GI and JP 5235 for NoV GII, respectively. PCR amplicons were electrophoresed in a 1.5% agarose gel, followed by staining with ethidium bromide (EtBr, 0.5 μg/ml) for 20 min and visualized under ultraviolet light.

2.3. Sequence analysis

Positive PCR products were subjected to sequencing by Big-Dye terminator cycle sequencing kit and an ABI prism 310 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA). We used first round PCR products for sequencing if first round PCR gave positive results. If only second round PCR was positive, we used second round PCR products. In both cases, we sequenced with the same primers used in each round on both strands. Sequence analysis was performed using CLUSTAL X software (Version 1.81). Reference NoV strains and accession numbers used in this study were as follows: Norwalk/68/US (M87661), Melksham/89/UK (X81879), SaitamaU201/98/J (AB067542), Arg320/95/AR (AF190817), Lordsdale/93/UK (X86557), Bristol/93/UK (X76716), and Manchester_Sapovirus (X86560).

2.4. Quantification of NoV

We performed real-time PCR to quantify NoV viral RNA in PCR-positive samples, as previously described.[17] Data were corrected by using ROX passive reference as an internal standard and recovery rate of the NoV genome was tested by highly purified NoV plasmid standards containing PCR products of the ORF1–ORF2 junction of the GII strain (Saitama U201, AB039782).[17] Half of the serum samples which were positive by RT-PCR in our laboratory were selected arbitrarily and tested for real-time PCR at the National Institute of Infectious Diseases in Tokyo in order to reconfirm the existence of NoV genes in serum.

2.5. Statistical analysis

SPSS software version 12.0J was used for data analysis. A p-value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. RT-PCR and patients' characteristics

Among the 56 stool samples collected, 26 were positive for NoV GII by first round PCR and 13 were positive by second round. Among the 90 serum samples collected from the 56 patients, 6 were positive for NoV GII by second round PCR and were confirmed by sequence analysis to be NoV. Neither of the two CSF samples contained NoV RNA even by second round PCR, although stool samples from these patients contained NoV GII RNA by first round PCR. All of the samples tested were negative for NoV GI.

3.2. Nucleotide sequence and phylogenetic analysis of NoV GII

The partial nucleotide sequences (282 bp) of the NoV capsid gene were analyzed by phylogenetic grouping according to the classification schemes described by Kageyama et al.[18] They were grouped into three distinct genotypes; two stool samples in genogroup II genotype 2 (GII/2), four stool samples and one serum sample in GII/3, and 33 stool and five serum samples in GII/4. The genotypes of stool and serum samples collected from the same patients were all matched and the identities of nucleotide sequences between these pairs ranged between 99.2% and 100%. The phylogenetic tree of the six pairs of stool and serum samples together with the reference strains is shown in Fig. 1.

The identities among strains in the same genotypes ranged from 96.8% to 99.6%, and each strain showed high identities (98.1–100%) with previously registered Japanese strains in the DDBJ DNA/GenBank database.

3.3. Quantification of NoV RNA

The median RNA viral load detected in the stool samples from patients with serum-positive and negative for NoV GII was 9.8×10^9 (range 4.2×10^8 to 4.0×10^{10}) and 1.1×10^9 (range 5.6×10^3 to 1.4×10^{11}) copies/g of stool, respectively. This difference was not statistically significant ($p = 0.117$, two-tailed Mann–Whitney U-test). The mean value for each group was 1.4×10^{10} (standard error, 8.1×10^9) and 1.0×10^{10} (standard error, 5.0×10^9) copies/g of stool with no statistical difference ($p = 0.722$, t-test). We also investigated any correlation between RNA viral load in the serum sample and that in the paired stool sample. Patient C was excluded from this analysis because no data by real-time PCR was available. With this small number of pairs ($n = 5$), no linear correlation between the serum and stool viral load was observed ($r = 0.071$, $p = 0.910$).

4. Discussion

Potential extra-intestinal spread is an important issue in understanding the pathogenesis of viral gastroenteritis. In this study, we observed that 15% of the NoV gastroenteritis patients (6/39) had NoV RNA in serum and could not detect NoV RNA in either of two CSF samples.

Our genetic analysis showed a very high homology between strains found in stool and serum, indicating that the viral RNA in blood had originated from the intestinal tract. The high homology between the strains in this study and those deposited in GenBank implied that no unique strains were associated with detection in blood stream.

Although the mean viral load in stool of serum-positive group was greater than that of serum-negative group, there was not statistically significant difference between these two groups. Several studies on rotavirus gastroenteritis showed that the antigen level in blood samples decreased as diarrhea subsided, implying a relationship between the presence of virus in the intestines and in the blood.[11,12] Hence, we may be able to observe a similar relationship in a larger sample sized study and/or samples collected consecutively from the same group.

Recently, experiments with murine NoV in a mouse model showed that infectious virus was detected in peripheral organs such as spleen, liver, and lung after oral inoculation, and that histopathological changes were observed not only in the intestines, but also in the spleen of immunocompetent mice.[19,20] These findings are of

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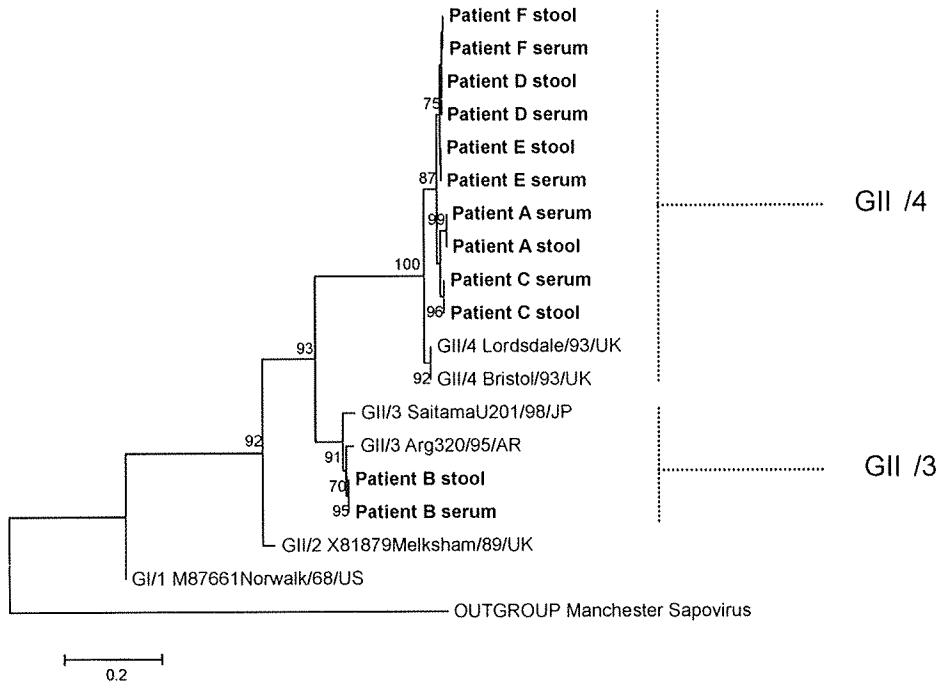

Fig. 1. Phylogenetic tree of NoV GII nucleotide sequences detected in pairs of stool and serum samples. The tree was constructed from partial nucleotide sequences of the capsid region. Reference strains of NoV were selected from the DDBJ DNA/GenBank database under the accession numbers indicated in the text. Strains detected in this study are highlighted in bold. Percentage bootstrap values above 70% are shown at the branch nodes. The scale indicates nucleotide substitutions per position.

significance in reviewing the current concept of human NoV, which focuses on intestinal infection. The murine model is consistent with our findings that suggest extr-intestinal spread of NoV during NoV gastroenteritis.

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Short communication

Immunochromatography test for rapid detection of norovirus in fecal specimens

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Japan

A B S T R A C T

An immunochromatography (IC) assay for rapid detection of norovirus (NoV) was evaluated with fecal samples collected from children who suffered from acute gastroenteritis during the winter season of 2007–2008 in Japan. A total of 75 fecal specimens were tested for NoV by the newly developed IC kit and by a gold standard RT-PCR method. The sensitivity, specificity, and agreement of this IC kit were 75.4%, 100%, and 80%, respectively. In addition, phylogenetic analysis revealed that the majority of NoV circulating in Japan during 2007–2008 belonged to the new variant GII/4 2006b genetic cluster. It was demonstrated that the IC kit evaluated in this study could detect these new variant NoV strains, which emerged recently in Japan. Therefore, it is suggested that this NoV IC kit could be used as an alternative method for the screening of NoV in fecal specimens, especially during the season of acute gastroenteritis outbreak.

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Viral enteric infections affect millions of people worldwide. The major agents include rotavirus, astrovirus, adenovirus, and enteric caliciviruses (norovirus and sapovirus) (Clark and McKendrick, 2004; Wilhelmi et al., 2003). These viruses are transmitted mainly via fecal–oral route through person-to-person contact or consumption of contaminated food. Recently, norovirus (NoV) has been emerged as significant etiologic agent and is recognized as the major cause of non-bacterial acute gastroenteritis in all age groups. The virus is a member of the family *Caliciviridae*, which is nonenveloped, positive-sense, single-stranded RNA virus (Green, 2007).

Because no readily available cell culture system exists for NoV, characterization and classification of NoV is based on reverse transcription-polymerase chain reaction (RT-PCR), genomic sequencing, and phylogenetic analysis (Kageyama et al., 2004; Zheng et al., 2006). In recent years, extensive molecular epidemiological studies of calicivirus infections in humans have been conducted. These detection techniques have markedly enhanced our understanding of the epidemiology and genetic diversity of NoVs. According to the latest scheme for NoV nomenclature, NoVs have been classified into five separate genogroups (GI to GV) and at least 29 genotypes (Zheng et al., 2006). Several epidemiological studies have clearly indicated that NoV GII/4 has been the most highly prevalent genotype in humans worldwide during the past decade (Buesa et al., 2008; Bull et al., 2006; Hansman et al., 2004; Khamrin et al., 2007; Malasao et al., 2008; Motomura et al., 2008; Reuter et al., 2008; Tu et al., 2007).

Recently, large outbreaks of NoV have occurred in various epidemiological settings in Japan and the main causative agent was the NoV GII/4 genotype (Morioka et al., 2006; Motomura et al., 2008; Okada et al., 2007; Sasaki et al., 2006). When patients are diagnosed with severe diarrhea, rapid virus identification is essential to ensure administration of the appropriate treatment and control. For this reason, a rapid and sensitive diagnostic tool, such as the immunochromatography (IC) test, is required for NoV detection.

Most recently, two reports on the evaluation of IC test (IP-Noro; Immuno Probe Co., Ltd., Saitama, Japan) for rapid detection of NoV directly from stool samples collected in Japan and Vietnam demonstrated the sensitivity, specificity, and overall agreement of the tests at 78.9% and 73.7%, 96.4% and 100%, 92.4% and 95.2%, respectively (Khamrin et al., 2008; Nguyen et al., 2007). However, that commercial IC test was developed by using specific antibodies against NoV GII/3 and GII/4, which previously showed broad reactivity with several NoV VLP genotypes (Shiota et al., 2007). In this report, we have evaluated the efficacy of the newly developed IC kit (Morinaga Milk

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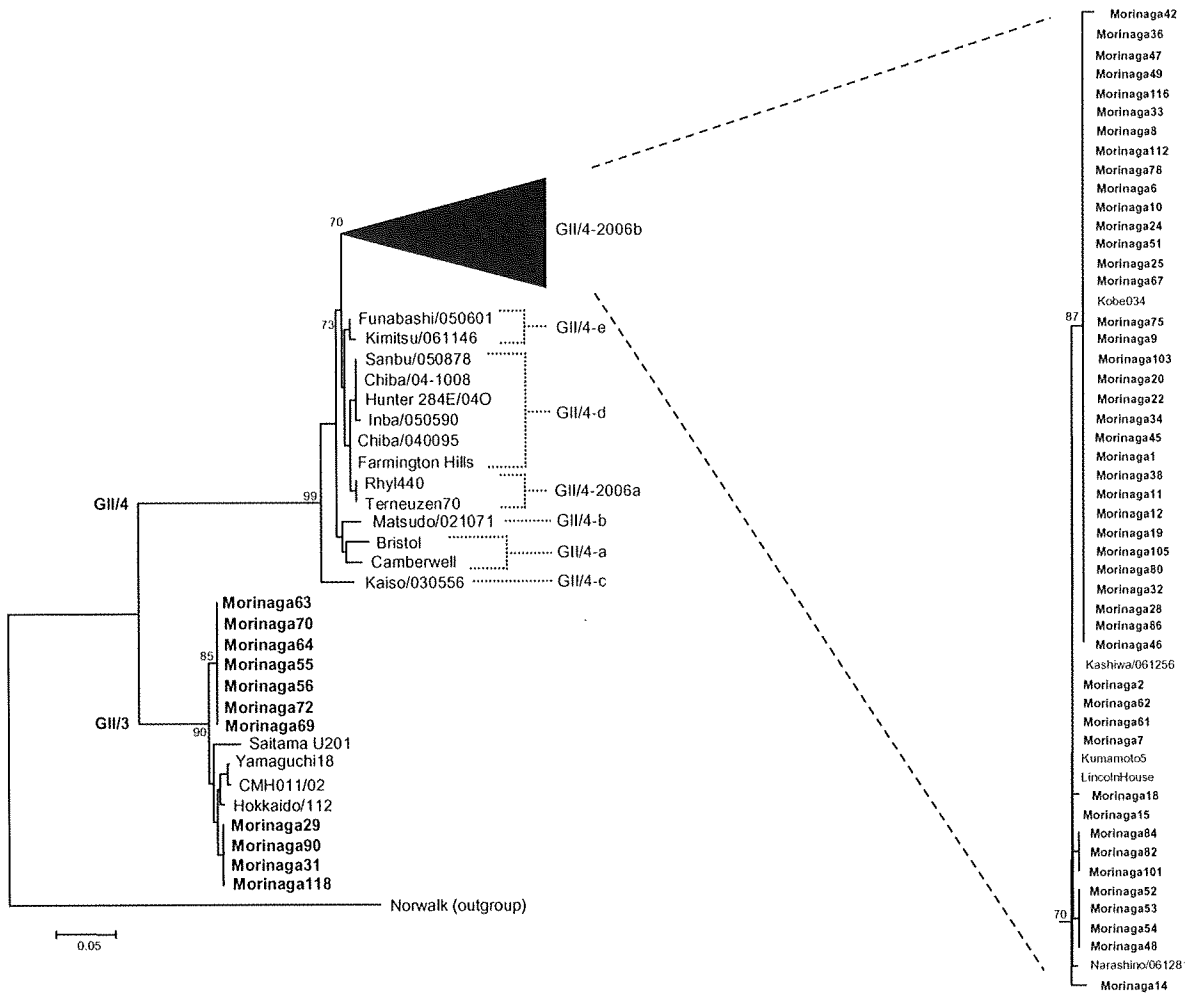

Fig. 1. Phylogenetic analysis of partial capsid sequences of NoV detected in Japan during December 2007 to January 2008. The tree was constructed on the basis of the neighbor-joining method using the MEGA 4 software package, and the numbers on each branch indicate the bootstrap values. The NoV strains detected in the present study are presented in boldface.

Industry Co., LTD.) which was developed for broad detection of several NoV genotypes including GI/1, GI/11, GII/2, GII/3, GII/4, GII/5, and GII/6.

A total of 75 fecal specimens were tested for rapid detection of NoV by the IC kit. All of these samples were collected from children who suffered from acute gastroenteritis in Shizuoka and Kyoto, Japan. The study period was during the peak season for NoV, winter, from December 2007 to January 2008. To evaluate the sensitivity and specificity of the IC test kit, all 75 fecal samples were tested. This IC test was performed according to the manufacturer's directions. Briefly, 10–20% of stool samples were prepared in tubes containing buffer. Then, stool suspensions were mixed by vortexing for 2 min and centrifuging at 3000 × *g* for 10 min. This was followed by adding the strip test into the tube which contained the stool mixture. It took only 15 min to obtain the result of the assay.

Table 1
Comparison of NoV detection in stool samples between the newly developed IC kit and the RT-PCR method.

		RT-PCR		Total (%)
		Positive	Negative	
IC	Positive	46	0	46 (61.3)
	Negative	15	14	29 (38.7)
	Total (%)	61 (81.3)	14 (18.7)	75 (100)

For the IC strip test, latex conjugated rabbit polyclonal antibodies against NoV GI/1, GI/11, GII/2, GII/3, GII/4, GII/5, and GII/6 genotypes were coated at the conjugate pad. The test line was also coated with immobilized polyclonal antibodies against NoV GI/1, GI/11, GII/2, GII/3, GII/4, GII/5, and GII/6 genotypes, while the control line was coded with anti-rabbit immunoglobulin.

For the reference test, the RNA genome of NoV was first extracted from 10% to 20% fecal supernatant using the QIAamp viral RNA Mini Kit (Qiagen, Germany). The presence of the NoV in fecal specimens was confirmed by RT-PCR using the 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 analyzed further for their genotypes by nucleotide sequence and phylogenetic analyses.

For NoV sequence and phylogenetic analysis, the PCR products were purified with a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI) and sequenced using the BigDye Terminator Cycle Sequencing kit (Perkin Elmer-Applied Biosystems, Inc., Foster City, CA) on an automated sequencer (ABI 3100; Perkin Elmer-Applied Biosystems, Inc.). The primers used for amplification

of the partial capsid genes were also used as sequencing primers. The sequences obtained were compared with those of NoV strains deposited in the GenBank using BLAST searches. The genotypes of NoV were classified using the phylogenetic clustering methods determined previously by Kageyama et al. (2004).

To evaluate sensitivity, specificity, and overall agreement of this newly developed IC kit, a panel of 75 stool samples was tested and the results were compared with those of the RT-PCR gold standard method and sequence analysis. As shown in Table 1, 46 out of 75 (61.3%) samples were positive for NoV by this IC kit, while the RT-PCR standard method showed NoV detection rate of 81.3%; 61 out of 75 samples were positive for NoV. This result indicated 75.4% sensitivity for the IC assay. No false positive for NoV detection was observed for this IC test. Fourteen samples that tested negative for NoV by RT-PCR were also negative by this IC kit, resulting in a specificity of 100%. In addition, by comparing this IC assay and the RT-PCR method, the total agreement of this newly developed IC kit was 80%.

For NoV detection by RT-PCR screening method, a total of 75 fecal specimens were tested and 61 (81.3%) were positive for NoV. Of these, all positive samples were identified as NoV GII genogroup, and only 2 genotypes, GII/3 and GII/4, were identified by sequence and phylogenetic analyses. Fig. 1 shows the phylogenetic analysis of partial NoV capsid sequences detected in this study. It was observed that GII/4 was the most predominant genotype and accounted for the majority (48 out of 61; 78.7%) of NoV detected.

Interestingly, all the NoV GII/4 analyzed in this study belonged to a new variant GII/4 2006b subtype, which differs from NoV subtypes GII/4-a–e, and from 2006a (Okada et al., 2007; Siebenga et al., 2008; Tu et al., 2008). All of the NoV GII/4 strains detected in this study were clustered together in the same branch and showed a close genetic lineage with the NoV 2006b reference strains recently circulating in Japan (Kobe034, Kashiwa/061256, Kumamoto5, and Narashino/061281). NoV GII/3 was detected at a lower prevalence in 11 of 61 samples (18.0%), while genotypes of NoV in 2 samples could not be identified because of insufficient stool samples.

In recent years, molecular techniques have revolutionized the diagnosis of NoV and led to the recognition that NoV is one of the major viruses responsible for non-bacterial gastroenteritis in humans of all age groups (Clark and McKendrick, 2004; Wilhelmi et al., 2003; Green, 2007). The conventional diagnostic methods to detect and identify NoV in stool samples (RT-PCR and sequence analysis) require experience, specialized laboratory equipment, and time. Therefore, a rapid, sensitive, and simple diagnostic tool, such as the IC test, is required, especially during an outbreak of NoV. The advantages of the IC assay are cost effectiveness, speed (15–20 min), and ease of use at the bedside without special laboratory equipment.

Recently, the sensitivity and specificity of the IC assay for detection of NoV in stool samples (IP-Noro; Immuno Probe Co., Ltd., Japan) have been evaluated (Khamrin et al., 2008; Nguyen et al., 2007). Those reports demonstrate that the sensitivity and specificity of the IP-Noro strip test ranged from 73.7% to 78.9% and 96.4% to 100%, respectively. Compared with this newly developed IC kit, the sensitivity and specificity are in good agreement with those previously reported (sensitivity; 75.4% and specificity; 100%). Moreover, this new IC kit was developed for the detection of several NoV genotypes using antibodies against NoV GI/1, GI/11, GII/2, GII/3, GII/4, GII/5, and GII/6 genotypes in the IC strip test.

Several NoV-VLP genotypes were used to test this IC kit to determine reactivity with NoV genotypes. It was found that this IC kit could detect other NoV genotypes of GI/1, GI/11, GII/2, GII/3, GII/4, GII/5, and GII/6 VLPs (data not shown). However, to clarify this point, additional testing with several other NoV genotypes from clinical samples is essential. The limitation of this study is that only a small number of negative samples were included. There were only 14 negative samples, which could affect the specificity test.

The data from phylogenetic analysis clearly demonstrate that this new IC assay can detect the new NoV variant GII/4 2006b strains which have recently begun circulating in Japan. Therefore, this study demonstrates that the new IC kit can be used as an alternative method for detecting NoV in fecal specimens and may be practical for NoV screening during outbreaks of viral gastroenteritis.

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SHORT COMMUNICATION

Throughput Expression of Multiple G-protein Coupled Receptors for HIV Infection in Choriocarcinoma Cells, Trophoblasts, and Breast Milk Cells

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SUMMARY

The chemokine receptors, which belong to G-protein coupled receptors (GPCRs) and become co-receptors when HIV enters the cell, have been mentioned in recent research. Numerous studies have reported that the cellular mechanism of HIV crossing the placental barrier is still not totally understood. This study was conducted to investigate whether the mRNAs of nineteen typs of GPCRs and CD4 were expressed in choriocarcinoma cell lines, trophoblasts, and breast milk cells by using RT-PCR. It was found that the expression of GPCRs varied in different cell lines. Of note is that CD4 could not be expressed in either choriocarcinoma cells or trophoblasts. It was noteworthy that mRNAs of multiple GPCRs were identified in choriocarcinoma cells, trophoblasts, and breast milk cells for the first time. The expression amounts of these mRNAs were further measured by quantitative RT-PCR. Interestingly, mRNAs of CCR9/CCR10 were strongly expressed in trophoblasts. This study provided further insights to the cellular mechanism of mother-to-child transmission of HIV.
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KEY WORDS

Expression, G protein coupled receptors, trophoblasts, choriocarcinoma, breast milk cells

INTRODUCTION

The HIV/AIDS epidemic has been rapidly increasing all over the world. The number of HIV infected pregnant women has also been increasing. The effective preventive measures of mother-to-child transmission of HIV have been established in many countries. Several factors have been reported to modify mother-to-child transmission of HIV such as the quality and quantity of maternal antibodies, viral strains, and antiretroviral therapies [Colognesi et al., 1997; Chermann, 1998; Yoshino et al., 1998; Dao et al., 2007; Volmink et al., 2007]. It is possible that HIV's entry-route exists in trophoblasts, presuming that HIV's co-receptors are expressed in syncytio-trophoblasts, which come in contact immediately with mother's blood and cyto-trophoblasts. Infection from an HIV infected woman to her child can occur at the beginning of pregnancy [Zachar et al., 1991; Soeiro et at., 1992, Kumar et al., 2004] but predominantly occurs at the end of pregnancy [Scarlatti, 2004; Tripathi et al., 2008]. On the other hand, HIV transmission via breast milk feeding can occur by the invasion of HIV into infant's body through his/her mouth [Coovadia et al., 2008; Read, 2008].

Eventhough numerous studies on HIV infection were conducted worldwide, the cellular mechanisms by which HIV-1 crosses the placental barrier are still unclear. To date, several G-protein coupled receptors were discovered as co-receptors of HIV entry. It has been recognized that four CC-CKRs (CCR2b, CCR3, CCR5, and CCR9) work as specific co-receptors for M-tropic or T-tropic HIV-1 strains; and another CXC-CKR known as CXCR4 works as specific co-receptor for T-tropic [Alkhatib et al., 1996; Doranz et al., 1996; Feng

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et al., 1996; Simmons et al., 1996; Sol et al., 1997; Chen et al., 1997 and 1998; Choe et al., 1996 and 1998]. On the other hand, numerous studies indicated that V28 (CX_3C-CKR), US28, APJ, GPR1, GPR15, STRL33, and RDC1 also play roles as co-receptors of HIV-1 and HIV-2 stock [Marchese et al., 1994; Chen et al., 1997; Deng et al., 1997; Farzan et al., 1997; Liao et al., Pleskoff et al., 1997; Shimizu et al 2000]. The previous study suggested that transmission of HIV-1 infection between trophoblast placental cells and T-cells takes place via an LFA-1 mediated cell to cell contact [Arias et al., 2003]. However, results of CD4 in placental trophoblasts is controversial. Some previous studies reported the lack of CD4 molecules or its mRNA in trophoblasts [Zachar et al., 1991; Esterman et al., 1996. Another study demonstrated that the HIV-1 receptors CD4 and CCR5 were not expressed in trophoblasts [Parry et al., 2006]. However, David et al., 1992 and 1995 suggested the possible expression of CD4 in trophoblasts in a small amount. Thus, the objective of the study was to investigate whether the mRNAs of nineteen GPCRs and the main receptor CD4 are expressed in choriocarcinoma cell lines, trophoblasts, and breast milk cells.

MATERIAL AND METHODS

In the study, three different choriocarcinoma cell lines (BeWo, JEG-3, and JAR), three placenta samples obtained from therapeutic abortions (7 to 9 weeks of pregnancy) , three breast milk samples obtained from mothers (1 to 5 weeks after delivery) provided by Dr. Takeshi Nagamatsu (Hospital of Tokyo University, Tokyo, Japan) were used. In choriocarcinoma cells, it is assumed that BeWo is derived from syncytio-trophoblasts because it forms syncytium. In addition, JEG-3 and JAR are derived from cytotrophoblasts because they have the ability to proliferate [Pattillo and Gey, 1968; Pattillo et al., 1971]. Each of the three cell lines was cultured using Eagle-MEM (Nissui Pharmaceutical, Tokyo, Japan) containing 10% FCS and incubated at 37°C in 5% CO_2. They were subcultured every two days. In trophoblasts, placenta samples were homogenized with an edge, washed with PBS, and broken to pieces with trypsin (Invitrogene, San Diego, CA) and DNase I (Sigma Aldrich, Saint-Louis, MD). The suspension was centrifuged for 10 min at 300×*g*. Then the upper layer containing trophoblasts was taken and the lower layer (blood cell layer) was removed. The blood cells and fibroblasts were removed from the suspension using the Percoll separation method [Nagamatsu et al., 2004]. Finally, trophoblasts were purified using the CD9 beads method [Yui et al., 1994; Nagamatsu et al., 2004]. The trophoblast preparation contained cytotrophoblasts and extra-villous trophoblasts. On the other hand, three breast milk samples were diluted with RPMI (Nissui Pharmaceutical, Tokyo, Japan) and centrifuged for 10 min at 300×g. Fat was removed and the breast cells were harvested. The breast cell preparation contained macrophages, lymphocytes, neutrophils, and epithelial cells.

RNA was extracted from cells using the RNA Extraction Kit (RNeasy Mini Kit; Qiagen, Germany) according to the manufacture's protocol. Approximately 1.7×10^7 cells of BeWo, 4.4×10^6 cells of JEG-3, and 1.1×10^7 cells of JAR, 8×10^4 cells of trophoblasts and 3×10^6 cells of breast milk were used. The extracted RNA samples were treated with DNase I to remove DNA. For RT-PCR, a 25 µl total volume of the reaction mixture 10×buffer, 2.5mM dNTP, Taq DNA polymerase (Promega, Madison, WI), forward and reverse primers, milli-Q water and cDNA solution was prepared. The thermal cycler program was: 2 min at 94°C; 38 cycles of 30 s at 93°C, 1 min at 50°C (58°C, 60°C, 65°C according to genes), 2 min at 72°C; and finally at 72°C for 7 min. For the positive control of the RNA extraction, the primers of β-actin were used. The sequences of the primers used and the lengths of PCR products were shown in Table 1. For the mRNAs of GPCRs recognized to be expressed, the quantitative PCR was performed. The expression amounts of the mRNAs were measured in comparison to those of β-actin.

RESULTS

In the study, mRNA of CD4 did not express in any choriocarcinoma cell lines, trophoblast samples, one breast milk cell sample, but it was expressed in the two remaining breast milk cell samples. As for GPCRs, it was recognized that mRNAs of C5a, CCR7, CCR9 /CCR10, CXCR4, GPR5, RDC1 were found in BeWo. In addition, mRNAs of C5a, CCR7, CCR9/ CCR10, CXCR4, CXCR5/BLR1, RDC1 expressed in JEG-3, and mRNAs of C5a receptor, CCR7, CCR9/ CCR10, CXCR5/BLR1, GPR5, RDC1 were detected in JAR. Also, the expression of mRNAs of C5a, CCR1, CCR7, CCR9/CCR10, CXCR4, CXCR5, GPR5, GPR12, RDC1 in the trophoblasts, and of C5a, CCR1, CCR5, CCR7, CCR9/CCR10, CXCR3, CXCR4, CXCR5, DEZ α, GPR5, GPR12, GPR25, RDC1 in the breast milk cells was identified as shown in Table 2, Figure 1, and Figure 2.

When the intensity of β-actin expression was assumed to be 100, in BeWo, the intensity of C5a was 0.892, CCR7 was 0.555, CCR9/CCR10 was 0.406, CXCR4 was 0.047, and RDC1 was 1.184. In JEG-3, the intensity of C5a receptor was 0.135, CCR7 was 0.008, CCR9/CCR10 was 0.163, CXCR4 was 0.008, CXCR5/BLR1 was 0.007, and RDC1 was 0.195. In JAR, the intensity of C5a was 0.285, CCR7 was 0.078, CCR9/CCR10 was 0.154, CXCR5/BLR1 was 0.004, and RDC1 was 0.372. In trophoblasts, the intensity of C5a was 0.102, CCR7 was 0.026, CCR9/CCR10 was 1.112, CXCR4 was 0.086, CXCR5 was 0.013, GPR5 was 0.074, GPR12 was 0.014, and RDC1 was 0.002.

Table 1: Primer DNA sequences of G-protein coupled receptors used in this study.

Human gene	Product length (bp)	Sequence
CD4	589	sense:5'-GGA TAC AGT GGA ACT GAC CTG-3'
		antisense:5'-TTC AAC TGT AAA GGC GAG TGG-3'
APJ	1143	sense:5'-ATG GAG GAA GGT GGT GAT TTT GAC AAC TAC-3'
		antisense:5'-CTA GTC AAC CAC AAG GGT CTC CTG GCT GTA G-3'
C5a receptor	1053	sense:5'-ATG AAC TCC TTC AAT TAT ACC ACC CCT GAT-3'
		antisense:5'-CTA CAC TGC CTG GGT CTT CTG GGC CAT AGT G-3'
CCR1	1068	sense:5'-ATG GAA ACT CAA AAC ACC ACA GAG GAC TAT G-3'
		antisense:5'-TCA GAA CCC AGC AGA GAG TTC ATG CTC CCC TG-3'
CCR4	1083	sense:5'-ATG AAC CCC ACG GAT ATA GCA GAT ACC ACC-3'
		antisense:5'-CGT CGC ATT CGC GGC CGC CTA CAG AGC ATC ATG AAG-3'
CCR5	243	sense: 5'-AAT TCT TTG GAC TGA ATA ACT GCA-3'
		antisense: 5'-GTG GAT CGG GTA TAG ACT GAG CTT A-3'
CCR7	573	sense:5'-CGC GTC CTT CTC ATC AGC AAG CTG TCC TGT G-3'
		antisense:5'-GTG CCG ACA GGA AGA CCA CTG CCG GAG CTG-3'
CCR9/CCR10	510	sense:5'-ATC CCT GAT ATG GTC TTT GTA CAG ACA CAT G-3'
		antisense:5'-GCT GGA TAA TGA GGC CTG GGC AGT GCC AGG-3'
CXCR3	555	sense:5'-CGG GGG CCC CCG GCC CGC GTG ACC CTC ACC TG-3'
		antisense:5'-CTG GAG CCC TCT CTG GTT GGG GCA GCC CAG GC-3'
CXCR4	570	sense:5'-CCA AGG AAG CTG TTG GCT GAA AAG GTG GTC TA-3'
		antisense:5'-TCC ACC TCG CTT TCC TTT GGA GAG GAT CTT-3'
CXCR5/BLR1	570	sense:5'-GGG ACC ATC TGG CTG GTG GGC TTC CTC CTT G-3'
		antisense:5'-GAG ACT GCT CCT GCG CCA GCT AGG GAA GAG-3'
DEZα	1122	sense:5'-ATG AGA ATG GAG GAT GAA GAT TAC AAC ACT TC-3'
		antisense:5'-TCA AAG CAT GCC GGT CTC CCT CTC ATT CAT AG-3'
DEZβ	1116	sense:5'-ATG GAG GAT GAA GAT TAC AAC ACT TCC ATC-3'
		antisense:5'-TCA AAG CAT GCC GGT CTC CCT CTC ATT CAT AG-3'
Duffy antigen	1017	sense:5'-ATG GCC TCC TCT GGG TAT GTC CTC CAG GCG GAG-3'
		antisense:5'-CTA GGA TTT GCT TCC AAG GGT GTC CAG ATG AG-3'
GPR-9-6	1074	sense:5'-ATG GCT GAT GAC TAT GGC TCT GAA TCC ACA TC-3'
		antisense:5'-TCA GAG GGA GAG TGC TCC TGA GGT TGT CTC C-3'
GPR5	1002	sense:5'-ATG GAG TCC TCA GGC AAC CCA GAG AGC ACC-3'
		antisense:5'-TCA GTA GAA GGA GGC GCC CTC ATA GGC GAA G-3'
GPR12	1005	sense:5'-ATG AAT GAA GAC CTG AAG GTC AAT TTA AGC GG-3'
		antisense:5'-CTA CAC ATC ACT GGG CGA GCG CGC TCT CTG GG-3'
GPR15	1083	sense:5'-ATG GAC CCA GAA GAA ACT TCA GTT TAT TTG-3'
		antisense:5'-TTA GAG TGA CAC AGA CCT CTT CCT CCT CCT GG-3'
GPR25	1083	sense:5'-ATG GCC CCC ACA GAG CCC TGG AGC CCC AGC CC-3'
		antisense:5'-CTA CCA GGA GGC CGA GGC AGT GTT CGC GGC C-3'
RDC1	281	sense:5'-AAG AAG ATG GTA CGC CGT GTC GTC TGC ATC CTG-3'
		antisense:5'-CTG CTG TGC TTC TCC TGG TCA CTG GAC GCC GAG-3'
β-actin	445	sense:5'-AGA GAT GGC CAC GGC TGC TT-3'
		antisense:5'-ATT TGC GGT GGA CGA TGG AG-3'

Table 2: Summary of RT-PCR products of mRNA of G-protein coupled receptors. Note: ±: faint, +: weak, ++: strong, - : not detected.

Gene	BeWo	JEG-3	JAR	Trophoblasts	Breast milk cells
CD4	-	-	-	±	+
APJ	-	-	-	-	-
C5a	+	±	+	+	++
CCR1	-	-	-	+	+
CCR4	-	-	-	-	-
CCR5	-	-	-	-	+
CCR7	++	+	++	+	+
CCR9/CCR10	++	++	++	++	+
CXCR3	-	-	-	-	+
CXCR4	+	+	-	++	++
CXCR5/BLR1	-	+	+	+	+
DEZα	-	-	-	-	±
DEZβ	-	-	-	-	-
Duffy antigen	-	-	-	-	-
GPR-9-6	-	-	-	-	-
GPR5	±	-	±	+	±
GPR12	-	-	-	+	±
GPR15	-	-	-	-	-
GPR25	-	-	-	-	±
RDC1	++	++	++	+	+

In breast milk cells, the intensity of CD4 was 0.065, C5a was 0.285, CCR5 was 0.232, CCR7 was 0.255, CCR9/CCR10 was 0.488, CXCR4 was 0.122, CXCR5 was 0.081, GPR25 was 0.631, and RDC1 was 0.053. It was obvious that the intensity of G-protein coupled receptor expression was low in comparison to β-actin expression which had been used as the internal control of RNA extraction and RT-PCR. But it was noteworthy to find out the direct evidence of these co-receptors' expressions in several different cells in this study that may increase our understanding of maternal-fetal HIV transmission because the co-receptors are very important for HIV-1 infectivity and tropism [Al-Husaini AM, 2008]. To date, mRNA expressions of C5a, CCR7, CCR9, CXCR5, GPR5, and RDC1 in the choriocarcinoma cell lines had not been fully investigated. In this study, these GPCRs were expressed in different cell lines for the first time. Also, mRNA expressions of C5a, CCR1, CCR7, CCR9, CXCR5, GPR5, GPR12, and RDC1 in trophoblasts and mRNA expressions of C5a, CCR1, CCR7, CCR9, CXCR3, CXCR5, DEZα, GPR5, GPR 12, GPR25 and RDC1 in breast milk cells were newly discovered. These results were in strong agreement with a previous study published by Shimizu et al., 2000 in which RDC1 was confirmed as co-receptor of HIV infection. In fact, mRNA of CD4 could be naturally confirmed to be expressed in breast milk cells, as it includes lymphocytes and macrophages.

However, mRNA of CD4 was not expressed in trophoblasts [Zachar et al., 1991; Esterman et al., 1996 Parry et al., 2006] which was consistent with our findings. Of note, our study also clearly indicated that the expression of mRNA of CD4 could not be identified in any choriocarcinoma cell line.

By analyzing mRNAs obtained, there were quantity differences among three choriocarcinoma cell lines. Regarding the amounts of expression, mRNA of CCR9/CCR10 was strongly expressed in trophoblasts. Conversely, the mRNAs of CXCR4 and CXCR5 were weakly expressed. In breast milk cells, mRNAs of CCR9/CCR10 and GPR25 were strongly expressed. On the other hand, CXCR4 was also identified to be the HIV co-receptor [Feng et al., 1996]. Interestingly, the amount of the CXCR4 expression in choriocarcinoma cells and trophoblasts was small. Therefore, it was possible that CXCR4 might have a function in HIV transmission from mother to child through the placenta during pregnancy [Ishii et al., 2000; Douglas et al., 2001]. Evidence of HIV-1 infection of trophoblasts is controversial [Al-Harthi et al., 2002]. Al-Harthi et al., 2002 reported that only full-term trophoblasts expressed CXCR4.

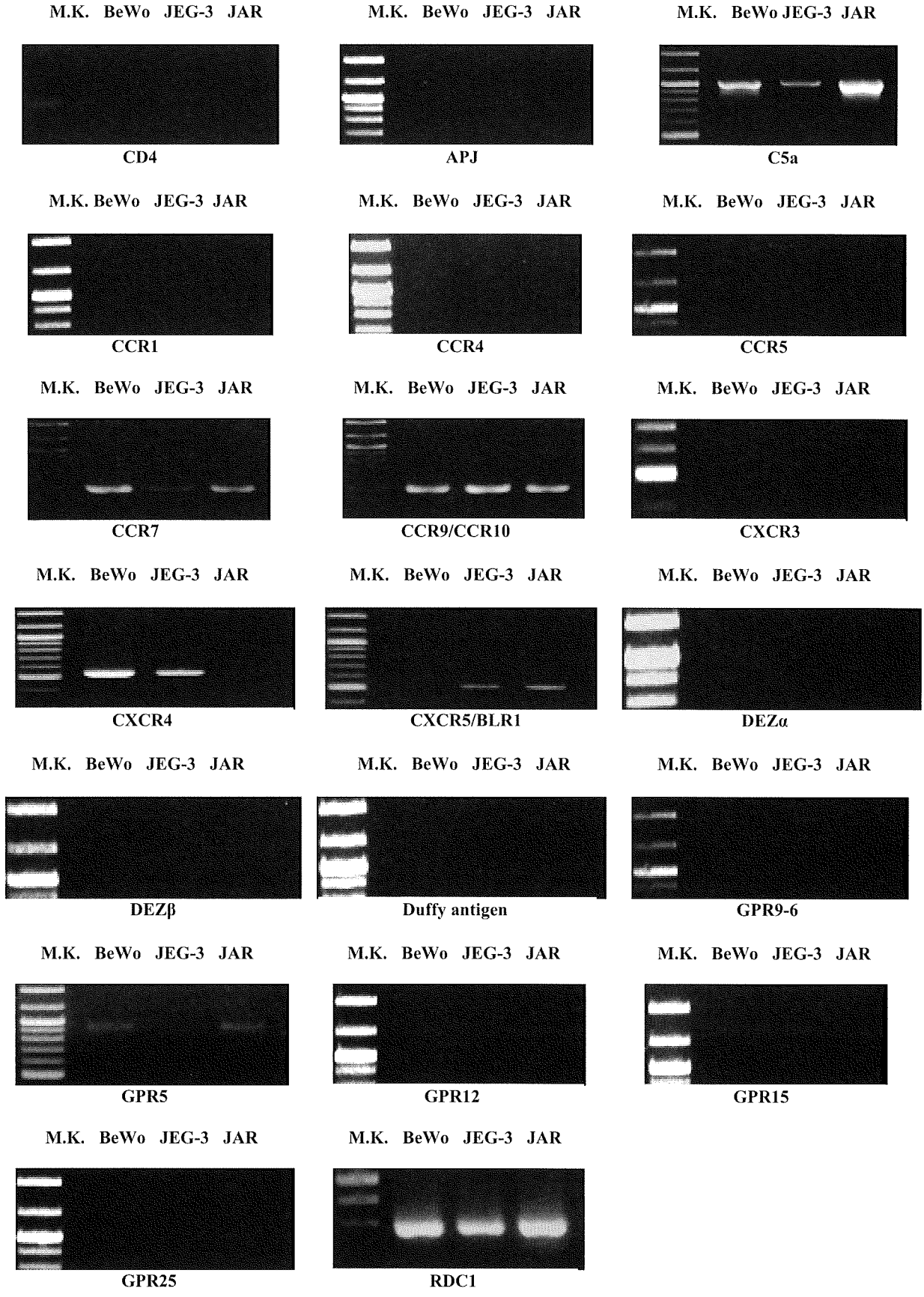

Figure 1: Electrophoresis of RT-PCR products of mRNA of G-protein coupled receptors in choriocarcinoma cell lines. From left to right: marker, BeWo, JEG-3, and JAR.