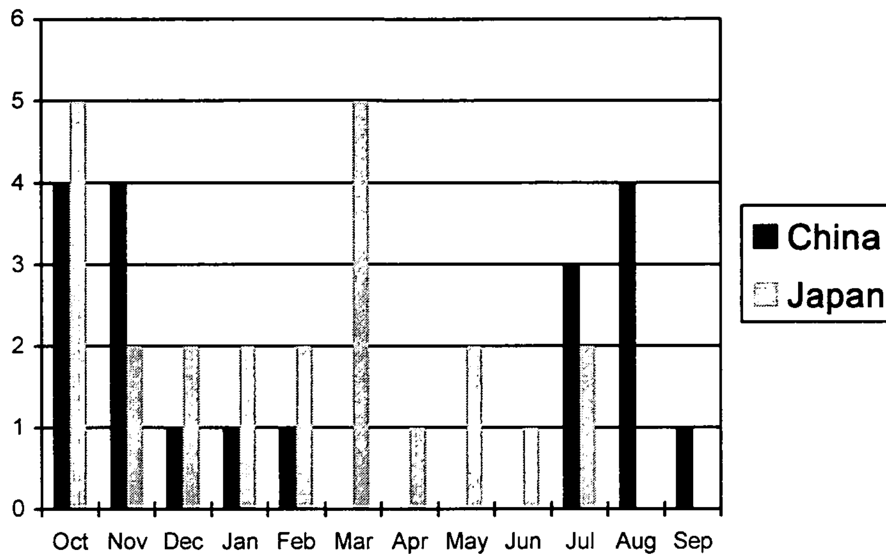

Figure 1: Monthly distribution of norovirus strains detected in oysters from China and Japan from October 2005 to September 2006.

CLUSTAL X. The genetic distance was calculated using Kimura's two-parameter method (PHYLIP). Reference norovirus strains and accession numbers used in this study were as follows: Manchester (X86560), Saitama T53GII/02/JP (AB112260), Girllington (AJ277606), Melksham (X81879), Chitta (AB032758), Wortley (AJ277618), Hillington (AJ277607), Alphatron (AF195847), Toronto (U02030), Seacroft (AJ277620), Leeds (AJ277608), Lordsdale (X86557), Idaho Falls/96/US (AY054299), Fayetteville/1998/US (AY113106), Erfurt/546/00/DE (AF42118), M7/99/US (AY130761), Saitama U1 (AB039775), Camberwell (AF145896), Snow Mountain (U70059), Paris Island/2003/USA (AY652979), Oberhausen 455/01/DE (AF539440), C14/2002/AU (AY845056), Herzberg 385/01/DE (AF539439), Arg320 (AF190817), VannesL169/2000/France (AY773210), Amsterdam (AF195848), White River/94/US (AF414423), Mexico (U22498), MD145 (AY032605), Mora/97/SE (AY081134), SaitamaT29GII/01/JP (AB112221), SaitamaKU80aGII/99/JP (AB058582), Mc37 (AY237415), Stockholm/IV4348/01/SE (AJ626633), Farmington Hills (AY502023), and Hunter284E/04O/AU (DQ078794).

RESULTS

Detection of norovirus contamination

A total of 225 oysters collected from China and Japan during October 2005 and September 2006 were examined for the presence of norovirus. Table 1 shows that norovirus was detected in 19 out of 130 oysters from China and in 24 out of 95 oysters from Japan, accounting for 14.6% and 25.3%, respectively. In China, norovirus in oysters was detected continuously from July to February with the highest prevalence in August, October and November (each of 21%, 4 of 19). On the other hand, norovirus in Japan was found year-round, except for August and September, with highest prevalence in March and October (each of 20.8%, 5 of 24). Figure 1 demonstrates that no norovirus in oysters was detected from March to June and from August to September in China and Japan, respectively.

Distribution of norovirus genotypes

The partial nucleotide sequences of the capsid gene of norovirus detected in this study were compared to each other as well as to those of norovirus reference strains available in GenBank by BLAST. Table 2 shows that

Table 2: Genetic characterization of norovirus detected in oysters from China and Japan.

Countries	Norovirus genotypes		
	GII/3	GII/4	
China	4 (21.1%, 4 of 19)	15 (78.9%, 15 of 19)	
Japan	12 (50%, 12 of 24)	12 (50%, 12 of 24)	
		Farmington Hills variant (%)	Hunter variant (%)
China		8 (53.3%, 8 of 15)	7 (46.7%, 7 of 15)
Japan		3 (25%, 3 of 12)	9 (75%, 9 of 12)

Table 3: Characterization of norovirus strains detected in oysters from China and Japan during 2005-2006

Number	Strain	Place	Collection date	Genogroup	Genotype	Variant	Genome copy per oyster
1	1/oyster/CN	China	October 2005	II	4	Farmington Hills	$1.2x10^3$
2	4/oyster/CN	China	October 2005	II	4	Hunter	$4x10^2$
3	7/oyster/JP	Japan	October 2005	II	4	Hunter	$3.3x10^2$
4	9/oyster/JP	Japan	October 2005	II	3	-	$7.8x10^5$
5	16/oyster/CN	China	October 2005	II	3	-	$2.5x10^3$
6	18/oyster/CN	China	October 2005	II	3	-	$9x10^4$
7	22/oyster/JP	Japan	October 2005	II	3	-	$2.1x10^3$
8	24/oyster/JP	Japan	October 2005	II	3	-	$8x10^2$
9	25/oyster/JP	Japan	October 2005	II	3	-	$4.2x10^2$
10	26/oyster/CN	China	November 2005	II	3	-	$1.1x10^3$
11	27/oyster/CN	China	November 2005	II	3	-	$1.1x10^5$
12	31/oyster/JP	Japan	November 2005	II	4	Hunter	$2.6x10^3$
13	38/oyster/CN	China	November 2005	II	4	Hunter	$1.3x10^3$
14	40/oyster/CN	China	November 2005	II	4	Hunter	$1.2x10^2$
15	45/oyster/JP	Japan	November 2005	II	4	Hunter	$3.8x10^2$
16	51/oyster/JP	Japan	December 2005	II	4	Farmington Hills	$4.2x10^3$
17	55/oyster/JP	Japan	December 2005	II	4	Hunter	$6x10^1$
18	56/oyster/CN	China	December 2005	II	4	Farmington Hills	$4.1x10^3$
19	72/oyster/JP	Japan	January 2006	II	4	Hunter	$2.6x10^2$
20	76/oyster/CN	China	January 2006	II	4	Farmington Hills	$3x10^3$
21	84/oyster/JP	Japan	January 2006	II	4	Farmington Hills	$2x10^5$
22	89/oyster/CN	China	February 2006	II	4	Farmington Hills	$2.5x10^2$
23	91/oyster/JP	Japan	February 2006	II	4	Farmington Hills	$5.7x10^2$
24	104/oyster/JP	Japan	February 2006	II	4	Hunter	$3.5x10^2$
25	111/oyster/JP	Japan	March 2006	II	4	Hunter	$1.7x10^2$
26	121/oyster/JP	Japan	March 2006	II	3	-	$7.5x10^3$
27	122/oyster/JP	Japan	March 2006	II	3	-	$3.5x10^3$
28	123/oyster/JP	Japan	March 2006	II	3	-	$7.3x10^5$
29	125/oyster/JP	Japan	April 2006	II	3	-	$5x10^3$
30	139/oyster/JP	Japan	May 2006	II	3	-	$6.6x10^2$
31	146/oyster/JP	Japan	May 2006	II	3	-	$6x10^2$
32	168/oyster/JP	Japan	June 2006	II	3	-	$2.9x10^3$
33	177/oyster/JP	Japan	July 2006	II	3	-	$6.7x10^2$
34	186/oyster/CN	China	July 2006	II	4	Hunter	$4.4x10^2$
35	187/oyster/CN	China	July 2006	II	4	Hunter	$1.5x10^3$
36	189/oyster/CN	China	July 2006	II	4	Hunter	$5.5x10^2$
37	191/oyster/JP	Japan	July 2006	II	4	Hunter	$1.5x10^2$
38	192/oyster/JP	Japan	August 2006	II	4	Hunter	$2.2x10^2$
39	201/oyster/CN	China	August 2006	II	4	Farmington Hills	$3x10^2$
40	202/oyster/CN	China	August 2006	II	4	Hunter	$1x10^1$
41	203/oyster/CN	China	August 2006	II	4	Farmington Hills	$6.4x10^2$
42	205/oyster/CN	China	August 2006	II	4	Farmington Hills	$2.3x10^2$
43	216/oyster/CN	China	September 2006	II	4	Farmington Hills	$3.4x10^2$

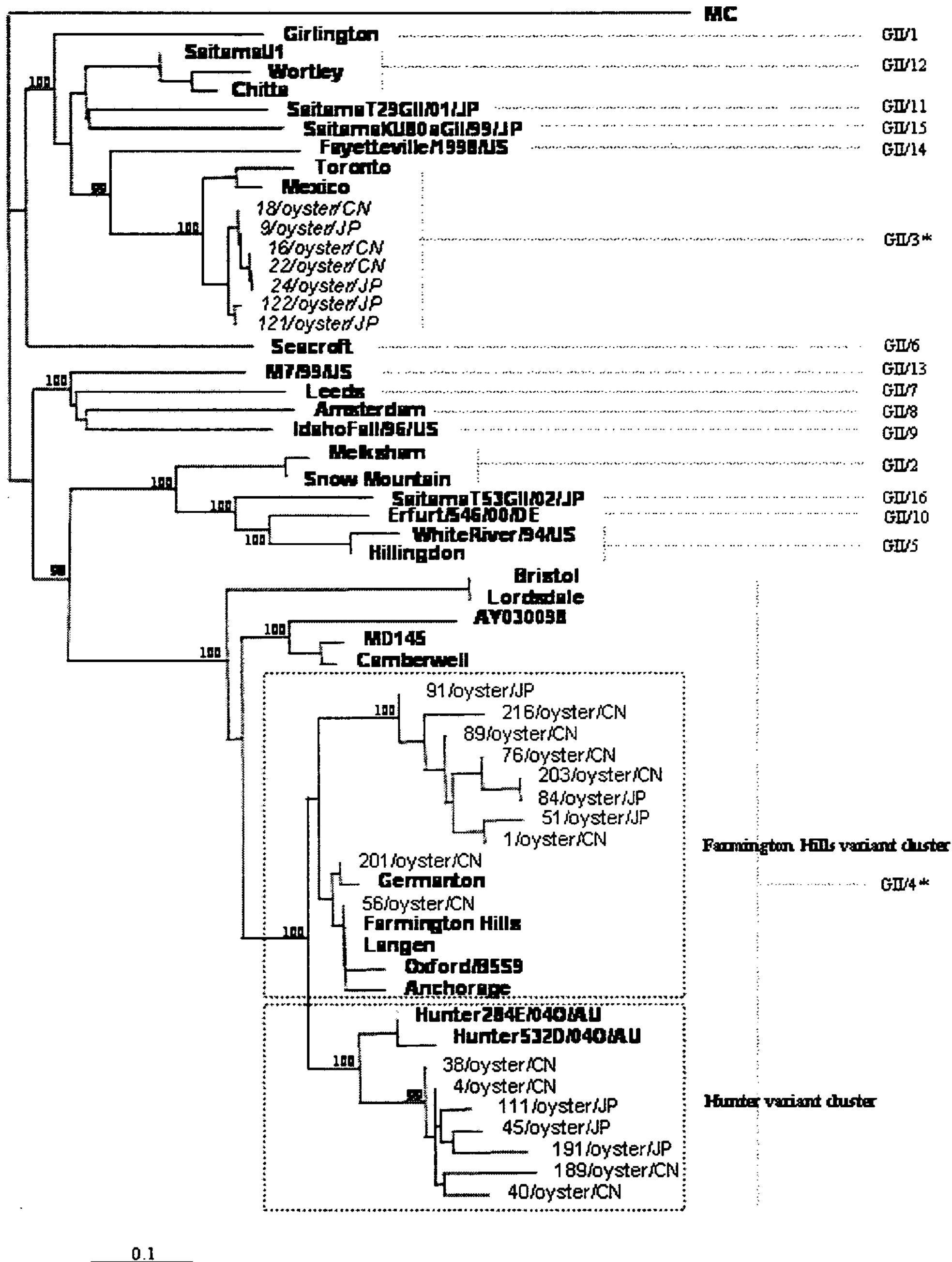

Figure 2: Phylogenetic tree obtained from nucleotide sequences of the norovirus capsid gene. Reference norovirus strains were selected from GenBank under the accession numbers indicated in the text. The norovirus strains detected in the study are highlighted in the italics. The scale indicates nucleotide substitutions per position. The numbers in the branches indicate the bootstrap values. * indicates the genotype containing the norovirus detected in this study.

based on the sequence analysis of the capsid gene of these norovirus strains, norovirus was divided into two distinct genotypes, 3 and 4, within genogroup II (GII). In China, the norovirus GII/4 (known as the Lordsdale virus cluster) was the most predominant, accounting for 78.9% (15 of 19), followed by the norovirus GII/3 (known as the Mexico virus cluster) with a lower prevalence of 21.1% (4 of 19). In contrast, it was interesting that both the norovirus GII/4 and the norovirus GII/3 were co-predominant with a prevalence of 50% (12 of 24) in Japan. Another interesting feature of the study was that the norovirus GII/4 strains in oysters from both countries were grouped into two distinct variant clusters known as the Farmington Hills variant and the Hunter variant (Figure 2). In China, the Farmington Hills-like norovirus predominated over the Hunter-like norovirus and these represented 42.1% (8 of 19) and 36.8% (7 of 19), respectively. In contrast, the detection rate of the Hunter-like norovirus was higher than that of the Farmington Hills-like norovirus in Japan, accounting for 37.5% (9 of 24) and 12.5% (3 of 24), respectively

Quantitation of the norovirus genome

Table 3 shows that more than 10^2 copies of the norovirus genome were found in the majority of oysters (95.3%, 41 of 43). In Japan, the lowest copy number was $6x10^1$ and the highest copy number was $7.8x10^5$. In China, the copy number of norovirus ranged from $1x10^1$ to $1.1x10^5$. It was found that the copy number of the norovirus GII/3 ranged from to $7.8x10^5$. The Farmington Hills-like norovirus strains had a copy number from $2.3x10^2$ to $2x10^5$. The Hunter-like norovirus strains had a copy number from $1x10^1$ to $2.6x10^3$. The number of oysters containing a large copy number (over 10^3) was identified in 7 cases (36.8%) and 10 cases (41.7%) in China and Japan, respectively. Of note, the highest copy number ($1.1x10^5$ to $7.8x10^5$) was found exclusively in norovirus GII/3 and the lowest copy number ($1x10^1$ to $6x10^1$) was only in the Hunter-like norovirus in both countries.

DISCUSSION

Norovirus is regarded as one of the most common causes of food-borne infections. Sporadic cases as well as outbreaks of acute gastroenteritis due to norovirus have been associated with the consumption of raw oysters grown in contaminated waters (19-23). In this study, the prevalence of norovirus contamination in oysters from China and in Japan was reported. Overall, the detection rate of norovirus was 19.1% in the total of 225 oyster samples included in this study. However, the detection rates of norovirus in oysters were quite differrent between China and Japan, representing 14.6% and 25.3%, respectively. Even though several studies conducted the detection on norovirus contamination in various different countries including Japan, reports documenting the occurrence of norovirus contamination in oysters from China are not available. To date, only one study on norovirus presence in oysters imported into Hong Kong from 11 countries over a 3-year period was published with the prevalence of 10.5% (24). Obviously, this is the first report, to the best of our knowledge, providing evidence for norovirus presence in oysters from China.

In some studies, the detection of norovirus contamination in oysters was predominant in the cold season, and several studies did not find a seasonal correlation (24-27). In this study it was found that norovirus was mainly detected continuously from October to May, months which are known as the cold season in Japan. In contrast, norovirus was mainly identified from July to November, known as the hot season in China. Taken together, these observations clearly indicate that the contamination of oysters with this virus occurred not only in the cold season but also in the hot season.

Genetic analysis revealed that the norovirus strains in our study belonged to only one distinct GII. This result was in line with other studies in which norovirus GII was the dominant genogroup in oysters (17, 28). It was found that the norovirus strains clustered into only two distinct genotypes, the GII/3 (known as the Mexico virus cluster) and the GII/4 (known as the Lordsdale virus cluster). In China, norovirus GII/4 was the most predominant, accounting for 78.9%, followed by norovirus GII/3 with a lower prevalence of 21.1%. In contrast, it was interesting that both the norovirus GII/4 and the norovirus GII/3 were co-predominant with a prevalence of 50% in Japan. Another interesting feature of the study was that the norovirus GII/4 strains in oysters from both countries were grouped into two distinct variant clusters known as the Farmington Hills variant and the Hunter variant. The Farmington Hills virus was associated with 64% of cruise ship outbreaks and 45% of land-based outbreaks in the United States in 2002 (15). Moreover, the Hunter virus was an important strain in causing 15.6% of sporadic cases as well as 42.9% of outbreaks of acute gastroenteritis during the year of 2004 in Australia (19). And the virus transmission by food was documented in these outbreaks (15). Quite possibly, these norovirus variants in oysters might play an important role in this diarrheal illness under these circumstances and our finding is the first to demonstrate evidence of the existence of norovirus variants in oysters.

To date, norovirus is still uncultivable by standard culture with different cell lines. The lack of a virus culture system has been a significant obstacle to the study of norovirus, but advances in the sequencing of norovirus have enabled its genomic characterization, and therefore genetic analysis becomes the principle method to classify norovirus. Recently, a quantitative real-time PCR method has been developed (14). In the previous reports, the sensitivity of nested PCR was higher than that of real-time PCR (27, 28). Therefore, the determination of the copy numbers of the norovirus genome was per-

formed only in norovirus-positive oysters by nested PCR. It was revealed that the majority of oysters (95.3%) had more than 10^2 copies of the norovirus genome. Interestingly, the number of oysters containing the large copy number (over 10^3) was found in 17 cases. Moreover, the highest copy number was 1.1×10^5 and 7.8×10^5 in Chinese and Japanese oysters. These findings were consistent with previous reports that oysters contained much smaller numbers of norovirus than the number of norovirus found in feces (30).

It has been reported that the distribution of norovirus genotypes in oysters was different to those in humans during the same period (24). However, it was interesting that the distribution of the norovirus genotype in Japanese oysters in this study was similar to the distribution of norovirus genotype in humans in Japan during 2005-2006, in which both norovirus GII/3 and GII/4 were co-predominant and only two, the Farmington Hills variant and the Hunter variant clusters, were identified within the GII/4 genotype (unpublished data). Obviously, there was an epidemiological link of the norovirus infection between humans and oysters in Japan.

In conclusion, these results are noteworthy because this is the first report, to the best of our knowledge, of the presence of norovirus variants in oysters. This observation improves our current knowledge of the genetic heterogeneity of norovirus in oysters as well as its epidemiology. Due to genetic diversity in different parts of the world, knowledge of molecular surveillance of norovirus in circulation in oysters is important in an effort to develop suitable and efficacious norovirus control strategies, and a continuous monitoring of the norovirus types in oysters is needed.

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References

1. Murray CJ, Lopez AD. 1997. Mortality by cause for eight regions of the world: Global burden of disease study. Lancet 1997; 349: 1269-76.
2. Parashar UD, Hummelman EG, Miller MA, Glass RI. Global illness and deaths caused by rotavirus disease in children. Emerg Infect Dis 2003; 9: 565-72.
3. Thapar N, Sanderson IR. Diarrhoea in children: an interface between developing and developed countries. Lancet. 2004; 363: 641-53.
4. Parashar UD, Glass RI. Public health. Progress toward rotavirus vaccines. Science 2006; 12: 851-2.
5. Chiba S, Sakuma Y, Kogasaka R, Akihara M, Horino K, Nakao T, Fukui S. An outbreak of gastroenteritis associated with calicivirus in an infant home. J Med Virol 1979; 4: 249-54.
6. McEvoy M, Blake W, Brown D, Green J, Cartwright R. An outbreak of viral gastroenteritis on a cruise ship. Commun Dis Rep CDR Rev 1996; 6: 188-92.
7. Marks PJ, Vipond IB, Carlisle D, Deakin D, Fey RE, Caul EO. Evidence for airborne transmission of Norwalk-like virus (NLV) in a hotel restaurant. Epidemiol Infect 2000; 120: 481-7.
8. McIntyre L, Vallaster L, Kurzac C, Fung J, McNabb A, Lee MK, Daly P, Petric M, Isaac-Renton J. Gastrointestinal outbreaks associated with Norwalk virus in restaurants in Vancouver, Bristish Columbia. Can Commun Dis Rep 2000; 28: 197-203.
9. White PA, Hansman GS, Li A, Dable J, Isaacs M, Ferson M, McIver CJ, Rawlinson WD. Norwalk-like virus 95/96-US strain is a major cause of gastroenteritis outbreaks in Australia. J Med Virol 2002; 68: 113-8.
10. Kageyama T, Shinoharam M, Uchidam K, Fukushi S, Hoshino FB, Kojima S, Takai R, Oka T, Takeda N, Katayama K. Coexistence of multiple genotypes, including newly identified genotypes, in outbreaks of gastroenteritis due to norovirus in Japan. J Clin Microbiol 2004; 42: 2988-995.
11. Green KY, Chanock RM, Kapikian AZ. 2001. Human caliciviruses, p. 841-874. *In*: D. M. Knipe., and P. M. Howley (ed.), Fields virology, Lippincott Williams & Wilkins, Philadelphia.
12. Seto Y, Iritani N, Kubo H, Kaida A, Murakami T, Haruki K, Nishio O, Ayata M, Ogura H. Genotyping of norovirus strains detected in outbreaks between April 2002 and March 2003 in Osaka City, Japan. Microbiol Immunol 2005; 49: 275-83.
13. Lopman BA, Brown DW, Koopmans M. Human caliciviruses in Europe. J Clin Virol 2002; 24; 137-60.
14. Kageyama T, Kojima S, Shinohara M, Uchida K, Fukushi S, Hoshino FB, Takeda N, Katayama K. Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR. Clin Microbiol 2003; 41: 1548-57.
15. Widdowson MA, Cramer EH, Hadley L, Bresee JS, Beard RS, Bulens SN, Charles M, Chege W, Isakbaeva E, Wright JG, Mintz E, Forney D, Massey J, Glass RI, Monroe SS. Outbreaks of acute gastroenteritis on cruise ships and on land: identification of a predominant circulating strain of norovirus--United States, 2002. J Infect Dis 2004; 190: 27-36.

16. Isakbaeva ET, Widdowson MA, Beard RS, Bulens SN, Mullins J, Monroe SS, Bresee J, Sassano P, Cramer EH, Glass RI. Norovirus transmission on cruise ship. Emerg Infect Dis 2005; 11: 154-8.

17. Myrmel M, Berg EM, Rimstad E, Grinde B. Detection of enteric viruses in shellfish from the Norwegian coast. Appl Environ Microbiol 2004; 70: 2678-84.

18. Phan TG, Nguyen TA, Yan H, Yagyu F, Kozlov V, Kozlov A, Okitsu S, Muller WE, Ushijima H. Development of a novel protocol for RT-multiplex PCR to detect diarrheal viruses among infants and children with acute gastroenteritis in Eastern Russia. Clin Lab 2005; 51: 429-35.

19. Lees D. Viruses and bivalve shellfish. Int J Food Microbiol 2000; 59: 81-116.

20. Lindqvist R, Andersson Y, de Jong B, Norberg P. A summary of reported foodborne disease incidents in Sweden, 1992 to 1997. J Food Prot 2000; 63: 1315-20.

21. Fankhauser RL, Monroe SS, Noel JS, Humphrey CD, Bresee JS, Parashar UD, Ando T, Glass RI. Epidemiologic and molecular trends of "Norwalk-like viruses" associated with outbreaks of gastroenteritis in the United States. J Infect Dis 2002; 186: 1-7.

22. Tauxe RV. Emerging foodborne pathogens. Int J Food Microbiol 2002; 78: 31-41.

23. Le Guyader FS, Bon F, DeMedici D, Parnaudeau S, Bertone A, Crudeli S, Doyle A, Zidane M, Suffredini E, Kohli E, Maddalo F, Monini M, Gallay A, Pommepuy M, Pothier P, Ruggeri FM. Detection of multiple noroviruses associated with an international gastroenteritis outbreak linked to oyster consumption. J Clin Microbiol 2006; 44: 3878-82.

24. Cheng PK, Wong DK, Chung TW, Lim WW. Norovirus contamination found in oysters worldwide. J Med Virol 2005; 76: 593-7.

25. Boxman IL, Tilburg JJ, Te Loeke NA, Vennema H, Jonker K, de Boer E, Koopmans M. Detection of noroviruses in shellfish in the Netherlands. Int J Food Microbiol 2006; 108: 391-96.

26. Costantini V, Loisy F, Joens L, Le Guyader FS, Saif LJ. Human and animal enteric caliciviruses in oysters from different coastal regions of the United States. Appl Environ Microbiol 2006; 72: 1800-9.

27. Nishida T, Nishio O, Kato M, Chuma T, Kato H, Iwata H, Kimura H. Genotyping and quantitation of noroviruses in oysters from two distinct sea areas in Japan. Microbiol Immunol 2007; 51: 177-84.

28. Nishida T, Kimura H, Saitoh M, Shinohara M, Kato M, Fukuda S, Munemura T, Mikami T, Kawamoto A, Akiyama M, Kato Y, Nishi K, Kozawa K, Nishio O. Detection, quantitation, and phylogenetic analysis of noroviruses in Japanese oysters. Appl Environ Microbiol 2003; 69: 5782-6.

29. Bull RA, Tu ET, McIver CJ, Rawlinson WD, White PA. Emergence of a new norovirus genotype II.4 variant associated with global outbreaks of gastroenteritis. J Clin Microbiol 2006; 44: 327-33.

30. Jaykus LA, De Leon R, Sobsey MD. A virion concentration method for detection of human enteric viruses in oysters by PCR and oligoprobe hybridization. Appl Environ Microbiol 1996; 62: 2074-80.

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Evaluation of Immunochromatography Tests for Detection of Rotavirus and Norovirus among Vietnamese Children with Acute Gastroenteritis and the Emergence of a Novel Norovirus GII.4 Variant

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Summary

A prospective study was conducted to evaluate two immunochromatography (ICG) tests for detection of group A rotavirus and norovirus GII, the commercial Dipstick 'Eiken' Rota kit (SA Scientific, USA) and the NV IC-1 stick (Immuno-Probe, Japan). Polymerase chain reaction (PCR) with specific primer pairs (Beg9 and VP7-1', for group A rotavirus; COG2F and G2SKR, for norovirus GII) was used as the reference method. The results of ICG tests were compared with those of reference method. The sensitivity, specificity and agreement between ICG tests and PCR were 87.8%, 93.3% and 89.4%, respectively, for rotavirus ICG test; and 73.7%, 100% and 95.2%, respectively, for norovirus ICG test. The immunochromatography assay for norovirus used in this study could detect not only common noroviruses, but also a novel norovirus GII.4 variant, which emerged in Ho Chi Minh City in 2006. Immunochromatography tests are easy, rapid and useful assays for detection of rotavirus and norovirus among pediatric patients with acute gastroenteritis in Vietnam.

Introduction

Rotavirus and norovirus are the two most common viral agents which cause acute gastroenteritis in infants and young children. In estimation, rotavirus causes ~22% of childhood diarrhea hospitalizations, and is responsible for about 611 000 childhood deaths per year worldwide, in which, most death cases belonged to the developing countries [1]. A review of food-related illness and death showed that norovirus accounted for ~23 million infections in all age groups in the United States [2]. Many epidemiological studies on rotavirus and recent reports about norovirus outbreaks in different countries confirm the disease burden of rotavirus and norovirus infections [3–9].

Rotavirus is a member of the *Reoviridae* family, which includes seven groups (A through G). Most diseases are due to group A rotavirus. Norovirus is one of the four members of the family *Caliciviridae*. Five genogroups of norovirus are thought to exist, though only norovirus GI, GII and GIV, are known to infect humans [10]. Epidemiological studies clearly indicated that norovirus GII is the main causative agent among noroviruses causing acute gastroenteritis [11–13].

After norovirus and rotavirus were discovered in stool sample by electron microscopic examination in 1972 and 1973, respectively [14, 15], several detection methods were developed to determine those viruses

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in fecal specimens. Enzyme-linked immunosorbent assays (ELISA) was the method of choice in many laboratories because it high sensitivity, and a built-in control for non-specific reaction. Recently, polymerase chain reaction (PCR) has been used broadly in epidemiological surveillances because of its ability to genotype samples that could not be typed by ELISA [16–20]. Those methods, however, need special equipment and are time consuming to perform. The development of rapid immunochromatography (ICG) based detection kits facilitates work in third-world countries, where there is lack of human and material resources for conducting laboratory tests, in hospital as well as in epidemiological researches. In this study, we evaluate two rapid ICG assays for detection of group A rotavirus and norovirus GII among pediatric patients with acute gastroenteritis in Ho Chi Minh City, Vietnam; and perform molecular analysis of norovirus strains detected.

Materials and Methods

Patients

A total of 30 out-patients and 74 in-patients who came to the Department of Gastroenterology, Children's Hospital 1, Ho Chi Minh City, with clinical diagnose of viral acute gastroenteritis in November 2006, were enrolled to this study. The patients were examined and followed-up by pediatricians. Severity of disease was assessed, based on the 20-point numerical score [21]. Another 10 healthy children were also recruited as controls.

Samples

One fecal specimen was collected from each child after coming to the out-clinic ward, for out-patients and controls, or after admitted to the department, for in-patients. Specimens were kept at +4°C, and checked for group A rotavirus and norovirus GII by ICG tests within less than 24 h after collecting. All stool samples were then stored at −20°C until checked further by monoplex PCR in the Department of Developmental Medical Sciences, The University of Tokyo, Japan.

Immunochromatography tests

Group A rotavirus was detected by using the Dipstick 'Eiken' Rota stick (SA Scientific, Texas, USA). This commercial kit has a capacity to detect a purified rotavirus antigen in a concentration of 100 ng/ml. Rotavirus G serotypes, including the five most common G-types: G1–G4 and G9, are detectable by this kit. The procedure was performed according to the manufacturer's instruction. Briefly, 12.5 mg of each fecal specimen was added to the reaction tube which contained 0.5 ml of reaction buffer, and was mixed with a mixer for 2 min. The mixture was then centrifuged at 2000 × g for 20 s before use.

Norovirus was determined by the NV IC-1 sticks lot 0609 (Immuno-Probe, Saitama, Japan), which was developed to determine norovirus GII/3 and GII/4, which are the most predominant norovirus genotypes globally. The 10% suspension was prepared with dilution buffer, then 80 µl of suspension and 20 µl of reaction buffer was mixed and left at room temperature for 3 min, before dropping onto the stick.

The reference test

Monoplex PCR was chosen as the reference test in this study. The fecal specimen was prepared as a 10% suspension in distilled water, and the viral RNA genomes were extracted from the fecal suspension with a QIAamp Viral RNA Mini kit (QIAGEN®, Hilden, Germany) according to the manufacturer's instruction. The procedure of reverse-transcription and PCR was carried out as previously described [22]. Briefly, 7.5 µl of the nucleic acid extract were mixed with a 7.5 µl reaction mixture, and then, the reaction tubes were incubated at 42°C for 1 h, followed by heating to 99°C for 5 min to inactivate the enzyme, and immediately cooling to 4°C.

Regarding PCR assay, 1 µl of cDNA was added to a reaction tube with 1.1 µl of 5X colorless GoTaq reaction buffer (Promega, WI, USA), 0.2 µl of each of the specific primers (33 µM each), 1.0 µl (2.5 mM/µl) of dNTPs (Roche, Mannheim, Germany) and 0.06 µl (5 U/µl) of GoTaq DNA polymerase (Promega), to achieve a total volume of 11.0 µl with the additional of MilliQ water. Each PCR cycle contained steps of 30 s at 94°C, 30 s at 55°C and 1 min at 72°C, for 35 cycles [23, 24]. All PCR products were electrophoresed in 1.5% agarose gel, followed by staining with ethidium bromide (0.5 µg/ml) and then visualized under ultraviolet light. Two specific primer pairs, Beg9–VP7-1′ for detection of group A rotavirus, and COG2F–G2SKR for norovirus GII [25–27], were used in this study (Table 1).

Sequence and phylogenetic analysis

PCR products of noroviruses detected were purified with the QIAquick PCR Purification Kit (Qiagen, Germany), and were sequenced by using Big Dye Terminator Cycle Sequencing kit version 3.1 and an ABI Prism 310 Genetic Analyzer (Applied Biosystems Inc.), according to the manufacturer's instruction. Sequence alignments were carried out by using the CLUSTAL W program, and the neighbor-joining method was used for the construction of the phylogenetic tree with MEGA2 version software [28], based on the classification of noroviruses by Okada *et al.* [29]. Nucleotide sequences of norovirus strains described in this study have been deposited in GenBank under accession numbers: EF198119–EF198137.

TABLE 1
Specific primers used in monoplex PCR

Primer	Target virus	Target region	Polarity	Sequence (5′ to 3′)[a]	Position	Amplicon
Beg9	Rotavirus group A	VP7 gene	+	GGCTTTAAAAGAGAGAATTTCCGTCTGG	1–28	395 bp
VP7-1'	Rotavirus group A	VP7 gene	–	ACTGATCCTGTTGGCCATCCTTT	395–373	
COG2F	Norovirus genogroup II	Capsid	+	CARGARBCNATGTTYAGRTGGATGAG	5003–5028	387 bp
G2SKR	Norovirus genogroup II	Capsid	–	TACATRTTRCCHRTACGNCCRCC	5389–5367	

[a]Nucleotide, B: C, G, or T; H: A, C, or T; N: any base; R: A or G; Y: C or T.

TABLE 2
Characteristics of norovirus positive patients[a]

Isolate	Result of IC	Mixed infection[b]	GII.4 variant	Hospitalization	Age group[c]	Dehydration	Vesikari's score
VN1/06	+	–	4d	Yes	2	None	10
VN9/06	+	–	4new	Yes	3	None	6
VN26/06	+	–	4new	Yes	3	None	14
VN41/06	+	–	4new	Outpatient	2	None	N/A[d]
VN59/06	–	+	4new	Yes	3	None	13
VN73/06	+	–	4new	Yes	1	None	11
VN74/06	+	–	4d	Yes	2	None	11
VN75/06	+	–	4d	Yes	2	None	10
VN77/06	+	–	4new	Yes	2	1–5%	17
VN78/06	–	+	4new	Yes	3	None	14
VN79/06	–	+	4new	Yes	2	None	14
VN80/06	+	+	4d	Yes	2	None	15
VN82/06	–	+	4new	Yes	1	1–5%	18
VN84/06	+	–	4new	Yes	3	None	15
VN85/06	+	–	4d	Yes	3	None	15
VN87/06	+	–	4new	Yes	3	None	9
VN88/06	–	–	4new	Yes	2	None	16
VN89/06	+	–	4d	Yes	3	None	15
VN95/06	+	–	4d	Yes	3	None	10

[a]All 19 cases were identified as norovirus positive by monoplex PCR.
[b]Mixed infection between norovirus and rotavirus group A by reference tests.
[c]Group 1, <6 months; group 2, 6–11 months; group 3, 12–23 months; group 4, 24–35 months; group 5, 36 months and older.
[d]N/A, not applicable.

Results

Detection of target viruses by immunochromatography tests
Sixty-seven of 104 (64.4%) samples were found positive with group A rotavirus by Dipstick 'Eiken' Rota sticks. Norovirus GII was determined in 14 of 104 (13.5%) fecal specimens. There was no specimen which contained more than one target viral agent by ICG tests. All the sticks clearly showed the control lines and test line (if positive) at the appropriate position. Of 10 fecal specimens from healthy controls, all ICG assays showed negative results with target viruses.

Results of reference test
Monoplex PCR assays were performed separately for detection of group A rotavirus and norovirus GII. Seventy-four of 104 (71.2%) samples showed positive with group A rotavirus, and norovirus GII was found in 19 of 104 (18.3%) fecal specimens. Five samples were determined as coinfection between group A rotavirus and norovirus GII (Table 2).

Sensitivity and specificity of immunochromatography tests
In comparison with the reference test, there were two samples showed positive with rotavirus by ICG assay and negative by PCR. For norovirus, all cases

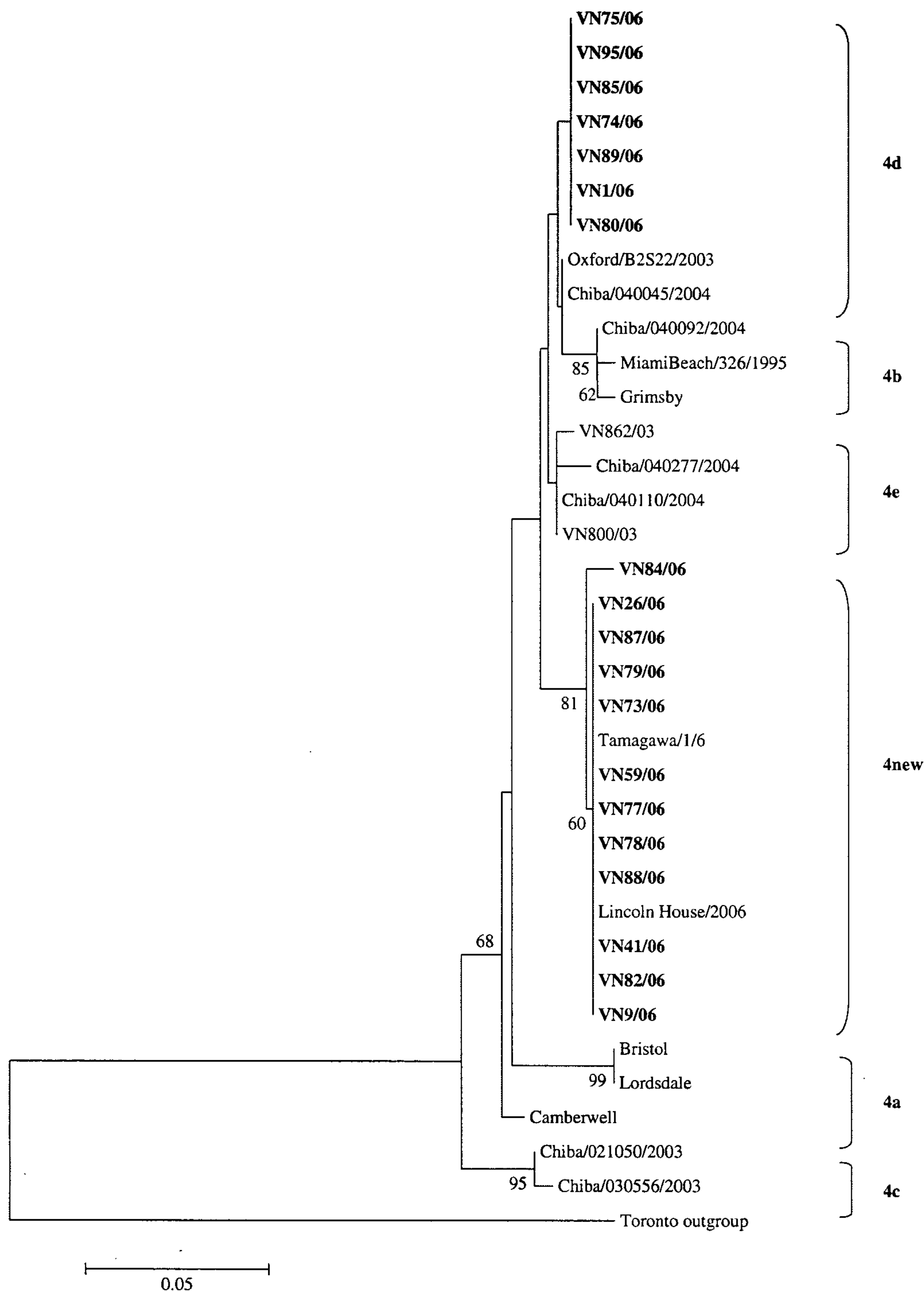

FIG. 1. Phylogenetic tree of a partial capsid gene (nt 5114–5321) of 19 Vietnamese noroviruses isolated in this study (in bold face) and other norovirus GII.4 strains. Bootstrap values >60% are indicated at the branch nodes. Vietnamese noroviruses detected in 2002–2003 surveillance, VN800/03 and VN862/03, are also shown. Toronto strain, GII.3 norovirus, is used as the out-group.

TABLE 3
Sensitivity, specificity and agreement of two IC tests for group A rotavirus and norovirus GII compared to reference test (monoplex PCR)

Parameter	Dipstick 'Eiken' Rota	NV IC-1 lot 0609
Sensitivity (%)	87.8	73.7
Specificity (%)	93.3	100
Agreement (%)	89.4	95.2

which were negative with norovirus by PCR also showed negative results with ICG test. The sensitivity, specificity and agreement of the two ICG tests, compared to PCR, are shown in Table 3.

Sequence analysis and phylogenetic tree of noroviruses detected

All 19 noroviruses detected by monoplex PCR were successfully sequenced at the capsid region. A BLAST search demonstrated that all studied isolates shared the greatest sequence identity with GII.4 norovirus strains. Interestingly, the phylogenetic tree of GII.4 noroviruses clearly showed that Vietnamese noroviruses isolated in this study belonged to two clusters, GII.4d and a novel variant which was distinct from previously described variants (Fig. 1).

Discussion

In this study, we conducted a prospective study to evaluate two immunochromatography tests for detection of group A rotavirus and norovirus GII, which were considered to be the two most common causes of acute gastroenteritis worldwide. To our knowledge, there has been no other report on evaluation of ICG test in viral enteropathogens among diarrheal children in Vietnam. The performance of ICG tests is easy (does not need special equipment), and rapid (15–20 min). This assay would be suitable for developing countries, where morbidity and mortality of diarrhea is high, and where laboratory assays to conduct epidemiological surveillances, as well as to monitor diarrhea outbreaks are limited.

The results of ICG test for rotavirus in this study was comparable with other evaluation studies. Bon *et al.* [30] evaluated seven ICG assays for the rapid detection of group A rotavirus, of them, four assays showed the sensitivity <90%. However, the reference method in that study was ELISA. PCR assay, the method of choice in detection of viral pathogens in many laboratories nowadays, was chosen as the reference test in this study, and made the results of comparison between test assay and reference assay more accurate.

Fourteen of 19 (73.7%) norovirus strains positive by PCR were detected by ICG test in this study. Of the five negative specimens, four were mixed infection between norovirus and rotavirus, which might cause the low concentration of target viruses in fecal specimens. Furthermore, five specimens which were negative by ICG test showed the faint bands when visualized under ultraviolet light, when amplified by PCR (data not shown). Although the ICG test using in this study has a capacity to detect norovirus in a concentration of 10^6 copies per 1 g of stool (Immuno-Probe, unpublished data), the low concentration of virus in fecal specimen might explain the fault negative results in rapid ICG tests. On the other hand, the results of ICG assay for norovirus detection in this study were comparable with that of other studies. Okame *et al.* [31] reported a comparison result between rapid ICG assay and PCR, with the sensitivity, specificity and concordance ratio 72.7%, 90.9% and 80.0%, respectively. It is noteworthy that ICG sticks using in this study could detect not only norovirus GII.4 which belonged to common variant (4d), but also novel norovirus GII.4 variant (Table 2).

The phylogenetic tree clearly showed that noroviruses isolated in Ho Chi Minh City were clustered into two different variants, and differed from noroviruses detected previously in this city, which belonged to variant GII.4e (Fig. 1). It is of interest that majority of the Vietnamese noroviruses in this study and the other two unique norovirus strains, Tamagawa/1/6 in Japan and Lincoln House/2006 in United Kingdom, made a novel cluster, tentatively called GII.4f. The mutation accumulation could cause a novel variant virus, and make it become the predominant strain in an outbreak [32]. There was a new norovirus GII.4 variant which caused Europe-wide outbreaks, and then became the major cause of norovirus outbreaks in many parts of the world [33, 34]. The mass detection of novel norovirus GII.4 variant in Vietnam, as well as in Japan and UK in the year 2006 highlights the ability of the spread of this norovirus variant worldwide.

Immunochromatography tests are easy, rapid and useful assays for detection of rotavirus and norovirus among pediatric patients with acute gastroenteritis in Vietnam.

References

1. Parashar UD, Gibson CJ, Bresse JS, *et al.* Rotavirus and severe childhood diarrhea. Emerg Infect Dis 2006;12:304–6.
2. Mead PS, Slutsker L, Dietz V, *et al.* Food-related illness and death in the United States. Emerg Infect Dis 1999;5:607–25.
3. Gleizes O, Desselberger U, Tatochenko V, *et al.* Nosocomial rotavirus infection in European countries: a review of the epidemiology, severity and economic

burden of hospital-acquired rotavirus disease. Pediatr Infect Dis J 2006;25:S12–21.
4. Jiraphongsa C, Bresee JS, Pongsuwanna Y, *et al.* Epidemiology and burden of rotavirus diarrhea in Thailand: results of sentinel surveillance. J Infect Dis 2005;192(Suppl 1): S87–93.
5. Orenstein EW, Fang ZY, Xu J, *et al.* The epidemiology and burden of rotavirus in China: a review of the literature from 1983–2005. Vaccine 2007;25:406–13.
6. Prato R, Lopalco PL, Chironna M, *et al.* Norovirus gastroenteritis general outbreak associated with raw shellfish consumption in south Italy. BMC Infect Dis 2004;4:37.
7. Sasaki Y, Kai A, Hayashi Y, *et al.* Multiple viral infections and genomic divergence among noroviruses during an outbreak of acute gastroenteritis. J Clin Microbiol 2006;44:790–7.
8. Tseng FC, Leon JS, MacCormack JN, *et al.* Molecular epidemiology of norovirus gastroenteritis outbreaks in North Carolina, United States: 1995–2000. J Med Virol 2007;79:84–91.
9. Uchida R, Pandey BD, Sherchand JB, *et al.* Molecular epidemiology of rotavirus diarrhea among children and adults in Nepal: detection of G12 strains with P[6] or P[8] and a G11P[25] strain. J Clin Microbiol 2006;44:3499–505.
10. Kageyama T, Shinohara M, Uchida K, *et al.* Coexistence of multiple genotypes, including newly identified genotypes, in outbreaks of gastroenteritis due to Norovirus in Japan. J Clin Microbiol 2004;42:2988–95.
11. Ike AC, Brockmann SO, Hartelt K, *et al.* Molecular epidemiology of norovirus in outbreaks of gastroenteritis in southwest Germany from 2001 to 2004. J Clin Microbiol 2006;44:1262–7.
12. Papadopoulos VP, Vlachos O, Isidoriou E, *et al.* A gastroenteritis outbreak due to Norovirus infection in Xanthi, Northern Greece: management and public health consequences. J Gastrointestin Liver Dis 2006;15:27–30.
13. Phan TG, Takanashi S, Kaneshi K, *et al.* Detection and genetic characterization of norovirus strains circulating among infants and children with acute gastroenteritis in Japan during 2004–2005. Clin Lab 2006;52:519–25.
14. Kapikian AZ, Wyatt RG, Dolin R, *et al.* Visualization by immune electron microscopy of a 27-nm particle associated with acute infectious nonbacterial gastroenteritis. J Virol 1972;10:1075–81.
15. Bishop RF, Davidson GP, Holmes IH, *et al.* Virus particles in epithelial cells of duodenal mucosa from children with acute non-bacterial gastroenteritis. Lancet 1973;2:1281–3.
16. Dove W, Cunliffe NA, Gondwe JS, *et al.* Detection and characterization of human caliciviruses in hospitalized children with acute gastroenteritis in Blantyre, Malawi. J Med Virol 2005;77:522–7.
17. Gallimore CI, Cheesbrough JS, Lamden K, *et al.* Multiple norovirus genotypes characterised from an oyster-associated outbreak of gastroenteritis. Int J Food Microbiol 2005;103:323–30.
18. Khamrin P, Peerakome S, Wongsawasdi L, *et al.* Emergence of human G9 rotavirus with an exceptionally high frequency in children admitted to hospital with diarrhea in Chiang Mai, Thailand. J Med Virol 2006;78:273–80.
19. Maneekarn N, Khamrin P, Chan-it W, *et al.* Detection of rare G3P[19] porcine rotavirus strains in Chiang Mai, Thailand, provides evidence for origin of the VP4 genes of Mc323 and Mc345 human rotaviruses. J Clin Microbiol 2006;44:4113–9.
20. Yoshinaga M, Phan TG, Nguyen TA, *et al.* Changing distribution of group A rotavirus G-types and genetic analysis of G9 circulating in Japan. 2006;151:183–92.
21. Ruuska T, Vesikari T. Rotavirus disease in Finnish children: use of numerical scores for clinical severity of diarrhoeal episodes. Scand J Infect Dis 1990;22:259–67.
22. Yan H, Yagyu F, Okitsu S, *et al.* Detection of norovirus (GI, GII), Sapovirus and astrovirus in fecal samples using reverse transcription single-round multiplex PCR. J Virol Methods 2003;114:37–44.
23. Phan TG, Nguyen TA, Yan H, *et al.* Development of a novel protocol for RT-multiplex PCR to detect diarrheal viruses among infants and children with acute gastroenteritis in Eastern Russia. Clin Lab 2005;51:429–35.
24. Yan H, Nguyen TA, Phan TG, *et al.* Development of RT-multiplex PCR assay for detection of adenovirus and group A and C rotaviruses in diarrheal fecal specimens from children in China. Kansenshogaku Zasshi 2004;78:699–709.
25. Ushijima H, Koike H, Mukoyama A, *et al.* Detection and serotyping of rotaviruses in stool specimens by using reversed transcription and polymerase chain reaction amplification. J Med Virol 1992;38:292–7.
26. Kojima S, Kageyama T, Fukushi S, *et al.* Genogroup-specific PCR primers for detection of Norwalk-like viruses. J Virol Methods 2002;100:107–14.
27. Shinohara M, Kageyama T. Rapid and efficient detection method of Norwalk virus. Nippon Rinsho 2002;60:1181–7.
28. Kumar S, Tamura K, Jakobsen IB, *et al.* MEGA2: molecular evolutionary genetics analysis software. Bioinformatics 2001;17:1244–5.
29. Okada M, Ogawa T, Kaiho I, *et al.* Genetic analysis of noroviruses in Chiba Prefecture, Japan, between 1999 and 2004. J Clin Microbiol 2005;43:4391–401.
30. Bon F, Kaplon J, Metzger MH, *et al.* Evaluation of seven immunochromatographic assays for the rapid detection of human rotaviruses in fecal specimens. 2006. Pathol Biol (Paris) [Epub ahead of print].
31. Okame M, Yan H, Akihara S, *et al.* Evaluation of a newly developed immunochromatographic method for detection of norovirus. Kansenshogaku Zasshi 2003;77:637–9.
32. Dingle KE. Norovirus infection control in Oxfordshire communities hospitals. Mutation in a Lordsdale norovirus epidemic strain as a potential indicator of transmission routes. J Clin Microbiol 2004;42:3950–7.
33. Bull RA, Tu ET, McIver CJ, *et al.* Emergence of a new norovirus genotype II.4 variant associated with global outbreaks of gastroenteritis. J Clin Microbiol 2006;44:327–33.
34. Lopman B, Vennema H, Kohli E, *et al.* Increase in viral gastroenteritis outbreaks in Europe and epidemic spread of new norovirus variant. Lancet 2004;363:682–8.

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Molecular and epidemiological trend of norovirus associated gastroenteritis in Dhaka City, Bangladesh

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Abstract

Background: Diarrhea, over the years, has killed millions of people and continues to be a major threat in Bangladesh.

Objectives: To determine the incidence of norovirus infection in infants and young children with acute gastroenteritis in Dhaka City, Bangladesh and to determine the genogroup and genotype in norovirus-positive stool specimens.

Study design: Fecal specimens were collected from infants and children with acute gastroenteritis in Dhaka City, Bangladesh from October 2004 to September 2005, and examined for norovirus by reverse transcription-polymerase chain reaction.

Results: Noroviruses were detected in 41 of 917 fecal specimens. Molecular analysis of norovirus was carried out by sequencing methods. Only norovirus GII/4 strains were detected during this study. The dominant genotype throughout the study period was GII/4. Norovirus infections were most commonly observed in winter and rainy seasons in Dhaka City. The common clinical symptoms in norovirus-infected patients were diarrhea (90%), vomiting (75%) and abdominal pain (46%).

Conclusions: This is the first epidemiological research of norovirus in Bangladesh. Norovirus is an important enteropathogen responsible for viral gastroenteritis among infants and children in Bangladesh.

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Keywords: Norovirus; RT-PCR; Dhaka; Genogroup; Genotype

1. Introduction

Many viral strains cause acute gastroenteritis in humans. Norovirus (NoV), rotavirus (RV) and astrovirus (ASV) are the most common causes of sporadic cases of this disease and have been responsible numerous outbreaks of nonbacterial gastroenteritis in a variety of settings, including hospitals, day care centers, nursing homes and schools (Atmar and Estes, 2001; Gallimore et al., 2004; Hale et al., 2000; Milazzo et al., 2002). The mortality of acute gastroenteritis among children is greater in developing than developed countries. Annual mortality associated with acute gastroenteritis was estimated to be 2.1 million in 2000 (Mulholland, 2004; Parashar et al., 2003). Group A rotaviruses (GARVs), family Reoviridae, are a major cause of acute diarrhea worldwide in infants (Parashar et al., 2003), while NoVs are recognized as the most important cause of nonbacterial gastroenteritis outbreaks in school-age children and adults worldwide (Koopmans et al., 2003; Parashar and Glass, 2003).

NoV, a member of the calicivirus family together with vesivirus, lagovirus and sapovirus (Farkas et al., 2004), contains single-stranded positive-sense RNA genomes of 7.6 kb excluding the poly-A tail that encodes three open reading frames (ORFs) (Jiang et al., 1993; Lambden et al., 1993). ORF1 encodes a nonstructural polyprotein, which is cleaved

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into functional proteins by a virus-encoded 3C-like protease. ORF2 and ORF3 encode the major capsid protein VP1 and minor capsid protein VP2, respectively (Glass et al., 2000; Jiang et al., 1992).

The detection of NoV infections has been limited by the inability to grow it in culture, but molecular detection methods are now available for use during epidemiological studies (Isakbaeva et al., 2005; Kageyama et al., 2004; Matsui et al., 1991). NoV can be divided into five distinct genogroups. NoVGI and NoVGII are mainly infectious to humans. A recent study indicated that NoV GI and GII strains consist of at least 15 and 18 genotypes, respectively (Okada et al., 2005; Seah et al., 1999).

2. Materials and methods

2.1. Study population and fecal specimens

One stool was collected from 917 infants and children with acute gastroenteritis in Dhaka City, Bangladesh from October 2004 to September 2005. Acute gastroenteritis was defined by three or more loose stools in a 24 h period. Stool samples were collected from those patients when viral gastroenteritis was clinically suspected and stored at −20 °C.

2.2. Viral RNA extraction

Fecal specimens were thawed, diluted with distilled water to 10% suspensions, and centrifuged at 10,000 × g for 10 min. The supernatant was removed and heated at 65 °C for 10 min. Viral RNA was extracted from 140 μl of the supernatant using a spin-column technique (QIAamp Viral RNA kit; Qiagen®, Hilden, Germany) according to the manufacturer's instructions.

2.3. Reverse transcription

For reverse transcription (RT), 4 μl extracted viral RNA as mixed with a reaction mixture consisting of 5× First strand buffer (Invitrogen, Carlsbad, CA, USA), 10 mM dNTPs (Roche, Mannnheim, Germany), 10 mM DTT (Invitrogen), superscript reverse transcriptase III (200 U/μl) (Invitrogen, Carlsbad, CA, USA), random primer (1 μg/μl) (hexadeoxyribonucleotide mixture) (Takara, Shiga, Japan), RNase inhibitor (33 U/μl) (Toyobo, Osaka, Japan) and MilliQ water. The total of reaction mixture is 8 μl. The RT step was carried out at 50 °C for 1 h, followed by heating at 99 °C for 5 min and then held at 4 °C (Phan et al., 2005).

2.4. Polymerase chain reaction (PCR)

NoV was detected by PCR analysis of cDNA with specific primers previously published (Yan et al., 2003) that target the junction between ORF1 and ORF2. We used G1-SKF (CTGCCCGAATTYGTAAATGA), G1-SKR (CCAACCC-ARCCATTRTACA) for NoVGI and COG2F (CARGA-RBCNATGTTYAGRTGGATGAG), G2-SKR (CCRCCN-GCATR-HCCRTTRTACAT) for NoVGII. The PCR was performed at 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s and a final extension at 72 °C for 7 min and then held at 4 °C.

2.5. Electrophoresis

The PCR products were electrophoresed in a 1.5% agarose gel, followed by staining with ethidium bromide (0.5 μg/ml) for 20 min and then visualized under ultraviolet (UV) light. The bands were recorded by photography (Ushijima et al., 1992).

2.6. Nucleotide sequence analysis

The nucleotide sequences of NoV-specific PCR products were determined with the Big-Dye terminator cycle sequencing kit and an ABI Prism 310 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA). Sequence analysis was performed using CLUSTAL X software (version 1.6). A phylogenetic tree was constructed with 100 bootstrap resamples of the nucleotide alignment data sets using the neighbor-joining method with CLUSTAL X. The genetic distance was calculated using Kimura's two-parameter method (Phylip).

2.7. Phylogenetic analysis

Phylogenetic and molecular evolutionary analyses were conducted using the MEGA version 3.2 software package (Kumar et al., 2001). The following reference strains and accession numbers were used in the phylogenetic analysis: Hawaii Calcivirus (U07611), Mexicovirus (U22498), Torontovirus (U02030), Lordsdale (X86557), Bristol (X76716), NLV/VA98387/1998 (AY038600), Hu/NV/Hokkaido/194/2004/JP (AB240180), Hu/NV/Hokkaido/231/2004/JP (AB240183), Hu/NV/Hokkaido/306/2005/JP (AB240187), Hu/G-II/Nagano/2004/H/JP (DQ095875) and Stockholm/97 (AF194182).

2.8. Nucleotide sequence submission

The nucleotide sequence data reported in this paper have been deposited in GenBank under the following accession numbers: DQ889461–DQ889501.

3. Results

3.1. Detection of noroviruses

NoVs were detected in 41/917 of the fecal specimens collected from infants and children with acute gastroenterits. All fecal specimens were also tested for the presence of sapovirus, astrovirus by RT-multiplex PCR. Among diarrheal

Table 1
Clinical features of norovirus-positive and norovirus-negative patients

Sign and symptoms	Norovirus-positive (N=41 (%))	Norovirus-negative (N=876 (%))
Diarrhea	37 (90%)	621 (72%)
Vomiting (≥3 times per day)	31 (75%)	309 (35%)
Abdominal pain	19 (46%)	248 (28%)
Fever (>100 °F)	11 (27%)	217 (24%)
Three to five times loose stools within 24 h	29 (70%)	578 (65%)
Six or more loose stools within 24 h	12 (30%)	298 (35%)
Age at hospitalization (≤24 months)	34 (82%)	824 (94%)

viruses detected, norovirus was the most prevalent (4.5%), followed by 2.7% of sapovirus and 0.3% of astrovirus (Dey et al., 2007). The youngest subject was 2-month-old and the oldest 38 months; the average age was 13 months. All fecal specimens were tested for the detection of NoV by RT-multiplex PCR method.

3.2. Clinical features of norovirus-positive and norovirus-negative patients

Clinical data of the 41 patients infected with NoV are listed in Table 1. The most common clinical symptoms were vomiting (75%), diarrhea (90%), abdominal pain (46%) and fever (27%). The most common clinical features of norovirus-negative patients were diarrhea (72%), vomiting (35%), abdominal pain (28%) and fever (24%). The percentage of vomiting, diarrhea and abdominal pain in norovitus-positive patients were higher than norovirus-negative patients.

3.3. Nucleotide sequence and phylogenetic analyses of norovirus

All the NoV amplicons were characterized for genogroup, genotypes and genetic relationship with the reference strains based on their capsid regions (Okada et al., 2005) classification scheme. Their partial nucleotide sequences were compared to each other as well as to reference NoV strains available in the DDBJ DNA/GenBank database by BLAST. The nucleotide sequence of the 5′ ends of the NoV capsid gene was determined by direct sequencing with the amplified fragments. All of the NoV sequences were classified into genogroup II (Fig. 1) and the NoV genogroup II clustered into one genotype (GII/4).

CLUSTAL X indicated that these NoVs were very similar at the amino acid level and were most closely related to the NLV/VA98387/1998, Hu/NV/Hokkaido/194/2004/JP, Hu/NV/Hokkaido/231/2004/JP, Hu/NV/Hokkaido/306/2005/JP and Hu/GII/Nagano/2004/H/JP strain.

3.4. Seasonal distribution of norovirus

The seasonal pattern of NoV infection in this study is shown in Fig. 2. Two peaks of NoV infection were observed. One peak occurred from November 2004 through March 2005 and another one occurred from August through September in 2005. Ninety percent (37 of 41) positive samples were collected during this peak time. It was found that the nucleotides as well as the amino acid sequences of capsid regions among norovirus isolates were of high identity (above 96%).

4. Discussion

NoV is the most common nonbacterial etiological agent for gastroenteritis outbreaks and a common cause of acute gastroenteritis in children. These outbreaks have a significant public health impact worldwide (Fankhauser et al., 2002; Pang et al., 1999). The NoV genogroup II viruses are dominant in all parts of the world. Several genotypes co-circulate, but the majority of infections are caused by a few genotypes (Koopmans et al., 2003). Predominance of GII strains over GI have been widely observed with sporadic cases of acute diarrhea and during outbreaks (Bon et al., 1999; Buesa et al., 2002; Farkas et al., 2000; Kirkwood and Bishop, 2001; Pang et al., 1999; Roman et al., 2002). This study showed that all Bangladeshi NoV sequences belonged only to genogroup II, which clustered into one genotype (GII/4). NoV detected in this study was closely homologous at the nucleotide and amino acid level. Nucleotide sequence analysis indicated that the common GII/4 strains were circulating among Bangladeshi children and infants.

The present study demonstrated two peaks of NoV infection. This result clearly indicated that norovirus infection was most commonly observed in late autumn to winter seasons (November–March) in Dhaka City.

Most of the norovirus-positive samples collected during this period. In this study, most of the patients (92%) were belonged to 1–24 months age group. The lowest age was 2 months, the highest was 3.2 years (38 months) and the average age was 1.1 years (13 months the NoV has been associated with gastroenteritis outbreaks in all age groups (Green et al., 2001). However, less is known of the age distribution of norovirus infection. In this study, infections were most commonly detected in children less than 3 years of age.

The most common signs and symptoms of infants and children infected with NoV were diarrhea (90%), vomiting (75%) and abdominal pain (46%). These results were consistent with other research. We found more patients with severe diarrhea (90%) than with vomiting (75%), which is in agreement with (Wyatt et al., 1974). Fever (body temperature more than 100 °F) was found in 27% of, which was similar to patients with acute gastroenteritis without. Number of loose stool per day was rather extended with most of the individuals having six to eight times (43%) stool per day.

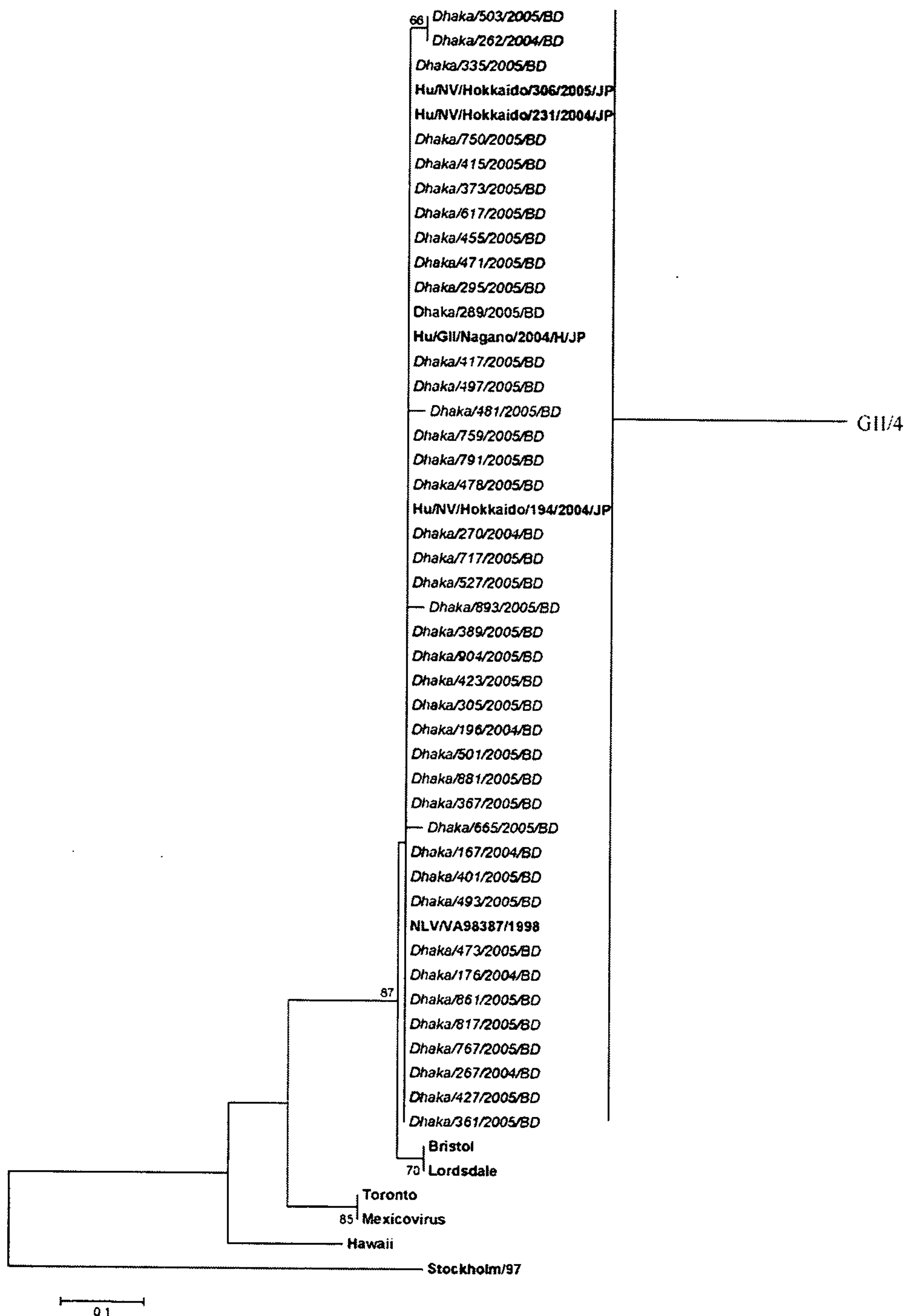

Fig. 1. Phylogenetic tree of nucleotide sequences of Bangladeshi norovirus. The tree was constructed from partial amino acid sequences of capsid region of norovirus. Reference strains of norovirus were selected from DDBJ/GenBank under the accession number indicated in bold. Bangladeshi norovirus was highlighted in italic. Stockholm/93 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. The GenBank accession numbers of this study are: DQ889461–DQ889501.

We found that NoV-infected persons developed symptoms of severe vomiting and watery diarrhea and typically remain symptomatic for 2–3 days.

Although the importance of viral gastroenteritis as a prime cause of morbidity and mortality in developing countries is well recognized, very few studies were conducted to evaluate the role of viral agents in childhood diarrhea in Bangladesh.

In conclusion, this is the first epidemiological research of norovirus in Bangladesh.

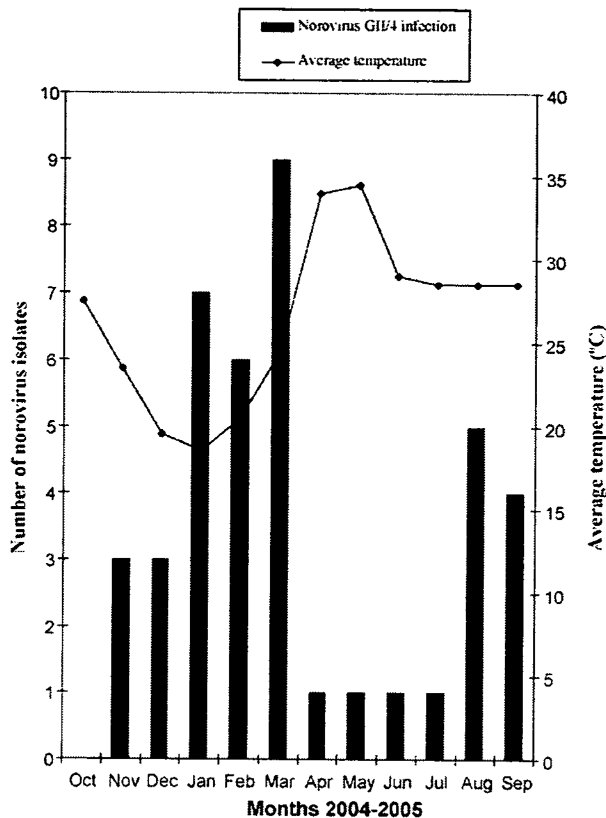

Fig. 2. Seasonal pattern of norovirus infection in infants and children with acute gastroenteritis in Dhaka City, Bangladesh during October 2004–September 2005. Average temperature data were obtained from the Metrological Institute, Dhaka.

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References

Atmar RL, Estes MK. Diagnosis of noncultivatable gastroenteritis viruses, the human caliciviruses. Clin Microbiol Rev 2001;14:15–37.

Bon F, Fascia P, Dauvergne M, Tenenbaum D, Planson H, Petion AM, et al. Prevalence of group A rotavirus, human calcivirus, astrovirus and adenovirus type 40 and 41 infection among children with acute gastroenteritis in Dijon. France J Clin Microbiol 1999;37:3055–8.

Buesa J, Collado B, Lopez-Andujar P, Abu-Mallouh R, Rodriguez Diaz J, Garcia Diaz A, et al. Molecular epidemiology of calciviruse causing outbreaks and sporadic cases of acute gastroenteritis in Spain. J Clin Microbiol 2002;40:2854–9.

Dey SK, Phan TG, Nguyen TA, Nishio O, Salim AF, Yagyu F, et al. Prevalence of sapovirus infection among infants and children with acute gastroenteritis in Dhaka City, Bangladesh during 2004–2005. J Med Virol 2007;79(5):633–8.

Fankhauser RL, Monroe SS, Noel JS, Humohrey CD, Bresee JS, Parashar UD, et al. Epidemiologic and molecular trends of "Norwalk-like viruses" associated with outbreaks of gastroenteritis in the United States. J Infect Dis 2002;186:1–7.

Farkas T, Jian X, Guerrero ML, Zhong W, Wilton N, Berk T, et al. Prevalence and genetic diversity of human calciviruses (HuCVs) in Mexican children. J Med Virol 2000;62:217–23.

Farkas T, Zhong WM, Jing Y, Huang PW, Espinosa SM, Martinez N, et al. Genetic diversity among sapoviruses. Arch Virol 2004;73: 1309–23.

Gallimore CI, Cub itt D, Plessis du N, Gray JJ. Asymptomatic and symptomatic excretion of noroviruses during a hospital outbreak of gastroenteritis. J Clin Microbiol 2004;42:2271–4.

Glass PJ, White LJ, Ball JM, Leparc-Goffart I, Hardy ME, Estes MK. Norwalk virus open reading frames 3 encode a minor structural protein. J Virol 2000;74:6581–91.

Green K, Chanock R, Kapikian A. Human caliciviruses. In: Knipe DM, Howley PM, et al, editors. Fields virology. 4th ed. Philadelphia: Lippincott Williams and Wilkins; 2001. p. 841–741.

Hale A, Mattick K, Lewis D, Estes M, Jiang X, Green J, et al. Distinct epidemiologicacal patterns of Norwalk-like virus infection. J Med Virol 2000;62:99–103.

Isakbaeva ET, Widdowson MA, Beard RS, Bulens AN, Mullins J, Monroe SS, et al. Norovirus transmission on cruise ship. Emerg Infect Dis 2005;11:154–8.

Jiang X, Wang MK, Graham DY, Estes MK. Expression, self assembly, and antigenicity of the Norwalk virus capsid protein. J Virol 1992;66:6527–32.

Jiang X, Wang MK, Estes MK. Sequence and genomic organization of Norwalk virus. Virology 1993;195:51–61.

Kageyama T, Shinohara M, Uchida K, Fukushi S, Hoshino FB, Kojima S, et al. Coexistence of multiple genotypes, including newly identified genotypes, in outbreaks of gastroenteritis due to norovirus in Japan. J Clin Microbiol 2004;42:2988–95.

Kirkwood CD, Bishop RF. Molecular detection of human calcivirus in young children hospitalized with acute gastroenteritis in Melbourne, Australia, during 1999. J Clin Microbiol 2001;39:2722–4.

Koopmans M, van Strien E, Vennema H. Molecular epidemiology of human calcivirus. In: Desselberger U, Gray J, editors. Viral Gastroenteritis. Amsterdam: Elsevier; 2003. p. 523–54.

Kumar S, Tamura K, jakobsen IB, Nei M. MEGA2: molecular evolutionary genetics analysis software. Bioinformatics 2001;17: 1244–5.

Lambden PR, Caul EO, Ashley CR, Clarke IN. Sequence and genome organization of a human small round-structured (Norwalk-like) virus. Science 1993;259:516–9.

Matsui SM, Kim JP, Greeenberg HB, Su W, Sun Q, Johnson PC, et al. The isolation and characterization of a Norwalk virus-specific cDNA. J Clin Investig 1991;87:1456–61.

Milazzo A, Tribe IG, Ratcliff R, Doherty C, Higgins G, Givney R. A large prolonged outbreak of human calicivirus infection linked to an aged-care facility. Commun Dis Intell 2002;26:261–4.

Mulholland EK. Global control of rotavirus disease. Adv Exp Med Biol 2004;549:161–8.

Okada M, Ogawa T, Kaiho I, Shinozaki K. Genetic analysis of Noroviruses in Chiba Prefecture, Japan, between 1999 and 2004. J Clin Microbiol 2005;43(9):4391–401.

Pang XL, Joensuu J, Vesikari T. Human calicivirus associated sporadic gastroenteritis in Finnish children less than two years of age followed prospectively during a rotavirus vaccine trial. Pediatr Infect Dis J 1999;18:420–6.

Parashar UD, Glass RI. Viral causes of gastroenteritis. In: Desselberger U, Gray J, editors. Viral Gastroenteritis. Amsterdam: Elsevier; 2003. p. 9–21.

Parashar UD, Hummelman EG, Bresee JS, Miller MA, Glass RI. Global illness and death caused by rotavirus disease in children. Emerg Infect Dis 2003;9:565–72.

Phan TG, Nguyen TA, Yan H, Yagyu F, Kozlov V, Kozlov A, et al. Development of a novel protocol for RT-multiplex PCR to detect diarrheal viruses among infants and children with acute gastroenteritis in Eastern Russia. Clin Lab 2005;51:429–35.

Roman E, Negredo A, Dalton RM, Wihelmi I, Sanchez-Fauquier A. Molecular detection of human calcivirus among Spanish children with acute gastroenteritis. J Clin Microbiol 2002;40:3857–9.

Seah E, Marshall JA, Wright PJ. Open reading frame 1 of Norwalk-like virus Camberwell: completion of sequence and expression in mammalian cells. J Virol 1999;73:10531–5.

Ushijima H, Mukoyama A, Hasegawa A, Nishimura S, Gentsch J. Detection and serotyping of rotaviruses in stool specimen by using reverse transcription and polymerase chain reaction amplification. J Med Virol 1992;38(4):192–297.

Wyatt RG, Dolin R, Blacklow R, Dupont HL, Buscho RF, Thornhill TS, et al. Comparison of three agents of acute infectious nonbacterial gastroenteritis by cross-challenge in volunteers. J Infect Dis 1974;129:709–14.

Yan H, Yagyu F, Okitsu S, Nishio O, Ushijima H. Detection of norovirus (GI, GII), sapovirus and astrovirus in fecal samples using reverse transcription single-round multiplex PCR. J Virol Methods 2003;14:37–44.

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Genetic Diversity of Noroviruses and Sapoviruses in Children Hospitalized With Acute Gastroenteritis in Chiang Mai, Thailand

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Human caliciviruses, including norovirus (NoV) and sapovirus (SaV), are recognized as common pathogens that cause acute viral gastroenteritis in children and adults throughout the world. To gain an overview of molecular epidemiology of human caliciviruses in children hospitalized with acute gastroenteritis in Chiang Mai, Thailand, from 2002 to 2004, NoV and SaV were detected and characterized molecularly for identification of their genotypes. From a total of 248 fecal specimens collected, 35 (14.1%) were positive for NoV GII genogroup. Among the 35 NoV GII, GII/4 was the most predominant genotype (22 strains), followed by GII/3 (7 strains), GII/1 (2 strains), GII/7 (2 strains), GII/2 (1 strain), and GII/16 (1 strain). In addition, only three specimens (1.2%) were positive for SaV, each of which was classified into two different genogroups. One isolate was clustered with GIV genogroup, while the other two belonged to two distinct genotypes of the SaV GI cluster, GI/1 and GI/2 genotypes. This study demonstrated that human caliciviruses are important enteric viruses that caused acute gastroenteritis in the hospitalized children in Chiang Mai, Thailand from 2002 to 2004. Moreover, a great genetic diversities of NoV and SaV were observed. ***J. Med. Virol. 79:1921–1926, 2007.*** © 2007 Wiley-Liss, Inc.

KEY WORDS: noroviruses; sapoviruses; acute gastroenteritis; Chiang Mai; Thailand

INTRODUCTION

Viral gastroenteritis is one of the most common illnesses in humans worldwide, and different agents such as rotavirus, astrovirus, adenovirus, and calicivirus have been associated with the disease [Clark and McKendrick, 2004]. Among different types of viruses that cause diarrhea, rotavirus is the most common, and is a major cause of severe gastroenteritis in infants and young children worldwide [Parashar et al., 2006]. Recently, however, human caliciviruses have emerged as significant etiologic agents of diarrheal disease across all age groups. Norovirus (NoV) and sapovirus (SaV) are two of the four genera of the family *Caliciviridae*, which is a nonenveloped, positive-sense, single-stranded RNA [Green et al., 2001]. These viruses are a leading cause of gastroenteritis worldwide and are responsible for outbreaks in various epidemiological settings, including restaurants, schools, day-care centers, hospitals, nursing homes, and cruise ships [McEvoy et al., 1996; Russo et al., 1997; McIntyre et al., 2002; Akihara et al., 2005].

Extensive molecular epidemiological studies of calicivirus infection in humans have been conducted. Application of RT-PCR and DNA sequencing techniques for the detection and characterization of NoV and SaV became the standard tests for detecting these pathogens [Yan et al., 2004]. These detection techniques have enhanced markedly our understanding of the epidemiology of NoV and SaV infections. Thousands of NoV and SaV strains have been identified, named, and classified into genogroups and genetic clusters.

Currently, based on the diversity of capsid sequences, NoVs are grouped into five genogroups (GI–GV), of which three have been found in humans; GI, GII, and GIV [Kageyama et al., 2004; Zheng et al., 2006]. Human NoV genogroups are subdivided further into at least

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15 genotypes in GI, 18 genotypes in GII, and only 1 genotype in GIV [Kageyama et al., 2004; Vinje et al., 2004; Okada et al., 2005]. Several epidemiological studies clearly indicated that NoV GII is the main causative agent among NoV, which causes acute diarrhea in humans [Hansman et al., 2004; Phan et al., 2006a,b; Tseng et al., 2007]. SaVs can be divided into five genogroups (GI–GV), of which GI, GII, GIV, and GV are known to infect humans, whereas SaV GIII infects porcine species [Farkas et al., 2004; Wang et al., 2006; Hansman et al., 2007].

In Thailand, very few molecular epidemiological studies of NoV and SaV have been conducted and various genotypes were circulated in different epidemiological settings [Guntapong et al., 2004; Hansman et al., 2004; Veeravigrom et al., 2004]. The frequency of NoV and SaV detection rates ranged from 8.6%–17.5% and 4.8%–15.0% of diarrheal disease in hospitalized cases. However, only two studies have been characterized further for their genotypes by sequence and phylogenetic analyses. The study carried out in Chiang Mai during 2000 to 2001 demonstrated that out of 105 specimens collected, 8 and 4 were found to be a single infection with NoV and SaV, while 1 was a mixed infection [Hansman et al., 2004]. The other study was conducted during 2002–2003. The stool specimens were collected from five different geographical areas in Thailand (Sa Kaeo, Chanthaburi, Songkhla, Nong Khai, and Tak). Of the 80 specimens examined, 11 and 9 of NoV and SaV single infections were identified, respectively, and three were found to be mixed infections [Guntapong et al., 2004].

With the aim of having an overview of molecular epidemiology of human caliciviruses in children hospitalized with acute gastroenteritis in Chiang Mai, Thailand, the prevalence of NoV and SaV infections were examined from 2002 to 2004. The genotypes of NoV and SaV were identified by sequence and phylogenetic analyses.

MATERIALS AND METHODS

Specimens Collection

A total of 248 fecal specimens were collected from pediatric patients aged less than 5-years-old, who were hospitalized with acute gastroenteritis at McCormick Hospital, Chiang Mai, Thailand. The study period was from March 2002 to December 2004.

RNA Extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The RNA genome of NoV and SaV were first extracted from 10% fecal suspension supernatant using the QIAamp viral RNA Mini Kit (Qiagen, Hilden, Germany). The presence of the NoV (GI and GII) and SaV in fecal specimens was detected by RT-PCR using the protocol described previously [Yan et al., 2004]. A forward primer, G1-SKF (nt 5342–5361) 5′-CTGCCCGAAT-TYGTAAATGA-3′, was used in combination with the reverse primer G1-SKR (nt 5653–5671) 5′-CCAACC-CARCCATTRTACA-3′, for the amplification of NoV GI, which specifically generated a PCR amplicon of 330 bp. For NoV GII identification, a forward primer, COG2F (nt 5003–5028) 5′-CARGARBCNATGTTYAGRTGGA-TGAG-3′, was used in combination with the reverse primer G2-SKR (nt 5367–5389) 5′-CCRCCNGCAT-RHCCRTTRTACAT-3′, which showed a PCR product size of 387 bp. For SaV detection, a 434 bp fragment was generated using forward primer SLV5317 (nt 5317–5339) 5′-CTCGCCACCTACRAWGCTTGGTT-3′ and reverse primer SLV5749 (nt 5727–5749) 5′-CGGRCYT-CAAAVSTACCBCCCCA-3′. All of the virus positive samples were analyzed further for their genotypes by nucleotide sequence and phylogenetic analyses.

Sequence and Phylogenetic Analyses

The PCR amplicons were purified with a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI) and sequenced by 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 to those of NoV and SaV strains deposited in the GenBank using the BLAST program. The genotypes of NoV and SaV were classified by using the clustering methods determined previously by Kageyama et al. [2004] and Phan et al. [2007b].

Nucleotide Sequence Accession Numbers

The partial nucleotide sequences of the capsid gene were deposited in GenBank under the accession number EF600759–EF600793 for NoV strains and EF600794–EF600796 for SaV strains.

RESULTS

Detection and Genotype Distribution of Noroviruses

Between March 2002 and December 2004, a total of 248 fecal specimens were collected from pediatric patients hospitalized with diarrhea, and 35 (14.1%) were found positive for NoV by using the RT-PCR screening method (Table I). Of these, all of the positive samples were identified as NoV GII genogroup, and none was a mixed infection between NoV and SaV. The age of the patients, who were infected with NoV, ranged from 4 months up to 3-years-old. All of the NoV positive samples were characterized further for their genotypes by sequencing of the partial capsid regions. The genotypes were classified according to the phylogenetic clustering method described by Kageyama et al. [2004]. A total of 35 NoV GII isolates were clustered exclusively with other GII reference strains and these GII strains were classified further into six distinct genotypes, including GII/1, GII/2, GII/3, GII/4, GII/7,