DETECTION AND GENETIC CHARACTERIZATION OF NOROVIRUS IN ENVIRONMENTAL WATER SAMPLES IN THAILAND

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Abstract. The aim of this study was to detect and characterize noroviruses (NoVs) in environmental water samples. One hundred and fourteen water samples were collected from a river and irrigation canals in central Thailand during 2006-2007. NoVs were detected by RT-nested PCR in 13% of the samples. The river samples (22%) contained NoVs at a higher frequency than the irrigation canal samples (4%). Among the 15 NoV-positive samples, 9 harbored genogroup (G) I, 2 samples with GII, and 4 samples with mixed GI and GII. DNA sequencing of PCR amplicons and phylogenetic analysis of partial capsid gene revealed that 5 samples were of genotype GI-2, 1 sample was GI-6, and 1 sample was a mix of GI-2 and GII-unclassified genotypes. NoVs in water samples quantified using quantitative RT-PCR were in the range of 4.91×10^2 -1.26×10^3 copies/ml for NoV GI and 3.51×10^3 copies/ml for NoV GII. This is the first study demonstrating the presence of NoV variants in water samples collected from a river and the adjacent canals of Thailand.

Keywords: norovirus, genotype, water, RT-nested PCR

INTRODUCTION

Noroviruses (NoVs) are causative agents of acute gastroenteritis in all age groups worldwide (Glass *et al*, 2009). NoVs belong to the Caliciviridae family and are divided into five distinct genogroups, of which genogroup I (GI), GII, and GIV infect humans. NoV GI and GII are the main human pathogens and can be classified further into 8 GI and 19 GII genotypes (Zheng *et al*, 2006). The viruses are transmitted via the fecal-oral route through contaminated food and water and person-to-person contact. Food-borne outbreaks of acute gastroenteritis caused by NoVs have been reported frequently (Barrabeig *et al*, 2010; CDC, 2010; Westrell *et al*, 2010). In addition, NoVs were associated with various water-borne outbreaks (Maunula *et al*, 2005; Hewitt *et al*, 2007; Lysén *et al*, 2009; Kvitsand and Fiksdal, 2010). NoVs have been detected in surface water (Hernandez-Morga *et al*, 2009), waste water (Aw and Gin, 2010), and even in drinking water (Victoria *et al*, 2010) thus leading to water contact and domestic use of NoV-contaminated being a matter of public health concern.

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Since human NoVs can not be cultivated in mammalian cells, molecular techniques have been used for detection of NoVs in clinical and environmental samples (Atmar and Estes, 2001). The development of a sensitive reverse transcription-nested polymerase chain reaction (RT-nested PCR) method has facilitated the detection of enteric viruses in water samples containing low amounts of viruses (Kittigul *et al*, 2005; Hernandez-Morga *et al*, 2009; La Rosa *et al*, 2010).

In Thailand, although reports of patients with acute gastroenteritis caused by NoVs have been documented extensively (Hansman *et al*, 2004; Khamrin *et al*, 2007; Malasao *et al*, 2008; Kittigul *et al*, 2010), data regarding NoVs in surface water are lacking thus, the objective of this study was to detect NoVs in water samples using an enrichment technique followed by RT-nested PCR. In addition, characterization of NoV genotypes by phylogenetic analysis was undertaken.

MATERIALS AND METHODS

Water sample collection

A total of 114 environmental water samples were collected monthly at different collection sites from Lop Buri River (55 samples) and irrigation canals (59 samples) in Lop Buri Province in January 2006, and from September 2006 to January 2007. Lop Buri Province is 155 km from Bangkok, the capital city of Thailand and has been reported to have a high prevalence of NoV gastroenteritis (Kittigul *et al*, 2010). The water collection sites were approximately 500 m apart. For each sample, 1 liter of water was collected from a depth of at least 30 cm below the water surface, kept in a chilled container, and transported immediately to laboratory for NoV analysis. Temperature and pH of water samples ranged 23-33ºC and 6.0-8.6, respectively.

Virus enrichment

All water samples were concentrated using a modified adsorption-elution technique previously described (Kittigul *et al*, 2005). In brief, the water samples were adjusted to pH 3.5 with 1 M HCl and aluminum chloride was added for a final concentration of 0.0015 M to flocculate the virus. Then water samples were stirred at room temperature for at least 30 minutes and passed through a anion membrane filter, 47 mm in diameter with 0.45 m pore size (Gelman, Ann Arbor, MI). After washing membrane with 0.14 M NaCl pH 3.5, virus was eluted with 2.9% tryptose phosphate broth containing 6% glycine, pH 9.0. The eluates were adjusted to pH 7.0-7.4 with 4 M HCl and concentrated using vacuum concentrator centrifuge (UniEquip Laborgeratebau-und Vertriebs GmbH, Planegg, Germany) to a final volume ranging from 1.5 to 4.2 ml and were stored at -80ºC until used for nucleic acid extraction.

RNA extraction and RT-nested PCR

A 140 l aliquot of concentrated water sample was for extraction of viral RNA using QIAamp® Viral RNA extraction kit (QIAGEN GmbH, Hilden, Germany). The RNA extract (60 l) was collected and kept at -80ºC until used. NoV was detected using RT-nested PCR previously described (Kittigul *et al*, 2010). The specific primer sets located in the capsid gene (region C) for NoV GI were COG1F (5′-CGY TGG ATG CGN TTY CAT GA-3′), G1SKF (5′-CTG CCC GAA TTY GTA AAT GA-3′) and G1SKR (5′-CCA ACC CAR CCA TTR TAC A-3′), and for NoV GII, COG2F (5′-CAR GAR BCN ATG TTY AGR TGG ATG AG-3′), G2SKF (5′-CNT GGG AGG GCG ATC GCA A-3′) and G2SKR (5′-CCR

CCN GCA TRH CCR TTR TAC AT-3') where Y = C or T, R = A or G, B = C, G or T, H = A, C or T, N = A, T, G or C (Kojima *et al*, 2002; Kageyama *et al*, 2003). RT-PCR was performed with 50 l reaction volume using SuperScript™ III One-Step RT-PCR System with Platinum® *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). In brief, RNA extract (2 l) was heated at 94ºC for 4 minutes, and placed on ice for 10 minutes. Then RNA was added to the RT-PCR mixture (48 l) consisting of 0.2 mM of each dNTP, 1.6 mM of $MgSO_4$, SuperScript™ III RT/Platinum® *Taq* Mix, 0.33 M of the specific primers (COG1F/G1SKR for NoV GI or COG2F/G2SKR for NoV GII), and nuclease-free water. Conditions for RT-PCR were as follows: 42ºC for 60 minutes; 94ºC for 2 minutes; 35 cycles of 94ºC for 1 minute, 50ºC for 1 minute, 72ºC for 1 minute; and a final heating at 72ºC for 3 minutes.

Nested PCR was performed in 50 l reaction volume. The RT-PCR amplification solution (2 l) was added to the reaction mixture (48 l) consisting of 2.5 mM $MgCl_2$, 1x *Taq* buffer, 0.2 mM of each dNTP, 0.33 M of the specific primers (G1SKF/G1SKR for NoV GI or G2SKF/G2SKR for NoV GII), 0.63 U *Taq* polymerase for NoV GI or 2.5 U for NoV GII, and nuclease-free water. Conditions for the nested PCR were as follows: 94ºC for 3 minutes; 35 cycles for NoV GI or 30 cycles for NoV GII of 94ºC for 1 minute, 50ºC for 1 minute, 72ºC for 2 minutes; and a final heating at 72ºC for 15 minutes. Nested PCR amplicons were analyzed by 1.5% agarose gel electrophoresis with ethidium bromide staining. Amplicons of 330 bp and 344 bp was indicative of NoV GI and NoV GII, respectively.

Standard precautions were utilized during the RT-nested PCR assay procedure, and the experiments were performed in three separate rooms. Negative control consisting of nuclease-free water and PCR mixture was included in each experiment. Positive control consisting of known NoV from a stool sample (positive by RT-nested PCR and identified by DNA sequencing) was included in the assays for test performance but was not included in sample assays to avoid cross-contamination.

Quantitative (q) RT-PCR

Norovirus Real Time RT-PCR kit (Shanghai ZJ Bio-Tech, Shanghai, China) containing NoV specific primers, Taqman probes and reagents for the simultaneous detection of NoV GI and GII was used with LightCycler 1.5 Instrument Real Time PCR System (Roche, Diagnostics, Mannheim, Germany). The qRT-PCR for detection of NoV was carried out following the manufacturer's instructions and performed with 20 l reaction volume. In brief, RNA extract (5 l) from the concentrated water sample was added to the RT-PCR mixture (15 l) consisting of NoV GI or GII super mix (13 l), RT-PCR enzyme mix (1 l) and internal control or molecular grade water (1 l). Conditions for RT-PCR were as follows: 45ºC for 10 minutes; 95ºC for 15 minutes; 40 cycles of 95ºC for 5 seconds, 60ºC for 30 seconds. Negative control was included in each qRT-PCR assay. NoV GI and GII positive control (1 x 10^8 copies/ml) provided with the kit was used for quantitation of NoVs. Standard curves of NoV GI and GII copy numbers (1 x 10^7, 1 x 10^6, 1 x 10^5, 1 x 10^4, and 1 x 10^3 copies/ml) versus threshold cycle (Ct) were generated. NoV GI and GII in the water samples were quantified from Ct values obtained and comparison with the standard curves.

Generation of phylogenetic relationship

PCR amplicons of NoV-positive

Table 1
Norovirus genogroups in environmental water samples from Lop Buri Province, Thailand.

Source of water	No. of samples	No. of norovirus (%)		
		GI	GII	GI+GII
River	59	9 (15)	1 (2)	3 (5)
Irrigation canals	55	0 (0)	1 (2)	1 (2)

samples from RT-nested PCR were purified using QIAquick PCR purification kit (QIAGEN GmbH, Hilden, Germany). Then DNA sequencing with G1SKR for NoV GI and G2SKR for NoV GII was performed at BioDesign Co, Ltd, Bangkok. The nucleotide sequences were compared to NoV strains deposited in NCBI (National Center for Biotechnology Information) GenBank database using BLAST (Basic Local Alignment Search Tool) program (Altschul *et al*, 1990). Phylogenetic relationships of NoVs were examined by aligning sequences using ClustalX program. A phylogenetic tree was constructed according to the neighbor-joining method using MEGA version 3.1 (Kumar *et al*, 2004). The nucleotide sequences of strains from this study have been deposited in GenBank (accession nos. HQ588794-HQ588800 for NoV GI and HQ588801 for NoV GII).

RESULTS

Norovirus contamination in environmental water samples

Of 114 water samples, 15 (13%) were positive for NoVs by RT-nested PCR. Regarding the sources of the water samples, NoVs were detected in 13/59 (22%) and 2/55 (4%) of river and irrigation canal samples, respectively. Of 15 NoV-positive water samples, 9 harbored GI, 2 samples GII, and 4 samples with mixed GI and GII (Table 1). NoV GI was found in water samples at a higher detection rate than NoV GII and the ratio of NoV GI to GII was 2.2:1. Both NoV GI and GII were detected in water samples collected in January, October, and December with the highest peak in October. Based on qRT-PCR assay, NoV GI was found in 8/13 NoV GI-positive water samples by RT-nested PCR concentrations were in the range of 4.91×10^2 -1.26×10^3 copies/ml, and NoV GII was detected in 1/6 RT-nested PCR NoV GII-positive water samples with a concentration of 3.51×10^3 copies/ml (Table 2).

Phylogenetic analysis of NoVs

Among the 15 NoV-positive samples, 7 river samples yielded sufficient amounts of amplicons for sequencing. Using BLAST program, 6 sequences from NoV GI partial capsid gene region showed 100% nucleotide identity (259 nucleotides) with the NoV sequence of urban wastewater from Singapore, Norovirus Hu/GI/UE/270607-3/2007/SGP (GQ925162) (Aw *et al*, 2009). Based on phylogenetic analysis of 240 nucleotides, these sequences belonged to GI-2, within the same cluster

Table 2
Characteristics of noroviruses detected in environmental water samples from Lop Buri Province, Thailand.

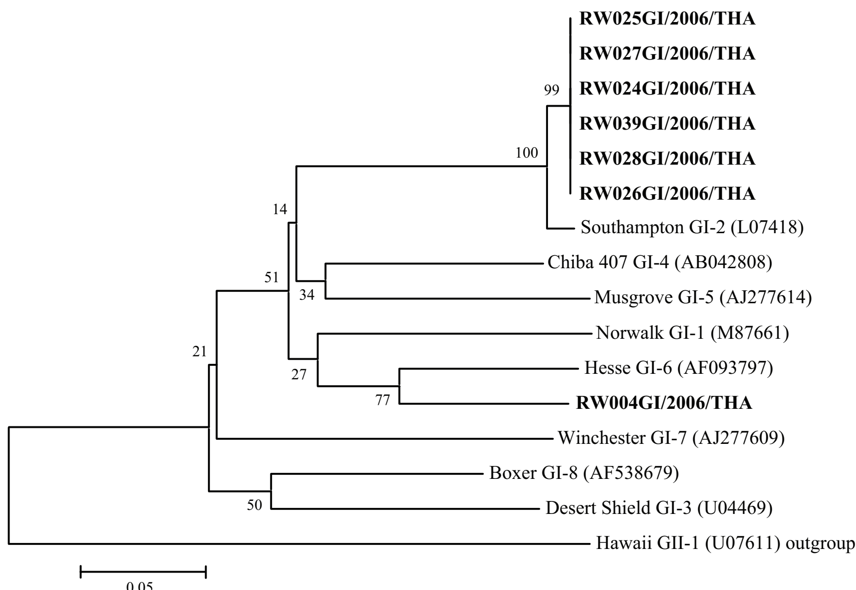
Sample ID	Source of water	Date of sampling	Genogroup[a]	Genotype[b]	Copies/ml[c]
004	River	January, 2006	GI	GI-6	4.91×10^2
023	River	October, 2006	GI	ND	1.26×10^3
024	River	October, 2006	GI	GI-2	6.86×10^2
025	River	October, 2006	GI	GI-2	1.09×10^3
026	River	October, 2006	GI	GI-2	1.01×10^3
027	River	October, 2006	GI	GI-2	1.02×10^3
			GII	ND	-[d]
028	River	October, 2006	GI	GI-2	7.02×10^2
034	River	October, 2006	GI	ND	-
039	River	October, 2006	GI	GI-2	7.49×10^2
			GII	GII-unclassified	3.51×10^3
070	Irrigation canal	December, 2006	GI+GII	ND	-
079	Irrigation canal	December, 2006	GII	ND	-
086	River	December, 2006	GI	ND	-
091	River	January, 2007	GI+GII	ND	-
098	River	January, 2007	GII	ND	-
100	River	January, 2007	GI	ND	-

[a]By RT-nested PCR; [b]By DNA sequencing and phylogenetic analysis; [c]By qRT-PCR; [d]Negative result; ND, not determined.

as the reference Southampton strain. One NoV sequence showed 99% nucleotide identity (263 nucleotides) with the NoV sequence from Japan, NV/Saitama T50GI/01/JP (AB112143) and belonged to GI-6, within the same cluster as the reference Hesse strain (Fig 1A). One NoV GII sequence showed 95% nucleotide identity (209 nucleotides) with the NoV sequences from urban wastewater in Singapore, Norovirus Hu/GII/KE/240407-1/2007/SGP (GQ925223), and from a Thai patient with acute gastroenteritis, Norovirus Hu/GII/Lop Buri112/2006/THA (GQ325682). This sequence belonged to the GII-unclassified genotype exhibiting separate lineage in the phylogenetic tree (Fig 1B). One river sample (sample ID, RW039) contained mixed sequences of NoV GI-2 and NoV GII-unclassified genotypes.

DISCUSSION

In this study, environmental water samples from 2 sources in Thailand, Lop Buri River and irrigation canals in Lop Buri Province, were examined for NoVs using RT-nested PCR and qRT-PCR, and these viruses were then analyzed for phylogenetic relationship. The river water samples were more frequently contaminated with NoVs than the water samples from irrigation canals. Human sewage from surrounding communities probably drained into the river, whereas irrigation canals were the source of water for agri-



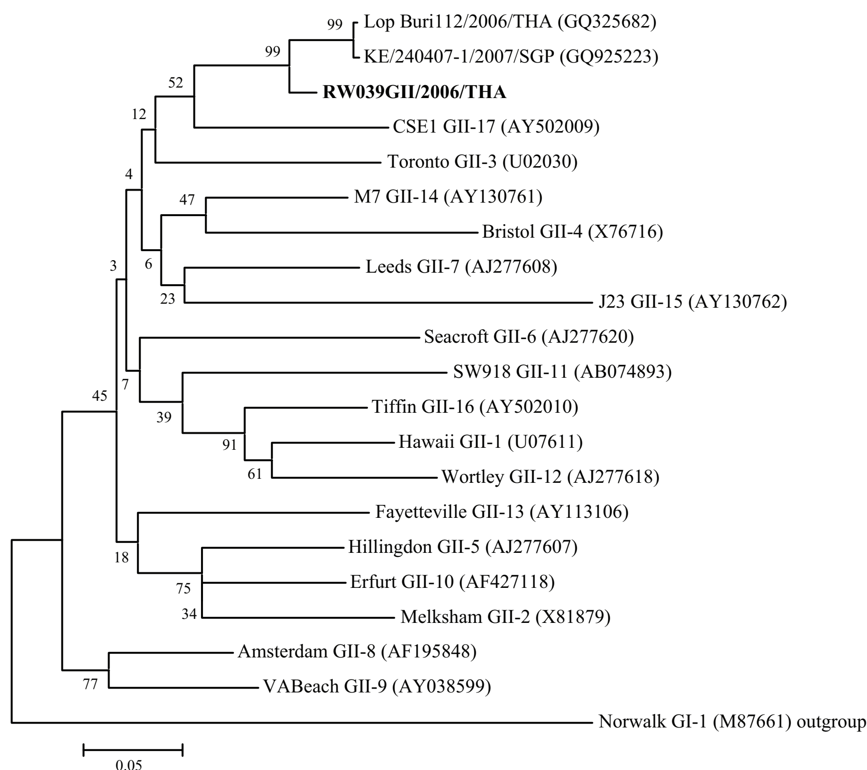


Fig 1-Phylogenetic analysis of partial capsid sequences of NoV GI (A) and NoV GII (B) from river water samples (RW004GI/2006/THA-RW039GI/2006/THA and RW039GII/2006/THA). The GenBank accession numbers for the 8 known GI and 17 known GII strains are shown in the bracket. The tree was generated based on the neighbor-joining method and the numbers on each branch indicate the bootstrap values.

cultural and domestic uses. The presence of NoVs in the river and irrigation canal water reflects the role of environmental water as a possible source of NoV transmission leading to a public health hazard.

This is the first report of NoVs circulating in environmental water in Thailand. The finding extends the reports documenting NoVs in environmental water (Hernandez-Morga *et al*, 2009; Aw and Gin, 2010; Victoria, *et al*, 2010). The predominance of NoV GI in water has been reported (Lee and Kim, 2008; Gentry *et al*, 2009; Kamel *et al*, 2010; La Rosa *et al*, 2010) and is associated with water-borne outbreaks of NoV gastroenteritis (Maunula *et al*, 2005; Lysén *et al*, 2009). It has been suggested that NoV GI might be more resistant to inactivation in water environments as compared to NoV GII (da Silva *et al*, 2007). On the other hand, others have noted that NoV GII is more prevalent than NoV GI in water samples (Lodder and de Roda Husman, 2005; Hernandez-Morga *et al*, 2009; Victoria *et al*, 2010) corresponding with the occurrence of NoV GII in patients with acute gastroenteritis. The high viral load in stool specimens would facilitate the spread of the virus, but NoV GI and GII strains might have different behaviors in a stable environment. In the current study, NoV GI and GII concentrations in water samples also were estimated using qRT-PCR. Data of NoV concentrations in environmental samples may provide information essential for public health risk assessment of NoV infections.

Contaminations of mixed NoV genotypes were found suggesting widespread circulation of both NoV genogroups in water environments A phylogenetic analysis of the sequences obtained from environmental water samples showed different genotypes of NoV circulating in the study locations. Multiple genotypes of NoVs present in water environments have been reported from various countries, *eg*, Singapore, Japan, Italy (Aw *et al*, 2009; Iwai *et al*, 2009; La Rosa *et al*, 2010). The co-circulation of NoV genotypes in water can cause outbreaks of acute gastroenteritis in humans, indicating the possibility of genetic recombination (Maunula *et al*, 2005), a common feature occurring mainly in the open reading frame (ORF)1/ORF2 overlapping region (Bull *et al*, 2007).

NoV GI and GII strains in this study exhibited the highest levels of nucleotide identity with NoV sequences from urban wastewater in Singapore and clinical specimens from Japan and Thailand. Interestingly, the presence of NoV GI-2 was predominant in environmental water, comparable to the study in Singapore over the same period, from August 2006 to January 2007 (Aw *et al*, 2009), thus indicating the widespread distribution of NoV genotypes in tropical surface waters. NoV GI-2 was also identified in sewage collected in Asian (Iwai *et al*, 2009) and European (La Rosa *et al*, 2010) countries. As well as in clinical isolates (Chhabra *et al*, 2009), NoV GI-2 was detected more frequently in foodborne outbreaks (Verhoef *et al*, 2010). Although NoV GI-6 was detected in water environments to a lesser than other genotypes, this genotype caused waterborne outbreaks (Maunula *et al*, 2005) and played a role in acute gastroenteritis of hospitalized patients (Malasao *et al*, 2008; Yoon *et al*, 2008; Chhabra *et al*, 2009). Of note, among the detected NoV GII genotypes, one genotype (GII-unclassified) could not be genotyped by the clustering method by Zheng *et al* (2006) used for full capsid sequence analysis. However, based on the phylogenetic analysis of partial capsid sequences described by Kageyama *et al* (2004), this NoV GII strain belongs

to NoV GII-16. The NoV GII-unclassified has been found previously in patients with acute gastroenteritis from Japan (Kageyama *et al*, 2004) and Thailand (Kittigul *et al*, 2010). The complete capsid gene sequence is required in order to classify this NoV into a new genotype.

NoV sequences identified in this study were aligned with other NoV sequences obtained from patients with acute gastroenteritis conducted in a previous study during the same period and from the same sites, but performed in separate experiments (Kittigul *et al*, 2010). The alignment of the nucleotide sequences (240 nucleotides) for NoV GI-2 and NoV GI-6 from water samples revealed 99% and 95% identity respectively, with NoV sequences obtained from patients with acute gastroenteritis. The alignment of the nucleotide sequences (209 nucleotides) for the NoV GII-unclassified genotype also showed 99% identity with the NoV sequence obtained from a patient with acute gastroenteritis.

Sequences resembling environmental water and clinical samples indicate epidemiological relationships of NoVs circulating in both sources. Thus, environmental water might be a possible source of NoV transmission and the cause of outbreaks of gastroenteritis.

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