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ORIGINAL ARTICLE

Genetic analysis of norovirus GII.4 variants circulating in Korea in 2008

K. S. Park · H. S. Jeong · K. A. Baek · C. G. Lee · S. M. Park · J. S. Park · Y. J. Choi · H. J. Choi · D. S. Cheon

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Abstract Noroviruses are the enteric pathogens most commonly responsible for infectious gastroenteritis and outbreaks of foodborne illness. The GII.4 norovirus, in particular, is responsible for the majority of epidemics. Here, we present data on the distribution of norovirus genotypes in Chungnam, Korea, in 2008, measure genetic variation among GII.4 strains, and compare Korean GII.4 variants with reference strains based on the 237-bp junction of ORF1 and ORF2. We detected 139 different strains, which formed two distinct genetic clusters with significant sequence diversity. One Korean cluster (2008-Korea_a) showed high similarity to the Sakai cluster that appeared in Japan and Europe in 2006. The other cluster (2008-Korea_b) was unique and unrelated to previously reported clusters. Genotype GII.4 was confirmed as the predominant cause of norovirus epidemics in Korea. Foodborne norovirus infections, on the other hand, were generally caused by emerging GII.4 genetic variants similar to those responsible for global epidemics.

K. S. Park · K. A. Baek · C. G. Lee · S. M. Park
Chungcheongnam-Do Institute of Health and Environmental Research, Daejeon, Korea

H. S. Jeong · D. S. Cheon (✉)
Division of Enteric and Hepatitis Viruses, Department of Virology, Center for Infectious Diseases, National Institute of Health, Korea Center for Disease Control and Prevention, 194, Tongil-Lo, Eunpyung-Gu, Seoul 122-701, Korea
e-mail: cheonds@hanmail.net

J. S. Park
Department of Pediatrics, Soonchunhyang University, Cheonan, Korea

Y. J. Choi
Department of Clinical Pathology College of Medicine, Soonchunhyang University, Cheonan, Korea

H. J. Choi
Department of Food and Nutrition, Daejeon Health Science College, Daejeon, Korea

Introduction

Gastroenteritis is a major public health issue worldwide, with 3.5–4.6 million gastroenteritis-associated deaths reported annually [37]. Acute gastroenteritis can be caused by a number of different agents, including bacteria, parasites, and viruses. Viral gastroenteritis is most commonly caused by rotavirus group A, enteric adenovirus, astrovirus, and norovirus [11]. Of these, norovirus is widely recognized as the major cause of non-bacterial acute gastroenteritis. Although the disease is usually mild and self-limiting, the viral agent is highly infectious [2, 36], it not only affects people who consume contaminated food or water, but it also can spread to those who come into close contact with infected individuals [37]. Norovirus outbreaks have been reported in various settings, including nursing homes, schools, cruise ships and restaurants [28].

Norovirus, which belongs to the family *Caliciviridae*, has a small round virion that is 27–35 nm in diameter and consists of a single-stranded, positive-sense, 7.4–7.7 kb polyadenylated RNA genome encoding three open reading frames [5, 8, 30]. Noroviruses have been classified into five major genetic groups: genogroup I (GI) through genogroup V (GV) [33]. GI, GII, and GIV generally infect humans, and GIII and GV infect bovine and murine species, respectively. GI, GII, and GIII have been subdivided into 14, 17, and 2 genetic clusters, respectively, whereas GIV and GV have one cluster each [1, 19].

Among the three genotypes that can infect humans, GII is the most common. Rapid spread of epidemics caused by the GII.4 strains has been reported worldwide since the 1990s [6, 17, 34, 35]. US95/96 was the first norovirus GII.4 variant linked to a general epidemic [21, 22]. This variant was identified as causing 55% of gastroenteritis outbreaks in the US from 1995 to 1996 [9], after which it spread to at least seven other countries [22]. In 2002, another GII.4, designated the Farmington Hills virus, was responsible for epidemics of gastroenteritis in Europe [7, 17], the US [35], and Australia [6]. The next wave of global epidemics occurred in 2004, when a remarkable increase of norovirus activity was associated with the development of another novel GII.4 variant, termed the Hunter virus [37]. Numerous outbreaks of disease throughout 2004 in Australia, New Zealand, Japan, Taiwan, and Europe were attributed to the Hunter virus [6, 16]. In 2006, an outbreak of the 2006a variant of norovirus GII.4 in Australia, New Zealand, and Europe followed a trend of infection similar to that observed for the Hunter virus in these countries during 2004 [16, 30, 31, 32]. Another variant, 2006b, was discovered in Europe in February 2006 and was associated with several outbreaks of gastroenteritis on cruise ships [28]. By July 2006, the 2006b variant had been identified in Australia, where it appeared to replace the 2006a variant as the etiological agent of outbreak-related gastroenteritis [31, 32].

Epidemics of gastroenteritis caused by norovirus GII.4 variants occurred in Chungnam, Korea, in 2008. Here, we have verified the genetic identities of these variants by comparing them with reference strains. Additionally, we have analyzed these samples phylogenetically.

Materials and methods

Stool specimens

We collected a total of 1,468 stool specimens from sporadic cases of acute gastroenteritis, while another 364 stool specimens were collected from 22 foodborne cases of acute gastroenteritis in Chungnam, Korea, during 2008. The fecal specimens were diluted to 10% suspensions with phosphate-buffered saline and clarified by centrifugation at 8,000×*g* for 15 min. The supernatants were collected and stored at −80°C until tested for the presence of norovirus.

RNA extraction

Viral RNA was extracted from the fecal supernatant using a viral nucleic acid prep kit (Greenmate Biotech, Seoul, Korea), according to the manufacturer's instructions. The extracted RNA was dissolved in 50 µL of nuclease-free water and stored at −80°C until used for RT-PCR.

Detection of norovirus RNA in clinical samples

We assayed for noroviruses in the stool samples using a single-step RT-PCR mixture (Promega) that consisted of the following: 2 µL of RNA template; 13 µL of 2× reaction mixture containing 0.2 mM dNTP mix, 1.5 mM $MgCl_2$, and thermostable *Taq* DNA polymerase (1.5 U/rxn); 10 µM each of forward (GI-F1M; 5′-CTGCCCGAATTYGTAAATGATGAT-3′) and reverse (GI-R1M; 5′-CCAACCCARCCATTRTACATYTG-3′) primers for GI, as well as forward (GII-F1M; 5′-GGGAGGGCGATCGCAATCT-3′) and reverse (GII-R1M; 5′-CCRCCIGCATRICCRTTRTACAT-3′) primers for GII [28]; and 1 µL of avian myeloblastosis virus reverse transcriptase (10 U/µL). The PCR proceeded as follows: 40 min of reverse transcription at 48°C; 3 min of denaturation at 94°C; 35 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 45 s; and a final extension at 72°C for 7 min.

For semi-nested PCR amplification of DNA, the reaction mixture (50 µL) consisted of the following: 2 µL of DNA template; 5 µL of 10× PCR buffer (100 mM Tris–HCl, 400 nM KCl, 15 mM $MgCl_2$, pH 9.0; Bioneer, Daejeon, Korea); 0.2 mM dNTP mix; dilution buffer for Taq DNA polymerase (20 mM Tris–HCl, 0.5 mM EDTA, 1 mM dithiothreitol, 100 mM KCl, stabilizers, 50% glycerol, pH 8.0), 1 µM each of forward primer (GI-F2; 5′-ATGATGATGGCGTCTAAGGACGC-3′) and GI-R1M reverse primer for GI, forward primer (GII-F3; 5′-TTGTGAATGAAGATGGCGTCGART-3′) and GII-R1M reverse primer for GII [28]; and 1 U of Taq DNA polymerase (Bioneer). Initial denaturation was carried out at 94°C for 3 min. This was followed by 25 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 45 s, with a final extension at 72°C for 7 min.

Nucleotide sequencing and molecular typing

Products from the semi-nested PCR were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany). The purified DNA was added to a reaction mixture containing 2 µL of BigDye Terminator reaction mix (ABI Prism BigDye Terminator cycle sequencing kit; Perkin–Elmer/Applied Biosystems, USA) and 2 pmol each of the GI-R1M and GII-R1M primers. Sequencing reactions were subjected to an initial denaturation at 96°C for 1 min, followed by 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min in a thermal cycler (Gene Amp PCR System 2700; Applied Biosystems). The products were purified by precipitation with 100% cold ethanol and 3 M

sodium acetate (pH 5.8) before being loaded onto an automated analyzer (Genetic Analyzer model 3100; Applied Biosystems).

A BLAST search of GenBank sequences was conducted to determine the molecular type of each isolate. This was defined as the genotype that was scored as having the most nucleotides in common with the query sequence [37].

Nucleotide comparison and phylogenetic analysis

Sequence analysis of the prevalent genotype GII.4 was performed for 29 isolates (14 isolates from sporadic cases and 15 from outbreak cases). Nucleotide and deduced amino acid sequences of candidate norovirus isolates were compared with the reference sequences (Table 1) using MegAlign (DNASTAR, Software, WI, USA) [29]. Phylogenetic relationships among the ORF2 sequences of the virus isolates were determined using MEGA software v. 4.0. Maximum Composite Likelihood was used as the substitution method, while the neighbor-joining method was used to reconstruct the phylogenetic tree [23]. The reliability of the phylogenetic tree was determined by bootstrap re-sampling of 1,000 replicates.

Nucleotide sequence accession numbers

The norovirus candidate sequences were deposited in the GenBank sequence database (accession numbers FJ986570 to FJ986598). Likewise, we also submitted the complete capsid gene sequence from one 2008-Korea_a and two 2008-Korea_b strains (accession numbers GU390900 to GU390902).

Results

Norovirus infections and the prevalence of norovirus genotypes

Norovirus was detected in 180 of 1,468 stool specimens (11.8%) collected from individuals with sporadic acute gastroenteritis. The monthly prevalence of norovirus-positive samples was as follows: 12.7% of gastroenteritis cases in February, 15.1% in March, 12.3% in October, 28.2% in November, and 34.6% in December (Table 2).

Foodborne outbreaks of norovirus were detected in 128 of 364 (35.2%) stool specimens collected from individuals with foodborne cases of acute gastroenteritis. All of these outbreaks were detected in November and December

Table 1 Reference strains of norovirus GII.4 used in nucleotide identity comparison and phylogenetic analysis

Cluster	Strain name	Accession number	Country	Year	Reference
CHDC	CHDC5191/1974/US	FJ537134	USA	1974	[4]
	CHDC2094/1974/US	FJ537135	USA	1974	[4]
	CHDC4871/1977/US	FJ537138	USA	1977	[4]
Camberwell	CHDC3967/1988/US	FJ537136	USA	1988	[4]
	Camberwell/101922/94/AUS	AF145896	AUS	1994	[24]
Grimsby	Burwash Landing/331/1995/US	AF414425	USA	1995	[20]
	Grimsby	AJ004864	UK	1995	[11]
	345/96002726/1996/SC	AF080549	USA	1996	[21]
Hunter	Hunter 284E/04O/AU	DQ078794	AUS	2004	[6]
	RotterdamP2D0/2005/NL	AB385628	NL	2005	[26]
	Dongen46/2006/NL	EF126961	NL	2006	[26]
	Hunter 273C/04O/AU	DQ078815	AUS	2004	[6]
	2004/NL	AY883096	NL	2004	Unpublished
	Yerseke38/2006/NL	EF126963	NL	2006	[25]
	Terneuzen70/2006/NL	EF126964	NL	2006	[25]
	RotterdamP7D119/2007/NL	AB385640	NL	2007	[26]
Farmington Hills	Oxford/B4S6/2002/UK	AY587985	UK	2002	[7]
	Germanton/2002/USA	AY502017	USA	2002	[36]
	Farmington Hills/2002/USA	AY502023	USA	2002	[36]
Sakai	DenHaag89/2006/NL	EF126965	NL	2006	[25]
	Sakai4/2006/JP	AB447450	JP	2006	[19]
	Kobe034/2006/JP	AB291542	JP	2006	[14]

Table 2 Monthly incidence of norovirus detected in sporadic cases of gastroenteritis in Chungnam, Korea, in 2008

Month	Number of samples	Number of Positive		Total number of positive	Positive rate (%)
		GI	GII		
January	121	1	9	10	8.3
February	55	1	6	7	12.7
March	73		11	11	15.1
April	88		10	10	11.4
May	101		4	4	4.0
June	155		3	3	1.9
July	170		2	2	1.2
August	134	1	5	6	4.5
September	136		11	11	8.1
October	114	1	13	14	12.3
November	142	2	38	40	28.2
December	179		62	62	34.6
Total	1,468	6	174	180	11.8

(Table 3). The mean number of patients per foodborne outbreak was 16.5, whereas the number of patients per norovirus-related foodborne outbreak was 25.6.

Prevalence of norovirus genotypes and phylogenetic analysis of GII.4 variants in Korea

Among the 180 isolates from sporadic acute gastroenteritis cases, a total of 15 genotypes were detected: Five GI genotypes and ten GII genotypes. In decreasing order of abundance, these were: GII.4 ($n = 139$, 77.2%), GII.3 ($n = 12$, 6.7%), GII.16 ($n = 10$, 5.6%), GII.2 and GII.8 ($n = 4$, 2.2%), and GI.4 ($n = 2$, 1.1%). The other nine genotypes (GI.3, GI.6, GI.7, GI.12, GII.1, GII.7, GII.11, GII.13 and GII.17) were responsible for the remaining 7.2% of cases, cumulatively.

Among isolates from foodborne outbreaks, a total of only three genotypes were detected: one GI genotype (GI.6) and two GII genotypes (GII.2 and GII.4). These data indicate that GII.4 is the most common cause of norovirus infections, regardless of whether they are sporadic or foodborne.

A phylogenetic analysis based on the partial nucleotide sequence of ORF2 revealed that the 2008 Korean GII.4 strains can be divided into two genetic clusters. The first variant (2008-Korea_a) clustered with Sakai and 2006b variants, while the second (2008-Korea_b) was found to be a new norovirus GII.4 cluster (Fig. 1).

We determined the complete capsid sequence of the Chungnam (12-107) strain from the 2008-Korea_b cluster and compared it with a representative reference strain from each of the six clusters. The sequence of the Chungnam (12-107) strain differs from the Sakai/2006/JP strain (Sakai cluster) by 8.3%.; the difference in the deduced amino acid sequence between these strains is 4.8%. Pairwise comparisons of nucleotide sequences of the complete capsid genes demonstrated that the sequence of the 2008-Korea_b strain differed from those of the other clusters by as little as 7.5% and as much as 13.6% (Table 4).

Discussion

Global outbreaks of norovirus-associated gastroenteritis have occurred since the late 1990s [5, 7, 17, 22, 30, 34, 35], and norovirus has been the etiological agent in many sporadic cases and foodborne outbreaks of gastroenteritis in Korea [14]. Foodborne outbreaks related to norovirus

Table 3 Summary of norovirus-related outbreaks in Chungnam, Korea, in 2008

Outbreak no.	Area	Day/month	Place	Age group	No. of specimens		Genotype
					Total	Norovirus positive	
out1	Boryeong	11/Nov	Condominium	Adults	38	6	GII.4
out2	Seocheon	17/Nov	Elementary school	Children	24	10	GII.2, GII.4
out3	Seosan	19/Nov	Military	Adults	36	8	GII.4
out4	Seosan	25/Nov	Elderly care facility	Old people	4	2	GII.4, GI.6
out5	Asan	1/Dec	High school	Young people	26	6	GII.4

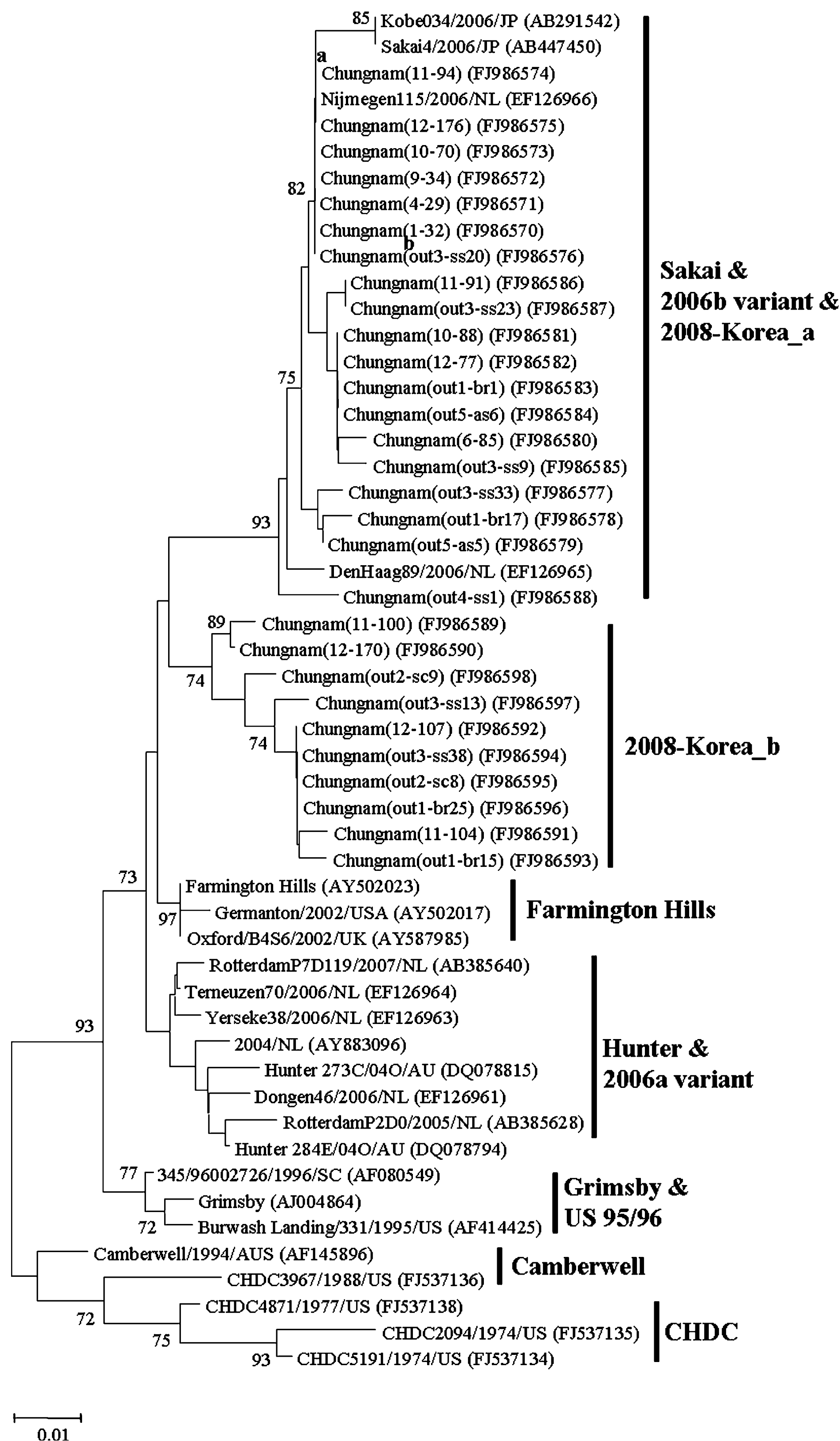

Fig. 1 Phylogenetic analysis based on a 237 bp sequence of the capsid region (ORF2) of norovirus GII.4. Nucleotide sequences were analyzed by the neighbor joining method. The *numbers at the branches* indicate bootstrap values for 1,000 replicas. [a]The "Chungnam" indicates district in Korea. In *parenthesis*, first digit means month of isolation and second digit diagnosis number of series. [b]The "out" represents foodborne outbreak by Table 2, and next character means region of outbreak as *br* Boryeong, *ss* Seosan, *sc* Seocheon, and *as* Asan

have generated more patients than outbreaks caused by any other infectious agent. Additionally, in our study, the highest rates of norovirus infection were in children younger than 5 years of age and in elderly people older than 60 years of age. Together, these statistics indicate that norovirus outbreaks are a major health concern. In order to

Table 4 Percent nucleotide and amino acid sequence divergence in the complete ORF2 region among seven clusters

Cluster	Strain	Sakai Sakai/2006/JP	2008-Korea_b Chungnam (12-107)	Faimington Hills Farmington Hills	Hunter Hunter 284E/04O/AU	Grimsby Grimsby	Camberwell Camberwell/1994/AUS	CHDC CHDC2094/1974/US
Sakai	Sakai/2006/JP	***	8.3	6.8	7.9	7.9	10.6	13.6
2008-Korea_b	Chungnam (12-107)	4.8	***	7.5	7.8	8.6	11.1	13.6
Farmington Hills	Farmington Hills	4.8	6.5	***	5.6	5.7	9.2	12.9
Hunter	Hunter 284E/04O/AU	5.9	6.1	5.2	***	7.2	9.9	12.4
Grimsby	Grimsby	6.7	7.2	5.0	6.5	***	6.9	10.6
Camberwell	Camberwell/1994/AUS	7.6	8.1	6.7	8.1	9.4	***	9.4
CHDC	CHDC2094/1974/US	9.4	9.6	8.1	9.4	5.9	3.5	***

Sequence were compared pairwise using the MegAlign (DNASTAR, Software, WI, USA), and the nucleotide and amino acid divergence values for the pairs of strains are shown as percentages. The upper triangle of the table represents nucleotide divergence, and the lower triangle of the table represents amino acid divergence

learn more about norovirus so that we could attempt to stop its spread, we determined the genotypic distribution and genetic diversity of the strains responsible for viral gastroenteritis in Chungnam, Korea, in 2008.

Norovirus-associated gastroenteritis occurs frequently during the winter [10, 24], but several recent studies in different countries have also found norovirus infections in the spring or summer [3, 13, 18]. We found that norovirus infections peaked in December, supporting a winter seasonality in Chungnam, Korea.

In the present study, we identified five GI and 10 GII genotypes. The high prevalence of GII.4 norovirus is consistent with recent clinical molecular epidemiological studies [6, 17, 35]. During the last decade, most epidemics of norovirus infection have been associated with the emergence of several novel GII.4 variants: US95/96 [22, 34], Farmington Hills virus [7, 17, 35], Hunter virus [5] and, more recently, 2006a and 2006b [13, 16].

Phylogenetic analysis identified two variants of Korean GII.4 strains: 2008a Korea variants and 2008b Korea variants. The 2008-Korea_b variant was especially interesting, as it formed a unique genetic cluster separate from the previously reported GII-4 variant.

New variants of GII.4 can emerge quickly, and there is no vaccine available thus far. Thus, it is of the utmost importance that affected individuals employ hygiene measures to control infections. Other than improved hygiene, one of our greatest tools in containing and/or preventing the spread of infection is a better understanding of the molecular epidemiology of norovirus. Our results indicate the continuing need for careful monitoring of gastroenteritis caused by GII.4 variants. In particular, it will be imperative for researchers to analyze a larger number of cases of norovirus infections in order to achieve a clearer understanding of how these infections spread and thus how they might be stopped.

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