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BRIEF REPORT

Molecular characterization of rotavirus diarrhea among children in South Korea: detection of an unusual G11 strain

Jung Ok Shim · In Hyuk Baek · Van Phan Le · Eun-Mi Ko · Won-Seok Seok · Young Uh · Ja Kyoung Kim · Sung Yeon Ahn · Hyeon Soo Lee · Eell Ryoo · So-Yeon Shim · Wonkeun Song · Inseok Lim · Wonyong Kim

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Abstract Among 312 rotavirus-positive samples collected from eight hospitals across South Korea during 2008 and 2009, the most prevalent circulating G genotype was G1 (35.9%), followed by G3 (24.7%), G2 (17.0%), G4 (7.7%), and G9 (2.6%). Notably, one unusual G11 lineage III strain—the first hypoendemic infection case in the world—was found. Of the P genotypes, P[8] (43.9%) was the most common, followed by P[6] (29.5%), P[4] (9.3%) and P[9] (0.6%). Determining G- and P-type combinations showed that G1P[8] was the most prevalent (20.5%), followed by G2P[6] (12.8%) and G3P[8] (12.8%). These findings provide new information concerning the current prevalence and spread of the rare G11 rotavirus.

Keywords Diarrhea · Genotype · G11 · Evolution

J. O. Shim and I. H. Baek contributed equally to this work.

J. O. Shim · J. K. Kim · S. Y. Ahn · H. S. Lee
Department of Pediatrics, Kangwon National University School of Medicine, Chuncheon, Korea

I. H. Baek · V. P. Le · W. Kim (✉)
Department of Microbiology, Chung-Ang University College of Medicine, Seoul, Korea
e-mail: kimwy@cau.ac.kr

E.-M. Ko · W.-S. Seok
Gangwon Institute of Health and Environment, Chuncheon, Korea

Y. Uh
Department of Laboratory Medicine, Yonsei University Wonju College of Medicine, Wonju, Korea

E. Ryoo · S.-Y. Shim
Department of Pediatrics, Gachon University of Medicine and Science, Incheon, Korea

W. Song
Department of Laboratory Medicine, KangNam Sacred Heart Hospital, Hallym University College of Medicine, Seoul, Korea

I. Lim
Department of Pediatrics, Chung-Ang University College of Medicine, Seoul, South Korea

Group A rotaviruses are the most commonly detected viral cause of severe diarrhea in infants and young children worldwide, infecting more than 125 million infants and young children every year, causing an estimated 611,000 deaths [24]. The virus, which belongs to the family *Reoviridae*, has a triple-layered capsid enclosing an 11-segment double-strand RNA (dsRNA) genome that encodes six viral and six nonstructural proteins [9]. Rotaviruses are classified into genotypes based on differences in the *VP7* (G genotype) and *VP4* (P genotype) capsid-protein-coding genes. Currently, 24 G genotypes and 34 P genotypes have been reported in humans and animals [34, 38]. However, a limited number of combined G/P genotypes are common in humans, such as G1P[8], G2P[4], G3P[8], G4P[8], and G9P[8] [32], and more recently, G12P[8] and G12P[6] have been reported [1].

The rare G11 genotype was first detected in pigs in 1988 in Mexico [28] and in 1994 in Venezuela [6]. More than a decade after the detection of these porcine strains, only seven strains have been reported in humans, found in areas of India, Bangladesh, Nepal, South Korea and Ecuador [3, 5, 15, 21, 25, 37]. In South Korea, molecular epidemiological studies have shown that G1–4 and G9 strains were prevalent in both the community and in hospitals, but significant distribution changes occur each year [33]. Recently, rare genotypes, such as G8, G11 and G12, have also emerged in South Korea [15, 19]. These distinct

changes in the prevalence of circulating rotavirus strains suggest that surveillance studies are important for efficacy testing and successful vaccine development in areas in which two kinds of rotavirus vaccines are available. The present study reports the distribution of G and P rotavirus genotypes and identifies the rare G11 strain in South Korea.

Four hundred eleven stool samples were collected from children younger than five years of age who were hospitalized for acute gastroenteritis in three university hospitals in Seoul and in five medical centers in Gangwon province between October 2008 and August 2009. All samples were diluted ten-fold with phosphate-buffered saline (PBS; pH 7.4) and tested for group A rotavirus antigen using ELISA with VP6 group-specific antibody (Dako Diagnostics, Cambridgeshire, UK).

Rotavirus dsRNA was extracted using TRI reagent (Molecular Research Center, Cincinnati, OH, USA) as described previously [19]. RT-PCR for *VP7* genotyping was performed using previously reported primers for genotypes G1–G6, G8 and G12 [2, 4, 7, 13, 14]. Three different primer sets for G genotyping (H1, C and A pools) were also used to analyze specimens that were not typed by ELISA or to confirm those that had been typed using ELISA [31]. RT-PCR was carried out with one cycle of reverse transcription at 42°C for 30 min, followed by 35 cycles of amplification (30 sec at 94°C, 30 sec at 42°C, 1 min at 72°C) and a final 7-min extension at 72°C in a GeneAmp PCR system 2700 (Applied Biosystems, Foster City, CA, USA). Each PCR product was separated in a 1.2% SeaKem LE agarose gel (FMC Bioproducts, Rockland, ME, USA), followed by ethidium bromide staining. The gels were viewed using a GelDoc XR image-analysis system (BioRad, Hercules, CA, USA). The RT-PCR for *VP4* was performed using a two-step amplification. The first step amplified gene 4 using con3 and con2 primers, and the second step used P-type-specific primers for genotypes P[4], P[6], P[8], and P[9]. The results were confirmed with alternative type-specific primers [11].

The genotypes of specimens that could not be typed using the currently available RT-PCR primers were determined using nucleotide sequencing and phylogenetic analysis. The amplified *VP7* or *VP4* gene was inserted into a pCR 2.1. cloning vector, introduced into *E. coli* TOP 10F' (Invitrogen, Carlsbad, CA, USA) by transformation, and sequenced using a BigDye terminator cycle sequencing kit and automatic DNA sequencer (Model 3730; Applied Biosystems). The resultant gene and deduced amino acid sequences were aligned using CLUSTAL_X [36] and Lasergene software (DNASTAR, Madison, WI, USA) with multiple alignment parameters against corresponding sequences of representative rotaviruses from the public database. Computer-assisted phylogenetic analysis was performed using neighbor-joining algorithms [29] from the PHYLIP suite of programs [10]. Evolutionary distances for the neighbor-joining analysis were based on the correction of Jukes and Cantor [16]. Tree topology was evaluated by bootstrap resampling of 1,000 replicates of the neighbor-joining dataset using the SEQBOOT and CONSENSE programs from the PHYLIP package.

The success of a rotavirus vaccination program depends on the development of robust regional surveillance systems that can provide comprehensive and systematic data on the disease burden [8]. Although there have been many recent reports on the molecular epidemiology of rotavirus diarrhea in children using nationwide or individual cohort studies across the globe, continuous surveillance studies are important for monitoring both new genotypes and changes in circulating genotypes.

In South Korea, G2 and G4 rotaviruses were the most frequent of the four common strains (G1–G4) between 1998 and 2004 [22, 35], while previous reports spanning 1987–1999 showed that G1 was the most predominant strain [32]. The G1 strain was again the most frequent during the 2005 through 2009 seasons [19, 20]. A similar trend, with the G1 strain being predominant (35.6%), continues in this study, clearly indicating that G1 is currently the most common circulating strain in South Korea. Of the 312 (75.9%) rotavirus-positive samples, G1 (35.9%, n=112) was the most common circulating genotype, followed by G3 (24.7%, n=77), G2 (17.0%, n=53), G4 (7.7%, n=24), and G9 (2.6%, n=8). Co-infections of G1/3 (1.6%, n=5), G1/9 (1.6%, n=5), G3/9 (0.6%, n=2) and G2/9 (0.3%, n=1) and a sample that was untyped by RT-PCR genotyping (0.3%, n = 1) were detected. Twenty-four samples (7.7%) were negative for full-length *VP7* amplification with G-type-specific primers but were positive with VP4-specific primers (Table 1).

Minor or uncommon rotavirus genotypes, such as G5, G8, G9, G11, and G12, have been documented across the world, and the results indicate that G9 strains are the most frequent causative agent of diarrhea [12, 21, 26]. Although G9 was not detected until 2002 in South Korea, its prevalence in rural health care centers has increased from 11% to 39% [17, 18] while remaining much lower (<0.8%) in urban hospitals [19, 22, 23, 33, 35]. This finding is consistent with the observations in this study, but we observed a slightly higher urban prevalence (2.6%). This difference in prevalence may be accounted for by factors such as different study population demographics, different study period, and different initiation times of the studies. The higher incidence in small rural provinces could be explained by the occurrence of an outbreak from a single clone. Alternatively, the geographic area and seasonal coincidence may play a role.

A rare G11 rotavirus strain, YM, was first detected in pigs in Mexico in 1988 [28], and later A253 was reported

Table 1 Distributions of human group A rotavirus G and P types in young children from 2008 and 2009 in eight South Korean hospitals

P genotype	G serotype											Total (%)
	G1	G2	G3	G4	G9	G11	G1/3	G1/9	G2/9	G3/9	NT[a]	
P[4]	14	9	3				1	1			1	29 (9.3)
P[6]	8	40	20	11					1		12	92 (29.5)
P[8]	64	1	40	11	6		4	1		1	9	137 (43.9)
P[9]	1		1									2 (0.6)
P[6/8]			1								2	3 (1.0)
NT	25	3	12	2	2	1		3		1		49 (15.7)
Total	112 (35.9)	53 (17.0)	77 (24.7)	24 (7.7)	8 (2.6)	1 (0.3)	5 (1.6)	5 (1.6)	1 (0.3)	2 (0.6)	24 (7.7)	312

NT: nontypeable

Table 2 *VP7* nucleotide and amino acid sequence identities between the Korean CAU-1 strain and other G11 rotaviruses

Strain	CAU-1		Country	Year reported	GenBank no.
	Nucleotide	Amino acid			
YM	91.2	95.3	Mexico	1988	M23194
A253	86.1	93.5	Venezuela	1994	L24163
Dhaka 6 10	98.4	98.1	Bangladesh	2005	AY773003
Dhaka 13-06	97.3	97.2	Bangladesh	2006	DQ482718
CRI 10795	97.2	97.2	India	2007	EF014906
CUK-1	99.6	100	South Korea	2007	EF121951
EC2184	84.9	93.0	Ecuador	2009	GQ149096
KTM368	98.6	98.6	Nepal	2010	GU199497
Maltab36-02	98.1	97.6	Bangladesh	2010	GU199508

in Venezuela in 1994 [6]. More than a decade after the detection of these two porcine strains, G11 strains were isolated from humans in many countries, including Dhaka6 [25], Dhaka13-06, and Maltab36-02 [21] strains from Bangladesh in 2005–2010; CRI 10795 from India in 2007 [3]; CUK-1 from South Korea in 2007 [15]; EC2184 from Ecuador in 2009 [5]; and KTM368 from Nepal in 2010 [37]. The sequence of one G11 strain in this study, CAU-1, which could not be typed using the current RT-PCR genotyping methods, was determined and compared to the *VP7* gene sequences of representative serotypes in the GenBank database. The *VP7* gene of CAU-1 was deposited into the NCBI GenBank database under accession number HQ198807.

Multiple alignment analysis showed that the strain shared high identity (>84.9% at the nucleotide level and >93.0% at the amino acid level) with nine reported human and porcine G11 strains, namely CUK-1, KTM368, Dhaka6, Matlab36-02, CRI 10795, YM, Dhaka13-06, A253, and EC2184 (Table 2). The identities between CAU-1 and two prototype porcine G11 strains, YM (GenBank accession number M23194) and A253 (GenBank accession number L24163) were lower (86.1–91.2% at the nucleotide level and 93.5–95.3% at the amino acid level) than those with the other human G11 strains, CUK-1 (GenBank accession number EF121951), KTM368 (GenBank accession number GU199497), Dhaka6 (GenBank accession number AY773003), Matlab36-02 (GenBank accession number GU199508), CRI10795 (GenBank accession number EF014906), and Dhaka13-06 (GenBank accession number DQ482718) (97.2–99.6% at the nucleotide level and 97.2–100% at the amino acid level).

Phylogenetic analysis based on the *VP7* gene sequence showed that CAU-1 belongs to lineage III, forming a tight genetic clade with six previous Asian human strains, Dhaka13-06, Matlab36-02, and Dhaka6, KTM368, CRI 10795 and CUK-1 (Fig. 1), while the South American human strain EC2184 (GenBank accession number GQ149096) was assigned to lineage IV. These observations imply that the Asian human G11 strains might have originated from a common ancestor, although the strains were isolated at different times, whereas Ecuadorian human G11 strain EC2184, which is most closely related to Venezuelan porcine strain A253 (91.8% nucleotide identity), might have been generated as a consequence of gene reassortment between porcine and human parental strains [5]. The

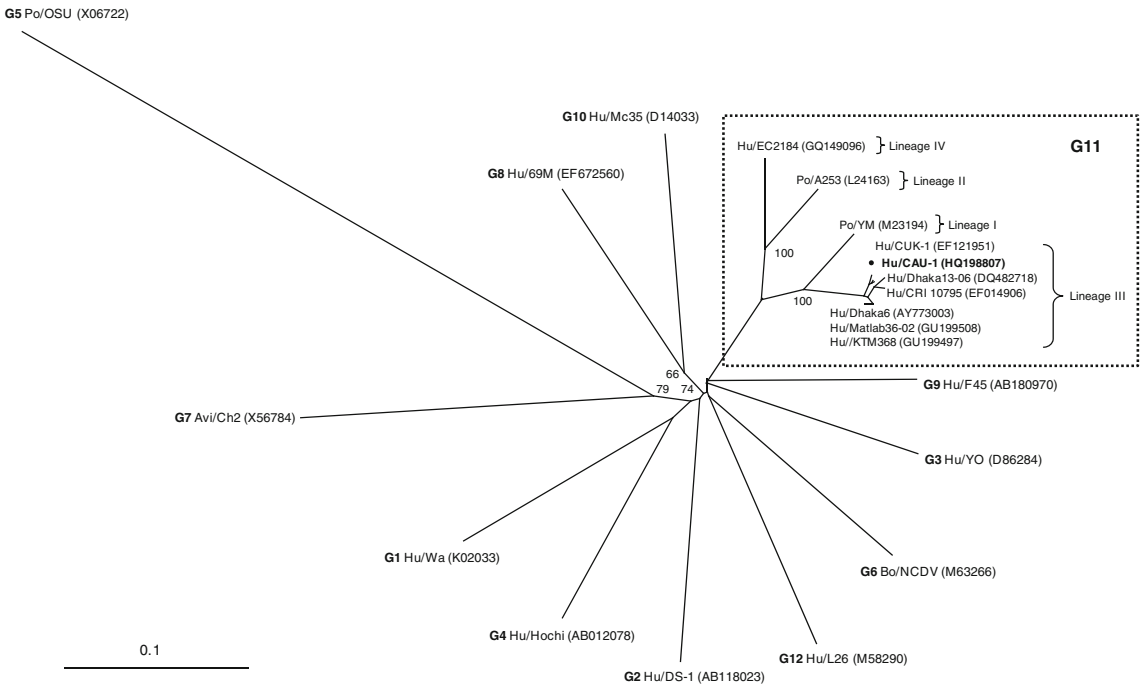

Fig. 1 An unrooted phylogenetic tree based on *VP7* gene nucleotide sequences shows relationships between G12 strains and other representative G genotypes of rotaviruses. Numbers at nodes indicate the level of bootstrap support (%) based on the neighbour-joining analysis of 1,000 re-sampled datasets; only values greater than 50% are given. The bar represents 0.1 substitutions per nucleotide position. Abbreviations: Avi, avian; Bo, bovine; Hu, human; Po, porcine

Korean CAU-1 strain, detected in Gangwon province, shared 99.6% nucleotide identity with the CUK-1 strain (GenBank accession number EF121951) reported in Seoul in 2007. These two Korean G11 strains were detected in different areas and seasons, suggesting that this rare genotype persists in this country. The *VP7* nucleotide sequence CAU-1 differed slightly from CUK-1, indicating genetic drift.

Currently, two rotavirus vaccines are available in this country: monovalent attenuated vaccine (Rotarix®; G1P[8]) and pentavalent human-bovine reassortment vaccine (Rotateq®; G1–G4P[8]). However, these vaccines may not be able to prevent all rotavirus infections, because of insufficient cross-protection against different serotypes [24]. G11 rotavirus, which is not included in present vaccine formulas, is believed to have originated from pigs. In South Korea, pigs are generally raised on industrial-scale farms, and it is not known whether pigs were the source of the infection of a human patient with the CAU-1 strain of G11 rotavirus or whether this strain was introduced from abroad. Thus, G11 human rotavirus strains should be monitored because they may become major emerging human pathogens.

To date, G11 rotavirus strains have been found combined with P[4], P[6], P[7], P[8], and P[25] genotypes [3, 5, 15, 21, 25, 37]. Notably, the new Korean G11 strain could not be typed using the current P typing method [11] despite repeated attempts. This strain may have undergone mutations in the untranslated region of the *VP4* gene, where the con2 and con3 primers bind.

Study data from this 2-year investigation indicate that P[8] (43.9%, n=137) was the most common circulating genotype, followed by P[6] (29.5%, n=92), P[4] (9.3%, n=29) and P[9] (0.6%, n=2). Co-infections of P[6/8] (1.0%, n=3) were detected using RT-PCR (Table 1). Most samples were typed using RT-PCR with con2 and con3 primers or with typing primers (1T-1, 2T-1, 3T-1, 4T-1, and con3) for the four common P genotypes, P[4], P[6], P[8], and P[9] [11]. Twenty-two samples were confirmed using the *VP4* gene sequence, and 59 samples (15.7%) were negative for full-length *VP4* gene amplification but positive with G-genotype-specific primers.

In a global rotavirus surveillance study, the G/P combinations G1P[8], G2P[4], G3P[8], G4P[8], G9P[8], and G9P[6] are the most prevalent in humans [30]. A similar trend has been reported in the recent Korean studies, which revealed a temporal change in the most common G/P type combinations of rotaviruses that is dependent on season and geography [19, 20, 22, 23, 35]. Determining G- and P-type combinations showed that the G1P[8] strain was the most prevalent (20.5%, n=64), followed by G2P[6] (12.8%, n=40), G3P[8] (12.8%, n=40), G3P[6] (6.4%, n=20), G1P[4] (4.5%, n=14), G4P[6] (3.5%, n=11) and G4P[8] (3.5%, n=11). Unusual combinations such as G2P[4]

(2.9%, n=9), G1P[6] (2.6%, n=8), G9P[8] (1.9%, n=6), G3P[4] (1.0%, n=3), G1P[9] (0.3%, n=1), G2P[8] (0.3%, n=1) and G3P[9] (0.3%, n=1) were also detected.

This study demonstrates the genotypic diversity of rotaviruses, and has revealed a hypoendemic G11 rotavirus case in South Korea. Further studies attempting to isolate and analyze the complete genomes of these rare rotaviruses would be useful for the study of genetic reassortment and the evolutionary origin of rotaviruses.

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