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2009년부터 2013년까지 서울의 일개 대학병원에서 동정된 로타바이러스 유전형의 분포

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Distribution of Human Rotavirus Genotypes in a Tertiary Hospital, Seoul, Korea During 2009-2013

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Purpose: Group A rotavirus (RV) is most common etiologic agent of acute gastroenteritis (AGE) in children worldwide. Recently, vaccination has been introduced in several countries to reduce the disease burden caused by RV infections, but continuous surveillance of RV strains is necessary to detect the emergence of potential variants induced by vaccine-immune pressure. This study aimed to investigate the changing pattern of RV genotypes in children with AGE, following the introduction of vaccination in Korea.

Methods: Genotyping of RVs by RT-PCR on the basis of VP7 and VP4 gene segment sequence was carried out on 201 rotavirus-positive stool samples, from children hospitalized with AGE between August 2009 and June 2013. We have directly sequenced PCR products and analyzed the phylogenetic tree.

Results: The most prevalent G genotype was G9 (33.3%), followed by G1 (22.4%), G3 (15.9%), G2 (6.0%), G4 (3.0%), G10 (1.5%), and mixed G-type (15.4%), with some nontypeable cases (2.5%). The detected P genotypes were P[4] (45.3%), P[8] (43.8%), mixed P-type (10.4 %), and P[2] (0.5%). The G9P[4] genotype was predominantly observed in hospitalized cases in Seoul in 2010/2011, however G1P[8] has been re-emerged as the predominant genotype in the following season (P=0.004).

Conclusions: It seems that the periodic fluctuation in predominance of the G1, G3, and G9 strains occurred in Korea during 2009-2013, following the introduction of RV vaccination.

Key Words: Rotavirus, Genotype, Gastroenteritis, Vaccine

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Introduction

Acute gastroenteritis (AGE) is the one of important causes of hospitalization in children in both developing as well as developed countries and Group A rotaviruses (RVA) are the most common cause of AGE among children. Recently, RV vaccines have been introduced in several countries including Korea to reduce the disease burden caused by RV infections. However, continuous surveillance of RV strains is necessary to detect the possible emergence of potential variants induced by vaccine-immune pressure because RV shows genetic diversity due to frequent point mutation and re-assortment. RVA belongs to the *Reoviridae* family, which is characterized by a segmented double-stranded RNA genome; RVA contains 11 gene segments encoding structural proteins (VP1-4, VP6, and VP7) and nonstructural proteins (NSP1-5/6)[1]. RVs are classified on the basis of their VP7 and VP4 gene sequences, and 27 G and 37 P genotypes have been reported[2]; the major genotypes reported worldwide are the G1P[8], G2P[4], G3P[8], G4P[8], G9P[8] combination genotypes, and G12[3]. In Korea, the G1 genotype was predominant in the 2000's, followed by G9, G3 and G4 genotypes[4]. Recently, re-emergence of G9 and subsequent increase of G1 and G3 have been reported[5]. A classification system that incorporates all 11 gene segments has been proposed[1], which would be useful for delineating the reassortment of genomes due to zoonotic transmission or immune selection pressure following the introduction of vaccination[6]. Although frequent detection of some genotypes such as G2 or G3 was has been reported after introduction of vaccines in some countries[7,8], it is uncertain whether these findings were due to natural seasonal variation or immune pressure induced by mass campaign. RV vaccines have been introduced in Korea in June 2007 (RotaTeq® vaccine) and March 2008 (Rotarix® vaccine). The overall vaccination rate was 30% in 2009, and 34.1 % (Rotarix® vaccine) and 26.1% (RotaTeq® vaccine) in 2012[9,10]. However, the data for the change of RV genotypes after introduction of vaccines is still lacking in Korea. The purpose of this study was to investigate the epidemiology and genotypic change of RV following the introduction of vaccination in Korea during 2009-2013.

Materials and Methods

Stool samples were obtained from children with AGE who were treated at Sanggyepaik Hospital from August 2009 to June 2013. The initial identification of RV was carried out using an enzyme immunoassay (EIA) kit, Rotaclone® (Meridian Bioscience, Cincinnati, OH, USA) and RV-positive stool samples were included in this study. The study was approved by the Institutional Review Board of Sanggyepaik Hospital, Inje University, Seoul, Korea. Samples were obtained after receiving informed written consent from a patient's parents. The stool samples were diluted 1:10 (weight/volume) with phosphate-buffered saline, homogenized using a vortex mixer, and subjected to centrifugation at 5,000× g for 30 min at 4℃. Supernatants were recovered and stored at -70℃ until use.

Total RNA was extracted from stool samples using the QIAamp Viral RNA kit (Qiagen GmbH, Hilden, Germany), and RV sequences were then amplified via reverse transcription-polymerase chain reaction (RT-PCR), using nested primers. RT-PCR was carried out on the 201 RV-positive samples in order to genotype the RV on the basis of VP7 and VP4 gene segment sequence. G genotyping was performed using a pool of different primers specific for G1-G4, G8, G9, G10, and G12[11,12]. The VP4 gene was characterized by using a pool of different P genotype-specific primers for P[4], P[6], and P[8]-P[10][13]. An 881-bp fragment of the VP7 gene amplified in the first round of PCR, using VP7 consensus primers, was sequenced[14]. For PCR amplification of the VP4 gene, an 876-bp fragment was generated using the Con3 forward primer and Con2 reverse primer [13]. For G9-positive samples (G9P[4]: 10 cases, and G9P[8]: 3 cases), which were chosen by implying the status and amounts of remnant samples, characterization of other gene segments that encode VP1-3, VP6, and NSP1-5 was carried out via RT-PCR, using specific primers for each gene segment[1]. Amplified products of

PCR reactions, which were randomly selected, were purified using the QIAquick® PCR Purification kit (Qiagen), and were then directly sequenced using the BigDye® Terminator Cycle Sequencing kit (version 3.1; Life Technologies, Carlsbad, CA, USA). Sequencing reaction products were resolved using an Applied Biosystems® ABI 3730 XL DNA analyzer (Life Technologies). The nucleotide sequences of the VP7 and VP4 genes were aligned using the Clustal W, and phylogenetic trees were constructed using the MEGA software, version 4.0[14]. We compiled the nucleotide sequences that were determined in this study with those available in the GenBank database by using the BLAST program (http://blast.ncbi.nlm.nih.gov/). The VP7 and VP4 sequences identified in this study have been submitted to the GenBank database under the accession numbers; VP7 from KF812568 to KF812615, and VP4 from KF812 616 to KF812663, respectively.

Categorical variables among study periods were compared using Chi-square test for trend. A P-value <0.05 was considered as statistically significant. All statistical analysis was performed by MedCalc version 15.6.1 (MedCalc software, Mariakerke, Belgium).

Results

1. Genotyping by RT-PCR

Stool samples were obtained from a total of 1,147 children with AGE who were treated at Sanggyepaik Hospital from August 2009 to June 2013. A total of 201 RV-positive stool samples were included in this study after screening of RV using an EIA kit. The G and P genotypes were determined by RT-PCR for 201 RV-positive samples that were obtained from children hospitalized with AGE between August 2009 and June 2013. During the period covered by this study, the most common G genotype was G9 (33.3%, 67 cases), followed by G1 (22.4%, 45 cases), G3 (15.9%, 32 cases), G2 (6.0%, 12 cases), G4 (3.0%, 6 cases), G10 (1.5%, 3 cases), and mixed G-type (15.4 %, 31 cases), with some nontypeable cases (2.5%, 5 cases) (Fig. 1A). The G3 genotypes were most frequently detected in cases hospitalized between August 2009 and July 2010, but the G9 genotype had rapidly emerged as the predominant genotype between August 2010 and July 2012; after August 2010, there was an increase in cases of the G1 genotype, which re-emerged as a major genotype between August 2012 and June 2013 (P=0.004) (Table 1). These data show periodic fluctuation of the G1 and G9 genotypes between September 2007 and June 2013 (Fig. 1A). During the period covered by the present study, the detected P genotypes were P[4] (45.3%, 91 cases), P[8] (43.8%, 88 cases), mixed P-type (10.4%, 21 cases), and P[2] (0.5%, 1 case). The most frequent combinations of G/P genotypes were G9P[4] (26.4%, 53 cases) and G1P[8] (19.4%, 39 cases), followed by G3P[8] (13.9%, 28 cases), and G9P[8] (7.0%, 14 cases) (Fig. 1B). Detec-

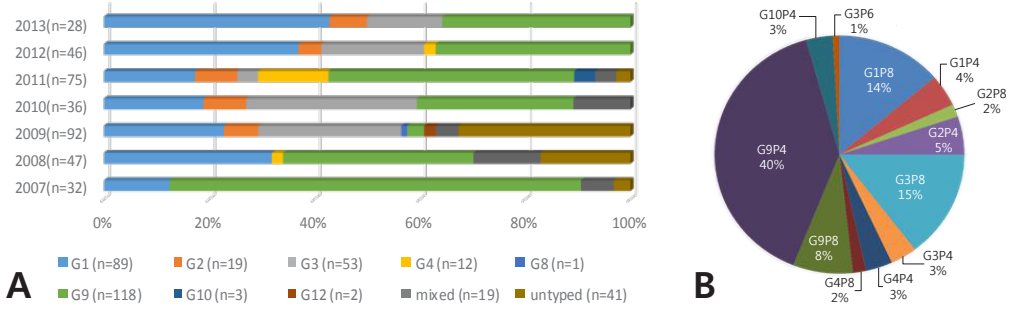


Fig. 1. (A) Distribution of VP7 genotypes of rotavirus from children hospitalized at Sanggyepaik Hospital between August 2007 and June 2013. (B) G/P combinations of rotavirus at Sanggyepaik Hospital between August 2009-June 2013. *The results of our previous study [5] between September 2007 and July 2009 was included to show the evolution of G type according to year.

tion of a high rate of unusual combination genotypes such as G9P[4] is notable, as these were not observed in our previous study that covered the period September 2007-July 2009.

2. Phylogenetic analysis

On the basis of VP7 gene sequence, the 14 G1 strains clustered in lineage I, and shared close sequence similarity with the previously reported RV strain from Korea (KOR/Seoul-669/2009 HM130947), as well as with strains from Japan (OH3592/2012 AB796447) and China (CHN/2678 EU708572) (Fig. 2). The six G2 isolates clustered in lineage IV and V, which showed that they were genetically close to other G2 isolates from Korea (Kor/Seoul-397/2009 HM130951 and Kor/Seoul-558/2009 HM130953) (Fig. 2). The 12 G3 isolates clustered in lineage III, and shared close sequence similarity with G3 isolates from Finland (FIN/HAL1166 L20882) and Korea (KOR/Seoul-502/2009 HM130957) (Fig. 2). The four G4 strains clustered in sublineage Ic and showed close sequence similarity to a G4 strain from the USA (GBR/ST3/1975/G4P6 EF672616) (Fig. 2). The nine G9 strains clustered in lineage III, and had high homology with strains from China (CHN/L463/2006 EU708596) and Korea (KOR/Seoul-378/2008 HM130979) (Fig. 3). The three G10 isolates clustered in lineage IX, and shared close sequence similarity with the G10 strains from Australia (for example, AUS/V585/2011/G10P14 JX567749) (Fig. 3). The genetic relationships of RV isolates from this study were also determined on the basis of VP4 gene sequence. Of the P[8] strains, 27 clustered in sublineage IIIA, and showed sequence similarity with previously reported P[8] strains from Korea (KOR/Seoul-291/2008/G1P HM130991 and KOR/KMR004/2000/G1P8 EF077330) (Fig. 3). Of the P[4] isolates, 21 clustered in lineage V, and showed high sequence similarity with strains isolated from Korea (KOR/Seoul-710/2009/G2P4 HM131053 and KOR/Seoul-620/2009/G3P4 HM131054) (Fig. 3). In thirteen G9 isolates, all gene segments belonged to the Wa-like genotype 1 constellation, but one isolate (SE-11-04-169) showed I2 and R2 genotypes for the VP6 and VP1 genes, respectively (Fig. 5).

Comparison of VP7 antigenic regions of Korean G1 isolates, identified in this study, with both vaccine strains (Rotarix® G1P[8] and RotaTeq® G1P[5]) revealed conservation of the six amino acid residues in the 7-1b antigenic site, but showed different amino acid residues at positions 91, 100, and 123 in the 7-1a region, and at position 217 in the 7-2 region (Fig. 4A). Among Korean G9 isolates identified in this study, there were 12 amino acid changes when compared to the Rotarix® G1 strain and the RotaTeq® G1 strain across the three antigenic sites. Korean G4 isolates showed different amino acid residues in five positions when compared to the RotaTeq® G4 strain across the three antigenic sites. Within the VP4 antigenic epitopes of the Korean P[8]

Table 1. G- and P-type Combinations Detected in Rotavirus isolates collected between Aug 2009 and June 2013 at Sanggyepaik Hospital, Seoul, Korea

Genotype	Number of samples				Total
	Aug 2009-Jul 2010 (N=39)	Aug 2010-Jul 2011 (N=86)	Aug 2011-Jul 2012 (N=44)	Aug 2012-Jun 2013 (N=32)	
G1P[8]	6	5	13	15	39
G1P[4]		5	1		6
G2P[8]		2			2
G2P[4]	1	5	2	2	10
G3P[8]	14	2	8	4	28
G3P[4]	3	1			4
G4P[8]		2			2
G4P[4]		4			4
G9P[8]	3	5	1	5	14
G9P[4]	7	26	14	6	53
G10P[4]		3			3
Unknown G P[8]		1			1
Unknown G P[4]	3				3
Unknown G P[2]		1			1
G1+G3P[8]	1	1			2
G1+G2P[4]		1			1
G2+G3P[4]		7			7
G1P[4]+P[8]		5			5
G2P[4]+P[8]		1			1
G3P[4]+P[8]		4	1		5
G4P[4]+P[8]			1		1
G9P[4]+P[8]	1	5	3		9

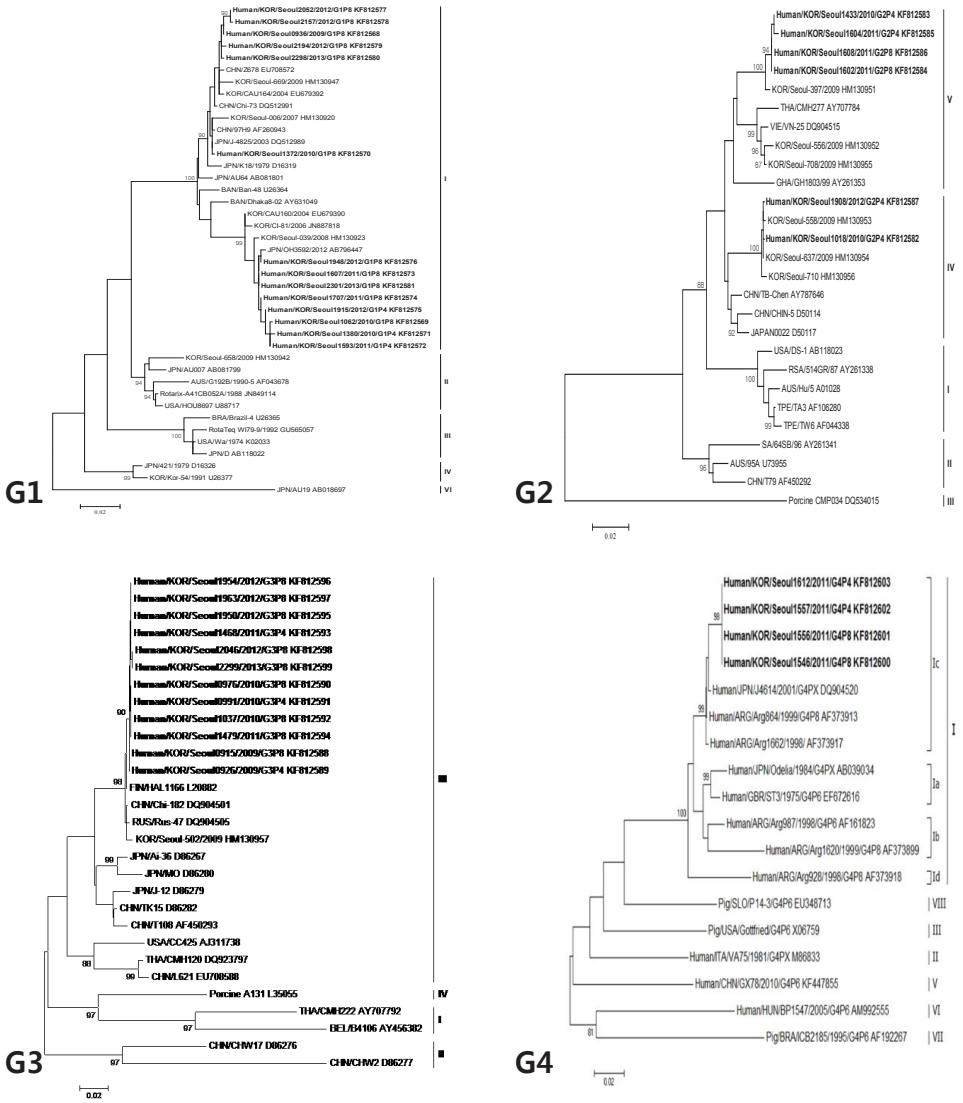


Fig. 2. Phylogenetic analysis of VP7 nucleotide sequences of genotype G1, G2, G3, and G4 strains. The tree was constructed using the Kimura 2-parameter and neighbor-joining methods in the MEGA software, version 4.0. Bootstrap values are shown at the branch nodes. Strains from other lineages and sublineages isolated worldwide are used for comparison. The lineages and sublineages are indicated at the right-hand side. The strains from this study are indicated in bold. The relevant GenBank accession numbers are indicated after the strain designation. The abbreviations are as follows: AUS Australia, BAN Bangladesh, BRA Brazil, CHN China, GHA Ghana, JPN Japan, KOR Korea, SA South Africa, THA Thailand, TPE Taipei, USA United States of America, VIE Vietnams, BEL Belgium, CHN China, FIN Finland, GBR Great Britain, HUN Hungary, ITA Italy, and RUS Russia.

isolates, amino acid differences were noted at four or five positions in comparison with the RotaTeq® strain, and at ten positions in comparison with the Rotarix® strain. Korean P[4] isolates showed divergent amino acid sequences when compared to the P[8] epitopes of the vaccine strains (17 amino acid differences with the Rotarix® strain, and 16 with the RotaTeq® strain) (Fig. 4B).

Discussion

Between August 2009 and June 2013, the predominant G genotype of human RV in Seoul, Korea successively

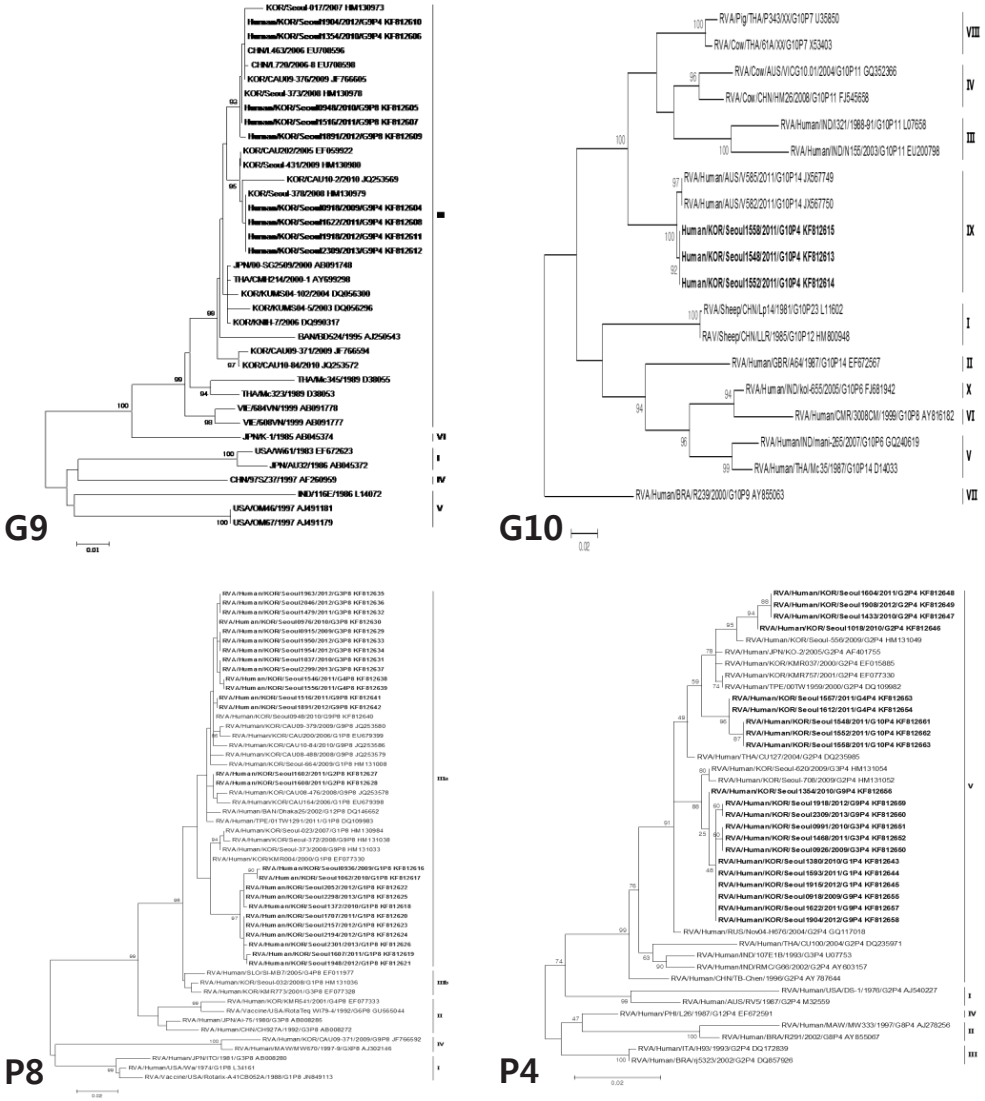
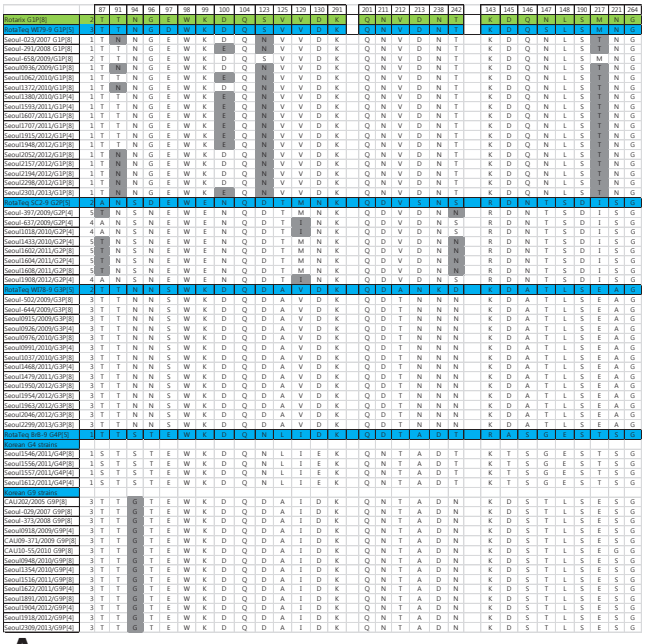


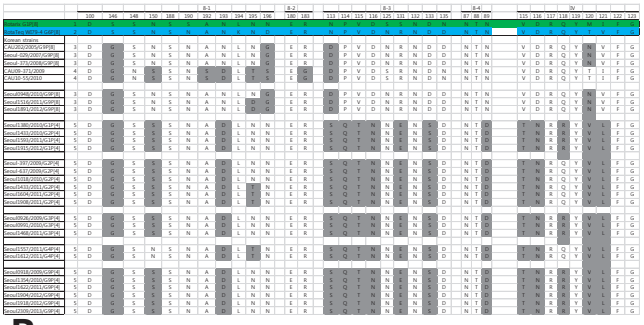
Fig. 3. Phylogenetic analysis of genotype G9, G10, P[8], and P[4] strains. The tree was constructed using the Kimura 2-parameter and neighbor-joining methods in the MEGA software, version 4.0. Bootstrap values are shown at the branch nodes. Strains from other lineages and sublineages isolated worldwide are used for comparison. The lineages and sublineages are indicated at the right-hand side. The strains from this study are indicated in bold. The relevant GenBank accession numbers are indicated after the strain designation. The abbreviations are as follows: AUS Australia, BAN Bangladesh, BRA Brazil, CHN China, CMR Cameroon, GBR Great Britain, IND India, JPN Japan, KOR Korea, THA Thailand, USA United States of America, VIE Vietnam, ITA Italy, MAW Malawi, PHI Philippines, RUS Russia, SLO Slovenia, and TPE Taipei.

changed. When compared to the results of our previous study[6], it appears that the predominant G genotypes (G1, G3, and G9) naturally fluctuated, despite RV vaccination being introduced to Korea in 2007. Following the introduction of vaccination, a statistically significant decrease of the disease burden due to RV was reported in Korea[16].

Interestingly, G1P[8] strains increased towards the end of this study. G1 lineage I was predominant throughout the study period and differences of amino acid residues at three positions (7-1a region) and one position (7-2 region) was revealed by comparison of VP7 antigenic regions of Korean G1 strains with both vaccine strains. Sublineage P[8]IIIA was most prevalent during the



A



B

Fig. 4. Alignment and comparison of amino acid sequences of antigenic regions of the VP7 (A) and VP4 (B) proteins from the Korean rotavirus strains and the two vaccine strains, Rotarix® (green) and RotaTeq® (blue). The changes of amino acid residues compared to those of both vaccine strains (gray).

	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
SE-09-12-105	G9	P[4]	I1	R1	C1	M1	A1	N1	T1	E1	H1
SE-10-01-118	G9	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
SE-10-11-079	G9	P[4]	I1	R1	C1	M1	A1	N1	T1	E1	H1
SE-11-03-065	G9	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
SE-11-04-169	G9	P[4]	I2	R2	C1	M1	A1	N1	T1	E1	H1
SE-11-04-170	G9	P[4]	I1	R1	C1	M1	A1	N1	T1	E1	H1
SE-12-01-088	G9	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
SE-12-02-021	G9	P[4]	I1	R1	C1	M1	A1	N1	T1	E1	H1
SE-12-02-039	G9	P[4]	I1	R1	C1	M1	A1	N1	T1	E1	H1
SE-12-02-055	G9	P[4]	I1	R1	C1	M1	A1	N1	T1	E1	H1
SE-12-02-061	G9	P[4]	I1	R1	C1	M1	A1	N1	T1	E1	H1
SE-12-05-063	G9	P[4]	I1	R1	C1	M1	A1	N1	T1	E1	H1
SE-13-03-045	G9	P[4]	I1	R1	C1	M1	A1	N1	T1	E1	H1

Fig. 5. Full genome characterization of thirteen G9 strains isolated in this study.

study period, consistent with previous findings in Brazil in which the definition of 1.5% cutoff value was proposed[17]. Continuous surveillance of the G1P[8] lineages are required to investigate any long term effect of vaccination on G1P[8] strain evolution.

In Brazil, a RV outbreak of a strain with the G2P[4] combination genotype was observed and initially attributed to immune pressure following the introduction of the Rotarix® vaccine[7], but it was later considered as a consequence of natural seasonal variation, in a study that showed frequent detection of the G2P[4] genotype in neighboring countries without a RV vaccination campaign[18,19]. In Korea, the G2P[4] genotype was detected in 4.0-18.3% of cases between 2000 and 2009[20-23]. In the present study, the G2P[4] genotype was detected in 4.9% of cases, which is similar to the incidence (4.0-4.3%) in other Korean studies that were carried out after the introduction of RV vaccination in 2007[5,24]. Frequent detection of the G3 genotype after the introduction of the RotaTeq® vaccine has been reported in Australia and the USA[8,25]. In the present study, the G3 genotype was mostly found in patients hospitalized in 2009/2010, but the detection rate of this genotype has fluctuated each year since the pre-introduction of RV vaccine in Korea[5,21]. These results suggest that the prevalence of the G3 genotype might be unaffected by immune pressure following the introduction of RV vaccine.

In this study, the G4 genotype clustered in sublineage Ic, which was of interest because worldwide outbreaks due to this sublineage have been reported in several countries[26,27]. The G12 genotype, a novel genotype recently detected in several countries, including Korea[2,5,28] was not found in the present study, however, another unusual genotype, G10, was found in three cases that occurred in 2011. This finding showed that re-assortment between human and bovine RV strains have occurred in Korea as in Africa[29]. The surveillance of

animal RV strains is important because unusual strains could affect the diversity of human RV population. Collectively, our results for cases from the years 2009-2013 suggest that the emergence of unusual G genotypes due to vaccine pressure has not yet occurred in Korea, but it is necessary to remind that this study could not be the representatives of RV strain in the whole Korea during the same period.

In this study, circulating rotavirus isolates were highly diverse and mixed infections was most frequently found (26.7%) between August 2010 and July 2011. These results suggest the possible association between high rate of mixed infections of RV and the extent of strain diversity of circulating RV, which was consistent with those of a recent study[30]. In this study, 2.5% (5 cases) was non-typeable, which might be attributed to low concentration of virus particles or PCR inhibitors. The dominance of nontypeable isolates in adults with acute gastroenteritis was observed in a recent Indian study[31].

Interestingly, the G9P[4] combination genotype was frequently detected in this study. Outbreaks of the unusual G9P[4] combination genotype have been reported in Mexico and Bangladesh[32,33], and were attributed to a possible reassortment in response to immune pressure. In Korea, the G9P[4] genotype was first identified in Gyeonggi province during 2003-2005 (detection rate: 1.7%)[20], but this genotype was rarely found (range of detection rate: 0.04-0.7%) in later studies[6,21,24] prior to this study; these findings are similar to those of a recent study in Latin America[18]. In the present study, phylogenetic analysis of the G9 and P[4] genotypes showed that they clustered in lineages III and V, respectively. Further characterization of remnant gene segments in 10 G9P[4] isolates and three G9P[8] isolates revealed that all of them, except one isolate, belonged to the Wa-like genotype 1 constellation. These findings differ from those of a previous study in Mexico, which showed the predominance of the E6 genotype in the NSP4 gene[33]; the human-porcine reassortment of the NSP2 and NSP3 genes was not observed here, either. In the present study, we compared the amino acid residues in antigenic epitopes of VP7 and VP4 between the circulating isolates and the vaccine strains in Korea, which revealed less antigenic variation than in a comparable recent study in Bangladesh[34]. In a recent Finnish study[35], there were no amino acid changes affecting the G1 genotype of VP7 and VP8*, despite RotaTeq® being exclusively used for vaccination after 2009, which suggests that there is no evidence of selection pressure. This study has some limitations: 1) lack of RV vaccination history and 2) using a primer amplifying about 40% of the VP4 segment. It had been suggested recently at least 50% of sequence were required from a segment for proper classification[1].

In conclusion, periodic fluctuation of RV genotypes such as G1, G3, and G9 has been observed in Korea during the period 2009-2013, following the introduction of vaccination. Predominance of the unusual G9P[4] combination genotype in 2011 was initially considered to be evidence of immune pressure due to vaccination, but the subsequent re-emergence of G1P[8] in 2013 suggests the possibility of natural fluctuation of genotypes. In addition, we could not detect any significant differences in sublineages of either G9 or P[4], or in the entire constellation of RV gene segments. However, it is still necessary to continuously monitor and characterize RV strains, in order to detect novel strains that emerge due to the immune pressure associated with mass vaccination.

Acknowledgements

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PEDIATRIC INFECTION & VACCINE PIV

요약

목적: 한국에 백신이 도입된 이후 장염으로 입원한 소아에서 확인된 로타바이러스 유전형의 분포를 알아보고자 하였다.
방법: 2009년 8월부터 2013년 6월 사이에 급성 위장관염으로 입원한 소아에게서 수집된 201개의 로타바이러스 양성 대변 검체를 대상으로 로타바이러스 유전형 분석이 시행되었다.
결과: G 유전형은 G9 (33.3%), G1 (22.4%), G3 (15.9%), G2 (6.0%), G4 (3.0%), G10 (1.5%)이었고 혼합형(15.4%) 및 분류불능(2.5%)이 관찰되었다. P 유전형은 P[4] (45.3%)과 P[8] (43.8%)가 주로 검출되었고 혼합형(10.4%)과 P[2] (0.5%)가 발견되었다. G9P[4] 유전형이 2010/2011년에 자주 검출되었으나 이후에 G1P[8] 유전형이 주로 검출되었다.
결론: 로타바이러스 백신 도입 이후 2009–2013년 동안 G1, G3 및 G9이 번갈아가며 주로 검출되는 것을 확인하였다.