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ORIGINAL ARTICLE

Phylogenetic Analysis of the Rotavirus Genotypes Originated from Children < 5 Years of Age in 16 Cities in South Korea, between 2000 and 2004

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Abstract
Objectives: The purpose of this study was to examine the diversity of the *G* and *P* types of human rotavirus strains isolated in South Korea during 2000 to 2004.
Methods: We selected 38 Group A rotavirus isolates among 652 fecal samples, which were collected from infants and children < 5 years of age with acute gastroenteritis or diarrhea admitted in 8 hospitals representative of five provinces of South Korea between 2000 and 2004. Rotavirus *P*- and *G*-genotypes were determined by nucleotide sequencing and phylogenetic analysis was performed.
Results: One *G1P[4]* consisted *G1-Id-P[4]-V*; one *G1P[6]* consisted *G1-Id-P[6]-Ia*; nine *G1P[8]* consisted *G1-Ib-P[8]-Ia* ($n = 3$), *G1-Ic-P[8]-Ia* ($n = 1$), and *G1-Id-P[8]-Ia* ($n = 5$); 13 *G2P[4]* consisted *G2-V-P[4]-V*; two *G3P[4]* consisted *G3-IIId-P[4]-V*; five *G3P[8]* consisted *G3-IIId-P[8]-Ia*; four *G4P[6]* consisted *G4-Ie-P[6]-Ia*; two *G4P[8]* consisted *G4-Ie-P[8]-II*; one *G9P[6]* consisted G9-*III-P[6]-Ia*.
Conclusions: A considerable amount of rotavirus genotypic diversity was detected in South Korea from 2000 to 2004. These findings are important to develop the effective vaccines and to undertake epidemiologic studies.

1. Introduction

Group A rotavirus, the most common etiologic agent of severe diarrhea in children, causes about 600,000 deaths per year [1]. Rotavirus, which is a genus belonging to the *Reoviridae* family, has a genome of 11 segments of double-stranded RNA surrounded by a triple-layered capsid consisting of a core, inner capsid, and outer capsid. The outer capsid is composed of two structural proteins, VP4 and VP7, which define virus *G*(VP4) or *P*(VP7) serotype specificity [2]. Although at least 15 *G* genotypes and 26 *P* genotypes are known [3–8], the most

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prevalent *P-G* combinations in humans are *G1P[8], G2P [4], G3P[8], G4P[8]* and *G9P[8]*. In Korea, rotavirus is still the most common viral agent of acute diarrhea in young children. Although *G1P[8]* was the most prevalent strain until 1997 regardless of geographic area or season [6,9], the predominant *G* type strain has shifted to other genotypes including *G4, G2* or *G9* [10−13]. In the present study, we examined the diversity of the *G* and *P* types of human rotavirus strains isolated in South Korea during 2000 − 2004 periods. As a result, we confirmed that total nine *P-G* genotypic isolates were identified.

2. Methods

2.1. Sample collection

A total of 38 rotavirus isolates were selected among 652 fecal samples, which were collected from infants and children < 5 years of age with acute gastroenteritis or diarrhea admitted in eight hospitals representative for five provinces of South Korea between 2000 and 2004. Human rotaviruses were detected in 354 of 652 (54.3%) fecal samples by enzyme-linked immunosorbent assay (ELISA). *G* and *P* genotypes were detected by multiplex polymerase chain reaction (PCR) in 316 (89.3%) and 327 (92.4%) of these sample, respectively. The location of these areas was plotted on the map of South Korea is shown Figure 1.

2.2. Nucleotide sequencing

Human rotavirus (HRV) double-stranded RNA (dsRNA) was extracted using a QIAamp Viral RNA kit (Qiagen GmbH, Hilden, Germany) in accordance with the manufacturer's instructions. The dsRNA samples were subjected to seminested multiplex Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) using conserved and type specific primers (VP7-*G1*, *G2*, *G3*, *G4*, and *G9*, and VP4-*P[4]*, *P[6]*, and *P[8]*)[14−18]. The PCR amplicons were purified using a commercial spin column method (Qiagen GmbH, Hilden, Germany) and sequenced automatically using the ABI PRISM 3100 automated DNA sequencer (Applied Biosystems, Inc, Foster city, California, USA).

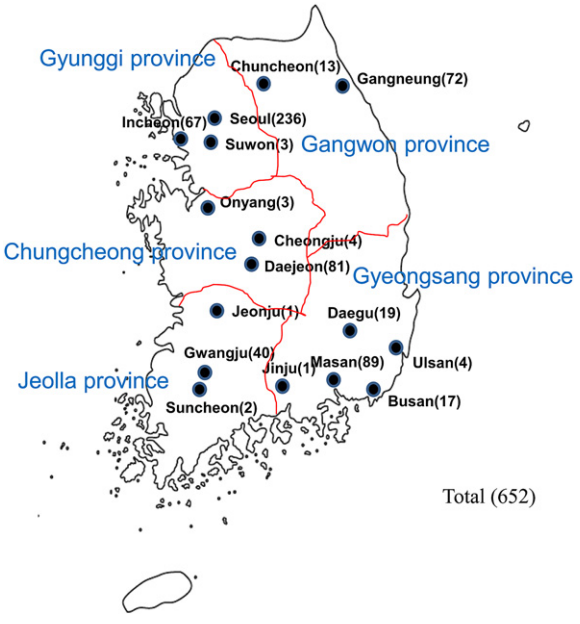

Figure 1. Location of the five provinces used in this study. Numbers in parentheses indicate the number of case.

2.3. Phylogenetic analysis of nucleotide sequences

The VP4 and VP7 sequences obtained were aligned and compared with others VP4 and VP7 sequences of rotaviruses available in the Genebank database (http://www.ncbi.nlm.nih.gov/genbank/). Phylogenetic trees of alignment were constructed using the neighbor-joining method by bootstrapping with 1000 replicates and phylogenetic distances were measured by the Tajima-Nei model [19] implements in the Molecular Evolutionary Genetics Analysis (MEGA) (http://www.megasoftware.net/) analytical package (version 5.05, Institute of Molecular Evolutionary Genetics and Department of Biology, Pennsylvania State University, University Park, Pennsylvania state, USA).

2.4. Nucleotide sequence accession numbers

The VP4 nucleotide sequences of the human rotavirus in this study were assigned accession numbers EF015885, EF077317-EF077322, EF077324-EF077342, EF077344, EF077345, and EF0773347-EF077356. The VP7 nucleotide sequences were deposited in NCBI GenBank under accession numbers HQ425255-HQ425292. The referenced sequences in the GenBank database are shown in Table 1.

3. Results

Most of the fecal samples were collected from Gyunggi province (306/652), followed by Gyeongsang province (130/652), Chungcheong province (88/652), Gangwon province (85/652), Jeolla province (43/652). These are shown in Figure 1. Among 652 stool samples, 354 (54.3%) children below 5 years of age were reactive in ELISA. *G* and *P* genotypes in parallel were detected by multiplex PCR in 314(88.7%) of these samples.

During the 2000−2004 period, nine *P-G* combinations ($N = 314$) are prevalent in South Korea. Overall, *G2P[4]* (53.5%) was the most dominant, followed by *G1P[8]* (16.6%), *G1P[6]*(13.7%), *G4P[6]*(7.3%), *G4P[8]*(5.7%), *G3P[8]*(1.3%), *G3P[4]*(1.0%), *G1P[4]* (0.6%) and *G9P [6]* (0.3%), which are shown Table 2. *G2P[4]* [Gyunggi (47.0%), Gangwon (58.1%), Chungcheong (46.9%), Jeolla (36.8%), and Gyeongsang (70.4%)] was the dominant combination of genotypes. However, subdominant combinations were different in five provinces. *G1P[6]* and

Table 1. Reference sequence of this study

Strains	VP4	VP7	Strains	VP4	VP7	Strains	VP4	VP7
MMC71	EU979382	EU839912	AK26	JF304929	JF304931	D205	JF304918	JF304920
mcs/13-07	EU753965	EU753963	Wa	M96825	M21843	NIV929893	DQ887060	DQ886957
GER31-08	GU979199	GU979198	GER198-08	GU393007	GU393006	GER173-08	GU392999	GU392998
GER125-08	GU392991	GU392990	GER96-08	GU392987	GU392986	GER84-08	GU392981	GU392980
GER67	GU392979	GU392978	GER15-08	GU392975	GU392974	CMH146/05	GU288635	288626
GER20-08	GU392977	GU392976	CMH032/05	GU288631	GU288623	CMH008/05	GU288627	GU288621
CMH054/05	GU288633	GU288624	GR846/86	AF161829	AF161822	NB123/86	AF161828	AF161821
NB187/86	AF161827	AF161820	GR442/86	AF161824	AF161817	Kagawa/88-349	AB039937	AB039033
Kagawa/88-104	AB039935	AB039030	Kagawa/90-544	AB039939	AB039026	Kagawa/90-554	AB039941	AB039025
Hochi/ Tokyo-1980	AB039943	AB039035	Odelia/ Tokyo-1984	AB039942	AB039034	YO	AB008279	D86284
CAU160	EU679396	EU679390	CAU164	EU679398	EU679392	KR/Seoul-661	HM131007	HM130944
KR/Seoul-697	HM131012	HM130949	OM67	HQ127436	AJ491179	F45	U30716	AB180970
AU19	AB017917	AB018697	ST3	EF672612	EF672616	TE56	AF183869	AF183856
Py9856	EU045216	DQ015681	DS-1	AB118025	AB118023	KR/Seoul-638	HM131021	HM130965
MW333	AJ278256	AJ278257	rj5323/02	DQ857926	DQ857953	MMC6	EU839950	EU839923
Py99449	EU045222	DQ015687	BP1227/02	AJ621505		BP271/00	AJ621502	
VN846/2003	EF179117		MW670	AJ302146		VN-281		DQ508167
Kor-64		U26378	PA5/90		DQ377573	CH631		AF183857
VN846/2003		EF545000	KR/Seoul-708		HM130955	KR/Seoul-710		HM130956
64SB/96		AY261341	MO		D86280	T108		AF450293
CC425		AJ311738	CHW17		D86276	A131		L35055
CMH222		AY707792	Arg928		AF373918	VA75		M86833
MW4086		FJ386453	KUMS00-74		DQ478420			

G1P[8] prevailed in Gyunggi province (20.1%, 17.2%), *G1P[8]* prevailed in Gangwon province (25.8%), *G1P[8]*, *G4P[6]* and *G4P[8]* prevailed in Chungcheong province (18.4%, 14.3% and 14.3%), and *G1P[6]* prevailed in Gyeongsang province (13.6%). Also, *G1P[8]* and *G3P[8]* prevailed in Jeolla province.

To examine the VP7 and VP4 nucleotide sequences for 38 isolates among 314 identified isolates, phylogenetic trees for *G* type (*G1*, *G2*, *G3*, *G4*, and *G9*) and *P* type [*P(4)*, *P(6)*, *P(8)*) were constructed by applying the neighbor-joining method. Sequences of VP7 and VP4 were determined from 38 representative rotaviruses, comprising the different genotypes and intra genotypic lineages detected by partial sequencing. Sequences of the representative isolates were submitted to GenBank (Table 3) and included in the phylogenetic analysis (Figures 2 and 3). In this study, the 11 *G1* rotavirus isolates showed that they are a part of the lineage I and are clustered into five minor lineages(*Ia-Ie*) in the phylogenetic analysis. The *G1* rotaviruses segregated into seven major lineages (*I−VII*) as reported by Arista and colleagues [20]. Most of these isolates are clustered in sub-lineage *Id* ($n = 7$), followed by sublineage *Ib* ($n = 3$) and sublineage *Ic* ($n = 1$). Sublineage *Id* isolates showed 97.8%∼99.2% nucleotide sequence similarity to strain GER15-08, and sublineage *Ib* isolates showed 99.3%∼99.6% nucleotide similarity with strains VN-281. KMR267 isolates in sublineage *Ic* showed 99.5% nucleotide similarity to Py9856. Among eleven *G1* rotavirus isolates, the three *G1-Ib* isolate was associated with *P[8]-IIIa*. One *G1-Ic* was associated with *P[8]-IIIa* and seven *G1-Id* isolates were associated with *P[4]-V*, *P[6]-Ia*, and *P[8]-IIIa*.

The *G2* rotaviruses segregated into five major lineages (*I−V*) [21]. The thirteen *G2* rotavirus isolates clustered under lineage *V*. They showed 99.2∼99.5% nucleotide similarity to strain GER84-08 from Germany. All *G2-V*

Table 2. Distribution of group A rotavirus P-G combination strains among infants and children below 5 years of age with diarrhea in five provinces of South Korea between 2000 and 2004

	Number of P-G combination strains (%)									
Provinces	G1P[4]	G1P[6]	G1P[8]	G2P[4]	G3P[4]	G3P[8]	G4P[6]	G4P[8]	G9P[6]	Total
Gyunggi	2(1.5)	27(20.1)	23(17.2)	63(47.0)	3(2.2)	0(0.0)	11(8.2)	5(3.7)	0(0.0)	134(42.7)
Gangwon	0(0.0)	1(3.2)	8(25.8)	18(58.1)	0(0.0)	0(0.0)	1(3.2)	2(6.5)	1(3.2)	31(9.9)
Chungcheong	0(0.0)	3(6.1)	9(18.4)	23(46.9)	0(0.0)	0(0.0)	7(14.3)	7(14.3)	0(0.0)	49(15.6)
Jeolla	0(0.0)	1(5.3)	6(31.6)	7(36.8)	0(0.0)	4(21.1)	1(5.3)	0(0.0)	0(0.0)	19(6.1)
Gyeongsang	0(0.0)	11(13.6)	6(7.4)	57(70.4)	0(0.0)	0(0.0)	3(3.7)	4(4.9)	0(0.0)	81(25.8)
Total	2(0.6)	43(13.7)	52(16.6)	168(53.5)	3(1.0)	4(1.3)	23(7.3)	18(5.7)	1(0.3)	314

Table 3. The G and P genotypes of the 38 representative rotavirus strains of this study are given. The VP7 and VP4 sequences were submitted to GenBank and the accordant accession numbers are provided in brackets. Patient age, gender, city of sample collection, and the year of sample collection are indicated

Isolates	G genotype (Acc.no.)	P genotypes (Acc.no.)	Patient age (month)	Patient sex	City of collection	Year of collection
KMR004	G1-Id (HQ425255)	P[8]-IIIa (EF077330)	11	M	Seoul	2002
KMR010	G3-IIId (HQ424279)	P[4]-V (EF077353)	11	F	Seoul	2003
KMR012	G3-IIId (HQ425280)	P[8]-IIIa (EF077336)	11	M	Seoul	2003
KMR021	G2-V (HQ422566)	P[4]-V (EF077344)	47	F	Seoul	2002
KMR023	G1-Id (HQ425256)	P[8]-IIIa (EF077350)	47	M	Seoul	2002
KMR024	G2-V (HQ425267)	P[4]-V (EF077345)	8	M	Seoul	2002
KMR028	G4-Ie (HQ425287)	P[6]-Ia (EF077334)	47	F	Seoul	2003
KMR025	G4-Ie (HQ425286)	P[6]-Ia (EF077348)	11	F	Seoul	2002
KMR029	G2-V (HQ425268)	P[4]-V (EF077354)	2	F	Seoul	2003
KMR037	G2-V (HQ425269)	P[4]-V (EF015885)	4	M	Seoul	2000
KMR044	G1-Id (HQ425257)	P[4]-V (EF077332)	6	M	Seoul	2003
KMR053	G4-Ie (HQ425288)	P[6]-Ia (EF077341)	23	F	Incheon	2004
KMR057	G2-V (HQ425270)	P[4]-V (EF077317)	31	F	Incheon	2000
KMR058	G1-Ib (HQ425258)	P[8]-IIIa (EF077342)	1	F	Incheon	2004
KMR060	G3-IIId (HQ425281)	P[4]-V (EF077347)	11	F	Incheon	2004
KMR101	G1-Id (HQ425259)	P[8]-IIIa (EF077338)	23	M	Seoul	2003
KMR106	G2-V (HQ425271)	P[4]-V (EF077356)	4	M	Seoul	2000
KMR126	G2-V (HQ425272)	P[4]-V (EF077340)	48	M	Seoul	2004
KMR184	G2-V (HQ425273)	P[4]-V (EF077318)	23	M	Daegu	2000
KMR267	G1-Ic (HQ425260)	P[8]-IIIa (EF077319)	23	F	Daejeon	2000
KMR294	G1-Id (HQ425261)	P[6]-Ia (EF077352)	1	M	Masan	2000
KMR419	G1-Id (HQ425262)	P[8]-IIIa (EF077320)	4	M	Gwangju	2000
KMR538	G4-Ie (HQ425289)	P[8]-II (EF077331)	36	M	Gangneung	2000
KMR541	G4-Ie (HQ425290)	P[8]-II (EF077333)	24	M	Gangneung	2000
KMR547	G2-V (HQ425274)	P[4]-V (EF077349)	48	F	Gangneung	2000
KMR548	G2-V (HQ425275)	P[4]-V (EF077351)	36	F	Gangneung	2000
KMR580	G1-Ib (HQ425263)	P[8]-IIIa (EF077335)	40	M	Gangneung	2000
KMR720	G9-III (HQ425292)	P[6]-Ia (EF077329)	1	F	Gangneung	2000
KMR733	G1-Ib (HQ425264)	P[8]-IIIa (EF077339)	19	M	Gangneung	2001
KMR748	G1-Id (HQ425265)	P[8]-IIIa (EF077325)	18	F	Gwangju	2001
KMR750	G2-V (HQ425276)	P[4]-V (EF077321)	3	F	Busan	2001
KMR751	G3-IIId (HQ425282)	P[8]-IIIa (EF077326)	19	M	Gwangju	2001
KMR757	G2-V (HQ425277)	P[4]-V (EF077337)	5	M	Masan	2001
KMR766	G3-IIId (HQ425283)	P[8]-IIIa (EF077327)	1	M	Gwangju	2001
KMR769	G2-V (HQ425278)	P[4]-V (EF077322)	3	M	Masan	2001
KMR773	G3-IIId (HQ425284)	P[8]-IIIa (EF077328)	6	M	Gwangju	2001
KMR787	G3-IIId (HQ425285)	P[8]-IIIa (EF077355)	3	M	Gwangju	2001
KMR792	G4-Ie (HQ425291)	P[6]-Ia (EF077324)	7	F	Daejeon	2001

Acc.no. = GenBank Accession Number.

isolates were associated with *P[4]-V*. Meanwhile, the seven *G3* strains are a part of the sublineage *IIId* and showed 99.3%～99.5% sequence similarity to strain GER198-08. The two *G3-IIId* were associated with *P[4]-V*, and five *G3-IIId* were associated with *P[8]-IIIa*. The 16 *P[4]-V* including *G1-Id*, *G2-V*, and *G3-IIId* shared more than 97.6% nucleotide similarity, and the six *G4* rotavirus strains clustered in sub-lineage *Ie* compared to strains KUMS00-74 has 98.7-99.4% nucleotide similarity. The four *G4-Ie* were associated with *P[6]-Ia* and two *G4-Ie* were associated with *P[8]-II*. Finally, the one *G9* isolate, KMR720 showed high sequence similarity to all lineage *III* strains (more than 98.3%) and associated with *P[6]-Ia*. While the five *P[6]-Ia* excluding *G9* isolate exhibited 98.4%～98.7% nucleotide sequence similarity to TE56, *G9* isolate, KMR720 exhibited 97.1% nucleotide sequence similarity to GR846/86. While *P[8]-IIIa* exhibited 97.6%～ 99.4% nucleotide similarity to strains CMH032/05, *P[8]-II* exhibited 99.4% nucleotide sequence similarity to strain Kagawa/88-104. As a result, we confirm that a total of nine *P-G* genotypic isolates were identified (11 *P-G* subgenotypes).

4. Discussion

Rotaviruses have been described as a major cause of severe diarrhea among infants and young children in

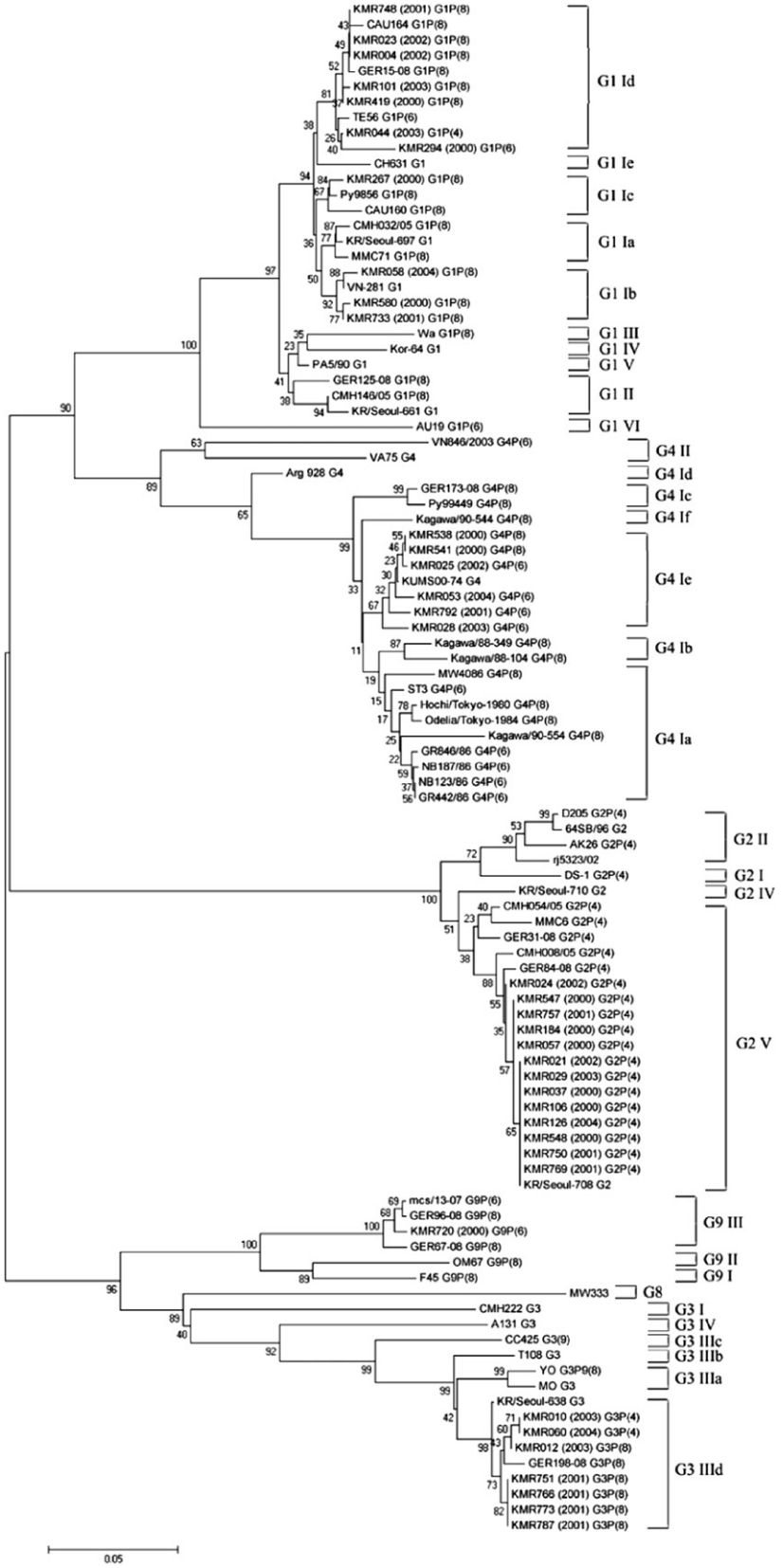

Figure 2. Phylogenetic analysis of VP7 gene nucleotide sequences of Group A rotavirus strains from Korea between 2000 and 2004. Phylogenetic trees of alignment were constructed using the neighbor-joining method by bootstrapping with 1000 replicates, and phylogenetic distances were measured by Tajima-Nei model. Only values > 50% are given. Numbers at nodes indicate the level of bootstrap support (%). Bar represents 0.05 substitutions per nucleotide position.

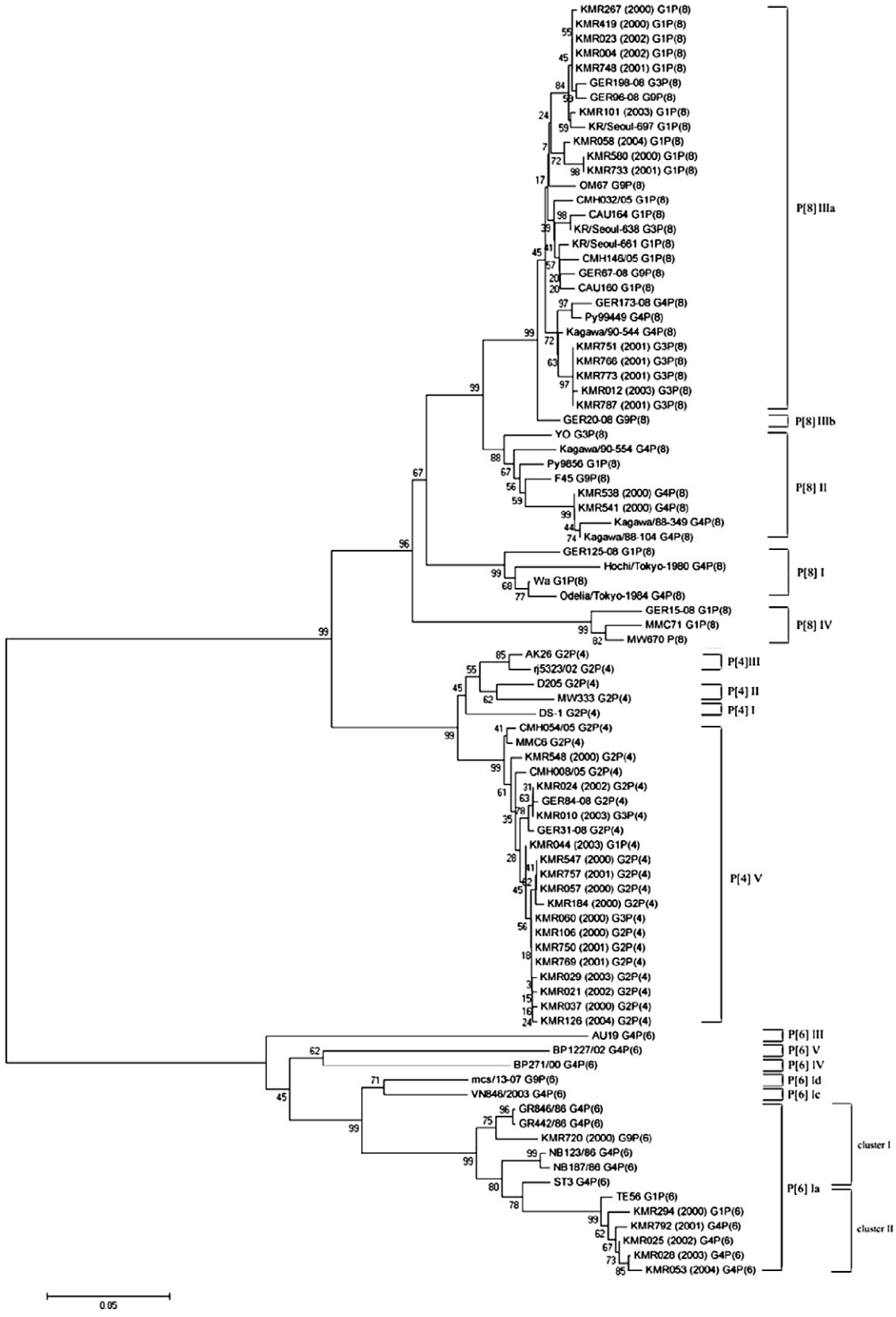

Figure 3. Phylogenetic analysis of VP4 gene nucleotide sequences of Group A rotavirus strains from Korea between 2000 and 2004. Phylogenetic trees of alignment were constructed using the neighbor-joining method by bootstrapping with 1000 replicates, and phylogenetic distances were measured by Tajima-Nei model. Only values > 50% are given. Numbers at nodes indicate the level of bootstrap support (%). Bar represents 0.05 substitutions per nucleotide position.

South Korea. Epidemiologic studies worldwide have revealed that five *P-G* combinations, *G1P[8]*, *G2P[4]*, *G3P[8]*, *G4P[8]*, and *G9P[8]*, have been linked to most of the cases of rotavirus diarrhea among infants and young children worldwide. In this study, we confirmed both uncommon *P-G* combinations [*G1P(4)*, *G1P(6)*, *G3P(4)*, *G4P(6)*, and *G9P(6)*] as well as most common *P-G* combinations [*G1P(8)*, *G2P(4)*, *G3P(8),* and *G4P(8)*)] There are two oral live vaccines available in Korea; Rotarix (GlaxoSmithKline, Rixensart, Belgium) is a monovalent vaccine that consists of the attenuated *G1P[8]* human rotavirus strain RIX4414 and has been in use in Korea since 2008. Rotateq (MERCK & CO.,INC, Pennsylvania, USA) is a pentavalent vaccine which consists of five attenuated human-bovine reassortant viruses [*G1* to *G4* and *P(8)]*; *G1*: human W179-bovine WC3 reassortant, *G2*: human SC2-bovine WC3 reassortant, *G3*: W178-bovine WC3 reassortant, *G4*: human BrB-bovine WC3 reassortant, *P1A[8]*: human W179-bovine WC3 reassortant. It has been available in Korea since 2007. Although these vaccines are effective on most common *P-G* combinations, *G2P[4]*, *G1P[8]*, *G3P[8]*, *G4P[8]*, and *G9P[8]*, those may fail to work on the other genotypes. All things considered, development of multivalent rotavirus vaccine including new ones must be required. Therefore, this study will provide useful information for the development of effective rotavirus vaccines in the future. Also, the international relationship has been limited by the geographic location in the past, but now it has expanded all over the world, and this expansion is thought to be the cause of the change in strains.

5. Conclusion

Consequently, the *P-G* combination genotypes of this study would serve as useful information for the development of effective rotavirus vaccines. Such *P-G* combinational phylogenetic study will also be necessary for international epidemiologic investigation of human rotaviruses providing novel insights into the interspecies transmission processes of rotaviruses. To strengthen our opinion, we highlight the need for continued monitoring of circulating rotavirus strains for effective prevention and vaccine development strategies.

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