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Original Article

Genetic Diversity of Echovirus 6 Strains Circulating in Korea

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Echovirus 6 (ECV6) is the prevalent serotype detected in aseptic meningitis cases in Korea. To analyze the genetic variation of ECV6 isolates recently circulating in Korea, we determined the partial sequence of the VP1 capsid gene from 22 Korean ECV6 isolates and performed pairwise analysis against 42 reference strains from the GenBank database using MegAlign. The 22 Korean ECV6 isolates formed 3 distinct genetic clusters: Kor-lineage I, II, and III. The Korean ECV6 strains showed significant genetic diversity with 14.8~22.8% nucleotide divergence among the 3 different lineages. These ECV6 Kor-lineages were demonstrated to belong to different genetic clusters using VP1 sequence-based phylogenetic analysis, implying that the recently circulating Korean ECV6 strains have potential antigenic variation.

Key Words: Echovirus 6, VP1 capsid gene, Phylogenetic analysis

INTRODUCTION

Human enteroviruses (HEV), RNA viruses from the *Picornaviridae* family, comprise more than 80 distinct serotypes and are divided into several subgroups: polioviruses and HEV-A, HEV-B, HEV-C, and HEV-D. HEV-B serotypes containing echovirus (ECV) 6 are coxsackie B viruses (CBVs) 1 to 6, coxsackievirus A9, ECV1 to ECV7, ECV9, ECV11 to ECV21, ECV24 to ECV27, ECV29 to 33, enterovirus (EV) 69, EV73 (1, 2).

ECV6 is associated with outbreaks of aseptic meningitis, mainly among young children. Other ECV6-associated symptoms include a rash, gastroenteritis, hepatitis and pneumonia (3~7). In the USA, ECV6 is the fifth most commonly identified enterovirus, presenting an epidemic pattern of circulation reminiscent of ECV11 and ECV30. Children less than 1 year old are most commonly affected by ECV6, while 5.6% of cases, with a known outcome, died (8). In Europe, ECV6 is one of the three main enteroviral serotypes (the others are ECV30 and ECV13) (9~11). In Korea, outbreaks of ECV6 occurred in 1998, 2002, and 2008 (12, 13).

The genome of ECV6 is a 7,500 nucleotide-long single-stranded polar RNA molecule. The 5' and 3' non-coding regions are generally highly conserved. The most variable regions of the genome are within the genes encoding the capsid proteins VP1, VP2, VP3, and VP4 that are partially exposed on the surface of the virus (14~16). The VP1 region is one of the mainly exposed regions of the viral capsid, and it has been suggested to include a serotype-specific antigenic neutralization site; therefore, the partial VP1 sequences of ECV6 isolates have been phylogenetically

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compared with a database of complete enterovirus VP1 sequences to determine whether they were genetically related to any known enterovirus serotypes (17, 18). In addition, phylogenetic analysis on sequence data of the VP1 region is a standard method of molecular analysis for epidemiological purposes (19, 20). On the basis of the ECV6 VP1 sequences, worldwide ECV6 isolates were divided into 3 genetic clusters (A, B, and C) with at least 15% diversity between the clusters, and cluster C was divided into the C1, C2, C3, and C4 sub-clusters (21, 22).

In this study, ECV6 isolates from Korea were analyzed by partial sequencing of the VP1 gene, and the sequences were phylogenetically compared with respective strains of the same serotype from other geographical regions. The major aim of this study was to investigate the genetic relationships among ECV6 isolates circulating in Korea.

MATERIALS AND METHODS

Viruses and cell lines

We used 22 ECV6 strains isolated from stool specimens of Korean patients with aseptic meningitis (Table 1). These viruses were cultured in the susceptible rhabdomyosarcoma, Vero, and buffalo green monkey kidney cell lines.

Reverse transcription-polymerase chain reaction (RT-PCR)

Cells exhibiting 70% cytopathic effects were frozen and thawed 3 times, and viral RNA was extracted from the supernatant of infected cells using magnetic beads (Toyobo,

Table 1. Echovirus 6 strains isolated from patients' stool with aseptic meningitis

Isolate	Gender	Age	Year	Area	Acession no.
Kor98-ECV6-278kj	M	1	1998	Kwangju	HM048849
Kor98-ECV6-270so	F	0	1998	Seoul	HM048853
Kor98-ECV6-276kj	M	5	1998	Kwangju	HM048850
Kor98-ECV6-277bs	F	2	1998	Busan	HM048851
Kor99-ECV6-286bs	F	0	1999	Busan	HM048864
Kor02-ECV6-082bs	M	1	2002	Busan	HM048843
Kor02-ECV6-081so	M	1	2002	Seoul	HM048844
Kor02-ECV6-192bs	M	0	2002	Busan	HM048845
Kor02-ECV6-148bs	F	1	2002	Busan	HM048846
Kor02-ECV6-268bs	M	5	2002	Busan	HM048847
Kor02-ECV6-263kj	F	0	2002	Kwangju	HM048848
Kor02-ECV6-279bs	M	4	2002	Busan	HM048852
Kor08-ECV6-03cn	F	0	2008	Cheonan	HM048854
Kor08-ECV6-18cn	M	0	2008	Yeongi	HM048855
Kor08-ECV6-13cn	M	1	2008	Hongsung	HM048856
Kor08-ECV6-05cn	F	2	2008	Cheonan	HM048857
Kor08-ECV6-01cn	F	7	2008	Yeongi	HM048858
Kor08-ECV6-14cn	M	0	2008	Cheonan	HM048859
Kor08-ECV6-09cn	F	2	2008	Cheonan	HM048860
Kor08-ECV6-16cn	M	0	2008	Cheonan	HM048861
Kor08-ECV6-15cn	M	9	2008	Cheonan	HM048862
Kor08-ECV6-11cn	M	1	2008	Yeongi	HM048863

Osaka, Japan). The extracted RNA was dissolved in 50 μl of nuclease-free water and stored at -70℃ until used for RT-PCR. Semi-nested RT-PCR with HEV specific primer sets was used to amplify the VP1 coding region as previously described (23).

For cDNA synthesis, a 20 μl reaction mixture containing 5 μl viral RNA, 0.2 μl primers (AN32, AN33, AN34, and AN35), 4 μl of 5× reverse transcriptase buffer, 2 μl of 0.1 M DTT, and 4 U M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) was incubated at 20℃ for 10 min, 37℃ for 120 min, 95℃ for 5 min, and then chilled on ice.

In the first-round PCR, a 50 μl reaction mixture containing 0.2 μM primers (224 and 222), 2 U *Taq* DNA polymerase (Promega, Madison, WI, USA), 100 μM dNTPs, and 2 μM $MgCl_2$ was amplified using 40 cycles of 95℃ for 30 s, 42℃ for 30 s, and 60℃ for 45 s. We added 1 μl from the first-round PCR to a second-round PCR for semi-nested amplification. The 50 μl reaction mixture containing 0.2 μM primers (AN89 and AN88), 2.5 U *Taq* DNA polymerase (Promega), 100 μM dNTPs, and 2 μM $MgCl_2$ was incubated at 95℃ for 6 min prior to 40 amplification cycles of 95℃ for 30 s, 60℃ for 20 s, and 72℃ for 15 s.

Nucleotide sequencing and molecular typing

The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany). Purified DNA was added to a reaction mixture containing 2 μl Big Dye terminator reaction mix (ABI Prism BigDye Terminator Cycle Sequencing Kit; Perkin-Elmer Applied Biosystems, Foster City, CA, USA) and 2 pmoles primers (AN88 and AN89). Sequencing reactions were subjected to an initial denaturation step at 96℃ for 1 min and 25 cycles of 96℃ for 10 s, 50℃ for 5 s, and 60℃ for 4 min in a GeneAmp PCR System 2,700 (Applied Biosystems). The products were purified by precipitation with 100% cold ethanol and 3 M sodium acetate (pH 5.8), and then loaded on an automated 3,100 Genetic Analyzer (Applied Biosystems).

The type of ECV6 isolates was determined from the highest scoring strain in GenBank using the Basic Local Alignment Search Tool (BLAST), *i.e.*, the enterovirus strain that gave the highest nucleotide similarity value with the query sequence (24).

Nucleotide comparison and phylogenetic analysis

The nucleotide sequences of the 22 Korean ECV6 isolates were compared with those of 42 reference strains using CLUSTALW (version 1.81) and MegAlign (DNASTAR) (25). Phylogenetic relationships among the VP1 sequences of the ECV6 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 (26). The reliability of the phylogenetic tree was determined by bootstrap re-sampling of 1,000 replicates.

Nucleotide sequence accession numbers

The enterovirus sequences reported here were deposited in the GenBank sequence database under the accession

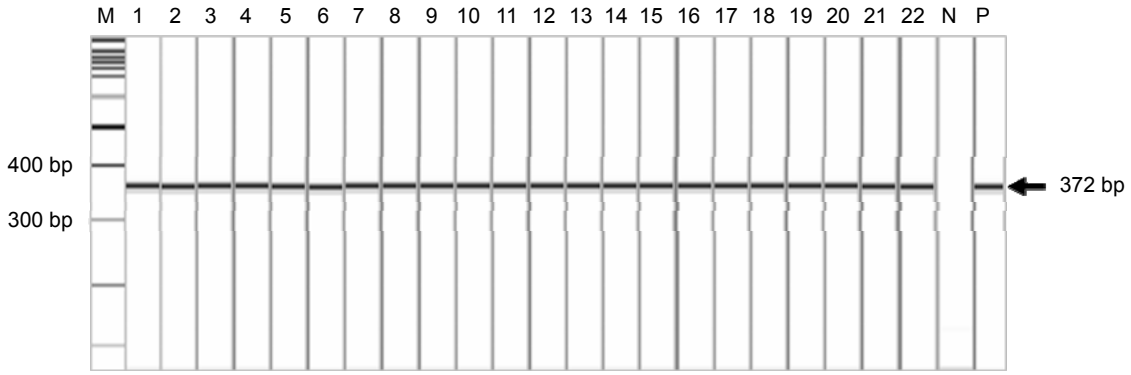

Figure 1. Gel electrophoresis of PCR products of VP1 gene of 22 ECV6 isolates from patients with aseptic meningitis; Lane M, 100 bp DNA ladder; Lane 1-22, 22 ECV6 isolates; Lane N, negative control; Lane P, positive control.

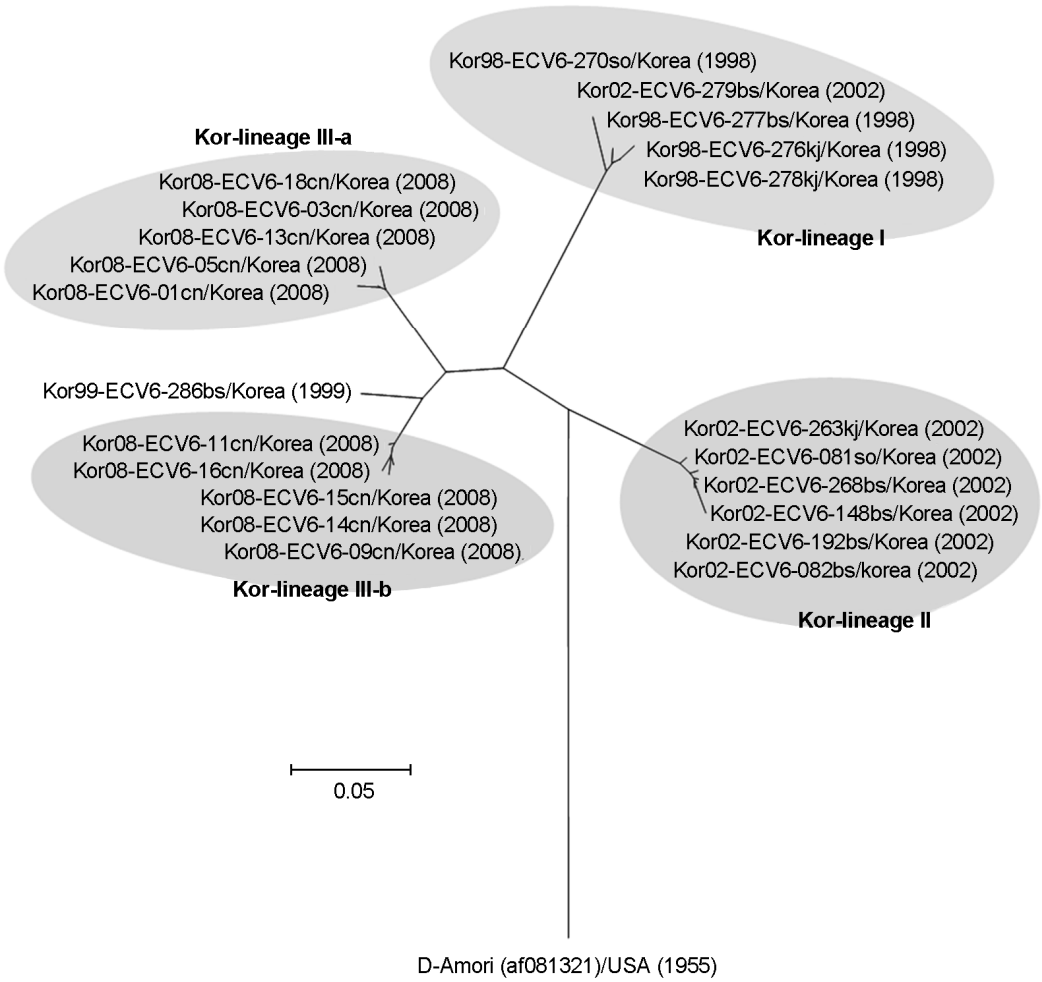

Figure 2. Phylogenetic analysis based on a 286 bp sequence of the VP1 region of Korean ECV6 isolates. Nucleotide sequences were analyzed using the neighbor-joining method.

numbers HM048843 to HM048864.

RESULTS

In order to analyze the genetic characteristics of ECV6, the VP1 gene of 22 strains isolated in Korea at 1998, 2002 and 2008 was amplified and sequenced (Fig. 1). The sequences were used to construct a phylogenetic tree with 42 reference strains from the GenBank database with the same serotypes.

Nucleotide sequence analysis demonstrated that the 22 strains isolated in Korea formed 3 distinct genetic lineages: Kor-lineage I, consisting of 5 isolates obtained in 1998 and 2002; Kor-lineage II, consisting of 6 isolates obtained in 2002; and Kor-lineage III, divided into 2 sub-lineages as Kor-lineage IIIa and Kor-lineage IIIb, consisting of 11 isolates obtained in 1999 and 2008 (Fig. 2). The nucleotide sequence identity was 97.5~99.3% in Kor-lineage I, 96.3~99.6% in Kor-lineage II, and 89.7~100% in Kor-lineage III. The sequences of Kor-lineage I, II, IIIa, and IIIb differed from the D'Amori (1955) ECV6 prototype strain by 24.2~24.5%, 21.0~22.2%, 23.8~25.2%, and 23.1~24.5% respectively. Kor-lineage I showed 14.0~18.9%, 17.3~18.7%, and 15.3~17.7% nucleotide divergence from Kor-lineage II, IIIa, and IIIb, respectively. Kor-lineage II shared 83.5~86.9% and 82.7~85.5% nucleotide identity with Kor-lineage IIIa and IIIb, respectively. Kor-lineage IIIa shared 90.2~92.8% nucleotide identity with Kor-lineage

Table 2. Percentage divergence of the nucleotide sequence of the VP1 coding region among the Korean lineage groups

		Kor-lineage I	Kor-lineage II	Kor-lineage III		D'Amori
				a	b	
I			14.0~18.9	17.3~18.7	15.3~17.7	24.2~24.5
II				13.1~16.5	14.5~17.3	21.0~22.2
III	a				7.2~9.8	23.8~25.2
	b					23.1~24.5
D'Amori						

The Kor-lineage I isolates were mainly isolated in 1998, Kor-lineage II isolates were obtained in 2002 and Kor-lineage III isolates were isolated in 2008.

Table 3. Percentage divergence of the nucleotide sequence of the VP1 coding region between the Korean lineage groups and cluster Cs

	Kor-lineage I	Kor-lineage II	Kor-lineage III	
			a	b
Cluster C1	16.8~18.9	0.7~6.6	12.6~16.8	13.6~15.7
Cluster C2	16.4~18.2	11.5~13.6	15.0~16.1	13.6~15.0
Cluster C3	8.4~12.9	15.0~18.2	12.6~16.8	12.2~16.1
Cluster C4	14.3~16.8	12.9~16.4	2.8~8.7	3.5~3.6

IIIb (Table 2).

Mao *et al*. segregated the sequences of ECV6 into three distinct clusters (A, B, and C), with some temporal and regional sub-clustering, and cluster C was sub-divided into the C1, C2, C3, and C4 sub-clusters (21). The Kor-lineage-I isolates were closer to ECV6 187602 (ay271508), which was isolated in Georgia in 2002, and belonged to cluster C3. The Kor-lineage-II isolates were closer to ver (ay697453), which was isolated in Greece in 2001, and belonged to cluster C1. The Kor-lineage III isolates belonged to cluster C4 (Fig. 3). The Kor-lineage IIIa isolates were closer to 25465 Tambov05 (ef397644), which was isolated in Russia in 2005, and the Kor-lineage IIIb isolates were closer to Anhui05-30 (fj542035) isolated in China in 2005.

The pairwise VP1 nucleotide sequence alignment of Kor-lineages and cluster Cs was compared (Table 3). Kor-lineage I showed 16.8~18.9%, 16.4~18.2%, 8.4~12.9%, and 14.3~16.8% nucleotide divergence from cluster C1 to C4, respectively. Kor-lineage II showed 0.7~6.6%, 11.5~13.6%, 15.0~18.2%, and 12.9~16.4% nucleotide divergence from cluster C1 to C4, respectively. Kor-lineage IIIa showed 12.6~16.8%, 15.0~16.1%, 12.6~16.8%, and 2.8~8.7% nucleotide divergence from cluster C1 to C4, respectively. Kor-lineage IIIb showed 13.6~15.7%, 13.6~15.0%, 12.2~16.1%, and 3.5~6.3% nucleotide divergence from cluster C1 to C4, respectively (Table 3).

DISCUSSION

There were large outbreaks of ECV6 in Korea in 1998, 2002, and 2008. Twenty-two Korean strains of ECV6 from the stool specimens of clinically defined enterovirus-associated patients were isolated and identified using molecular typing (24, 27). Some previous studies have demonstrated the genetic diversity among strains of the ECV6 serotype. Molecular investigations based on VP1 sequence analysis demonstrated that the genetic diversity of ECV6 was characterized by sequential displacements among multiple genetic variants (21, 28). The sequences of ECV6 isolates analyzed in this study formed three lineages: the strains from Kor-lineage I were isolated in 1998 except for "Kor02-ECV6-279bs"; Kor-lineage II isolates were obtained in 2002; and Kor-lineage III strains were isolated in 2008.

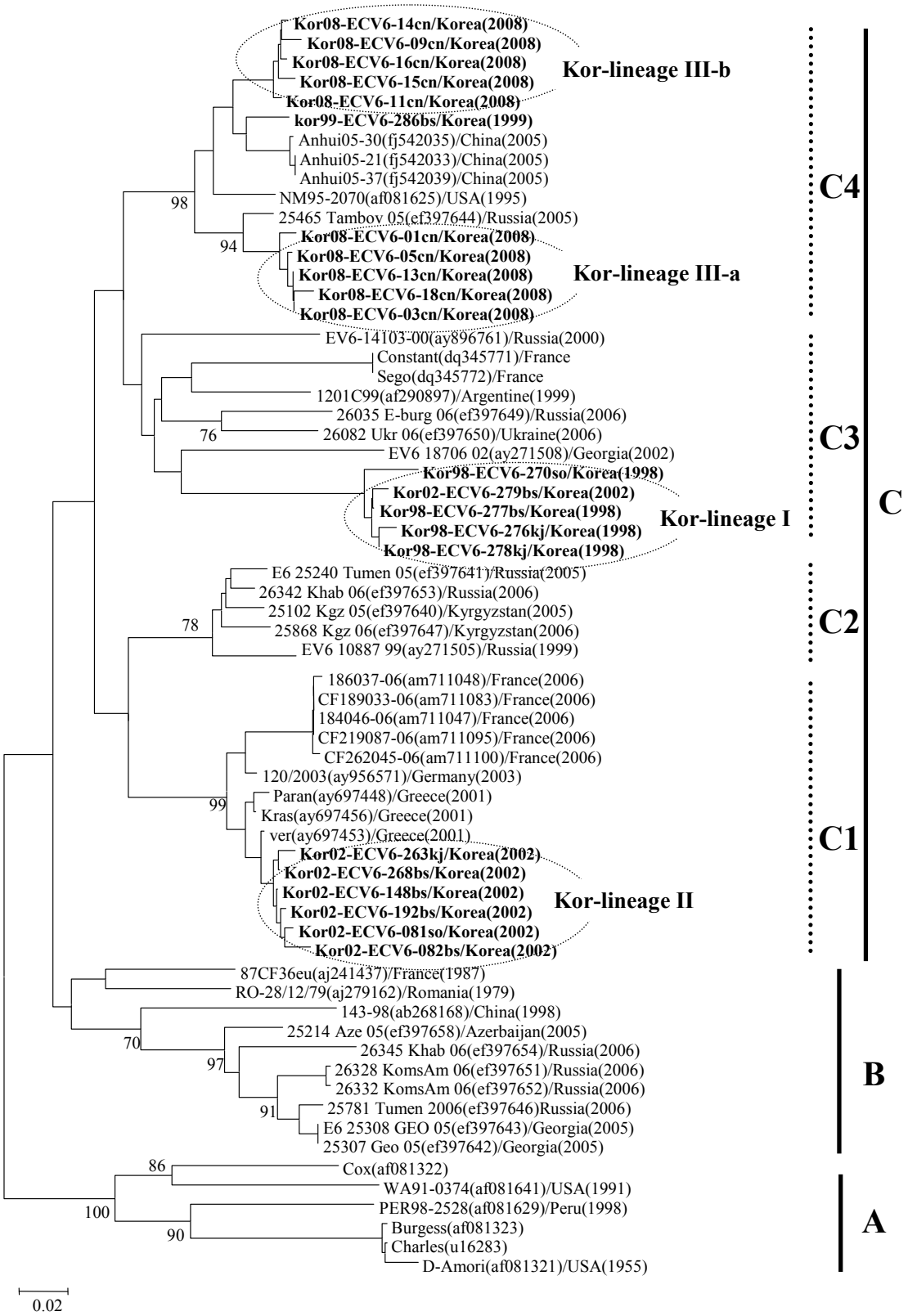

Figure 3. Phylogenetic analysis based on a 286 bp sequence of the VP1 of ECV6 isolates. Nucleotide sequences were analyzed by the neighbor-joining method. The numbers at the branches indicate the bootstrap values for 1,000 replicates.

Almost ECV6 used in this study detected in Korea during 2002 season formed Kor-lineage II but only one "Kor02-ECV6-279bs" has unique genetic identity compared with other ECV6 circulating in 2002. Diversity between two genetic cluster of ECV6 detected in 2002 is about 14 to 18%, suggesting the two possibility of its origin. One is different importing sources of ECV6 from other country and the other is ECV6 circulating in 1998 had existed in the environment until 2002 epidemic season. In addition, ECV6 isolates belonged to Kor-lineage III were sub-divided into two sub-lineages as Kor-lineage IIIa and IIIb; nevertheless, these strains were all isolated in 2008, and the divergence between these sub-lineages was as high as 7.5~10.0%. The ECV6, like other RNA viruses, has a high mutation rate due to the absence of proofreading activity during genome replication. It is estimated that approximately one mutation is generated per the newly synthesized genome (29).

ECV6 showed high level of genetic diversity over 74.8% nucleotide sequence identity among the strains of intra-serotype, which is similar phenomenon observed in other serotypes of enteroviruses. The significant level of genetic diversity among intra-serotype depending on circulating periods and regions was reported previously (27).

Worldwide ECV6 isolates are divided into 3 genetic clusters (A, B and C) with at least 15% diversity between the clusters; cluster C is divided into the C1, C2, C3, and C4 sub-clusters (21). The Kor-lineage-I isolates belonged to cluster C3, the Kor-lineage-II isolates belonged to cluster C1, and the Kor-lineage-III isolates belonged to cluster C4.

The phylogenetic tree generated in this study indicated that different ECV6 strains co-circulated in Europe during the same season, similar to the observations of a previous study, but it is not known whether ECV6 strains of the same clusters were epidemiologically linked. In this study, we compared the divergence of ECV6 strains isolated in Korea, Europe, Russia, China, and the USA; however, we did not find any association between year or region between each cluster. In conclusion, the present study provides the first report describing the molecular genetic characterization of Korean ECV6 isolates.

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