J. Microbiol. Biotechnol. (2009), **19**(9), 1055–1064
doi: 10.4014/jmb.0810.584
First published online 16 July 2009

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Molecular and Epidemiological Characterization of Enteroviruses Isolated in Chungnam, Korea from 2005 to 2006

Baek, Kyungah[1,7], Kwisung Park[1], Eunhye Jung[1], Eunhee Chung[2], Joonsoo Park[3], Hwajung Choi[4], Seunghwa Baek[5], Youngmee Jee[6], Doosung Cheon[6], and Gwangsook Ahn[7]*

[1]*Chungcheongnam-Do Health and Environment Research Institute, Daejeon 300-801, Korea*
[2]*Department of Pediatrics, College of Medicine, Dankook University, Cheonan 330-714, Korea*
[3]*Department of Pediatrics, College of Medicine, Soonchunhyang University, Cheonan 330-090 , Korea*
[4]*Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-333, Korea*
[5]*Department of Herbal Resources, Professional Graduate School of Oriental Medicine, Wonkwang University, Iksan 570-749, Korea*
[6]*Division of Enteric and Hepatitis Viruses, Department of Virology, National Institute of Health, Korea Center for Disease Control and Prevention, Seoul 122-701, Korea*
[7]*Department of Biology, Daejeon University, Daejeon 300-716, Korea*

Received: October 21, 2008 / Revised: May 11, 2009 / Accepted: June 3, 2009

Enteroviruses were identified and characterized from patients with aseptic meningitis and other enterovirus-related diseases in Chungnam, Korea from 2005 to 2006. Enteroviruses were isolated from 79 of 519 cases (15.2%) in 2005, and 37 of 386 cases (9.6%) in 2006. Based on partial VP1 sequencing, a total of 116 enterovirus isolates were resolved into 13 types. Prevalent among the Chungnam isolates were echovirus 18 and coxsackievirus B5 in 2005, and echoviruses 5 and 25 in 2006. This is the first time echoviruses 5 and 18 have been identified in Korea since enterovirus surveillance began there in 1993. The temporal distribution of enterovirus epidemics in Chungnam showed a remarkable seasonal pattern, with cases occurring during most of the three months of the summer from June to August. The highest rate of enterovirus-positive cases occurred in patients less than 1 year of age. The ratio of male to female enterovirus-positive patients was approximately 1.8:1. Comparison of the VP1 amino acid sequences of the 15 coxsackievirus B5 isolates with reference strains revealed that all Chungnam isolates are substituted at positions 23 (V23I), 19 (S19G), 75 (Y75F), and 95 (N95S). Upon comparing the nine ECV5 isolates with foreign strains, it was found that only the Chungnam isolates, with the exception of Kor06-ECV5-239cn, have P at position 153 and F at position 146. The three ECV9 isolates from 2006 show alterations at amino acids 36, 148, and 154 outside of the BC-loop and at position 84 in the BC-loop, whereas the seven isolates from 2005 and the other ECV9 strains in the database only show the alteration at position 84 (D, I, N, S). The five ECV25 isolates have an S residue at position 134, whereas most of the foreign strains have an N residue.

Keywords: Enterovirus, RT–PCR, VP1 gene, phylogenetic tree

*Corresponding author
Phone: +082-42-280-2433; Fax: +082-42-283-2481;
E-mail: micbaek@korea.kr

Human enteroviruses are RNA viruses of the Picornaviridae family. They show themselves clearly through a variety of clinical symptoms, such as aseptic meningitis, common cold, hand-foot-mouth disease, acute hemorrhagic conjuncitivitis, myocarditis, encephalitis, and poliomyelitis. Aseptic meningitis, which mainly affects young children, is the most commonly encountered illness associated with enteroviral infections and often appears in the form of outbreaks [30, 34, 38]. Sixty-five immunologically distinct serotypes are known to cause infections in humans, and they are grouped into polioviruses (PV), echoviruses (ECV), coxsackieviruses A (CVA) and B (CVB), and enterovirus types 68–71. These viruses are also genetically classified into five species (A–D and PVs) [7, 16, 23, 31, 32].

Outbreaks of enterovirus infections typically peak in the summer and early fall, and various serotypes are often associated with a single outbreak [34]. The predominant enterovirus types vary yearly among ECV 9, 13, 18, and 30, with CVB5 being the type most frequently isolated in Europe and the United States in recent years [1, 4, 5, 8]. Since 1993, when nationwide surveillance began in Korea, there have been reports of summer outbreaks of various enteroviruses, such as ECV 6, 9, 13, 30, and CVA24 [9].

The enterovirus genome consists of a 7,500-nucleotide-long single-stranded polar RNA molecule. In general, the 5' and 3' noncoding regions (NCRs) are highly conserved, whereas the most variable region of the genome lies within the genes encoding the capsid proteins, VP1, VP2, VP3, and VP4, which are partially exposed on the virus surface [2, 6, 10]. Laboratory diagnosis of enterovirus infections is based on amplification of highly conserved regions of the genome. The 5' NCR seems to be the most conserved region among enteroviruses and is therefore targeted widely in diagnostic procedures [33, 39]. In addition to traditional virological methods that are used to identify enterovirus serotype, RT–PCR methods have been recently developed based on amplification of the VP1 region [3, 20, 21, 24, 27], which is the most exposed protein of the viral capsid and includes a serotype-specific antigenic neutralization site [15, 17]. In addition, phylogenetic analysis of VP1 sequence data is considered to be a standard method of molecular analysis for epidemiological purposes [18, 26].

In this study, we analyzed enteroviruses isolated from patients with diagnosed enteroviral disease in Chungnam, Korea during the 2-year period from 2005 to 2006. In order to determine the enterovirus serotypes of the epidemic, viral culture was carried out by inoculating susceptible cells, and then examining them for the appearance of cytopathological effects. In addition, molecular detection was performed using 5' NCR RT–PCR and sequencing of the enterovirus VP1 region. Partial VP1 sequences were compared with a database of complete enterovirus VP1 sequences of all serotypes to determine whether the isolates are genetically related to any known enterovirus serotypes in the GenBank database. In doing so, we aim to determine the epidemiological and molecular characteristics of the enterovirus strains that are prevalent in Chungnam.

Materials and Methods

Fig. 1 shows the testing algorithm for the detection and molecular typing of enteroviruses.

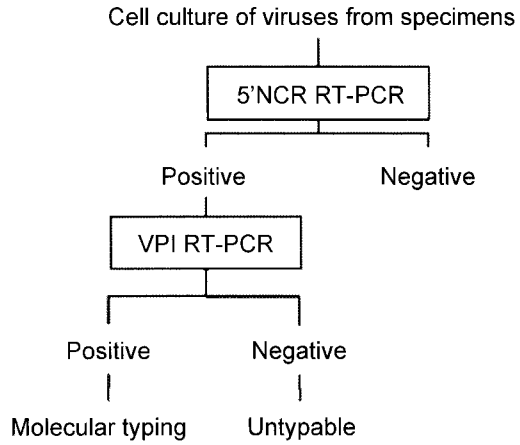

Fig. 1. Testing algorithm for the detection and molecular typing of enteroviruses.

Virus Isolation

Susceptible rhabdomyosarcoma (Rd), Vero, and buffalo green monkey (BGM) cell lines were used to isolate enteroviruses from 905 clinical specimens (stool or cerebrospinal fluid) obtained from patients having a clinical association with enterovirus infection in Chungnam, Korea from January 2005 to December 2006.

RT–PCR

Cells exhibiting 70% cytopathic effects were frozen and thawed three times, and then viral RNA was extracted from the supernatant using magnetic beads (Toyobo, Japan). The extracted RNA was dissolved in 50 μl of nuclease-free water and stored at −70°C until use in RT–PCR.

For cDNA synthesis, the 20-μl reaction mixture contained 5 μl of viral RNA, 0.2 μM primer (AN32, AN33, AN34, and AN35) (Table 1) 4 μl of 5× reverse transcriptase buffer, 2 μl of 0.1 M DTT, and 4 U of 10 mM M-MLV reverse transcriptase (Invitrogen). The reaction mixture was incubated at 20°C for 10 min, 37°C for 120 min, and 95°C for 5 min, and then chilled on ice.

PCR using a primer set specific for the 5' NCR of the enterovirus was conducted as described previously [41]. Briefly, a 50 μl reaction mix containing 0.2 μM of primers ENT-F and ENT-R [41] (Table 1), 2 U of *Taq* DNA polymerase (Promega, Madison, WI, U.S.A.), 100 μM dNTPs, and 2 μM $MgCl_2$ was amplified for 35 cycles of

Table 1. Primers used for 5' NCR and VP1 RT–PCR amplification and sequencing.

Primer	Sequence[a]	Region	Location
ENT-F	5'-AAG CAC TTC TGT TTC CCC GG-3'	5' NCR	161–181
ENT-R	5'-ATT GTC ACC ATA AGC AGC CA-3'	5' NCR	577–569
AN32	5'-GTY TGC CA-3'	VP1	3,009–3,002
AN33	5'-GAY TGC CA-3'	VP1	3,009–3,002
AN34	5'-CCR TCR TA-3'	VP1	3,111–3,104
AN35	5'-RCT YTG CCA-3'	VP1	3,009–3,002
224	5'-GCI ATG YTI GGI ACI CAY RT-3'	VP3	1,977–1,996
222	5'-CIC CIG GIG GIA YRW ACA T-3'	VP1	2,969–2,951
AN89	5'-CCA GCA CTG ACA GCA GYN GAR AYN GG-3'	VP1	2,602–2,627
AN88	5'-TAC TGG ACC ACC TGG NGG NAY RWA CAT-3'	VP1	2,977–2,951

[a]IUB ambiguity codes; I, deoxyinosine.

94°C for 1 min, 52°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 7 min.

Semi-nested PCR amplification of the VP1 coding region was performed as described previously [20]. In the initial PCR, a 50-μl reaction mix containing 0.2 μM of primers 224 and 222 (Table 1), 2 U of *Taq* DNA polymerase (Promega), 100 μM dNTPs, and 2 μM $MgCl_2$ was subjected to amplification for 40 cycles of 95°C for 30 sec, 42°C for 30 sec, and 60°C for 45 sec. One μl of the first PCR was added to a 50-μl reaction mix containing 0.2 μM of primers AN89 and AN88 [20] (Table 1), 2.5 U of *Taq* DNA polymerase (Promega), 100 μM dNTPs, and 2 μM $MgCl_2$, which was incubated at 95°C for 6 min prior to 40 amplification cycles of 95°C for 30 sec, 60°C for 20 sec, and 72°C for 15 sec.

Nucleotide Sequencing and Molecular Typing

PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Germany). Purified DNA was added to 2 μl of reaction mix (ABI Prism BigDye Terminator Cycle Sequencing Kit; Perkin-Elmer Applied Biosystems, U.S.A.) and 2 pmoles of primers AN88 and AN89 (Table 1). Sequencing reactions were subjected to an initial denaturation at 96°C for 1 min, and then 25 cycles consisting of 96°C for 10 sec, 50°C for 5 sec, and 60°C for 4 min in a GeneAmp PCR System 2700 (Applied Biosystems). Products were purified by precipitation with 100% cold ethanol and 3 M sodium acetate (pH 5.8), and then loaded onto an automated 3100 Genetic Analyzer (Applied Biosystems).

The molecular type of each isolate was determined to be the serotype of the highest scoring strain in GenBank using the Basic Local Alignment Search Tool (BLAST); that is, the enterovirus strain whose sequence had the highest nucleotide similarity to the query sequence [25].

Sequence Analysis

Among 90 strains of prevalent enterovirus types, 51 strains having a more informed profile were selected for sequence analysis, as shown

Table 2. Candidate enteroviruses isolated in this study.

Isolate[a]	Diagnosis	Gender	Age	Isolated month	Specimen[b]	Accession no.	type
Kor05-CVB5-237cn	Meningitis	F	5	Jun. 2005	CSF	EU590798	
Kor05-CVB5-182cn	Meningitis	F	7	Jun. 2005	CSF	EU604650	
Kor05-CVB5-203cn	Fever, acute gastroenteritis	M	2	Jun. 2005	Stool	EU604651	
Kor05-CVB5-263cn	Meningitis	M	0	Jun. 2005	Stool	EU604652	
Kor05-CVB5-293cn	Fever, vomiting, headache			May. 2005	CSF	EU604653	
Kor05-CVB5-323cn	Sepsis-like	M	0	Jul. 2005	CSF	EU604654	
Kor05-CVB5-335cn	Sepsis-like	M	0	Jul. 2005	CSF	EU604655	
Kor05-CVB5-342cn	Sepsis-like	F	0	Jun. 2005	CSF	EU604656	Coxsackievirus B5
Kor05-CVB5-349cn	Sepsis-like	M	0	Jul. 2005	CSF	EU604657	
Kor05-CVB5-381cn	Meningitis	F	6	Jul. 2005	CSF	EU604658	
Kor05-CVB5-388cn	Meningitis	F	0	Jul. 2005	Stool	EU604659	
Kor05-CVB5-420cn	Meningitis	F	11	Aug. 2005	CSF	EU604660	
Kor05-CVB5-437cn	Fever, vomiting			Aug. 2005	CSF	EU604661	
Kor05-CVB5-438cn	Fever, vomiting, headache			Aug. 2005	CSF	EU604662	
Kor05-CVB5-466cn	Meningitis	M	0	Aug. 2005	Stool	EU604663	
Kor06-ECV5-2228cn	Fever, vomiting	M	12	Jul. 2006	Stool	EU590801	
Kor06-ECV5-153cn	Fever, vomiting, diarrhea	M		Jun. 2006	Stool	EU590802	
Kor06-ECV5-220cn	Fever, vomiting	F	0	Jul. 2006	Stool	EU590803	
Kor06-ECV5-234cn	Fever, vomiting	M	6	Jul. 2006	Stool	EU590804	
Kor06-ECV5-237cn	Fever, vomiting	M	0	Jul. 2006	Stool	EU590805	Echovirus 5
Kor06-ECV5-239cn	Sepsis-like	F	0	Jul. 2006	CSF/Stool	EU590806	
Kor06-ECV5-253cn	Sepsis-like	F	1	Jul. 2006	CSF/Stool	EU590807	
Kor06-ECV5-257cn	Sepsis-like	F	0	Jul. 2006	CSF	EU590808	
Kor06-ECV5-203cn	Fever, vomiting	F	1	Jul. 2006	Stool	EU604666	
Kor05-ECV9-288cn	Meningitis	M	0	Jul. 2005	Stool	EU590809	
Kor05-ECV9-296cn	Meningitis	F	4	Jul. 2005	Stool	EU590810	
Kor05-ECV9-320cn	Sepsis-like	F	0	Jul. 2005	Stool	EU590811	
Kor05-ECV9-352cn	Meningitis	F	0	Jul. 2005	CSF	EU590812	
Kor05-ECV9-360cn	Sepsis-like	F	0	Jul. 2005	Stool	EU590813	
Kor05-ECV9-443cn	Fever, vomiting	F		Aug. 2005	Stool	EU590814	Echovirus 9
Kor06-ECV9-243cn	Meningitis	M	2	Jul. 2006	CSF/Stool	EU590815	
Kor06-ECV9-289cn	Fever, vomiting	F	2	Aug. 2006	Stool	EU590816	
Kor06-ECV9-161cn	Fever, vomiting, headache	M	0	Jun. 2006	Stool	EU590817	
Kor05-ECV9-245cn	Meningitis	M	0	Jun. 2005	Stool	EU604665	

Table 2. Continued.

Isolate[a]	Diagnosis	Gender	Age	Isolated month	Specimen[b]	Accession no.	type
Kor05-ECV18-055cn	Fever, vomiting	F	6	Apr. 2005	Stool	EU590818	Echovirus 18
Kor05-ECV18-060cn	Meningitis	M	6	Apr. 2005	Stool	EU590819	
Kor05-ECV18-075cn	Meningitis	F	6	May. 2005	Stool	EU590820	
Kor05-ECV18-109cn	Acute gastroenteritis	M	6	May. 2005	Stool	EU590821	
Kor05-ECV18-132cn	Meningitis	M	10	Jun. 2005	Stool	EU590822	
Kor05-ECV18-134cn	Fever, vomiting	M	6	Jun. 2005	Stool	EU590823	
Kor05-ECV18-216cn	Meningitis	M	5	Jun. 2005	Stool	EU590824	
Kor05-ECV18-267cn	Sepsis-like	M	0	Jun. 2005	Stool	EU590825	
Kor05-ECV18-274cn	Meningitis	M	8	Jul. 2005	Stool	EU590826	
Kor05-ECV18-315cn	Meningitis	M	6	Jul. 2005	CSF	EU590827	
Kor05-ECV18-347cn	Meningitis	M	9	Jul. 2005	Stool	EU590828	
Kor05-ECV18-054cn	Meningitis	M	5	Apr. 2005	Stool	EU604664	
Kor06-ECV25-248cn	Sepsis-like	M	0	Jul. 2006	CSF/Stool	EU590799	Echovirus 25
Kor06-ECV25-291cn	Fever, vomiting	M	0	Aug. 2006	Stool	EU590800	
Kor06-ECV25-229cn	Fever, vomiting, headache	M	0	Jul. 2006	Stool	EU604667	
Kor06-ECV25-279cn	Fever, vomiting	M	0	Aug. 2006	Stool	EU604668	
Kor06-ECV25-281cn	Fever, vomiting	M	2	Aug. 2006	CSF/Stool	EU604669	

[a]"Kor00", isolated in Korea in year 00; "-XXX-", type of enterovirus; "000cn", isolation number in Chungnam.
[b]CSF, cerebrospinal fluid.

Table 2. Nucleotide and deduced amino acid sequences of candidate enterovirus isolates were compared with reference strains using CLUSTAL W (version 1.81) and Megalign (DNASTAR, Inc.) [40], both of which are applied Needleman–Wunsch algorithms (M-NW similarity test). Sequential pairwise alignment was performed, and then a similarity score for each pair of sequences was obtained manually. However, the quality of the alignment could not be measured, and the gap opening penalty (GOP) and the gap extension penalty (GEP) values were used as default values [14, 19].

The phylogenetic relationships between the VP1 sequences of each virus isolate were inferred through the neighbor-joining method as implemented in the CLUSTAL W program [35]. The resulting trees were plotted using Treeview (version 1.6.6) [29]. The reliability of the phylogenetic tree was determined by the bootstrap resampling test with 1,000 replicates.

Nucleotide Sequence Accession Numbers

The candidate enterovirus sequences reported here were deposited in the GenBank sequence database, under the accession numbers EU590798 to EU590828, and EU604650 to EU604669 (Table 2).

Results

Enterovirus Detection and Molecular Typing

Enteroviruses were cultured from 905 samples obtained from patients with aseptic meningitis and other enterovirus-related diseases and subjected to a diagnostic 5' NCR RT–PCR that generated a 436-bp amplicon. Consequently, we isolated a total of 116 enteroviruses: 79 from 519 cases (15.2%) in 2005, and 37 from 386 cases (9.6%) in 2006. For molecular typing and phylogenetic analysis, the VP1 amplicons generated in the semi-nested RT–PCR were sequenced and were found to correspond to a 372-bp region of the VP1 gene. The 116 isolates were identified as ECV18 (n=38, 32.8%), CVB5 (n=21, 18.1%), ECV5 (n=14, 12.1%), ECV9 (n=10, 8.6%), ECV25 (n=7, 6.0%), CVB3 (n=3, 2.6%), and CVB4 (n=2, 1.7%). CVA4, 6, 9, 13, 16, and CVB1 were also identified. Fifteen samples did not generate VP1 amplicons in semi-nested PCR and were therefore considered to be untypable. (Table 3)

Table 3. Number of enterovirus types isolated in Chungnam from 2005 to 2006.

Type of enterovirus	Number isolated in 2005	2006	Total	Percentage of total
Coxsackievirus A4	-	1	1	0.9
Coxsackievirus A6	1	-	1	0.9
Coxsackievirus A9	-	1	1	0.9
Coxsackievirus A13	-	1	1	0.9
Coxsackievirus A16	-	1	1	0.9
Coxsackievirus B1	1	-	1	0.9
Coxsackievirus B3	1	2	3	2.6
Coxsackievirus B4	-	2	2	1.7
Coxsackievirus B5	21	-	21	18.1
Echovirus 5	-	14	14	12.1
Echovirus 9	7	3	10	8.6
Echovirus 18	38	-	38	32.8
Echovirus 25	-	7	7	6.0
Untypable	10	5	15	12.9
Total	79	37	116	-

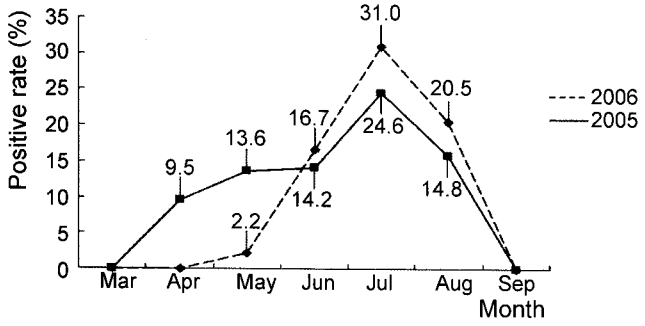

Fig. 2. Temporal distribution of enterovirus-positive cases in Chungnam, Korea from 2005 to 2006.

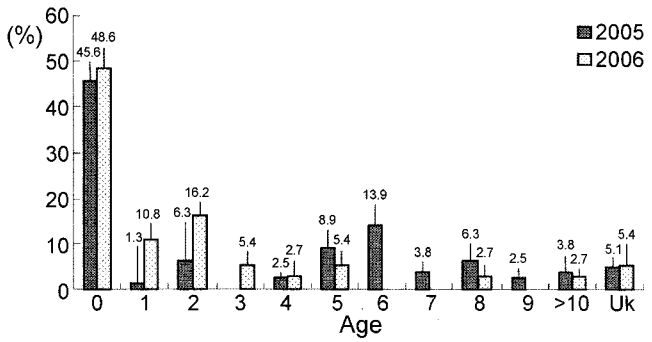

Fig. 3. Age distribution of enterovirus-positive patients in Chungnam, Korea from 2005 to 2006.
Uk, Unknown.

Epidemiological Features of Enteroviruses in Chungnam

The temporal distribution of enterovirus epidemics in Chungnam in both 2005 and 2006 showed an obvious seasonal pattern during the short period from June to August. The enterovirus detection rate was 14.2% and 16.7% in June, 24.6% and 31.0% in July, and 14.8% and 20.5% in August 2005 and 2006, respectively, as shown Fig. 2.

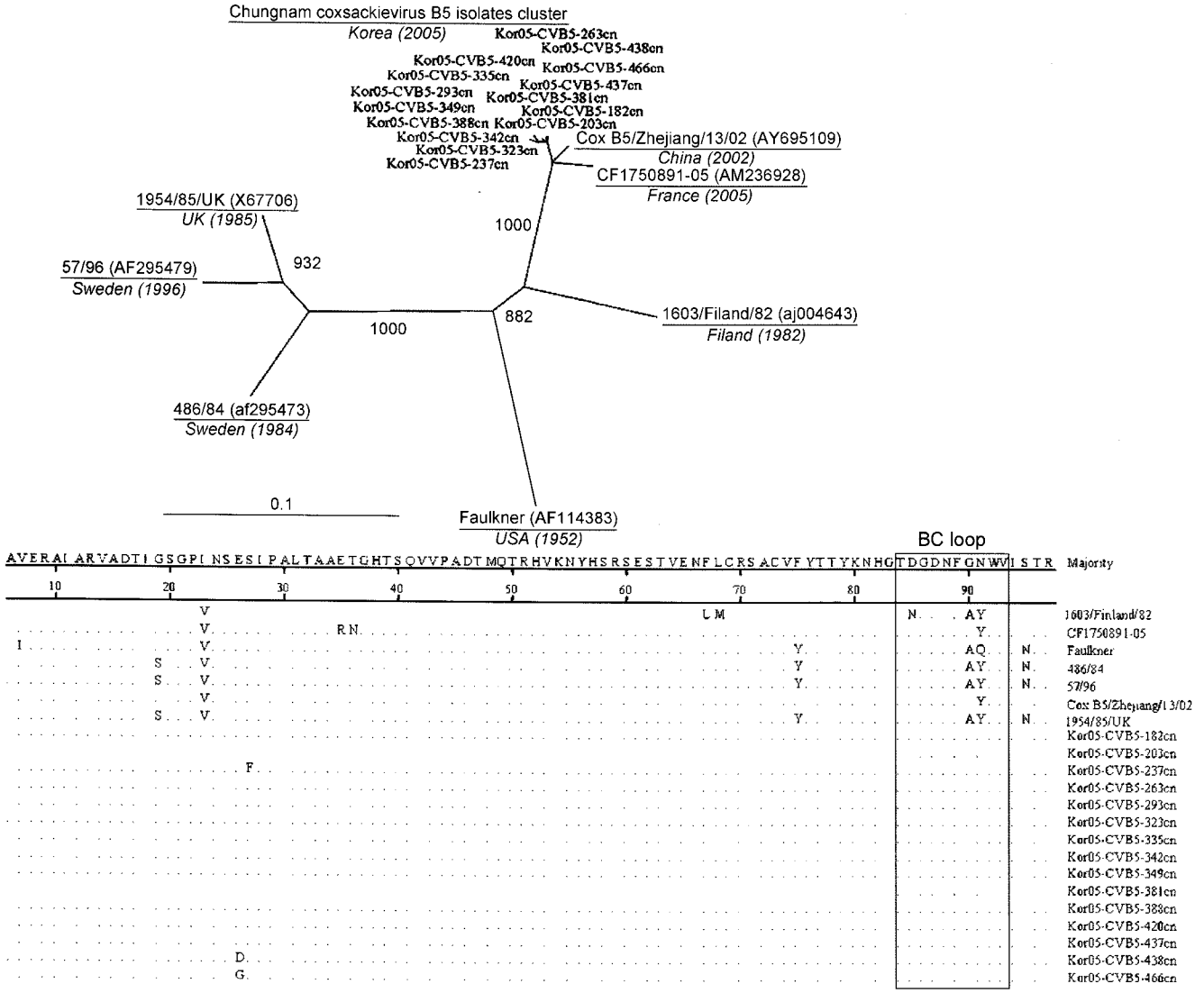

Fig. 4. Phylogenetic analysis and comparison of deduced amino acid sequences of the VP1 region in Chungnam coxsackievirus B5 strains.
Nucleotide sequences were analyzed by the neighbor-joining method. The numbers at the branches indicate bootstrap values for 1,000 replicates.

Patient age was indicated for 75 of 79 enterovirus-positive samples (94.9%) in 2005, and 35 of 37 (94.6%) in 2006. Of these cases, 71/75 (94.7%) cases with known age in 2005 and 32/35 (91.4%) cases with known age in 2006 occurred in individuals under 10 years of age, whereas 4/75 (5.3%) cases with known age in 2005 and 2/35 (5.7%) cases with known age in 2006 occurred in individuals over 10 years of age. The highest rate of enterovirus-positive cases occurred in individuals less than 1 year of age, with 45.6% in 2005 and 48.6% in 2006, as shown Fig. 3. Of the 116 total isolates, 74 were from males and 42 were from females, resulting in a sex ratio of about 1.8:1.

Sequence Comparison and Phylogenetic Analysis

The similarity of the partial VP1 nucleotide sequences of the 15 Chungnam CVB5 isolates was established to be very high, ranging from 98.7% to 100%. These isolates share 78.6–98.0% VP1 nucleotide sequence identity with those of seven foreign CVB5 strains. The closest relationships were found with a Chinese strain (CoxB5/Zhejiang/13/02) that has been isolated since 2002 and a French strain (CF1750891-05) that has been isolated since 2005, as shown in Fig. 4. In contrast to reference strains, all Chungnam CVB5 isolates are substituted at positions 23 (V23I), 19 (S19G), 75 (Y75F), and 95 (N95S). Most of the Chungnam isolates have identical VP1 amino acid sequences, with the exception of Kor05-CVB5-237cn, Kor05-CVB5-438cn, and Kor05-CVB5-466cn. AY was substituted for GN at positions 90 and 91 in the BC-loop, as shown in Fig. 4 [13].

Upon comparing the VP1 amino acid sequences of the nine ECV5 isolates from Chungnam to those of foreign strains, we found that only the Chungnam isolates, with the exception of Kor06-ECV5-239cn, have P at position 153 and F at position 146. There is 97.6% to 99.3% identity among the Chungnam isolates, and they show 81.5% to 96.6% identity with the foreign ECV5 strains. The nine Chungnam isolates group together with two recently deposited reference strains, BE02-3792 isolated in Belgium in 2002 and Germany/312/2003, as shown Fig. 5.

Pairwise VP1 sequence alignments revealed 98.3% to 100% identity among the seven ECV9 Chungnam isolates from 2005, which, with the exception of Kor05-ECV9-

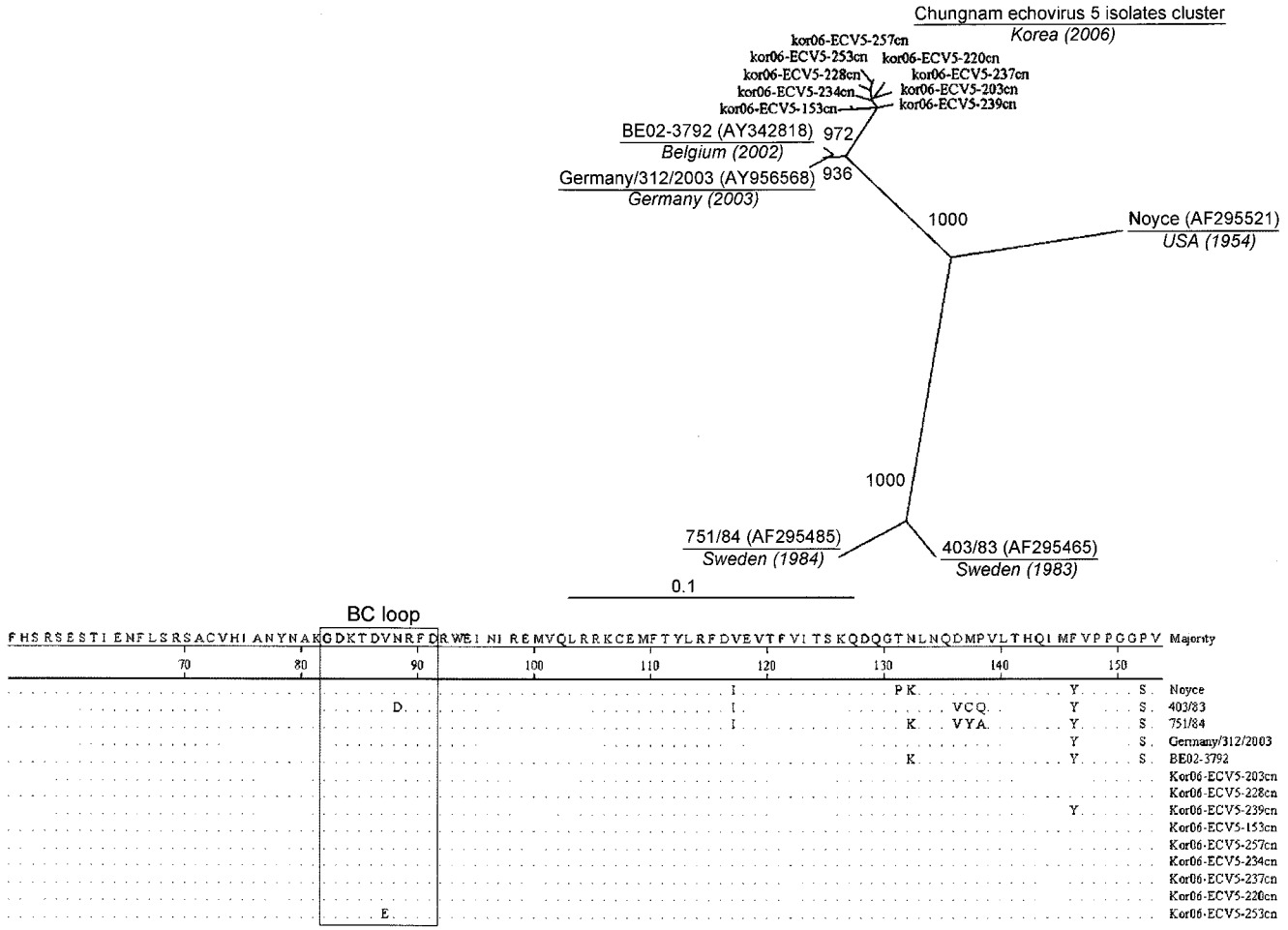

Fig. 5. Phylogenetic analysis and comparison of deduced amino acid sequences of the VP1 region in Chungnam echovirus 5 strains.
Nucleotide sequences were analyzed by the neighbor-joining method. The numbers at the branches indicate bootstrap values for 1,000 replicates.

443cn, are most similar to strain Type 9/Kurume/97 isolated in Japan in 1997. Moreover, there is 99.4% to 100% identity among the three isolates from 2006, which show 96.1% identity to Kor05-ECV9-443cn, indicating that these isolates are more closely related to the latter than to the reference strains in Fig. 6. Furthermore, whereas these isolates show alterations at amino acids 36, 148, and 154 outside of the BC-loop, as well as at position 84 within the BC-loop, the seven isolates from 2005 and the other ECV9 strains in the database show the alteration at position 84 (D, I, N, S) (Fig. 6).

Comparison of partial VP1 amino acid sequences (residues 1–181) revealed that the Chungnam ECV18 isolates, with the exception of Kor05-ECV18-216cn, Kor05-ECV18-347cn, and Kor05-ECV18-132cn from 2005, share a clear consensus sequence with CF1400191-05, which was isolated in France in 2005. All 12 Chungnam ECV18 isolates show 79.7–97.4% identity with foreign ECV18 strains and are 98.2–100% identical to one another, as shown Fig. 7.

In comparison to the five Chungnam ECV25 isolates, the foreign strains show considerable differences in the VP1 BC-loop, and the Chungnam isolates have an S residue at amino acid 134, whereas most of the foreign strains have an N residue. In comparison with 501-3/Mog/03 isolated in Mongolia in 2003, the Chungnam ECV25 isolates have substitutions at amino acids 86 (N86D) and 91 (A91T) of the BC-loop, whereas the remaining regions show consensus, with the exception of Kor06-ECV25-229cn. The Chungnam ECV25 isolates show 80.7–92.0% identity to foreign ECV25 strains and 99.3–100% identity to one another. In terms of phylogenetic relationships, the Chungnam isolates are more closely related to strain 501-3/Mog/03 than to any other foreign strain in Fig. 8.

DISCUSSION

Various enterovirus epidemics have been monitored in Korea over the past few years but, unfortunately, analytical research was not conducted in the Chungnam area [9, 12, 13]. In this study, we identified 116 enterovirus isolates

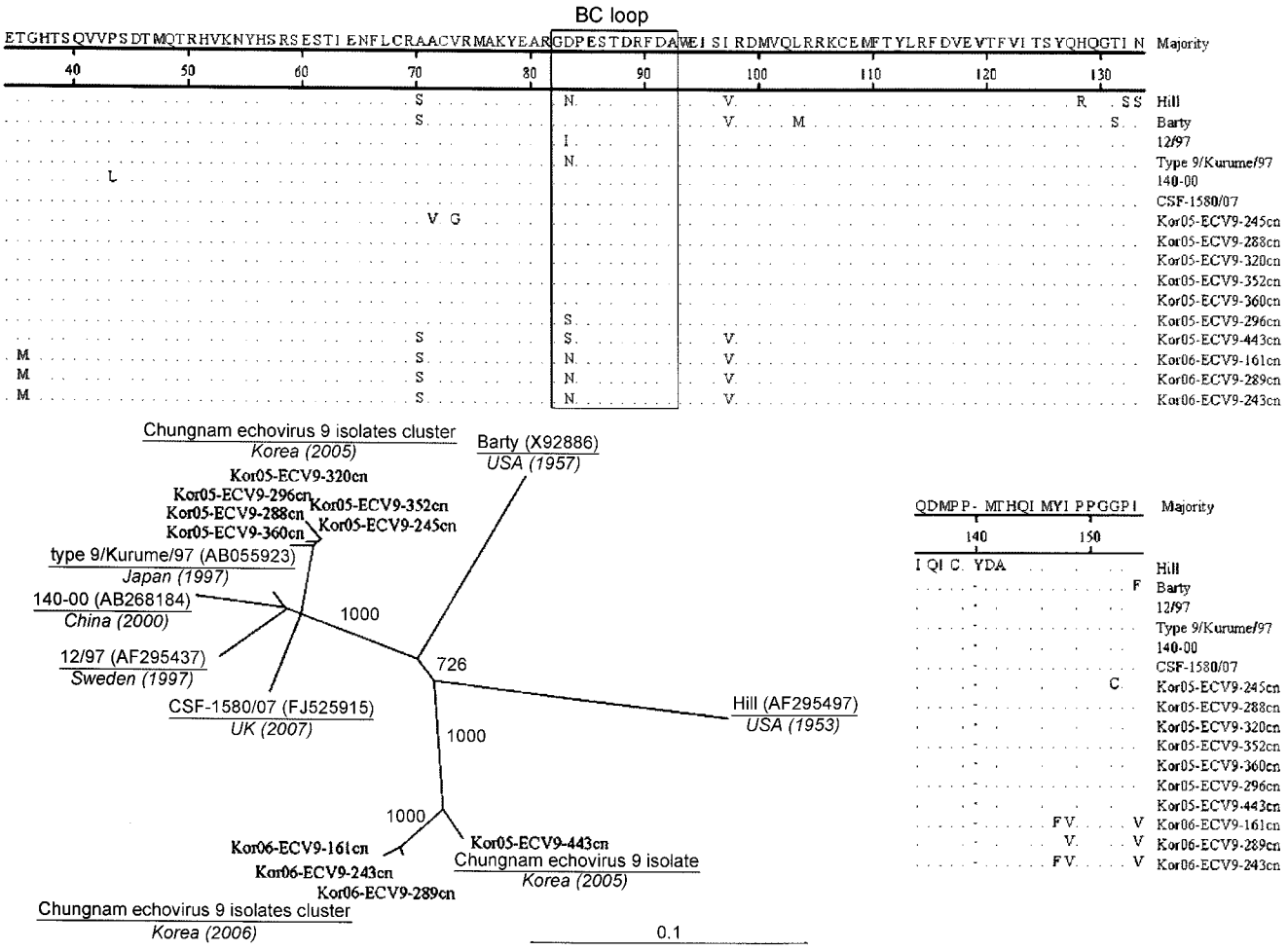

Fig. 6. Phylogenetic analysis and comparison of deduced amino acid sequences of the VP1 region of Chungnam echovirus 9 strains.
Nucleotide sequences were analyzed by the neighbor-joining method. The numbers at the branches indicate bootstrap values for 1,000 replicates.

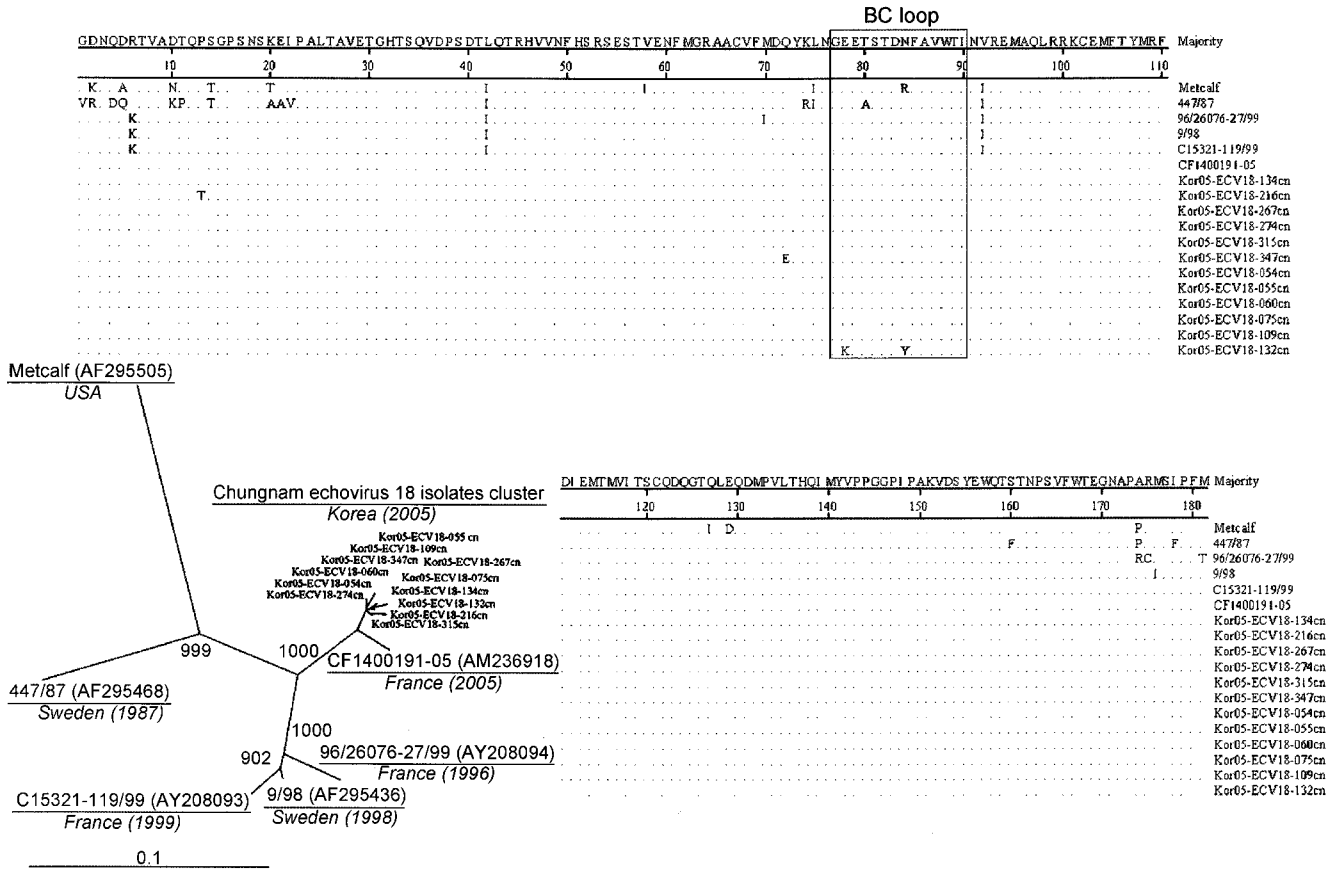

Fig. 7. Phylogenetic analysis and comparison of deduced amino acid sequences of the VP1 region of Chungnam echovirus 18 strains.
Nucleotide sequences were analyzed by the neighbor-joining method. The numbers at the branches indicate bootstrap values for 1,000 replicates.

(12.8%) from 905 cases, belonging to 13 types, in Chungnam from 2005 to 2006. The highest rate of enterovirus-positive samples occurred in children less than 1 year of age, implicating enteroviruses as causative agents of the aseptic meningitis epidemic. In the United States, the peak age for children with aseptic meningitis is reported to be < 1 year old [36]. ECV18 and CVB5 were the most prevalent molecular types in 2005, whereas ECV5 and ECV25 were the most prevalent in 2006. This is the first time ECV18 and ECV5 have been identified in Korea since surveillance began in 1993 [9, 12].

Enterovirus epidemics typically commence in southern regions in the spring and then gradually move to northern regions, reaching a peak incidence in the summer and then waning by the fall [12, 28]. The results of the present study are in accordance with this general pattern. Enterovirus epidemics in Korea will be affected by environmental shifts induced by climate change due to global warming, etc. Therefore, systematic long-term epidemiological studies will be carried out to prevent the spread of enteroviruses.

There have been recent attempts to develop a new method for typing enteroviruses by RT–PCR amplification followed by sequencing of the VP1 region of the genome. Different parts of the VP1 region have been targeted and have been proven to contain serotype-specific information [3, 11, 24]. The BC-loop in the VP1 region is associated with viral antigenicity, and substitutions of amino acid sequences in this region are believed to be important in host infection of enteroviruses [13, 22, 37]. In this study, it was found that the VP1 region of the Chungnam isolates showed notable variation in amino acid sequences. Of particular note are the V23I substitution in CVB5, the Y146F and S153P substitutions in ECV5, and the I42L and I92V substitutions in ECV18 isolates.

The Chungnam CVB5 strains clustered with CoxB5/Zhejiang/13/02 isolated in China in 2002, and CF1750891-05 isolated in France in 2005. Comparison of BC-loop sequences showed that the Korean CVB5 isolates have substitutions at positions 90 and 91 (AY to GN) [13]. Comparison of Chungnam ECV9 strains isolated in the two different years of the study reveals that the 2006 strains have substitutions at positions 36, 71, 84, 98, 148, and 154 relative to the 2005 strains. According to phylogenetic analysis, Kor05-ECV9-443cn seems to be the source of the 2006 Chungnam ECV9 epidemic strains. In general, the Chungnam isolates are more closely related to more

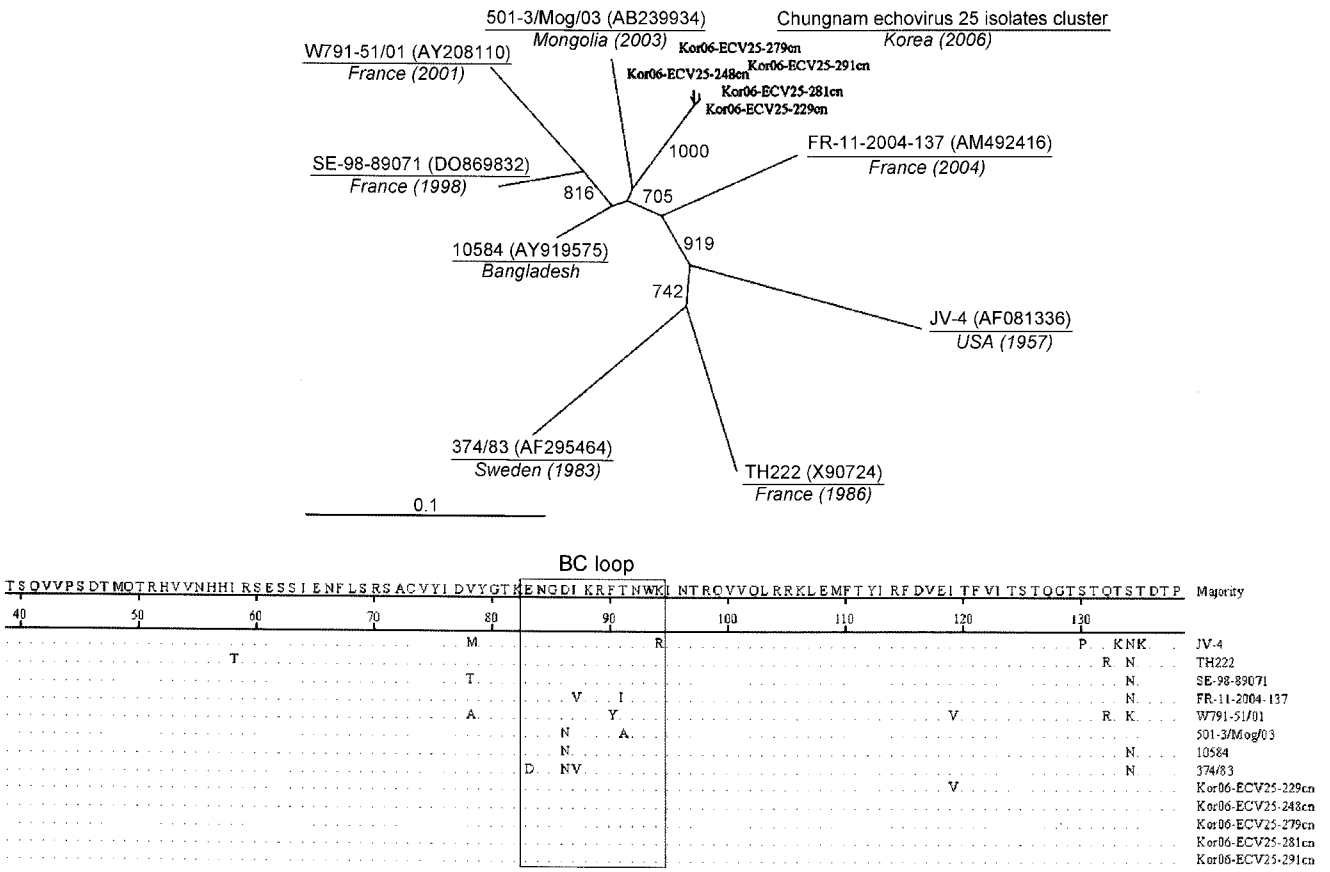

Fig. 8. Phylogenetic analysis and comparison of deduced amino acid sequences of the VP1 region of Chungnam echovirus 25 strains. Nucleotide sequences were analyzed by the neighbor-joining method. The numbers at the branches indicate bootstrap values for 1,000 replicates.

recently isolated reference strains. BE02-3792 isolated in Belgium in 2002 and Germany/312/2003 showed the closest relationships to Chungnam ECV5 isolates. In addition, Chungnam ECV 18 and ECV 25 isolates are more closely related to CF1400191-05 isolated in France in 2005, and 501-3/Mog/03 isolated in Mongolia in 2003, respectively.

Having identified the causative viruses, the prevailing strains, and their epidemiological patterns in patients with enterovirus-related diseases in Chungnam in 2005 and 2006, we suggest that the results of this study might reflect national trends of enterovirus outbreaks in Korea during those 2 years [13]. Molecular characterization of the Chungnam isolates also revealed patterns of variation that may prove useful in future studies.

REFERENCES

1. Böttner, J. L., S. Daneschnejad, W. Handrick, V. Schuster, U. G Liebert, and W. Kiess. 2002. A season of aseptic meningitis in Germany: Epidemiologic, clinical and diagnostic aspects. *Pediatr. Infect. Dis. J.* **21:** 1126–1132.
2. Caggana, M., P. Chan, and A. Ramsingh. 1993. Identification of a single amino acid residue in the capsid protein VP1 of coxsackievirus B4 that determines the virulent phenotype. *J. Virol.* **67:** 4797–4803.
3. Caro, V., S. Guillot, F. Delpeyroux, and R. Crainic. 2001. Molecular strategy for serotyping of human enteroviruses. *J. Gen. Virol.* **82:** 79–91.
4. Centers for Disease Control and Prevention. 2006. Enterovirus surveillance -- United States, 1970–2005. *Morb. Mortal. Wkly Rep.* **55:** 1–20.
5. Chomel, J. J., D. Antona, D. Thouvenot, and B. Lina. 2003. Three ECHOvirus serotypes responsible for outbreak of aseptic meningitis in Rhône-Alpes region, France. *Eur. J. Clin. Microbiol. Infect. Dis.* **22:** 191–193.
6. Dunn, J. J., N. M. Chapman, S. Tracy, and J. R. Romero. Genomic determinants of cardiovirulence in coxsackievirus B3 clinical isolates: Localization to the 5' nontranslated region. *J. Virol.* **74:** 4787–4794.
7. Huttunen, P., J. Santti, T. Pulli, and T. Hyypiä. 1996. The major echovirus group is genetically coherent and related to coxsackie B viruses. *J. Gen. Virol.* **77:** 715–725.
8. Jacques, J., H. Moret, D. Minette, N. Lévêque, N. Jovenin, G Deslée, F. Lebargy, J. Motte, and L. Andréoletti. 2008. Epidemiological, molecular, and clinical features of enterovirus

respiratory infections in French children between 1999 and 2005. *J. Clin. Microbiol.* **46:** 206–213.
9. Jee, Y. M., D. S. Cheon, W. Y. Choi, J. B. Ahn, K. S. Kim, Y. S. Chung, *et al.* 2004. Updates on enterovirus surveillance in Korea. *Infect. Chemother.* **36:** 294–303.
10. Knowlton, K. U., E. S. Jeon, N. Berkley, R. Wessely, and S. J. Huber. 1996. A mutation in the puff region of VP2 attenuates the myocarditic phenotype of an infectious cDNA of the Woodruff variant of coxsackievirus B3. *J. Virol.* **70:** 7811–7818.
11. Künkel, U. and E. Schreier. 2000. Genetic variability within the VP1 coding region of echovirus type 30 isolates. *Arch. Virol.* **145:** 1455–1464.
12. Lee, K. Y., D. Burgner, H. S. Lee, J. H. Hong, M. H. Lee, J. H. Kang, and B. C. Lee. 2005. The changing epidemiology of pediatric aseptic meningitis in Daejeon, Korea from 1987 to 2003. *BMC Infect. Dis.* **97:** 2–5.
13. Lee, S. T., C. S. Ki, and N. Y. Lee. 2007. Molecular characterization of enteroviruses isolated from patients with aseptic meningitis in Korea, 2005. *Arch. Virol.* **152:** 963–970.
14. Martinez, H. M. 1983. An efficient method for finding repeats in molecular sequences. *Nucleic Acids Res.* **11:** 4629–4634.
15. Mateu, M. G. 1995. Antibody recognition of picornaviruses and escape from neutralization: A structural view. *Virus Res.* **38:** 1–24.
16. Mayo, M. and C. R. Pringle. 1998. Virus taxonomy. 1997. *J. Gen. Virol.* **79:** 649–657.
17. McPhee, F., R. Zell, B. Y. Reimann, P. H. Hofschneider, and R. Kandolf. 1994. Characterization of the N-terminal part of the neutralizing antigenic site I of coxsackievirus B4 by mutation analysis of antigen chimeras. *Virus Res.* **34:** 139–151.
18. Minor, P. D. 1990. Antigenic structure of picornaviruses. *Curr. Top. Microbiol. Immunol.* **161:** 121–154.
19. Needleman, S. B. and C. D. Wunsch. 1970. A general method applicable to the search for similarities in the amino acid sequence of two proteins. *J. Mol. Biol.* **48:** 443–453.
20. Nix, W. A., M. S. Oberste, and M. A. Pallansch. 2006. Sensitive, seminested PCR amplification of VP1 sequences for direct identification of all enterovirus serotypes from original clinical specimens. *J. Clin. Microbiol.* **44:** 2698–2704.
21. Norder, H., L. Bjerregaard, and L. O. Magnius. 2001. Homotypic echoviruses share aminoterminal VP1 sequence homology applicable for typing. *J. Med. Virol.* **63:** 35–44.
22. Norder, H., L. Bjerregaard, L. Magnius, B. Lina, M. Aymard, and J. J. Chomel. 2003. Sequencing of 'untypable' enteroviruses reveals two new types, EV-77 and EV-78, within human enterovirus type B and substitutions in the BC loop of the VP1 protein for known types. *J. Gen. Virol.* **84:** 827–836.
23. Oberste, M. S., K. Maher, and M. A. Pallansch. 1998. Molecular phylogeny of all human enterovirus serotypes based on comparison of sequences at the 5' end of the region encoding VP2. *Virus Res.* **58:** 35–43.
24. Oberste, M. S., K. Maher, D. R. Kilpatrick, and M. A. Pallansch. 1999. Molecular evolution of the human enteroviruses: Correlation of serotype with VP1 sequence and application to picornavirus classification. *J. Virol.* **73:** 1941–1948.
25. Oberste, M. S., K. Maher, M. R. Flemister, G. Marchetti, D. R. Kilpatrick, and M. A. Pallansch. 2000. Comparison of classic and molecular approaches for the identification of untypeable enteroviruses. *J. Clin. Microbiol.* **38:** 1170–1174.
26. Oberste, M. S., W. A. Nix, K. Maher, and M. A. Pallansch. 2003. Improved molecular identification of enteroviruses by RT–PCR and amplicon sequencing. *J. Clin. Virol.* **26:** 375–377.
27. Oberste, M. S., K. Maher, A. J. Williams, N. Dybdahl-Sissoko, B. A. Brown, M. S. Gookin, *et al.* 2006. Species-specific RT–PCR amplification of human enteroviruses: A tool for rapid species identification of uncharacterized enteroviruses. *J. Gen. Virol.* **87:** 119–128.
28. Oh, S. H., M. S. Lee, J. H. Kang, C. H. Kim, J. Y. Park, Y. M. Shon, W. J. Lee, C. S. Chun, and S. M. Shin. 1996. Report of nationwide epidemiology of aseptic meningitis outbreaks in 1993 in Korea. *J. Korean Pediatr. Soc.* **39:** 42–52.
29. Page, R. D. 1996. TreeView: An application to display phylogenetic trees on personal computers. *Comput. Appl. Biosci.* **12:** 357–358.
30. Pallansch, M. A. and M. S. Oberste. 2003. Molecular detection and characterization of human enteroviruses, pp. 245–257. *In* A. Matsumori (ed.). *Cardiomyopathies and Heart Failure: Biomolecular, Infectious and Immune Mechanisms*. Kluwer Academic Publishers, Boston, U.S.A.
31. Pringle, C. R. 1999. Virus taxonomy at the XIth International Congress of Virology, Sydney, Australia, 1999. *Arch. Virol.* **144:** 2065–2070.
32. Pulli, T., P. K. Oskimies, and T. Hyypiä. 1995. Molecular comparison of coxsackie A virus serotypes. *Virology* **211:** 30–38.
33. Romero, J. R. 1999. Reverse-transcription polymerase chain reaction detection of the enteroviruses. *Arch. Pathol. Lab. Med.* **123:** 1161–1169.
34. Rotbart, H. A. 1995. Meningitis and encephalitis, pp. 271–289. *In* H. A. Rotbart (ed.). *Human Enterovirus Infections*. ASM Press, Washington, D. C.
35. Saitou, N. and M. Nei. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4:** 406–425.
36. Sawyer, M. H. 1999. Enterovirus infections: Diagnosis and treatment. *Pediatr. Infect. Dis. J.* **18:** 1033–1039.
37. Stirk, H. J. and J. M. Thornton. 1994. The BC loop in poliovirus coat protein VP1: An ideal acceptor site for major insertions. *Protein Eng.* **7:** 47–56.
38. Thoelen, I., P. Lemey, I. van der Donck, K. Beuselinck, A. M. Lindberg, and M. van Ranst. 2003. Molecular typing and epidemiology of enteroviruses identified from an outbreak of aseptic meningitis in Belgium during the summer of 2000. *J. Med. Virol.* **70:** 420–429.
39. Thoelen, I., E. Moës, P. Lemey, S. Mostmans, E. Wollants, A. M. Lindberg, A. M. Vandamme, and M. van Ranst. 2004. Analysis of the serotype and genotype correlation of VP1 and the 5' noncoding region in an epidemiological survey of the human enterovirus B species. *J. Clin. Microbiol.* **42:** 963–971.
40. Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. Clustal W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22:** 4673–4680.
41. Zoll, G. J., W. J. Melchers, H. Kopecka, G. Jambroes, H. J. van der Poel, and J. M. Galama. 1992. General primer-mediated polymerase chain reaction for detection of enteroviruses: Application for diagnostic routine and persistent infections. *J. Clin. Microbiol.* **30:** 160–165.