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# Molecular Characterization of Echovirus 30-Associated Outbreak of Aseptic Meningitis in Korea in 2008

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**Evaluation of the primary etiologic agents that cause aseptic meningitis outbreaks may provide valuable information regarding the prevention and management of aseptic meningitis. In Korea, an outbreak of aseptic meningitis caused by echovirus type 30 (E30) occurred from May to October in 2008. In order to determine the etiologic agent, CSF and/or stool specimens from 140 children hospitalized for aseptic meningitis at Soonchunhyang University Cheonan Hospital between June and October of 2008 were tested for virus isolation and identification. E30 accounted for 61.7% (37 cases) and echovirus 6 accounted for 21.7% (13 cases) of all the human enteroviruses (HEVs) isolates (60 cases in total). For the molecular characterization of the isolates, the VP1 gene sequence of 18 Korean E30 isolates was compared pairwise using the MegAlign with 34 reference strains from the GenBank database. The pairwise comparison of the nucleotide sequences of the VP1 genes demonstrated that the sequences of the Korean strains differed from those of lineage groups A, B, C, D, E, F, and G. Reconstruction of the phylogenetic tree based on the complete VP1 nucleotide sequences resulted in a monophyletic tree, with eight clustered lineage groups. All Korean isolates were segregated from other lineage groups, thus suggesting that the Korean strains were a distinct lineage of E30, and a probable cause of this outbreak. This manuscript is the first report, to the best of our knowledge, of the molecular characteristics of E30 strains associated with an aseptic meningitis outbreak in Korea, and their respective phylogenetic relationships.**

**Keywords:** Enterovirus, echovirus 30, VP1 gene, phylogenetic tree

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Aseptic meningitis is a clinical syndrome characterized by meningeal inflammation that is not caused by any identifiable bacterial pathogen in the cerebrospinal fluid (CSF) [5]. Human enteroviruses (HEVs), small RNA viruses belonging to the *Picornaviridae* family, have been identified as the major etiologic agent of aseptic meningitis. Over 90% of viral meningitis cases are caused by HEVs, and more than 10,000 cases of aseptic meningitis are reported annually to The Centers of Disease Control and Prevention [5, 20]. HEVs can be transmitted by fecal–oral and respiratory routes, as well as by indirect contact such as *via* fomites and contaminated water [9].

Echovirus 30 (E30), one of the distinct serotypes of HEVs, is a commonly isolated agent that causes sporadic to large outbreaks of aseptic meningitis in many regions of the world. In the last 15 years, multiple outbreaks and nationwide epidemics related to E30 have occurred worldwide [1, 9, 26, 29]. In the U.S.A., E30 was a primary cause of meningitis outbreaks in 2003 and 2004 [9, 12]. Moreover, several outbreaks of E30-associated meningitis have occurred in Asia in the last decade – in Taiwan (2001), China (2003 and 2004), and Japan (2004 and 2006) [1, 26, 27, 29]. In Korea, several outbreaks of HEV-associated meningitis have been reported since 1993, but E30 was the sole cause of the aseptic meningitis outbreak in 1997 [4, 17].

HEVs are non-enveloped viruses with a single positive-stranded RNA genome of approximately 7.5 kb. Sixty-eight distinct serotypes of HEVs have been identified among more than 90 serotypes [9]. These viruses are genetically classified into four species: HEV-A, HEV-B, HEV-C, and HEV-D. The capsids of HEVs are composed of four structural proteins (VP1, VP2, VP3, and VP4) and VP1–VP3 proteins are partially exposed on the capsid surface [18]. The VP1 region, which is one of the main exposed regions of the viral capsids and the most variable

region of the genome, provides the virus with distinct antigenic properties. In order to determine the genetic relationship between the E30 isolates and any known enterovirus serotypes, the partial VP1 sequences have been compared with a database of complete enterovirus VP1 sequences of all E30 viruses [3, 13, 14]. Additionally, phylogenetic analyses of sequence data of the VP1 region have been used extensively in molecular evaluations for epidemiology investigations [7, 8, 15]. In previous studies based on VP1 sequence analyses, E30 has evidenced a pattern of monophyletic evolution in which lineage displacement is correlated with the temporal dynamics of strains of this serotype [12, 13, 16, 22].

E30 appears to circulate widely throughout Asia. Molecular characterizations are important to the epidemiology of E30; the determination of the VP1 sequences for isolated strains during an E30 meningitis outbreak may generate valuable information regarding enteroviral epidemiology. However, little information is currently available regarding the molecular characteristics of E30-associated aseptic meningitis in Korea. In this study, we have evaluated the genetic diversity and molecular characteristics of the isolates *via* sequence analysis of the VP1 regions of the viral genome. This database is anticipated to prove useful for molecular epidemiologic investigations of echovirus disease outbreaks.

## Materials and Methods

### Specimens Collection and Virus Isolation

Clinical specimens were obtained between June and October of 2008 from patients admitted to the Department of Pediatrics at the Soonchunhyang University Cheonan Hospital, Korea. The CSF and/or stool specimens were collected in sterile containers from children who had been hospitalized for aseptic meningitis. All specimens were centrifuged for 20 min at 3,000 ×*g* at 4°C and were filtered prior to inoculation into the cell culture.

Viral isolation was conducted using susceptible cell lines such as rhabdomyosarcoma (Rd), Vero, and Buffalo green monkey (BGM) cells. Each culture tube containing susceptible cell lines in Eagle's minimum essential medium was inoculated with 0.2 ml of clinical specimens and then incubated at 37°C with 5% $CO_2$ until the appearance of enterovirus-like cytopathic effects or until 14 days after inoculation with no detectable cytopathic effects.

### RT–PCR and Sequencing

The infected cells, in which a 70% cytopathic effect was noted, were frozen and thawed three times for viral RNA extraction. The viral RNA was extracted from the supernatants of the infected cells using Magnetic-beads (Toyobo, Japan). The extracted RNA was then resuspended in 50 µl of distilled water and stored at −70°C until use in RT–PCR.

The RT–PCR for the VP1 gene of E30 was conducted in a single step with Ready2Use RT–PCR one-step redplus mastermix (GenDEPOT, CA, U.S.A.). The RT–PCR mixture (50 µl) consisted of 10 µl of RNA template; 25 µl of 2× reaction mixture containing 0.2 mM dNTP mix and 1.5 mM $MgCl_2$ and GenDEPOT's magic buffer optimized for one-step RT–PCR, and thermostable *Taq* DNA polymerase with red dye (1.5 U/rxns); 10 µM (each) primers E30-2490F (5'-AAA GTG CAC TCA ACA AG-3') and FD3KR (5'-GAG GAT TAT GGG TTG ATC G-3') [30]; and 1 µl of AMV reverse transcriptase (1 U/µl). Reverse transcription was conducted for 90 min at 42°C followed by 5 min of denaturation at 95°C and 35 cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min. A final extension step was performed for 7 min at 72°C.

The PCR products were gel-isolated by electrophoresis and purified using the QIAquick PCR purification kit (Qiagen, Germany). In

**Table 1.** E30 isolate used for phylogenetic analysis.

| Isolation | Sampling date | Specimen | Patient age | Clinical symptom | Access. No. |
|---|---|---|---|---|---|
| kor08-ECV30-01cn | 16-Jun-08 | Stool | 4.2 | Fever | FJ848344 |
| kor08-ECV30-02cn | 19-Jun-08 | Stool | 0.3 | Fever | FJ848345 |
| kor08-ECV30-03cn | 20-Jun-08 | Stool | 9.7 | Headache | FJ848346 |
| kor08-ECV30-04cn | 24-Jun-08 | CSF | 11.6 | Headache | FJ848347 |
| kor08-ECV30-05cn | 29-Jun-08 | CSF | 9.7 | Headache | FJ848348 |
| kor08-ECV30-06cn | 26-Jun-08 | Stool | 6.7 | Headache | FJ848349 |
| kor08-ECV30-08cn | 8-Jul-08 | CSF | 8.3 | Headache | FJ848350 |
| kor08-ECV30-09cn | 11-Jul-08 | CSF | 12.2 | Headache, fever, vomiting | FJ848351 |
| kor08-ECV30-10cn | 15-Jul-08 | Stool | 6.1 | Headache | FJ848352 |
| kor08-ECV30-11cn | 15-Jul-08 | CSF | 3.0 | Fever | FJ848353 |
| kor08-ECV30-12cn | 15-Jul-08 | Stool | 3.7 | Headache | FJ848354 |
| kor08-ECV30-13cn | 17-Jul-08 | CSF | 3.6 | Headache | FJ848355 |
| kor08-ECV30-14cn | 28-Jul-08 | Stool | 8.7 | Headache | FJ848356 |
| kor08-ECV30-15cn | 25-Jul-08 | Stool | 4.2 | Headache | FJ848357 |
| kor08-ECV30-16cn | 3-Aug-08 | Stool | 3.5 | Fever | FJ848358 |
| kor08-ECV30-17cn | 31-Jul-08 | Stool | 6.9 | Fever | FJ848359 |
| kor08-ECV30-19cn | 8-Aug-08 | Stool | 0.2 | Fever | FJ848360 |
| kor08-ECV30-20cn | 22-Aug-08 | Stool | 5.4 | Headache | FJ848361 |

brief, 30 to 90 ng of purified DNA and 2 pmoles of E30-2490F and FD3KR primer were mixed in a reaction tube containing 2 μl of Big Dye terminator reaction mix (ABI Prism BigDye Terminator Cycle Sequencing Kit; Perkin-Elmer Applied Biosystems, U.S.A.). After the reaction, the products were purified *via* precipitation with 100% cold ethanol and 3 M sodium-acetate (pH 5.8), and then loaded onto an automated 3100 Genetic Analyzer (Applied Biosystems).

### Sequence Analysis

For sequence analysis, 18 E30 isolates were selected randomly from aseptic meningitis patients (Table 1). Nucleotide and deduced amino acid sequences of the E30 isolates were compared with the reference strains using CLUSTAL X (version 1.81) and Megalign (DNASTAR) [24]. The phylogenetic relationships among the VP1 sequences of each of the viral isolates could be inferred *via* the neighbor-joining method as implemented in the CLUSTAL X program [21]. The resultant trees were constructed with MEGA (version 4.0).

### Nucleotide Sequence Accession Numbers

The E30 candidates sequences reported here were deposited in the GenBank sequence database under accession numbers FJ848344 to FJ848361 (Table 1).

## RESULTS

### General Characteristics of patients and HEVs Identification

An outbreak of aseptic meningitis occurred in Korea from May to October of 2008. During that period, hospitalization (298 cases) for aseptic meningitis increased markedly over the same period in the previous year (65 cases, May to October in 2007) at the Soonchunhyang University Cheonan Hospital in Korea. The majority of patients (88%) were younger than 16 years of age and had been admitted to the pediatric department. The sex ratio of male to female was 1.89:1 (195:103). The most frequent clinical manifestations were fever, headache, and vomiting.

In order to determine the etiologic agent, CSF and/or stool specimens from 140 hospitalized children with aseptic meningitis at the Soonchunhyang University Cheonan Hospital between June and October of 2008 were examined for the purposes of virus isolation and identification. E30 accounted for 61.7% (37 cases) and echovirus 6 accounted for 21.7% (13 cases) of all HEV isolates (60 cases). The age of the E30-infected patients ranged between 2 months and 13 years and the median age was 5.8 years. The ratio of males to females was 1.31 (21:16). Among the 103 stool samples, E30 was isolated in 33 samples and echovirus 6 in 11 samples. Nine E30 isolates and five echovirus 6 isolates were obtained from 83 CSF specimens (Table 2).

**Table 2.** Enterovirus types isolated from patients with aseptic meningitis between June and October in 2008.

| Virus type | No. of case (%) | No. of isolated (isolation rate, %) according to specimens | |
|---|---|---|---|
| | | Stool, n=103 | CSF, n=83 |
| E30 | 37 (61.7) | 33(32.0) | 9(10.5) |
| Echovirus 6 | 13 (21.7) | 11(10.7) | 5(5.8) |
| Echovirus 7 | 2 (3.3) | 2(1.9) | |
| Echovirus 9 | 1 (1.7) | 1(1.0) | |
| Echovirus 16 | 1 (1.7) | 1(1.0) | |
| Coxsackievirus B3 | 1 (1.7) | 1(1.0) | |
| Coxsackievirus B1 | 1 (1.7) | 1(1.0) | |
| Coxsackievirus A4 | 1 (1.7) | 1(1.0) | |
| Untypable enterovirus | 3 (5.0) | 3(2.9) | |
| Total | 60 (100) | 54(52.4) | 14(16.3) |

### Sequence Analysis of VP1 Gene

In order to analyze the genetic characteristics of E30, 18 strains isolated from aseptic meningitis patients were selected and examined, and are listed in Table 1. The VP1 sequences of 18 Korean isolates were determined and compared with 34 reference strains from the GenBank

**Table 3.** Percentage divergence of nucleotide and amino acid sequence of the VP1 coding region (nt 2,481–3,308) among eight E30 lineage groups (A–E, except for E30 prototype).

| | A | B | C | D | E | F | G | H[a] |
|---|---|---|---|---|---|---|---|---|
| A | *** | 7.0–9.2 | 7.4–12.1 | 8.5–10.7 | 11.5–12.7 | 10.0–11.8 | 9.8–13.4 | 10.6–11.8 |
| B | 1.8–2.9 | *** | 4.7–8.1 | 5.8–6.9 | 10.4–12.0 | 8.0–9.8 | 8.0–11.8 | 10.4–11.8 |
| C | 1.4–3.3 | 0.7–3.3 | *** | 4.0–17.5 | 9.8–11.7 | 6.0–9.3 | 5.9–10.9 | 9.3–12.6 |
| D | 2.5–3.6 | 1.4–2.9 | 0.7–2.9 | *** | 9.2–11.2 | 5.6–7.2 | 6.9–9.9 | 9.1–10.5 |
| E | 2.5–4.3 | 1.8–3.3 | 1.4–4.0 | 2.2–2.5 | *** | 9.5–11.6 | 10.7–12.8 | 11.7–13.5 |
| F | 2.2–4.0 | 0.7–2.9 | 0.4–3.3 | 0.7–1.4 | 1.4–2.9 | *** | 8.9–11.7 | 8.8–11.2 |
| G | 2.5–4.0 | 1.4–3.3 | 1.1–4.0 | 1.1–2.5 | 1.1–2.2 | 1.1–2.5 | *** | 12.0–13.9 |
| H[a] | 2.2–2.9 | 1.8–.3.6 | 1.1–3.3 | 1.1–2.2 | 2.5–3.6 | 1.1–2.2 | 1.8–2.9 | *** |

Sequence were compared pairwise using the MegAlign (DNASTAR, Software, WI, U.S.A.), and the percentage nucleotide and amino acid divergence values for the pairs of strains are shown. The upper triangle of the table represents nucleotide divergence, and the lower triangle of the table represents amino acid divergence.

[a]Korean isolates group.

database. The identity of the nucleotide sequence for the VP1 region within 18 E30 isolates was quite high (98.2~99.9%) in the 876 bp. The nucleotide sequence of Kor08-ECV30 differed from the Group G lineage of E30 isolated in Russia between 1999~2001 and in China in 2003, by 12.0% to 13.9%. The differences in the deduced amino acid sequence between the Korean isolates and the Group G lineage were 1.8~2.9%. Pairwise comparison of the nucleotide sequences of the VP1 genes demonstrated that the sequences of the Korean strains differed from those of the isolates of lineage groups A, B, C, D, E, and F by 10.6% to 11.8%, 10.4% to 11.8%, 9.3% to 12.6%, 9.1% to 10.5%, 11.7% to 13.5%, and 8.8% to 11.2%, respectively (Table 3). None of the Korean isolates harbored substituted amino acids within the BC loop that were associated with the reactivity of type-specific antibodies. However, on the amino acid sequence comparison of the VP1 region of the Korean isolates to the reference strains, all of the Korean isolates were substituted at the position 9 amino acid (R9K) and 133 (T133V) (Fig. 1). Nucleotide sequences encoding for VP1 (nt 2,481–3,308) were analyzed *via* the neighbor-joining methods and the trees were constructed using the MEGA software package, version 4.0. The reconstruction of the phylogenetic tree based on the complete VP1 nucleotide sequences of the Korean isolates and the E30 reference strains evidenced a monophyletic VP1 tree (Fig. 2). According to the clusters formed *via* phylogenetic analysis, E30 was clustered into eight lineage groups, consistent with previously published E30 phylogenies [30]. All Korean isolates were segregated from the other

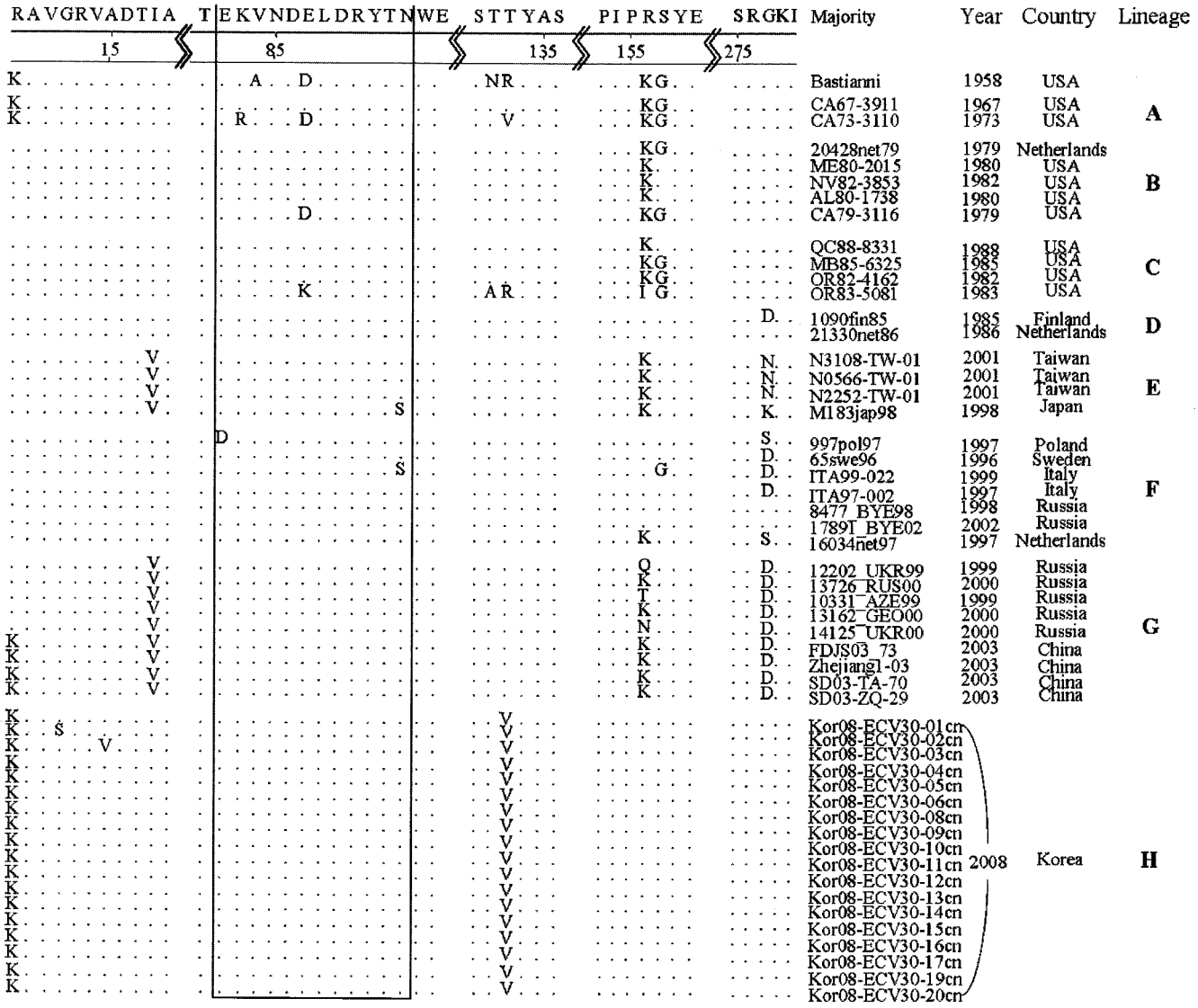


**Fig. 1.** Comparison of the deduced amino acid sequences of 52 E30 strains in the VP1 region.
The BC loop is boxed.

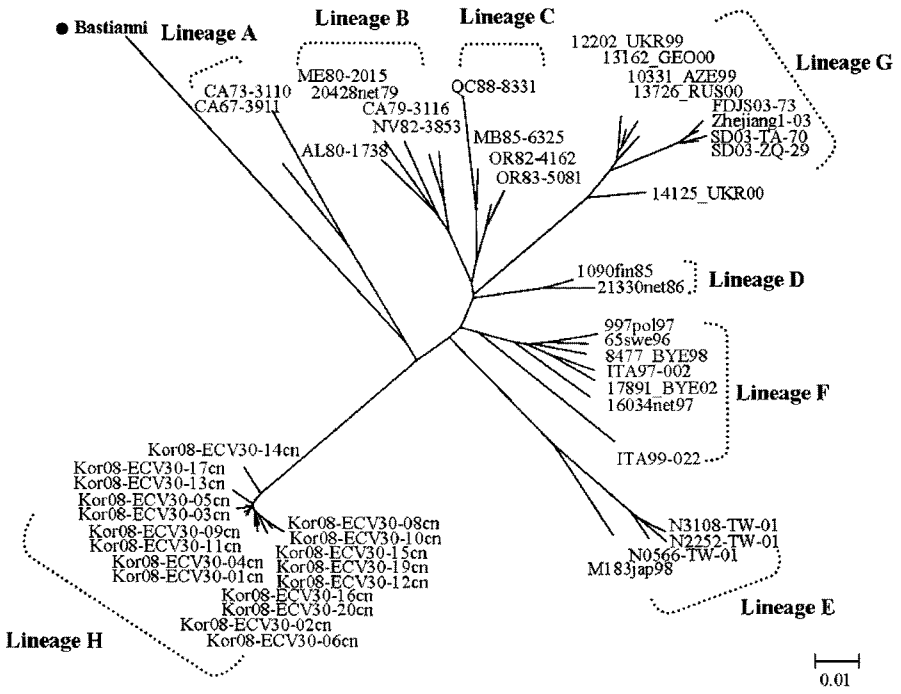


**Fig. 2.** Phylogenetic analysis of Korean isolates and reference strains of E30.
Nucleotide sequences encoding for the VP1 (nt 2,481–3,308) were analyzed *via* the neighbor-joining methods and the trees were constructed using the MEGA, version 4.0, software package. The nucleotide position is relative to the Bastianni strain (AF311938) sequence. ●; Bastianni strain of E30 prototype.

previously reported lineage groups, thus suggesting that the Korean strains were a distinct lineage of E30, and may plausibly have been the cause of this outbreak.

## DISCUSSION

Since echoviruses were initially isolated from the stools of asymptomatic individuals in 1951 [19], many researchers have asserted that echoviruses are associated with a variety of human diseases, including asymptomatic infections, febrile illness, aseptic meningitis, and severe diseases in newborns [10]. E30, a member of the HEV-Bs, has caused a number of outbreaks of aseptic meningitis in many different countries. Several outbreaks of E30-associated meningitis have been reported in Asia over the past decade. In Korea, a variety of enteroviral epidemics have been reported since 1993 [6]. A large outbreak of aseptic meningitis was induced by echovirus 9 between April and August of 1993 in Korea, and several outbreaks were reported later. Since the E30-associated viral meningitis outbreak of 1997 occurred in Korea, no outbreak of E30-associated meningitis was again detected until April of 2008. In 2008, E30 (60%) and echovirus 6 (30%) caused large outbreaks of aseptic meningitis throughout South Korea from May to August, as reported by the Division of Enteric and Hepatitis Viruses, National Institutes of Health in Seoul, Korea [23]. We also observed that, during the same period, the hospitalization of aseptic meningitis patients increased more than 4-fold that of the previous year at the Soonchunhyang University Cheonan Hospital. In our etiologic evaluation, E30 was the principal isolate from 140 children hospitalized for aseptic meningitis, which suggests that E30 was in fact the main etiologic agent of this outbreak.

Many previous studies have demonstrated the genetic diversity among strains of the E30 serotype [12, 14, 16]. Molecular investigations based on VP1 sequence analysis demonstrated that the genetic diversity of E30 has been characterized by sequential displacements among multiple genetic variants [9]. Point mutations of E30, which have been associated with meningitis outbreaks, involve substantial genetic diversity in the gene encoding for the VP1 polypeptide [2, 25, 26, 28]. Whereas previous molecular epidemiological investigations have suggested that different E30 variants can exist among circulating strains in local meningitis outbreaks and that a given variant can be associated with several outbreaks in distant geographical areas, each outbreak was generally associated with only one or a few viral variants [14, 22]. Although some studies have reported that genotypes of E30 bearing distinct amino acid variations may possibly have been associated with different antigenic patterns, opinions as to how they should be defined are still a matter of controversy among many investigators [16, 22, 23]. The genetic diversity of E30 provided us with different classifications of the genetic

variants of E30. Recent phylogeography investigations of human E30 have demonstrated that seven viral lineages were closely related to the time period in which they were isolated [9, 16, 30]. Over the past 10 years, the groups E, F and G lineages of E30 included the most divergent viruses thus far isolated. The G lineage E30, which was initially isolated in the Community of Independent States in 1999, caused aseptic meningitis outbreaks in the Chinese provinces of Zhejiang and Shandong during 2002–2004, and viruses in the E30 E lineage circulated throughout Japan in 1997–1998 and throughout Taiwan in 2000–2001.

The principal objective of this study was to conduct a molecular characterization of E30 circulating in Korea in 2008. Owing to the paucity of information regarding the VP1 sequence of E30 isolated in Korea in 1997, we were unable to assess genetic diversity among E30 strains isolated in Korea in 1997 and 2008. Pairwise comparisons of VP1 sequences and phylogenetic tree analysis demonstrate that a distinct lineage of E30 strains isolated from Korea in 2008 differs from the E30 strains isolated previously in Asia. The nucleotide sequence of the Korean isolate differed from the G lineage of E30 isolated in China in 2003 and the E lineage of E30 isolated in Taiwan in 2001 by 1.8% to 2.9% and 2.5% to 3.6%, respectively. The BC loop within the VP1 region was identified as crucial for the reactivity of type-specific antibodies [11]. None of the Korean isolates harbored substituted amino acids within this region, but all of the Korean isolates were substituted at the position 9 amino acid (R9K) and 133 (T133V). Reconstruction of the phylogenetic tree based on the complete VP1 nucleotide sequences of Korean isolates and the reference strains of E30 resulted in a monophyletic tree, with eight clustered lineage groups. All Korean isolates were segregated from other previously reported lineage groups.

Consequently, E30 strains, the VP1 sequences of which are distinct from strains previously isolated from Asia, appear to have been responsible for an outbreak of aseptic meningitis in Korea in 2008. This manuscript is the first report of the molecular characteristics of E30 strains associated with the outbreak of aseptic meningitis in Korea and their respective phylogenetic relationships. As the E30 surveillance data for Korea are incomplete, we were unable to provide a comprehensive description of the genetic diversity and dynamics of E30 in Korea. Thus, it is necessary to establish an enteroviral molecular surveillance system in Korea in order to gain a better understanding of viral transmission and evolution.

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