Korean Journal of Pediatrics Vol. 52, No. 11, 2009
DOI : 10.3345/kjp.2009.52.11.1234
■ Original article : Clinical study ■

Molecular identification and clinical features of enteroviral infection in children of central Korea: An overview of enteroviral epidemiology between spring 2005 and autumn 2006

Eui Jung Roh, M.D., Yong Man Jin, M.D.*, Eun Hee Chung, M.D.*, Young Pyo Chang, M.D.*, Woo Sung Park, M.D.*, Kwisung Park†, and Young Mee Jee, M.D.‡

Department of Pediatrics, College of Medicine, Konyang University Daejeon,
Department of Pediatrics*, College of Medicine, Dankook University, Cheonan, Chungcheongnam-Do
Health and Environment Research Institute†, Daejeon,
Division of Enteric and Hepatitis Viruses, Department of Virology‡, National Institute of Health,
Korea Center for Disease Control and Prevention, Seoul, Korea

= Abstract =

Purpose : Enteroviruses (EVs) are commonly known to cause infection, especially in infants and children. This report presents an overview of enterovirus epidemiology in central Korea.

Methods : From the spring of 2005 to the autumn of 2006, we collected the cerebrospinal fluid (CSF) and stool samples from the pediatric patients with a febrile illness or suspected meningitis who were admitted to hospitals in central Korea. In order to test for EVs, cell lines were derived from pretreated susceptible specimen, and the cytopathic effects were observed. Seminested real time-polymerase chain reaction (RT-PCR) and direct sequencing were performed for genotypic and phylogenetic analyses.

Results : Of the 305 patients examined, 51 (16.7%) tested positive for EV. Of these 51 patients, 44 showed the following serotypes: Echovirus (ECV) 18 (18 cases, 35.2%), Coxsackievirus B (CVB) 5 (13 cases, 25.4%), ECV25 (5 cases, 9.8%), ECV9 (4 cases, 7.8%), ECV5 (3 cases, 5.8%), and EV74 (1 case, 1.9%). In 2005, between June and August, ECV18 and CVB5 were mostly responsible for the enteroviral infections among the patients in central Korea. In 2006, between July and August, ECV25 was mostly the cause of enteroviral infection.

Conclusions : There is a need for continuous surveillance of enteroviral infection and its clinical manifestations, particularly for EV74, which was first identified in Korea. **(Korean J Pediatr 2009;52:1234-1240)**

Key Words : Children, Enterovirus, RT-PCR, Enterovirus 74

Introduction

Enterovirus, a member of the *Picornoviridae* family, is a 7.4 kb single-stranded positive-sense RNA non-enveloped virus. Presently, there are about 70 serotypes including poliovirus, coxsackievirus A (CVA), coxsackievirus B (CVB), echovirus (ECV) as well as other types of enterovirus (EV). Most EV infections are asymptomatic or result in only mild symptoms, e.g. non-specific febrile illness or mild upper respiratory symptoms. In addition, EVs can cause a wide variety of other clinical illnesses, including acute haemorrhagic conjunctivitis, aseptic meningitis, undifferentiated rashes, acute flaccid paralysis, myocarditis and neonatal sepsis-like disease[1].

The standard method for isolation of EV is with the use of a special neutralizing antibody after culturing a specimen. Several molecular methods have been developed, and reverse transcription-polymerase chain reaction (RT-PCR) assays with primers designed to target the 5'noncoding region (NCR) or VP1 region of the enterovirus genome can rapidly detect the presence of EVs[2-4]. The most variable regions of the genes encoding the capsid proteins VP1, VP2 and VP3 are exposed partially on the virion surface.

Received : 11 May 2009, Revised : 14 August 2009
Accepted : 5 October 2009
Address for correspondence : Eun Hee Chung, M.D., Ph.D.
Department of Pediatrics, College of Medicine, Dankook University,
Anseo-dong San 16-5 Cheonan, Chungcheongnam-Do, 330-715, Korea
Tel : +82-41-550-3935, Fax : +82.41-565-6167
E-mail : ehchung@dankook.ac.kr

Because the VP1 gene contains major antigenic sites as well as receptor recognition sequences, the VP1 sequence reflects the serotype of the EV. Sequence analysis of the VP1 region is thus useful for molecular epidemiological studies of EV disease outbreaks[5, 6].

The purpose of this study was to evaluate the recent epidemiology of EV in the central region of Korea to provide vital statistics for future diagnosis and prevention of EV infection.

Materials and methods

1. EVs isolation

EVs were isolated from clinical specimens (CSF, stool) that were obtained from hospitalized pediatric patients with febrile illness or suspected meningitis at Dankook university hospital from April 2005 to October 2006 by using susceptible cell lines such as rhabdomyosarcoma (Rd), Vero and buffalo green monkey (BGM) cells. Determination of their genotype was carried out by sequence analysis of the VP1 gene.

2. RNA extraction and seminested RT-PCR

After three cycles of freezing and thawing of the cells showing 70% cytopathic effect, viral RNA was extracted from the supernatant of infected cells using the TRI-reagent (Molecular Research Center, Inc., Cincinnati, USA). Extracted RNA was dissolved in 10 μL of nuclease-free water and then stored at 70°C until used for the reverse transcription reaction.

For cDNA synthesis, a 20 μL reaction mixture comprising of 5 μL of each viral RNA, 4 μL of 5 transcriptase buffers, 2 μL of 0.1 M DTT, 4 μL of 10 mM dNTP mix (Invitrogen, Carlsbad, USA) and 200 U of M-MLV reverse transcriptase (Invitrogen, Carlsbad, USA) were used. The reaction mixture was incubated at 20°C for 10 min, 37°C for 120 min, 95°C for 5 min and then chilled on ice. Seminested RT-RCR was performed as described by Nix et al[4].

3. Nucleotide Sequencing and Genetic Analysis

The PCR products were purified using the QIA quick PCR purification kit (Qiagen, Hilden, Germany). Purified DNA was added in a reaction mixture containing 2 μL of Big Dye terminator reaction mix (ABI Prism BigDye Terminator Cycle Sequencing Kit; Perkin-Elmer Applied Biosystems, Inc., Foster City, USA) and 2 pmoles of sense or antisense primer. Sequencing reactions were subjected to the initial denaturation at 9°C for 1 min and 25 cycles consisting of 96°C for 10 sec, 50°C for 5 sec, and 60°C for 4 min in a Gene Amp PCR system 2700 (Applied Biosystems). The products were purified by precipitating them with 100% cold ethanol and 3 M sodium-acetate (pH 5.8) and then loaded on an automated 3100 Genetic Analyzer (Applied Biosystems).

Sequencing data were aligned using the program SeqMan (DNASTAR, Software, Madison, WI, USA). Nucleotide sequences of the VP1 coding region of ECV18 isolates were compared with the prototype strains by the Clustal-W methods and a phylogenetic tree was constructed from the alignments by Megalign (DNASTAR, Software).

Results

1. Enterovirus isolation and RT-PCR

After pretreatment of the RD cell, Vero cell and BGM cell samples were inoculated and the cytopathic effects observed seminested RT-PCR was carried out on the VP1 portions using cell culture fluid.

From April 2005 to October 2006, 51 patients tested positive for EV in CSF and stool samples collected from 305 patients. Among 305 patients, CSF was collected from 279 patients and stool samples were collected from 173 patients. Of 279 CSF samples, 13 (4.6%) were positive of 173 stool samples, 41 (21.7%) were positive for EV. In 2005, among 152 CSF samples, 12 (7.9%) tested positive for EV; for 96 stool samples, 27 (28.1%) tested positive. In 2006, among 127 CSF samples, 1 (0.78%) tested positive for EV; for 77 stool samples, 14 (18.2%) tested positive.

2. Clinical manifestations and serotypes of EV

Among 51 patients that tested positive for EV, 40 were less than 4 months of age, and 11 were greater then 4 months of age. The average age was 1.7 years. The average age for patients less than 4 months was 1.9 months, and the average age for patients greater than 4 months was 7.7 years. There were 32 males and 19 females, males outnumbered females by 1.7 times.

Of 51 patients that tested positive for EV, 18 cases

(35.2%) were ECV18, 13 cases (25.4%) were CVB5, 5 cases were ECV25, 4 cases (7.8%) were ECV9, 3 cases (5.8%) were ECV5 and 1 case (1.9%) was EV74.

In 2005, ECV18 (50%) and CVB5 (27.8%) were the most common serotype. Among all EV types identified, 83.4% were found between June and August. In 2006, ECV25 (31.3%), CVB5 (18.8%) and ECV5 (18.8%) were the most common serotype. Among all EV types identified, 86.6% were found between July and August (Fig. 1).

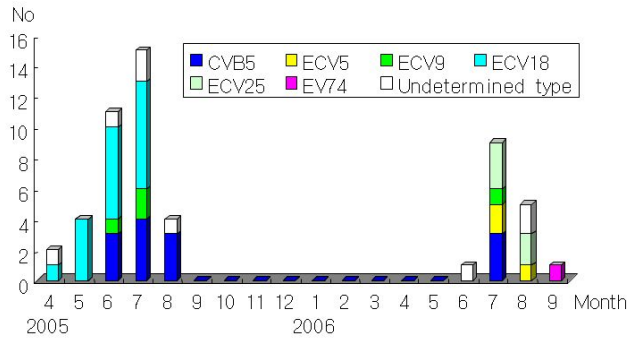

Fig. 1. Monthly distribution of enterovirus-infected children in central Korea. CVB5, Coxsackie virus B5; ECV, Echovirus; EV, Enterovirus.

Clinical symptoms were recorded only in the cases where patients were able to express themselves. Among the 51 patients that tested positive for EV all except one case, 98%, presented with a fever of 11 patients with headache, 98.9% or 10 cases were positive for EV. In addition, diarrhea was found in 37.3% (19/51), abdominal pain in 36.4%, (4/12), poor oral intake in 34.6% (17/51), vomiting in 27.5% (14/51), rash in 17.6% (9/51), cough in 7.8% (4/51), rhinorrhea in 5.9% (3/51), and seizures in 2.0% (1/51) were the most common symptoms in patients with enteroviral infections. When comparing the frequency of symptoms by the most common EV serotypes, there were no siginificant differences observed. EV74 was initially isolated from the patient's stool, in a 14-year-old boy that presented with headache and fever. His neurolo-

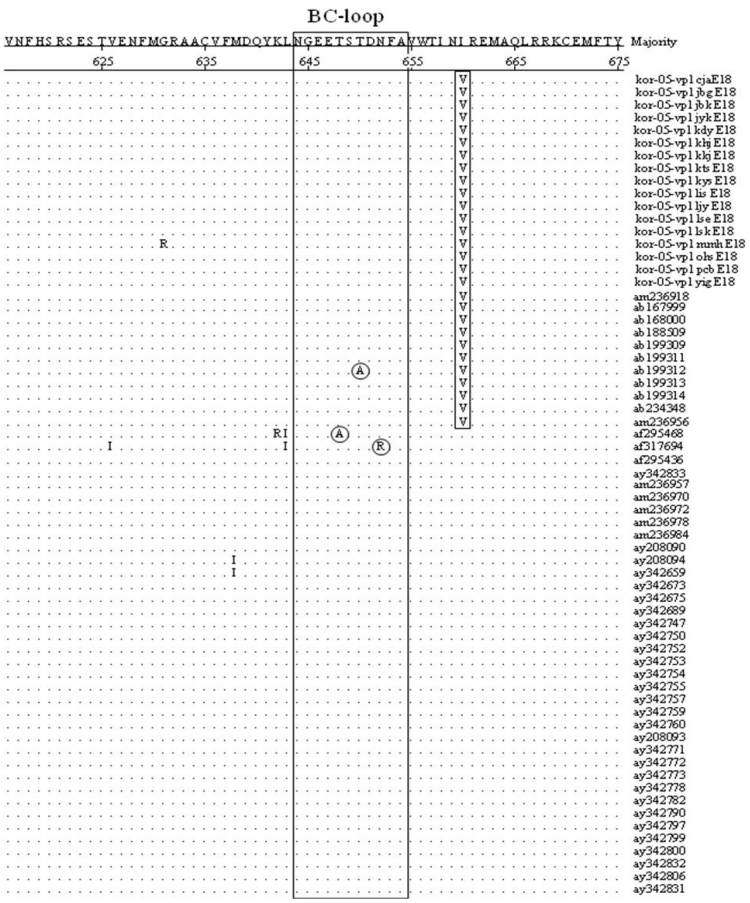

Fig. 2. Comparison of the amino acid sequences of the VP1 gene shows the presence of a BC-loop region in about 65 ECV18 strains.

gical examination was unremarkable and the CSF analysis showed no evidence of meningitis. The patient was hospitalized for 4 days and improved with conservative care.

3. Phylogenetic analysis of ECV18 and EV74

We analyzed the VP1 sequences of ECV18 that were isolated in central area of Korea. This data was compared with representative strains in the GenBank database (Fig. 2). From the amino acid sequence of the ECV strains, there was one amino acid substitution found at 660I to V. The Korean strains of ECV18 showed 98.4–100% nucleotide sequence identity with one another and 80.2–91.5% identity with other foreign isolates of the intra-types. Phylogenetic relationships of ECV18 were studied and the genetic diversity investigated (Fig. 3). Basis on the sequence variations, the ECV18 strains contained four genogroups and the Korean strains could be grouped into genogroup-III.

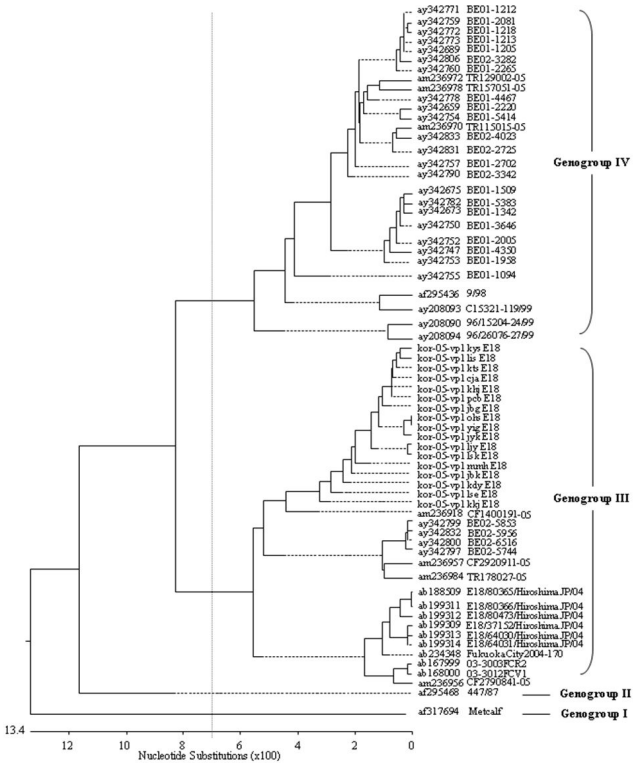

Fig. 3. Phylogenetic analysis made on the basis of the VP1 gene of the Korean ECV18 strains and that of the foreign strains.

The VP1 gene sequence, of the Korean EV74 isolate shared 81.0–84.2% nucleotide and 96.5–98.2% amino acid sequence identities with those of the foreign EV74 strains. The closest relationships were observed with the Bangladesh strain (BAN00-10217) isolated in 2000, respectively (Fig. 4).

DISCUSSION

A national surveillance program for enterovirus was started in Korea in 1991. Since then, many EV outbreaks have been reported, and several new serotypes of EV have been isolated[7]. For example, an ECV9 epidemic was reported in 1993, CVB1 and ECV9 were isolated in 1996, ECV30 in 1997, EV71 in 1999, and CVB5 in 2001. In 2002, ECV13 and CVA9 were first isolated from a patient with aseptic meningitis and CVA24 was first isolated from

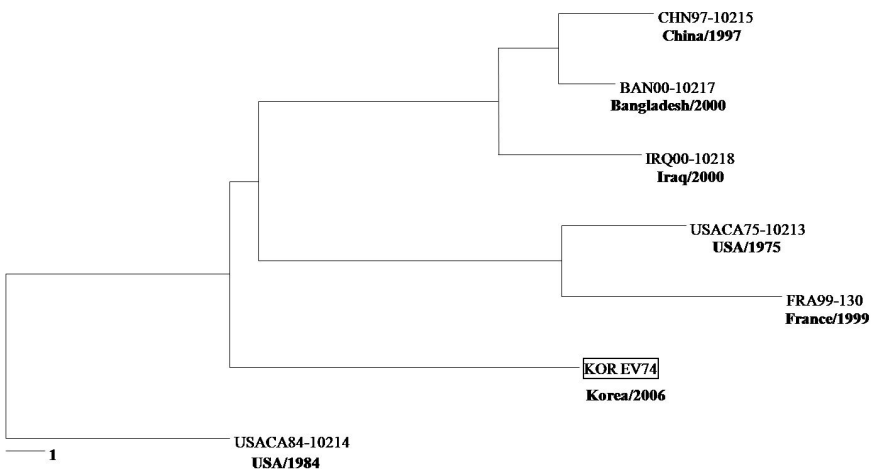

Fig. 4. Phylogenetic relationships between the Korean EV74 strains isolated in 2006, determined on the basis of a partial sequence of the VP1 gene. Sequences of the strains used for comparison were obtained from the GenBank database.

patients with keratoconjunctivitis[3, 7]. These epidemics of EVs in Korea were similar to epidemics in other countries with a climate similar to that of Korea. However, within Korea, EV detection can differ in each province, even within the same year[7].

In this study, enteroviruses were isolated from April to August in 2005 and from June to September in 2006. Most of the viruses were isolated from June through August. In this study, ECV18 and CVB5 were mainly isolated from June to August in 2005 and ECV25 was mainly isolated from July to August in 2006. In particular, ECV18 was first isolated in Korea after the initiation of the national surveillance program for EV. Similar serotype epidemics were reported in Japan[8, 9], and other countries[10, 11] at more or less the same time.

In 2005, the phylogenetic analysis showed a 98.2–100% homology of the Korean ECV18 strain. However, foreign strains showed 10–21% diversity in 2005. A very close homology between Japanese strains isolated in 2004 and Korean strains isolated in 2005 was noted (Fig. 2, 3). It is likely that there was propagation of the EVs between the countries.

The BC–loop is one of the regions associated with viral antigenicity, and substitutions resulting in conformational changes in this region are believed to play a role in host adaptation to the picornaviruses. Mutants with only one or a few amino acid changes might be at least partially resistant to neutralization by the sera from natural hosts[12, 13]. Compared to the BC–loop regions of the 17, ECV18 strains and the representative ECV18 strains isolated elsewhere, except for the three strains (ab199312, af295468, af317694), usually had the same amino acid sequences. In the VP1 region, the amino acid sequence of ECV18 strains had one amino acid substitution found at 660I to V; this was assumed to be a point mutation that was carried over from 2003.

Sequencing of the gene that encodes the capsid protein VP1 has been used as a surrogate for antigenic typing in order to distinguish enterovirus serotypes; EV73, EV74, and EV75 serotypes were identified recently using this method[14, 15]. Currently, 74 enterovirus strains have been isolated over a 25–year period from five different countries (USA, China, France, Bangladesh, Iraq). EV74 was first isolated in 2004 from six patients. Three of those patients had acute flaccid paralysis[15]. In this study, EV74 was first isolated from the stool of a 14–year–old boy who presented with headache and fever. His neurological examination was normal and the CSF analysis showed no evidence of meningitis. The patient was hospitalized and improved after 4 days of conservative care.

Phylogenetic analysis of the EV74 strains showed that the Korean strain was close to the Bangladesh strain (BAN00–10217) isolated in 2000. Additional isolation of EV73 confirmed its worldwide distribution[14–16], and three new types, EV76, EV77 and EV78 were also identified using similar methods[15, 17, 18]. The discovery of these new types suggested that numerous additional enterovirus types await identification by sustained surveillance[15].

The clinical characteristics present in affected patients showed that most EV infected patients were infants less than three months of age (40/51). Neonatal EV infections lead to a wide range of clinical manifestations, from mild febrile illness to severe, sometimes fatal, sepsis like disease[19]. According to data reported from the National Enterovirus Surveillance System (NESS) during 1983–2003[19] in the USA, the most common serotypes were ECV11 (14.0%), CVB2 (8.9%) and CVB5 (7.5%). ECV18 was ranked ninth. A fatal outcome was noted in 3.3% of cases. Neonates were at a higher risk for death than persons aged ≥1 month, as well as patients infected with CVB4 as opposed to other EV strains[19]. A fatal outcome was noted for approximately 1.6–3.2% of cases aged ≥1 month infected with CVB5, ECV5, ECV18, and ECV25[19]. In this study, there were no fatalities reported in patients infected with CVB4. Recently, the Korean data from the national surveillance showed that most of the patients with were meningitis were older children. It is necessary to continue the surveillance of EV in neonates with sepsis like symptoms in order to be able to monitor the fatal outcomes.

Among 168 infants under three months of age, enterovirus was isolated from 40 of them (23.8%). In 2005, 27 cases of enteroviral infection were identified, ECV18 (40.7%) and CVB5 (33.3%) were the most common. In 2006, 13 cases of enteroviral infection were identified, ECV25 (38.4%) and ECV5 (23.1%) were the most common. This distribution was similar in infants older than four months of age.

Enteroviral infected patients had fever, irritability, vomiting and diarrhea without prodromal symptoms. In older children fever and headache were the most common symptoms. Clinical manifestations did not correlate with the

serotype and most of the clinical manifestations disappeared within 48 hours, including fever. The clinical manifestations of enterovirus (the four serotypes or the two groups of CBV5) were similar, and no complications were associated with the enteroviral infections in this study.

In this study, the EV74 serotype was first isolated in Korea. We confirmed that EV was a cause of fever and irritability in infants younger than three months of age. These clinical manifestations are difficult to distinguish from bacterial infection. Therefore, a more systematic enterovirus surveillance program is needed. Mutant strains appeared to have spread among different countries even quite recently. Therefore, more epidemiological and biological studies are needed to determine the relationships between the antigenic properties and the epidemiology of these strains.

한 글 요 약

2005년 봄부터 2006년 가을까지 국내 중부지역의 enterovirus 감염의 분자유전학 조사와 임상양상

건양대학교 의과대학 소아청소년과학교실,
단국대학교 의과대학 소아청소년과학교실,*
충청남도 보건환경연구회†,
질병관리본부 간염, 장바이러스과‡

노의정 · 진용만* · 정은희* · 장영표*
박우성* · 박귀성† · 지영미‡

목 적: Enteroviruse (EV)는 영아와 소아에서 가장 흔한 감염의 원인으로 경한 발열성 질환에서 뇌수막염이나 치명적인 심근염까지 다양한 질환을 일으키는 원인이다. 본 연구에서는 국내 중부지역의 EV 유병율에 대해 알아보고자 하였다.

방 법: 2005년 4월부터 2006년 10월까지 국내 중부지역에서 발열성 질환이나 뇌수막염이 의심되어 입원한 소아에서 얻은 검체를 모아 RT-PCR을 시행하여 EV를 확인하였고, genotype과 phylogenetic 분석을 위해 direct sequencing을 시행하였다.

결 과: 뇌척수액과 대변검체에서 PCR을 시행한 305명의 환아 중 51명(16.7%)에서 EV가 양성을 보였다. 그중 다음의 serotype이 44명에서 확인되었다: Echovirus (ECV) 18 (18 cases, 35.2%), Coxsackievirus (CVB) 5 (13 cases, 25.4%), ECV25 (5 cases, 9.8%), ECV9 (4 cases, 7.8%), ECV5 (3 cases, 5.8%), EV74 (1 case, 1.9%). 2005년 6월과 8월 사이에는 ECV18과 CVB5가 가장 많이 검출되었다. 2006년 7월과 8월 사이에는 ECV25가 가장 많이 검출되었다.

결 론: 지속적인 enterovirus 감염의 감시가 필요하며 임상적인 의의가 밝혀져야 할 것이다. 특히 EV74가 처음으로 국내에서 발견되었기에 이를 보고하는 바이다.

References

1) Muir P, Kämmerer U, Korn K, Mulders MN, Pöyry T, Weissbrich B, et al. Molecular typing of enteroviruses: current status and future requirements. Clin Microbiol Rev 1998;11:202-27.
2) Romero JR. Reverse-transcription polymerase chain reaction detection of the enteroviruses. Arch Pathol Lab Med 1999; 123:1161-9.
3) Park K, Lee K, Lee J, Yeo S, Lee S, Cheon DS, et al. Acute hemorrhagic conjunctivitis epidemic caused by coxsackievirus A24 variants in Korea during 2002-2003. J Med Virol 2006; 78:91-7.
4) Nix WA, Oberste MS, Pallansch MA. Sensitive, seminested PCR amplification of VP1 sequences for direct identification of all enterovirus serotypes from original clinical specimens. J Clin Microbiol 2006;44:2698-704.
5) Künkel U, Schreier E. Genetic variability within the VP1 coding region of echovirus type 30 isolates. Arch Virol 2000; 145:1455-64.
6) Caro V, Guillot S, Delpeyroux F, Crainic R. Molecular strategy for 'serotyping' of human enteroviruses. J Gen Virol 2001;82:79-91.
7) Jee YM. Laboratory surveillance and research activities on enteric viruses in NIH, KCDC. Infection and Chemotherapy (Korean) 2005;37:S174-81.
8) Kusuhara K, Saito M, Sasaki Y, Hikino S, Taguchi T, Suita S, et al. An echovirus type 18 outbreak in a neonatal intensive care unit. Eur J Pediatr 2008;167:587-9.
9) Takao S, Wakatsuki K, Yoshida H, Shimizu H, Wakita T. Neutralization assays for echovirus 18 isolates in 2006. Jpn J Infect Dis 2007;60:65-6.
10) McLaughlin JB, Gessner BD, Lynn TV, Funk EA, Middaugh JP. Association of regulatory issues with an echovirus 18 meningitis outbreak at a children's summer camp in Alaska. Pediatr Infect Dis J 2004;23:875-7.
11) Kelly H, Brussen KA, Lawrence A, Elliot E, Pearn J, Thorley B. Polioviruses and other enteroviruses isolated from faecal samples of patients with acute flaccid paralysis in Australia, 1996-2004. J Paediatr Child Health 2006;42:370-6.
12) Stirk HJ, Thornton JM. The BC loop in poliovirus coat protein VP1: an ideal acceptor site for major insertions. Protein Eng 1994;7:47-56.
13) Norder H, Bjerregaard L, Magnius L, Lina B, Aymard M, Chomel JJ. 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 2003;84:827-36.
14) Oberste M, Schnurr D, Maher K, al-Busaidy S, Pallansch M. Molecular identification of new picornaviruses and characterization of a proposed enterovirus 73 serotype. J Gen Virol 2001;82:409-16.
15) Oberste MS, Michele SM, Maher K, Schnurr D, Cisterna D, Junttila N, et al. Molecular identification and characterization

of two proposed new enterovirus serotypes, EV74 and EV75. J Gen Virol 2004;85:3205-12.
16) Norder H, Bjerregaard L, Magnius LO. Open reading frame sequence of an Asian enterovirus 73 strain reveals that the prototype from California is recombinant. J Gen Virol 2002; 83:1721-8.
17) Norder H, Bjerregaard L, Magnius L, Lina B, Aymard M, Chomel JJ. 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 2003;84:827-36.
18) Bailly JL, Cardoso MC, Labbe A, Peigue-Lafeuille H. Isolation and identification of an enterovirus 77 recovered from a refugee child from Kosovo, and characterization of the complete virus genome. Virus Res 2004;99:147-55.
19) Khetsuriani N, Lamonte A, Oberste MS, Pallansch M. Neonatal enterovirus infections reported to the national enterovirus surveillance system in the United States, 1983-2003. Pediatr Infect Dis J 2006;25:889-93.