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Genetic analysis of the VP1 region of Human enterovirus 71 strains isolated in Korea during 2000

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Summary. We have isolated Human enterovirus 71 (EV71) from stool and CSF samples taken from patients with acute flaccid paralysis, herpangina, or hand, foot and mouth disease in 2000. Both the cell culture-neutralization test and RT-PCR were used to detect enteroviruses. Rhabdomyosarcoma (RD), HEP2c, and BGM cells were used for the isolation of viruses, and serotypes were determined by the neutralization test using EV71-specific antiserum. For genomic analysis, we amplified a 437-bp fragment of the 5′-noncoding region of the enterovirus genome and a 484-bp fragment of the VP3/VP1 region of EV71 by RT-PCR, with positive results. Products amplified using an EV71-specific primer pair were sequenced and compared with other isolates of EV71. Analysis of the nucleotide sequences of the amplified fragments showed that the EV71 isolates from patients were over 98% homologous and belonged to the genotype C.

Introduction

Human enterovirus 71 (EV71) belongs to the family *Picornaviridae*, the genus *Enterovirus*, and the species *Human enterovirus A* which includes more than 68 serotypes. It is a distinct member of the genus, and is associated with a high rate of neurological complications, including meningoencephalitis and pulmonary complications such as pulmonary oedema and haemorrhage. Like other enteroviruses, EV71 can be transmitted by a faecal-oral route or a respiratory route.

EV71 infection was recognized in California in 1969 and was first reported by Schmidt et al. [15]. Since then, there have been four major epidemics of EV71

infection involving fatalities. These occurred in Bulgaria in 1975, Hungary in 1978, Malaysia in 1997, and Taiwan in 1998 [1, 5], and caused many deaths: 44 deaths in Bulgaria, 45 in Hungary, 31 in Malaysia, and 78 in Taiwan [12, 16]. A recent outbreak of EV71 in Taiwan showed that this virus can invade the brainstem, leading to encephalitis, and can also manifest pulmonary complications [8, 11, 18, 19].

Hand, foot and mouth disease (HFMD) and herpangina are two major clinical manifestations of EV71 infection. Human coxsackievirus A16, which is also a member of the species *Human enterovirus A*, is the other major causative agent of HFMD. EV71 infection has various clinical manifestations such as acute flaccid paralysis, aseptic meningitis, and encephalitis, as well as HFMD and herpangina. Neurological and pulmonary complications of EV71 infection can occur within a few days, leading to death [9, 10, 20, 21]. There has been also a report of fatal cases of EV71 infection in Japan. Hand, foot and mouth disease and herpangina prevailed in Korea during 2000, with an outbreak of HFMD-herpangina in Cheju Province, although no fatality was associated with this outbreak.

EV71 can be detected from stool, cerebrospinal fluid (CSF), throat swab, and vesicular swab samples by the cell culture-neutralization test, indirect immunofluorescence assay, or RT-PCR using EV71-specific primers. The cell culture-neutralization test is the method recommended by the World Health Organization (WHO), as this test can both isolate the virus and identify its serotype. Rhabdomyosarcoma (RD), HEP2c, Vero, and Buffalo Green Monkey Kidney (BGM) cells are commonly used for enterovirus isolation and EV71-specific antiserum is used to identify EV71, together with enterovirus-specific antisera provided by WHO. However, classical serological typing of enteroviruses has raised some problems insofar as there have been many enteroviruses isolated during the last few years that could not be typed by neutralization. Therefore, efforts have been made to complement this classical method of enterovirus serotyping with molecular approaches using RT-PCR with amplicon sequencing [14]. Indirect immunofluroscence assay, a rapid diagnostic method that identifies the viruses within cells, can also be applied to the detection of EV71.

Molecular detection techniques using RT-PCR followed by DNA sequencing can identify the molecular epidemiological relationships between particular viruses. On the basis of a partial VP1 sequence, EV71 has been classified into genotypes A, B, and C. Genotypes B and C are further divided into two clusters (B1/B2, C1/C2) [3, 4].

Although HFMD or herpangina has been detected in Korea every year, the incidence of HFMD or herpangina increased in 2000. However, the actual number of cases of HFMD or herpangina in Korea is unknown and the causative viral agents for HFMD or herpangina in Korea were not confirmed by laboratory tests. In this study, we have isolated EV71 from samples collected from patients with various clinical manifestations including aseptic meningitis, HFMD, herpangina, and acute flaccid paralysis for the first time in Korea.

Materials and methods

Specimens

Cerebrospinal fluid and stool samples from Korean patients with HFMD, herpangina, aseptic meningitis, or acute flaccid paralysis (Table 1), or cell-culture supernatants showing cytopathic effects (CPE) were used for the isolation and identification of EV71. Viruses were isolated by the cell culture-neutralization test and also detected by indirect immunofluorescence assay and RT-PCR.

Cell culture-neutralization test

Rhabdomyosarcoma (RD), Vero, and BGM cells were used for the isolation of enteroviruses. Stool specimens were resuspended in phosphate-buffered saline (PBS) to a 10% suspension and clarified by centrifugation at 3,000 × g for 20 min. The supernatant was mixed with 1/10 volume of chloroform and centrifuged again at 3,000 × g for 20 min. The supernatant was used to inoculate susceptible cells. CSF was used directly to inoculate cells. Inoculated cells were examined daily for 5–7 d for CPEs and tested by neutralization using the WHO/Rijksinstituut voor de Volksgezondheid en Milieuhygiene (RIVM, Netherlands) antisera set. Cells with no CPE were passaged twice, at intervals of 7 d, to completely rule out the presence of virus. Supernatants from the inoculated wells showing CPE were harvested and mixed with 1/10 volume of chloroform for 15 min at room temperature, then centrifuged for 15 min at 1500 × g. The supernatant was collected carefully and used for the microneutralization test. Rabbit anti-EV71 antiserum kindly provided by the Victorian Infectious Diseases Reference Laboratory (VIDRL), Australia, and the WHO Western Pacific Regional Polio Reference Laboratory, was used for the neutralization test using the protocol described in the WHO manual for the virological investigation of poliomyelitis (WHO 1990). Each isolate was titrated and 100 $TCID_{50}$ of each virus was mixed with EV71 antiserum and incubated at 37 °C in a CO_2 incubator for 1 h. During the incubation period, the cell suspension from a culture flask was trypsinized and diluted in medium to a concentration of $1 - 2 \times 10^5$ cells/ml. The appropriate number of cells was distributed in a 0.1 ml volume into wells containing serum-virus mixture

Table 1. Clinical diagnosis of patients from whom enterovirus 71 was isolated

Isolate	Age/ Gender	Region	Specimens	Name of isolates	Patient Symptoms/Diagnosis	GenBank access no.
1	2 mo/F	Kyunggi	Stool/CSF	EV71-2000-PYJ	fever, acute flaccid paralysis/poliomyelitis	–
2	2 y/F	Seoul	Stool	EV71-2000-Kim	HFMD/fever	AY125966
3	6 mo/M	Seoul	Stool	EV71-2000-559	HFMD/encephalitis	AY125967
4	2 y/M	Seoul	Stool	EV71-2000-560	HFMD/fever	AY125968
5	1 y/M	Seoul	Stool	EV71-2000-789	HFMD/fever	AY125969
6	4 y/F	Seoul	Stool	EV71-2000-790	HFMD/fever	AY125970
7	5 y/M	Kyunggi	Stool	EV71-2000-793	HFMD/fever	AY125971
8	2 y/M	Seoul	Stool	EV71-2000-795	HFMD/fever	AY125972
9	2 y/F	Cheju	Serum	EV71-2000-863	HFMD/fever	AY125973
10	7 mo/M	Cheju	Stool	EV71-2000-864	HFMD/bronchiolitis	AY125974
11	6 y/M	Cheju	Stool	EV71-2000-865	HFMD/fever	AY125975
12	6 ml/M	Seoul	Stool	EV71-2000-941	HFMD/fever/encephalitis	AY125976

or virus only, or into wells containing medium only, and incubated at 37 °C. The plates were examined daily for CPE for 5–7 d to assess the neutralizing effects of specific antiserum.

Indirect immunofluorescence assay

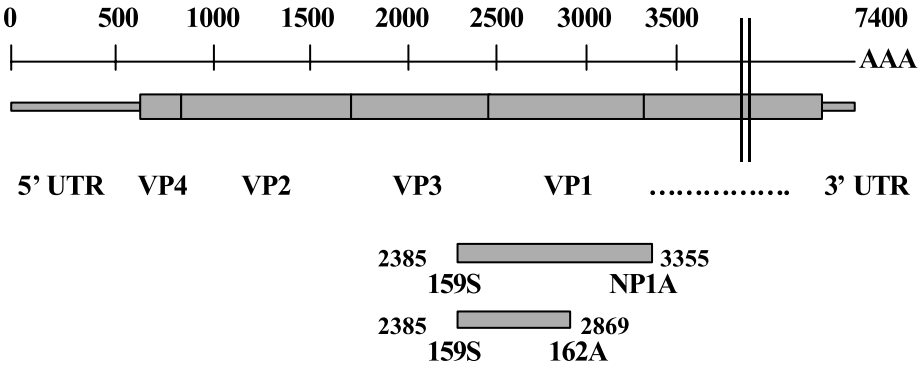
Cells were grown to a monolayer and inoculated with $1 \times 10^{6-7}$ $TCID_{50}$ viruses. For the immunofluorescent staining of cells, 10 μl mouse anti-EV71 monoclonal antibody (Chemicon International Inc. MAb 979) diluted 1:1000 in PBS-Tween 20 and then examined for fluorescent signal using fluorescence microscopy. Strong fluorescence within the cytoplasm was considered a positive reaction.

Extraction of viral RNA and reverse transcription-polymerase chain reaction (RT-PCR)

To extract RNA, Tri-reagent (Molecular Research Centre, Inc. U.S.A.) was used. For reverse transcription, an enterovirus 5′ non-coding region specific reverse primer, ENTR, or EV71 VP1-specific reverse primer (NP1A), or an oligo dT primer was used. The reaction mixture, in a total volume of 2 μl was incubated at 20 °C for 5 min, 42 °C for 60 min, and the MmLV reverse transcriptase (Gibco BRL) was inactivated at 95 °C for 5 min. To amplify a 437-bp region of the 5′-non-coding region of the enterovirus, primers ENTF and ENTR were used. The reaction mixture was incubated at 94 °C for 1 min, 52 °C for 90 s, and 72 °C for 90 s for 35 cycles. To amplify the VP1 region of EV71, we followed the method of Brown et al. [4]. Briefly, semi-nested PCR was performed using primers NP1A and 159S or 159S and 162A to amplify the 971 bp fragment or the 484 bp fragment, respectively. Primers 159S and 162A were designed to accommodate codon degeneracy. Primers used for the RT-PCR are shown in Fig. 1. Negative control reactions were included for all PCR procedures, and PCR procedures were performed under strict laboratory conditions to avoid cross-contamination and carryover.

Cloning and nucleotide sequencing

Amplified PCR products were cloned using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA 92008, U.S.A.), according to the manufacturer's protocol. Two μl TOPO Cloning reaction was added to One Shot competent *E. coli* cells (Invitrogen, Carlsbad, CA 92008, U.S.A.). The mixture was incubated on ice for 15–30 min and heat-shocked for 30 s at 42 °C without shaking. The tubes were immediately transferred to ice, and 250 μl SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose; Invitrogen, Carlsbad, CA 92008, U.S.A.) was added and incubated with horizontal shaking at 200 rpm at 37 °C for 1 h. For each transformation, 10–50 μl was spread on a pre-warmed selective plate and incubated overnight at 37 °C. White colonies were picked and cultured overnight in LB medium containing 50 μg/ml ampicillin or kanamycin. Plasmid DNA was purified from overnight cultures using the Wizard *Plus* SV Minipreps DNA Purification System (Promega, Madison WI, U.S.A.) according to the manufacturer's instructions. Sequencing reactions were carried out using the ABI PRISM Big-dye Terminator Ready Reaction Kit (Perkin Elmer Applied Biosystems, Foster City, CA 94404, U.S.A.). Automatic sequencing was performed with an ABI 377 sequencer (Perkin Elmer Applied Biosystems, Foster City, CA 94404, U.S.A.). Nucleotide sequences were analysed using the Megalign program of Lasergene, DNAStar. Megalign can align multiple sequences by drawing a histogram of consensus strength at the top of each alignment panel using one of two alogarithms: the Jotun Hein method or the Clustal method. The Jotun-Hein algorithm constructs alignments restricted on the basis of evolutionary assumptions, whereas the Clustal algorithm makes no *a priori* assumption of relatedness. The Clustal method is used to align sequences for which the similarity is not necessarily based on evolutionary relatedness. We



Primer	Sequence	Position (region, nucleotides, direction)
159S	5'-ACYATGAAAYTGTGCAAGG-3'	Enterovirus 71 VP3, 2385–2403, sense
162A	5'-CCRGTAGGKGTRCACGCRAC-3'	Enterovirus 71 VP1, 2869–2850, antisense
NP1A	5'-GCICCICAYTGITGICCRAA-3'	Enterovirus 71 VP1, 3355–3336, antisense
ENTF	5'-AAGCACTTCTGTTTCCCCGG-3'	Enterovirus 5'-noncoding region, 161–181, sense
ENTR	5'-ATTGTCACCATAAGCAGCCA-3'	Enterovirus 5'-noncoding region, 596–577, antisense

Y = C or T; R = A or G; I = inosine; K = G or T.

Fig. 1. Primers and PCR scheme for specific amplification of EV71. Hybridization sites for primers 159S, 162A, and NP1A, used for amplifications, are indicated

used the Clustal method to group sequences into clusters by examining the distance between all pairs. The relationship of the sequences is shown in dendrograms (Figs. 3 and 4). The length of each pair of branches represents the distance between sequences. Units indicate the numbers of substitution events.

Results

Patients' clinical information

EV71 was detected in samples from 12 patients by RT-PCR and indirect immunofluoroscence assay, and isolated by the cell culture-neutralization test. Basic data for 12 patients are described in Table 1. Patients' ages ranged from 2 months to 6 y. Most patients had HFMD and two patients also had either encephalitis or bronchiolitis. One patient with acute flaccid paralysis had fever for 5 days before the admission and the brain MR imaging of the patient showed the tissue destruction in the bilateral posterior portions of the medulla oblongata and bilateral anterior horns of cervical spinal cord from C3–C6 level suggesting polio-like encephalomyelitis.

Detection of EV71 by RT-PCR

Samples from the 12 patients described in Table 1 were examined by RT-PCR. All samples showed a clear fragment of 437 bp when amplified using enterovirus-specific primers and a 484 bp amplification product when primers specific for EV71 were used.

Identification of EV71 by neutralization test and immunofluorescence assay

Rhabdomyosarcoma, Vero, and BGM cells were used to isolate EV71. Among three cell lines which were used for virus isolation, Vero cells showed the most distinct CPEs. However, there was no significant difference in susceptibility among cell lines depending on the origin of cells. All 12 samples showed CPEs characteristic of enteroviruses and were screened for enteroviruses using the neutralization test with the WHO/RIVM antisera set. Since WHO/RIVM antisera set can neutralise most echoviruses and but does not include the neutralisation antiserum for the EV71, EV71 is identified as an untypable virus with WHO/RIVM antiserum. Therefore, we used EV71-specific rabbit antibody for the neutralization of viruses. Wells that showed enterovirus-specific CPE but were not neutralized with the WHO/RIVM enterovirus set could be neutralized by rabbit anti-EV71 antiserum. EV71-specific immunofluorescence was detected within the cytoplasm of infected cells using a monoclonal antibody specific to EV71 (MAb 979, Chemicon International Inc.) shown in Fig. 2.

Molecular analysis

A 484 bp fragment was amplified from the VP1 region of EV71 using semi-nested PCR. The VP1 region correlates with enterovirus serotypes, and the primers used in this study have been shown to specifically amplify EV71 with no cross-reactivity

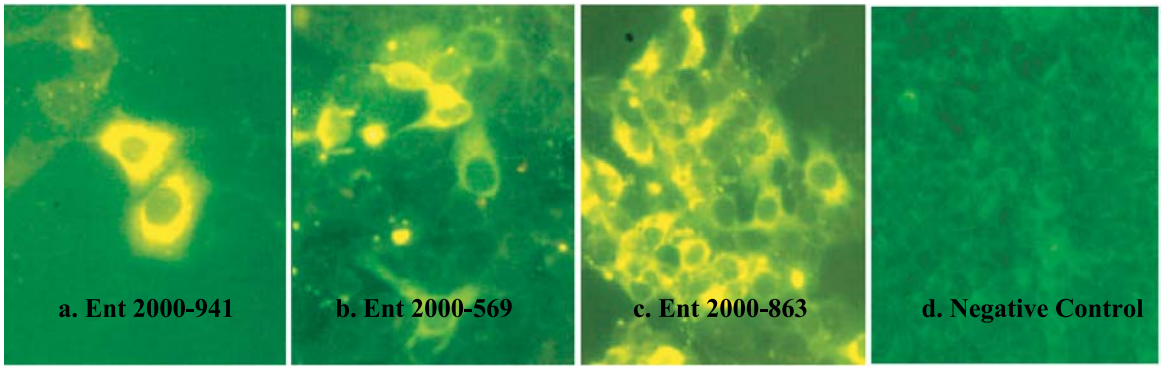


Fig. 2. Immunofluorescence assay of 2000 isolates. **a**. Ent 2000-941(× 400), **b**. Ent 2000-569 (× 200), **c**. Ent 2000-863 (× 200), **d**. negative control. In contrast to the negative control well (**d**), EV71-inoculated wells (**a**, **b**, and **c**) show bright green fluorescence within the cytoplasm of infected Vero cells

with coxsackievirus A16 [4]. The amplified fragments were cloned and sequenced for further analysis. The nucleotide sequences of the amplified fragments revealed that the EV71 isolates of patients were over 98% homologous and belonged to genotype C.

The VP1 region of the Korean isolates showed 98–99% nucleotide sequence identity with one another and 95–97% identity with other foreign isolates of genogroup C (Fig. 3). More divergence was observed when these isolates were compared with foreign isolates such as EV71 strains 9166-TX 98 or 2246-NY 87 although these all belong to the same genogroup C. From the dendrogram, it is clear that strain EV71pyj has the most divergent VP1 sequence among Korean isolates. It is significant that this sequence was isolated from a patient with symptoms of acute flaccid paralysis (AFP), unlike the other isolates, which were isolated from patients with HFMD. We can assume that EV71 has been circulating in this country for some time and that strain EV71pyj, which causes AFP, has accumulated mutations that can induce neurological symptoms. The sequences of EV71-2000-Kim, EV71-2000-559, EV71-2000-560, EV71-2000-789, EV71-2000-790, EV71-2000-793, EV71-2000-795, EV71-2000-863, EV71-2000-864,

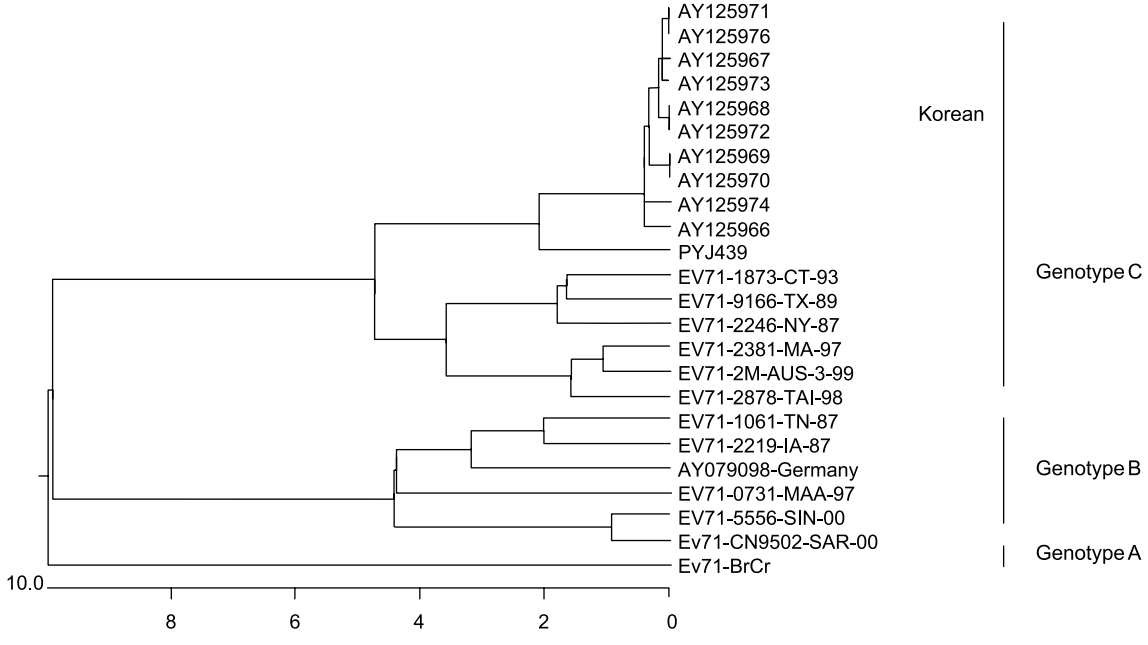


Fig. 3. Dendrogram showing the genetic relationships of EV71 Korean isolates with foreign isolates based on the partial sequence (439 bp) of VP1. AY125971, AY125976, AY125967, AY125973, AY125968, AY125972, AY125969, AY125970, AY125974, AY125966, PYJ 439 constitute Korean EV71 strains. The GenBank Accession numbers for EV71-1873-CT-93, EV71-9166-TX-89, EV71-2246-NY-87, EV71-2381-MA-97, EV71-2M-Aus-3-99, EV71-2878-TAI-98, EV71-1061-TN-87, EV71-2219-IA-87, EV71-0731-MAA-97, EV71-5556-SIN-00, EV71-CN9502-SAR-00 and EV71-BrCr are AF009559, AF135954, AF009542, AF135943, AF376013, AF286511, AF009528, AF009539, AF135911, AF376124, AF376071 and U22521, respectively

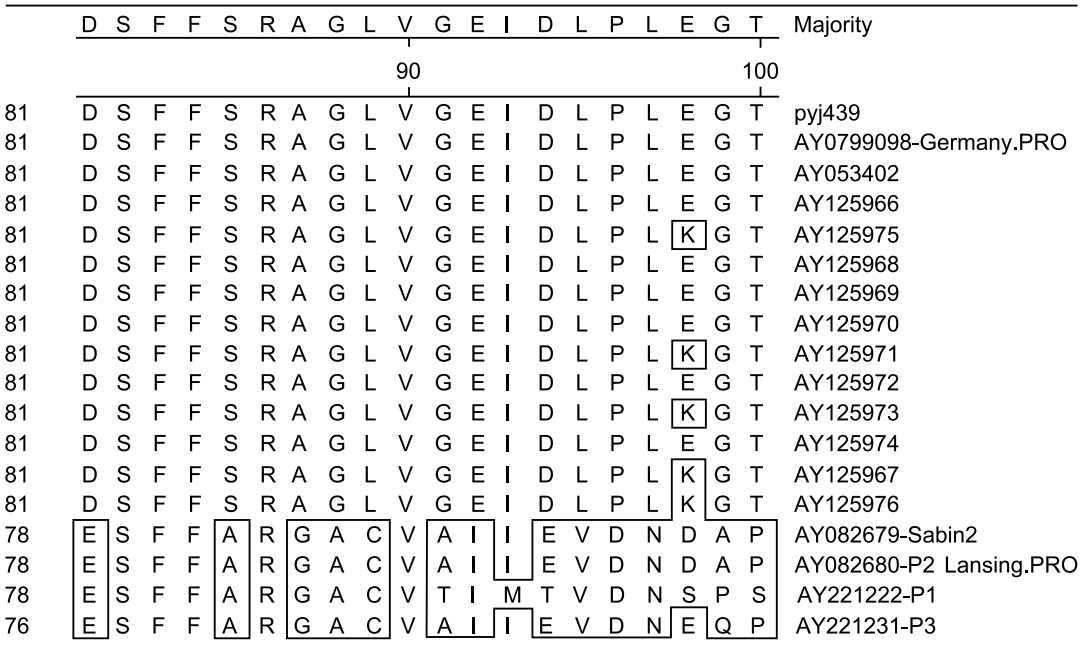


Fig. 4. Amino acid substitution from glutamic acid to lysine in the VP1 region which is shared by some Korean strains. This substitution results from G to A nucleotide change

EV71-2000-865 and EV71-2000-941 were deposited in the GenBank sequence database under accession numbers AF276912, AF276913, and AF276914, respectively.

By comparing VP1 sequences of EV71 strains, we found some sequence variations shared by a few strains such as nt G to A change in the VP1 region as shown in the Figure 4. Although this nucleotide change also led to amino acid E to K substitution, it did not correlate with other nucleotide substitution of known neurovirulent strains (Fig. 4). This amino acid residue of both EV71 and poliovirus belongs to surface-exposed region when Protean (Lasergene) was used for the prediction of hydrophilicity and surface probablity. This amino acid residue also belongs to the neutralising antigenic epitope of the VP1 region of polioviruses. Also the strains AY125969 and AY125970 were closely related and shared some nucleotide changes. The AY125974 strain which manifested bronchiolitis as well as HFMD showed some nucleotide substitutions shared with the AY079098 strain which showed some neurovirulence in a German patient. The strain from acute flaccid paralysis showed most significant difference from other EV71 isolates in 2000.

Discussion

The incidence of EV71 has increased dramatically during the last few years and outbreaks, which have included fatal cases with severe complications, have occurred in Malaysia and Taiwan in 1997 and 1998. The various clinical features

of EV71 seem to be based on genetic diversity, as they are rapidly evolving viruses. The genetic variation within genotypes (<12%) is less than the variation between genotypes (16.5–19.7%) of EV71 [4]. However, genogrouping of EV71 varies across research groups. Chu et al. [7], Brown et al. [3], and Singh et al. [17] have classified EV71 strains by comparing the sequences of the VP1 or VP4 regions.

Chu et al. [7] identified A, B, C1, C2, and C3 genotypes on the basis of a 650-bp VP4 region. They analysed 44 EV71 strains and found that most Taiwanese strains belonged to genotype C3, whereas strains from Japan belonged to C2 or C1. Genotype C was again divided into three clusters: C1, C2, and C3. This research group analysed the VP4 and VP1 regions but found no significant nucleotide difference between the fatal and non-fatal infections in Taiwan. However, a more rigorous study is required to identify the virulence factor associated with the pathogenicity of EV71. Brown et al. [3, 4] grouped EV71 into groups A, B, and C on the basis of phylogenetic analysis of the complete VP1 sequence. Genogroups B and C were further divided into clusters B1/B2 and C1/C2/C3. The prototype EV71 strain, BrCr CA-70, a strain isolated in California in 1970, is the only member of genotype A. The strains isolated in the U.S.A. and Australia from 1972 to 1988, and all isolates from a large HFMD outbreak in Malaysia, belong to genotype B. All strains isolated after 1985 belong to genotype C.

Based on the classification of Brown et al. [3], the Korean strains isolated in 2000 belong to cluster C, which includes strains isolated in the U.S.A. and Australia from 1986 to 1995 and isolates collected in 1997 from Sarawak, Malaysia. Most strains of genotype B were isolated before 1990. Isolates from a large outbreak in Taiwan in 1998 belong to genotypes B and C3, and specimens isolated from fatal cases of EV71 were caused by both genotypes B and C3. Brown et al. [4] divided 113 strains of EV71 into genogroups A, B, and C. Most of the analysed strains originated in the U.S.A. These researchers amplified and sequenced the 485-bp fragment from the VP1 region. Their results show that there is no clear correlation between the severity of the disease and the genetic lineage of the virus isolated, because strains of all genogroups caused severe disease.

Singh et al. [17] used a different part of the VP1 region to classify EV71 into genotypes A, B, C, D, and E strains. Therefore, we need to clearly establish the genotyping system for EV71 to avoid confusion in the classification of this virus.

EV71 infections in Korea were first reported by Cho et al. [6] and Yoo et al. [22]. Cho et al. [6] sent 200 samples that had been collected between 1989 and 1990 in Seoul to the Special Reference Laboratory in Tokyo to be examined for enterovirus-specific antibodies. Among children under the age of five years, the antibody titre to EV71 was high, especially before mid-July, whereas Human echovirus 30 was the prevalent strain after mid-July during this period. Yoo et al. [22] collected specimens from patients with aseptic meningitis between May and August 1993 in Incheon. They also sent their specimens to the Special Reference Laboratory in Tokyo for viral culture and identification because enterovirus culture was not readily available in Korea. EV71 was isolated from seven samples. In this study, we have isolated EV71 for the first time in Korea, using rabbit EV71-specific antiserum.

Our results indicate that the nucleotide identities of over 98% are present in the Korean isolates collected in 2000. Therefore, we can assume that the HFMD outbreak was possibly caused by strains of identical origin. There was a significant difference between our 2000 isolates and the Taiwanese isolates collected in 1998, which caused many fatalities. We have mainly collected and examined specimens from patients with aseptic meningitis since 1993, and we have not considered samples from patients with HFMD to any degree. Because we cannot analyse our isolates together with previous Korean isolates of EV71 at this point, it is not possible to determine the proportion of predominant genotypes in Korea. Extended examination of specimens from throughout the country over at least a few years will be required to confirm the changing pattern of prevailing genotypes in Korea.

To monitor the emergence of new strains and to facilitate the early detection of strains during outbreaks, it is important to understand the epidemiology of EV71 infections. It is significant that one strain was isolated from a patient with acute flaccid paralysis symptoms because this is evidence that a strain of EV71 in Korea is neurovirulent. Two fatal cases following HFMD or herpangina were found in 2000 (personal communication with Dr. Chung Hee Chung, 2000); however, we could not conduct laboratory confirmation of EV71 in samples from those patients.

It is necessary to analyse this strain together with other foreign EV71 isolates that have caused AFP symptoms to identify the region responsible for AFP. Brown and Pallansch (1995) compared a neurovirulent isolate with the prototype strain (EV71/BrCr) and found that they shared only 81% nucleotide identity and 95% amino acid identity. Comparison of the sequence of Korean isolate EV71pyj with poliovirus may also be useful to predict possible evolution to neurotrophic strains. This EV71pyj strain which caused AFP was found to be the most divergent from other EV71 strains which were found in 2000. When we compared 439 bp of the VP1 region of EV71 strains, it was found that some of nucleotide changes of this neurovirulent strain were shared with nucleotide sequences of polioviruses although the relationship to neurotropism of this substitution is not known. Sequencing the complete genome of EV71 isolates will provide more information on the genetic and epidemiological relationships between isolates. Although the genetic determinants of neurovirulence of EV71 remain unknown, the VP1 gene has been suggested to express a virulence factor (McMinn et al., 2001). The data of McMinn et al. indicate that substitution of VP1 residue 170 from alanine to valine may be associated with the increased neurovirulence of EV71. However, one of neurovirulent strains from Germany (AY0799098) which was compared with Korean EV71 strains did not have 170 alanine to valine substitution. They could not rule out the possibility that attenuating mutations within other regions or different host susceptibility factors are involved. They have also suggested that viruses belonging to genogroup C1 may have lower epidemic and neurovirulent potential than other genogroups because they have only been isolated from patients with uncomplicated cases of HFMD. The fact that we have isolated 12 strains of EV71 from patients with uncomplicated HFMD and only one strain associated with AFP is consistent with their findings.

A seroprevalence study based on the neutralization test would be useful to evaluate the substantial incidence of EV71 infection in Korea.

In conclusion, we have isolated and sequenced EV71 for the first time in Korea. The sequence analysis of our isolates shows that these strains are clustered into genotype C although they tend to form a separate cluster from other strains which belong to genotype C. We need to accumulate further sequence data from more Korean EV71 isolates to establish a strain bank for this virus. This will help us to understand the molecular epidemiology of EV71 infection and also to develop a more sensitive and specific detection method based on EV71 strains circulating in Korea.

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