Sequence analysis of the first B5 subgenogroup strain of enterovirus 71 isolated in Korea

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Enterovirus A71 (EV71), the main etiological agent of hand-foot-mouth disease (HFMD), circulates in many areas of the world and has caused large epidemics since 1997, especially in the Asia-Pacific region. In this study, we determined the full-genome sequence of CMC718, a newly isolated EV71 strain in Korea. The CMC718 genome was 7,415 nucleotides in length and was confirmed by whole-genome phylogenetic analysis to belong to the B5 genotype. In particular, CMC718 demonstrated maximum identity with strain M988 of the B5 genotype and numerous amino acid variants were detected in the 3D domain of the viral protein P3, which is consistent with the mutation pattern of a B5 strain isolated in 2012–2013. Comparison of the CMC718 sequence with other EV71 reference strains confirmed the relationship and genetic variation of CMC718. Our study was a full-genome sequence analysis of the first EV71 strain of the B5 genotype isolated in South Korea. This information will be a valuable reference for the development of methods for the detection of recombinant viruses, the tracking of infections, and the diagnosis of EV71.

Keywords: human enterovirus A71, B5 subgenogroup, whole-genome sequencing, phylogenetic analysis, Korea

Introduction

Hand, foot, and mouth disease (HFMD) is an acute infectious disease characterized by vesicular eruptions on the palms of the hands, the feet, and in the oral cavity and is common worldwide in children under the age of 5, especially in developing countries (Xie *et al.*, 2016; Li *et al.*, 2018). HFMD is caused by enteroviruses with the principal etiologic agents being enterovirus A71 (EV71), coxsackievirus A16 (CA16), and some serotypes of echovirus (Wang *et al.*, 2014). The majority of patients with HFMD show mild clinical symptoms such as fevers and skin rashes. However, those infected with EV71 may suffer severe clinical manifestations ranging from aseptic meningitis to acute flaccid paralysis and brainstem encephalitis, which may even lead to death (Jang *et al.*, 2013). EV71 was first isolated in 1969 from a patient with HFMD in California that presented with neurological disease and then described in 1974 (Kim *et al.*, 2016b). Since then, EV71 has caused numerous outbreaks and epidemics worldwide, particularly in Asian-Pacific regions such as Malaysia in 1997, Taiwan in 1998, South Korea in 2000, Singapore in 2000, Vietnam in 2003, and China in 2008 (Wang *et al.*, 2012; Donato *et al.*, 2016; Kim *et al.*, 2016b).

EV71 is a member in the *enterovirus A* species of the genus *Enterovirus* in the family *Picornaviridae*. The virus particles of EV71 are small (20–30 nm in diameter), non-enveloped, icosahedral capsids that contain positive-sense, single-stranded RNA genomes of approximately 7.5 kilobases (kb) in length (Yi *et al.*, 2017). The genome of EV71 contains 5′- and 3′-untranslated regions (UTRs) that are essential for viral gene expression and replication. The genome contains one open reading frame (ORF) encoding a polyprotein that includes three regions, P1, P2, and P3. The P1 region encodes four structural viral proteins (VP1–VP4), P2 encodes three nonstructural proteins (2A–2C), and P3 encodes four nonstructural proteins (3A–3D) (Zhang *et al.*, 2013).

VP1, the major antigenic and neutralizing domain, is the most variable protein of the virus. EV71 is classified into three different genotypes (A–C) based on the gene sequence of VP1. Genotype A consists solely of the prototype EV71 strain (BrCr), while genotypes B and C are each classified into five subgenotypes, B0–B5 and C1–C5, respectively (Noisumdaeng *et al.*, 2018). EV71 strains of the same genotype share > 92% nucleotide sequence identity, whereas isolates with a diversity > 15% are considered to be different genotypes (Duong *et al.*, 2016).

The first EV71 infection in Korea was reported in 2000 and a national enterovirus surveillance system has subsequently been established to monitor enteroviruses (Lee *et al.*, 2016). All enteroviruses reported in Korea to date have been genotype C with the epidemic subgenotypes differing during the years such as C1 in 2009, C2 in 2009, C3 in 2000–2003, C4 in 2007–2011, and C5 in 2009 (Hyeon *et al.*, 2013; Lee *et al.*, 2016; Yi *et al.*, 2017). Interestingly, there have no outbreaks of genotype B reported. Therefore, we confirmed the need for research on various EV71 genotypes and performed a complete nucleotide sequence analysis of B genotypes found

Table 1. Diagnosis Primer sequences of the RT-PCR assays

Primer	Sequence (5′→3′)	Region	Position	Reference
EntF	CAA GCA CTT CTG TTT CCC CGG	5′-NCR	160–180	Hong *et al.* (2010)
EntR	ATT GTC ACC ATA AGC AGC CA		580–599	

in Korea. We expect that the results from this study will be useful to not only advance our understanding of the molecular genetics and biology of EV71, but also to help in the analysis of basic mechanics and the international spread of the virus.

Materials and Methods

Ethics statement

The stool sample used in the study was provided by the Waterborne Virus Bank (WAVA). The institutional review board of Songeui Medical Campus, The Catholic University of Korea reviewed and approved the use of this sample for the purpose of research in this study. The use of the sample did not directly affect the patient and consent was not required. The sample collection and all experimental work were supervised by the Catholic Medical Center Office of Human Research Protection Program (CMC OHRP) of South Korea (approval no. MC19SESI0090).

Sample preparation and viral RNA extraction

An enterovirus-positive stool specimen was isolated in August 2012 from a 2-year-old female patient with HFMD from South Korea. The patient presented with symptoms of skin rashes on the hands and feet along with oral ulcers. The stool sample was stored at -70°C until RNA extraction. The frozen stool sample was thawed and diluted with 10% phosphate-buffered saline (PBS) after which it was centrifuged at $13,000 \times g$ for 10 min at 4°C. The supernatant was collected and stored at -80°C until analysis. Viral RNA was extracted from 140 µl of supernatant using a QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. The isolated RNA was stored at -70°C until further use.

Reverse transcription polymerase chain reaction (RT-PCR)

For the detection of enteroviruses, reverse transcription polymerase chain reaction (RT-PCR) was performed using a One-Step RT-PCR Kit (Qiagen) with EntF and EntR primers based on the sequence of the 5′-non-coding region (5′-NCR) as previously described (Hong *et al.*, 2010). Primer details are provided in Table 1. To analyze the whole-genome sequence of the detected enterovirus strain, RT-PCR was performed using a OneStep RT-PCR Kit (Qiagen) with 13 pairs of newly designed primers based on the TW51135-2012 strain (GenBank accession no. MG756753) and 2716Yamagata03 strain

Table 2. Newly designed primers used in this study

Primer	Sequence (5′→3′)	Position[a]	Size (bp)	Reference
GSP1	GAATTGGAGTTTTCATGAGATCCGG	776–800		
GSP2	CAAAGTAGTCGGTTCCGCTGCAGAG	531–555		This study
NestedGSP	CGGATGGCCAATCCAATAGCTA	623–644		
EV71-1-F	TTA AAA CAG CCT GTG GGT TG	1–20	764	
EV71-1-R	GAC ACT TGT GAG CCC ATG TT	745–764		
EV71-2-F	CCG GTG TGC AAT AGA GCT	642–659	750	
EV71-2-R	GGG TGA CTG TCT TCT GT	1375–1391		
EV71-3-F	GCT AGC AAG TTC CAC CA	1294–1310	778	
EV71-3-R	AAG TGA CCT CTA GTG ACC	2054–2071		
EV71-4-F	CAT GGC AGT CAA CGA TGC	2000–2017	781	
EV71-4-R	ATG TCT ATA TCC CAG TTG GC	2761–2780		
EV71-5-F	CAG TAC AGC AGA GAC TAC	2661–2678	805	
EV71-5-R	GGA CGA CAC TAA TAG GTC	3448–3465		
EV71-6-F	GGC AAC TTC AGA GTG GTT A	3370–3388	751	This study
EV71-6-R	CACTCGCCATATCATTGAAC	4101–4120		
EV71-7-F	GAT CAA AGC CAA GAC AGC AT	4029–4048	761	
EV71-7-R	CGTCAGAGTCAGACACTGT	4771–4789		
EV71-8-F	GTT ATC GCA TCC ACC AAC G	4732–7450	753	
EV71-8-R	CCTGACACCTAACATGGTAA	5465–5484		
EV71-9-F	GGT CCA AGT CTC GAC TTT G	5392–5410	783	
EV71-9-R	CAGCTTGTGTCACGTATTC	6154–6174		
EV71-10-F	GGA GGT CGA CTT TGA GCA	6090–6107	820	
EV71-10-R	GTTCAACTCATCCAGGTCT	6891–6909		
EV71-11-F	GTG CTC TGG TAC TTC CAT C	6810–6828	575	
EV71-11-R	TTT TTT TTT TTT GCT ATT CTG GTT ATA ACA AAT			

[a] According to GenBank accession number TW51135-2012 (MG756753), 2716Yamagata03 (LC375766)

(GenBank accession no. LC375766). The new primers are detailed in Table 2. RT-PCR was performed using an S1000 thermal cycler (Bio-Rad). The amplification protocol included an initial RT step at 50°C for 30 min followed by PCR activation at 95°C for 15 min and 40 cycles of amplification, each consisting of 1 min at 95°C, 1 min at 52–54°C, and 1 min at 72°C, with a final extension step of 10 min at 72°C. The PCR products were then electrophoresed and visualized on an ethidium bromide-stained 2% agarose gel.

Determination of the 5′- and 3′-ends of the EV71 genomic RNA

To determine the 5′-ends of the enteroviral genomic RNA, the RNA was reverse transcribed in to complementary DNA (cDNA) and rapid amplification of cDNA ends (RACE) was performed using the 5′ RACE System for RACE Version 2.0 Kit (Invitrogen) according to the manufacturer's recommendations. Three primers, GSP1, GSP2, and nested GSP, were designed for the 5′-end RACE PCR based on the VP4 region sequence (Table 2). To obtain the exact sequence of the 3′-end of the genomic RNA, cDNA was synthesized by reverse transcription using 3′-oligo (dT)-anchor-R (Table 2). The second PCR amplification was conducted using the EV71-11-F and 3′-anchor-R primers (Table 2) under the following conditions: 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min followed by 72°C for 7 min.

Whole-genome cloning and sequencing

The PCR products were cloned into the pGEM-T Easy Vector (Promega) according to the manufacturer's recommendations and transformed into competent *Escherichia coli* DH5α cells (RBC). Transformants were selected at 37°C for 16–18 h on Luria-Bertani (LB) agar media (Duchefa) containing 40 mg/ml X-gal, 0.1 mM isopropyl-β-D-thiogalactoside, and 50 mg/ml ampicillin. Selected clones were inoculated into LB broth (Duchefa) containing 50 μg/ml ampicillin and incubated overnight in an IS-971R shaking incubator (Jeiotech) at 37°C. The cultures were then centrifuged at 800 × *g*, the pellets resuspended in 600 μl fresh LB media with 10% glycerol, and stored at -80°C until required for further use. Plasmid DNA was purified using a HiYield Plasmid Mini Kit (RBC) according to the manufacturer's recommendations.
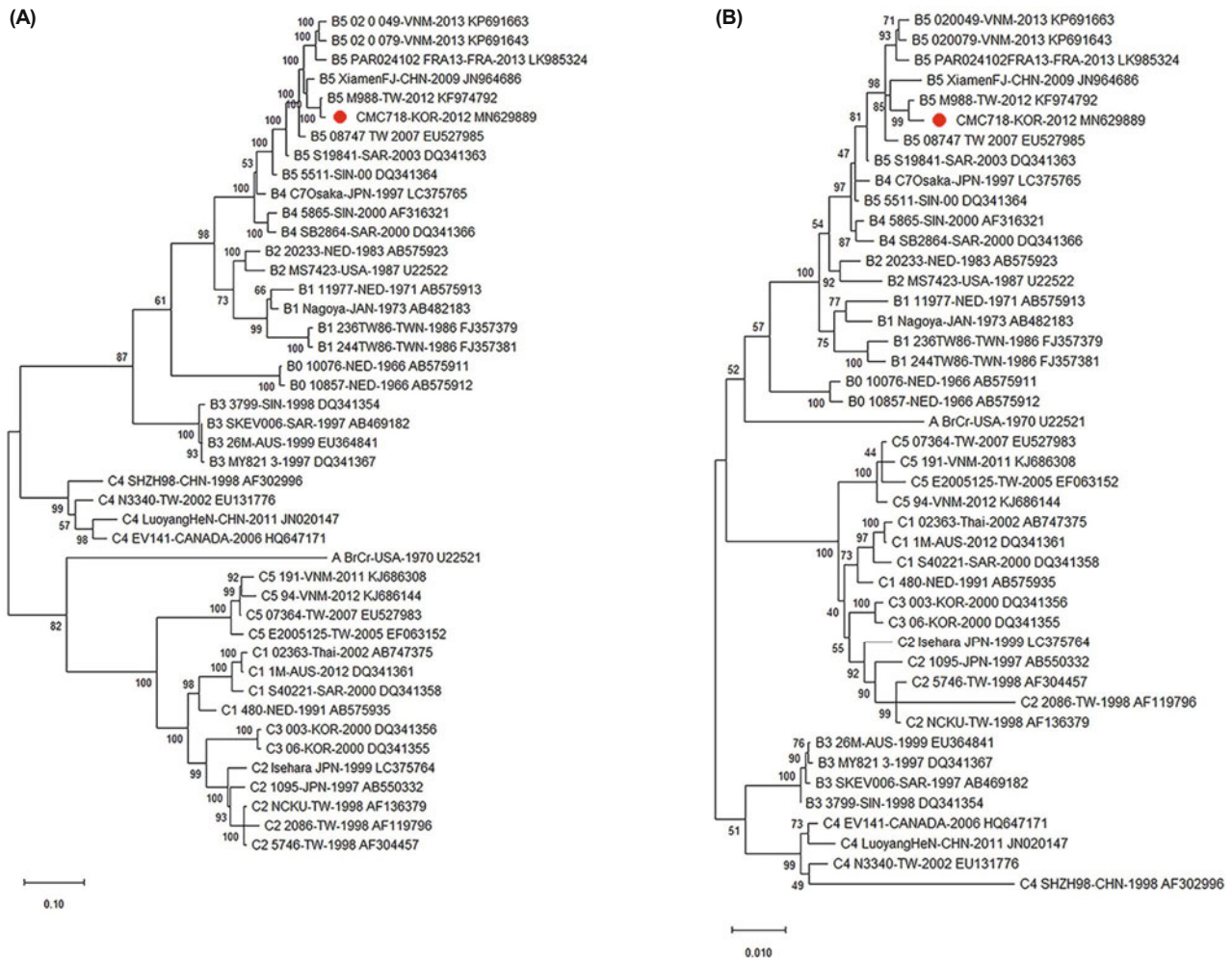
Fig. 1. Phylogenetic analysis of the complete nucleotide (A) and amino acid (B) sequences of the Enterovirus A71 detected determined by genotyping and reference strains isolated worldwide.

Fig. 2. Phylogenetic analysis of enterovirus A71 based on amino acid sequence. The tree were constructed with the neighbor-joining method. Phylogenetic trees base on (A) amino acid sequence of P1, (B) amino acid sequence of P2, (C) amino acid sequence of P3.

The DNA was sequenced by Macrogen and the results were analyzed using the Basic Local Alignment Search Tool (BLAST) made available through the National Center for Biotechnology Information (NCBI).

Phylogenetic analysis

Comparative sequence analyses, including sequence alignments and estimation of genetic distances, were performed with Clustal W using the Molecular Evolutionary Genetic Analysis software (MEGA soft version 6.0). Phylogenetic trees were constructed using the neighbor-joining method with a Kimura two parameter model in MEGA (Saitou and Nei, 1987) and branch support was calculated based on 1,000 bootstrap replicates. The complete genome sequences and partial genome sequences were obtained from the NCBI database.

Results

Genomic characterization of the EV71 isolate

The EV71 viral RNA was extracted from the stool sample collected and provided by WAVA. The isolated RNA from the EV71 strain, designated as CMC718, had a total length of 7,415 nucleotides and was flanked by a 747-nucleotide 5′-UTR and an 85-nucleotide 3′-UTR preceding the poly (A) tail. The CMC718 strain contained a single ORF of 6,579 nucleotides predicted to encode a polyprotein of 2,193 amino acids. The complete genomic sequences of CMC718 have been deposited in the GenBank database (accession number MN629889).

Phylogenetic analyses of CMC718 and EV71 reference genomes

Phylogenetic analyses were performed to evaluate the genetic relationships between strain CMC718 and 40 reference strains isolated worldwide and deposited into GenBank. A phylogenetic tree was constructed based on the nucleotide sequences comprising the whole genomes. The phylogenetic tree revealed that the EV71 reference strains could be clearly grouped into twelve subgenogroups, corresponding to A,

B0–B5, and C1–C5. According to the analyses of the whole-genome sequences, the average nucleotide sequence divergence among the EV71 strains was 79.2–98.9%. In particular, the whole-genome sequence of strain CMC718 showed a maximum identity of 93.9–98.9% with the members of the B5 subgenogroup (Fig. 1).

P domains analysis

Consistent with other enteroviruses, the genome of CMC718 consisted of three separate P domains (P1, 2,586 nt; P2, 1,734 nt; P3, 2,259 nt). A phylogenetic tree was constructed by analysis of the amino acid sequences of each P domain (Fig. 2). Domain P1 of CMC718 was clustered with five B5 reference strains and exhibited nucleotide and amino acid similarities ranging from 93.5–99.1% and 99.3–99.9%, respectively, while similarities between P1 of CMC718 and the other 40 reference strains ranged from 80.6–91.7% and 95.7–99.7%, respectively (Table 3). Domain P2 of CMC718 also clustered with five B5 reference strains and exhibited nucleotide and amino acid similarities ranging from 95.0–99.1% and 99.1–99.8%, respectively, while similarities between P2 of CMC718 and the other 40 reference strains ranged between 77.9–93.5% and 92.0–99.3%, respectively. Similar to P1 and P2, domain P3 of CMC718 also clustered with five B5 reference strains and exhibited nucleotide and amino acid similarities ranging from 93.4–98.8% and 97.9–99.3%, respectively, while similarities between P3 of CMC718 and the other 40 reference strains ranged from 77.9–92.5% and 90.7–97.5%, respectively.

Nucleotide and amino acid polymorphisms

Comparison of predicted amino acid sequences of various B5 genotypes was performed to identify amino acid variations according to country and year of isolation. Table 4 shows the amino acid substitution pattern of CMC718 compared with 34 strains collected from 2000 to 2015 based on sequence alignment. CMC718 was classified with B5 strains collected from 2012–2013. In addition, whole-genome sequencing indicated that 15 amino acid variants existed in the CMC718 strain. The variants included four substitutions ($VP3_{68}$, $2C_{41}$, $3D_{78}$, and $3D_{204}$) that were unique and not

Table 3. Amino acid sequence identities between CMC718 strain and other EV71 subgenogroups

Region	A	B0	B1	B2	B3	B4	B5	C1	C2	C3	C4	C5
P1	96.9	98.5–98.7	98.6–99.2	99.2	99.3–99.5	99.3–99.7	99.3–99.9	97.7–97.8	97.2–97.7	97.7	97.6–97.8	97.3–97.7
VP4	100	98.6	98.6–100	87.1–100	100	100	98.6–100	98.6–100	100	100	100	100
VP2	98	99.2–99.2	99.2–99.6	99.2–99.6	99.2–99.6	99.6	99.2–100	97.6–98	96.9–98.0	98	96.1–98.0	98
VP3	97.9	98.3–98.8	98.3–99.2	98.8–99.2	99.2–99.6	99.6	99.6	96.3–96.7	96.3–96.7	96.7	93.8–96.7	96.7
VP1	94.3	97.6–98.7	98.0–98.7	99.3	99.0–99.3	98.7–99.7	99.0–100	98.0–98.3	97.3–98.3	97.6	96.0–98.3	96.6–97.6
P2	94.5	96.4–96.9	97.4–98.6	97.6–99.3	97.1–97.2	98.8–99.1	99.1–99.8	95.3–95.7	92.0–95.5	95.0–95.2	93.3–96.9	94.8–95.2
2A	94.1	96.8	96.3–98.9	97.3–98.9	97.3–97.9	98.4–98.9	98.9–100	94.7–96.3	94.7–95.2	93.6–94.1	94.7–96.8	94.1–94.7
2B	93.9	96.0–97.0	97.0–100	100	98	100	100	91.9–92.9	80.9–91.9	92.9	91.9–97.0	90.9–92.9
2C	94.5	96.0–96.7	87.0–98.2	97.3–99.4	96.0–96.4	98.8–99.1	99.1–99.7	95.7–96.0	90.0–96.0	95.7	92.7–96.4	95.4–95.7
P3	93.8	95.8–95.9	96.1–96.8	96.3–96.5	93.8–93.9	97.2–97.5	97.9–99.3	93.2–93.5	90.7–93.0	93.2–93.4	90.8–94.2	93.0–93.6
3A	100	98.8	98.9–100	97.7–100	87.7–98.8	97.7–100	98.8–100	94.2–94.2	88.4–94.2	93	94.2–98.8	93.0–94.2
3B	90.9	95.5	95.5–100	90.9–95.5	95.5	95.5–100	100	95.5	95.5	95.5	90.9–100	93.5–95.5
3C	92.3	96.2	96.2–97.3	96.7	94.5	96.2–97.8	98.4–99.5	91.8	85.8–92.3	90.2–90.7	91.3–94.5	90.7–91.8
3D	95	95.2	95.7–96.3	92.6–96.1	92.6–97.2	97.0–97.2	93.9–99.1	93.1–93.7	92.9–94.4	92.6–94.4	90.0–93.3	93.7–94.2

Table 4. Comparison major amino acid substitutions in B5 strains of 2000-2015

Strain	P1	P2		P3												
	VP3	2A	2C	3C	3D											
	68	102	41	257	182	8	22	78	126	132	140	143	170	204	228	251	383
2015-VNM-B5 94	R	A	K	D	M	P	R	A	K	.	R	F	R	T	S	V	H
2015-VNM-B5 90	R	A	K	D	M	P	R	A	K	.	R	F	R	T	S	V	H
2015-VNM-B5 50	R	A	K	D	M	P	R	A	K	.	R	F	R	T	S	V	H
2015-VNM-B5 118	R	A	K	D	M	P	R	A	K	.	R	F	R	T	S	V	H
2014-THA-A1570	R	A	K	D	M	.	R	A	K	.	R	F	G	T	S	V	H
2014-THA-A1564	R	A	K	D	M	.	R	A	K	.	R	F	R	T	S	V	H
2014-CHN-J098	R	A	K	D	M	P	R	A	K	.	R	F	R	T	S	V	H
2014-VNM-15.2	R	.	K	D	M	P	R	A	K	.	R	F	R	T	S	V	H
2014-VNM-15.1	R	.	K	D	M	P	R	A	K	.	R	F	R	T	S	V	H
2013-THA-A1083	R	A	K	D	M	.	R	A	K	.	R	F	R	T	S	V	H
2013-THA-A1048	R	A	K	D	M	.	R	A	K	.	R	F	R	T	S	V	H
2013-FRA-PAR024102	R	A	K	D	M	.	R	A	K	.	R	F	R	T	S	V	H
2013-VNM-136	R	.	K	D	M	P	R	A	K	.	R	F	R	T	S	V	H
2013-VNM-135	R	.	K	.	T	P	.	A	.	.	.	.	.	T	.	.	H
2013-VNM-134	R	.	K	.	T	.	.	A	.	.	.	.	.	T	.	.	H
2012-KOR-CMC718	**K**	**V**	**R**	**E**	**A**	**S**	**H**	**P**	**R**	**N**	**K**	**L**	**K**	**A**	**A**	**I**	**Y**
2012-TW-M988	R	.	K	.	T	P	.	A	.	.	.	.	.	T	.	.	.
2012-TW-96015	R	.	K	.	T	P	.	A	.	.	.	.	.	T	.	.	.
2012-THA-A851	R	A	K	D	.	P	R	A	K	D	.	F	.	T	S	V	.
2012-THA-A847	R	A	K	D	.	P	R	A	K	D	.	F	.	T	S	V	.
2012-THA-A761	R	A	K	D	.	P	R	A	K	D	.	Y	.	T	S	V	.
2012-THA-A577	R	A	K	D	.	P	R	A	K	D	.	F	.	T	S	V	.
2011-TW-N1745	R	A	K	D	M	P	R	A	K	.	.	F	.	T	S	V	H
2011-THA-SiICRC13	R	A	K	D	V	.	R	A	K	D	.	F	.	T	S	V	H
2009-TW-02877	R	A	K	D	M	P	R	A	K	.	.	F	.	T	S	V	H
2009-CHN-Xiamen	R	.	K	D	M	P	R	A	K	.	.	F	.	T	.	V	H
2008-TW-M0380	R	A	K	D	M	P	R	A	K	.	.	F	.	T	S	V	H
2007-TW-08747	R	A	K	D	M	P	R	A	K	.	.	F	.	T	S	V	H
2007-TW-07364	R	.	K	D	E	P	R	A	K	D	.	F	.	T	S	.	H
2006-BRU-35334	R	A	K	D	V	P	R	A	K	D	.	F	.	T	S	.	H
2003-TW-N2838	R	A	K	D	V	P	R	A	K	D	.	F	.	T	S	.	H
2003-MYS-S19841	R	A	K	D	V	P	R	A	K	D	.	F	.	T	S	V	H
2003-JPN-Yamagata-03	R	A	K	D	V	P	R	A	K	D	.	F	.	T	S	V	H
2000-SIN-5511	R	A	K	D	V	P	R	A	K	D	.	F	.	T	S	V	H

found in any other B5 strains. At position 862 aa of the structural protein coding region, only one amino acid substitution was detected in CMC718, that being a change from Arg to Lys at $VP3_{68}$. A total of 14 amino acid substitutions were detected in the 1,331 amino acids of the non-structural protein coding region of CMC718. The specific changes are as follows: $2A_{102}$ (Ala→Val), $2C_{41}$ (Lys→Arg), $2C_{257}$ (Asp→Glu), $3C_{182}$ (Met→Ala), $3D_8$ (Pro→Ser), $3D_{22}$ (Arg→His), $3D_{78}$ (Ala→Pro), $3D_{126}$ (Lys→Arg), $3D_{132}$ (Asp→Asn), $3D_{143}$ (Phe→Leu), $3D_{204}$ (Thr→Ala), $3D_{228}$ (Ser→Ala), $3D_{251}$ (Val-Ile), and $3D_{383}$ (His→Tyr).

Discussion

EV71 is one of the main etiological agents of HFMD worldwide and since its first isolation in California, USA in 1969, various forms of outbreaks have occurred worldwide (Lin *et al.*, 2003). EV71 exhibits cyclic patterns of genotypic di-

versity with different strains appearing every 2–3 years in various countries (Horwood *et al.*, 2016; Kim *et al.*, 2016a; NikNadia *et al.*, 2016). Among these, the B5 genotype was first discovered in Singapore in 2000 (Noisumdaeng *et al.*, 2018) and later proved to be the primary cause of HFMD outbreaks in Asia-Pacific countries such as Vietnam and Taiwan (Yi *et al.*, 2017).

The current study isolated EV71 strains from clinical samples collected from patients with HFMD in Korea and confirmed through full-genome sequence analysis to be of the B5 genotype. This is the first analysis reported of the B5 gene type in South Korea. The P1 domain showed extremely high amino acid identity (99.9%) with strain 0874 isolated in Taiwan in 2007. The amino acid sequences of domains P2 and P3 each showed the greatest identity with those domains of strain M988 isolated in Taiwan in 2012, with 99.8 and 99.3% identity, respectively. In effort to understand the evolution of EV71, this study focused on the P3 region where mutations occurred most frequently. The P3 region of enterovi-

ruses are divided into four sections, 3A–3D. In the case of strain CMC718, 11 of 15 amino acid variants occurred in the P3 domain. Specifically, one variant was confirmed in 3C and 10 variants in 3D. The 3C region contains the proteolytic activity that cleaves the viral precursor polyprotein into structural and functional proteins and activates the 3D protein (Cao *et al.*, 2019). Position $3C_{182}$ can exhibit various amino acid variants, but has appeared to be stable in isolates since 2013 with a Met residue being found at this position. Because the 3D region encodes an RNA-dependent RNA polymerase (RdRp) that lacks proofreading activity, it exhibits a high error rate when replicating RNA (Ferrer-Orta *et al.*, 2015; Cox *et al.*, 2017). The analysis of region 3D revealed an unusual amino acid variant pattern in the B5 reference strains. Variants are observed at positions $3D_{22}$, $3D_{126}$, $3D_{143}$, and $3D_{228}$ in strains isolated from the 2012 and 2013 outbreaks and at position $3D_{383}$ in the strain isolated from the 2012 outbreak, but not in strains isolated from outbreaks of other years. In contrast, variants at positions $3D_{140}$ and $3D_{170}$ have been seen in strains isolated in 2013. This has also been observed in strains isolated from later years. Consistent with this, strain CMC718, which was isolate from a sample collected in 2012, had a pattern similar to the strain-specific amino acid variants discovered in 2012–2013.

Although rare, the accompaniment of neurological manifestations in HFMD caused by EV71, such as aseptic meningitis, acute flaccid paralysis, and encephalitis, may prove lethal in the most severe cases (Jung *et al.*, 1998; Ooi *et al.*, 2010). Prompt and accurate identification of the specific pathogen is crucial in order for clinicians to be able to appreciate the possibility of such life-threatening conditions. However, it should be noted that the B5 reference strains used in this study were limited to Thailand, Vietnam, and Taiwan and it was difficult to analyze possible interactions between the reference strains. In order to analyze the relationship between strains from different countries, additional reference strains must be analyzed.

This is the first study to report the full-length sequence of an EV71 B5 strain isolated from a clinical sample from South Korea. This sequence data should be useful in comparing full-length B5 sequences of other strains identified globally. Moreover, the information acquired from the whole-genome sequence of strain CMC718 may prove useful for obtaining more accurate diagnoses of enteroviruses, as well as for advancing basic research aimed at elucidating genetic functions and predicting new pandemic variants. This also provides important data needed for comparing enteroviruses as part of the process of vaccine development.

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