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Enteroviruses isolated from herpangina and hand-foot-and-mouth disease in Korean children

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Abstract

Hand-foot-and-mouth disease (HFMD) and herpangina are commonly prevalent illness in young children. They are similarly characterized by lesions on the skin and oral mucosa. Both diseases are associated with various enterovirus serotypes. In this study, enteroviruses from patients with these diseases in Korea in 2009 were isolated and analyzed. Demographic data for patients with HFMD and herpangina were compared and all enterovirus isolates were amplified in the VP1 region by reverse transcription-polymerase chain reaction and sequenced. Among the enterovirus isolates, prevalent agents were coxsackievirus A16 in HFMD and coxsackievirus A5 in herpangina. More prevalent months for HFMD were June (69.2%) and May (11.5%), and June (40.0%) and July (24.0%) for herpangina. Age prevalence of HFMD patients with enterovirus infection was 1 year (23.1%), 4 years (19.2%), and over 5 years (19.2%). However, the dominant age group of herpangina patients with enterovirus infection was 1 year (48.0%) followed by 2 years (28.0%). Comparison of pairwise VP1 nucleotide sequence alignment of all isolates within the same serotypes revealed high intra-type variation of CVA2 isolates (84.6–99.3% nucleotide identity). HFMD and herpangina showed differences in demographic data and serotypes of isolated enteroviruses, but there was no notable difference in amino acid sequences by clinical syndromes in multiple comparison of the partial VP1 gene sequence.

Keywords: Herpangina, Hand-foot-and-mouth disease, Enteroviruses

Introduction

Hand-foot-and-mouth disease (HFMD) and herpangina, which commonly affect young children, are enterovirus infections causing a variety of exanthems. HFMD is a self-limiting exanthematous eruption characterized by vesicles in the oral cavity, mainly in the buccal mucosa and tongue, and peripherally distributed cutaneous lesions on the hands and feet. Herpangina produces multiple oral ulcers affecting predominantly the posterior part of the oral cavity only [1-3]. These diseases are associated with different strains of enteroviruses, such as coxsackievirus A (CVA) 2, 5, 6, 10, 16; coxsackievirus B (CVB) 1, 2, 5; and enterovirus (EV) 71 [4-8].

Human enterovirus (HEV) genera containing the CVA, CVB, echovirus (ECV), and EV serotypes are transmitted mainly via the fecal-oral route and by contact with throat discharges or fluid from blisters [9]. Generally, HEV outbreaks peak during the summer and early fall, and various serotypes are often associated with a single outbreak [10]. Since 1993, when nationwide surveillance began in Korea, there have been reports of summer outbreaks of enteroviruses caused by ECV 5, 6, 7, 9, 13, 18, and 30; CVA 24; CVB 3 and 5; and EV 71 [11,12]. Especially, outbreaks of HFMD and herpangina caused by HEV infection were reported in 2009 in Korea [13,14].

Diagnosis of HEV infections is based on amplification of a highly conserved 5' non-coding region (NCR) that is widely-targeted in diagnostic procedures [15,16]. In addition to traditional virological methods to serotype HEV, reverse transcription-polymerase chain reaction (RT-PCR) based on amplification of the VP1 region have been recently developed [17-19]. Because the VP1 region is one of the main exposed regions of the viral capsid and

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has been suggested to include a serotype specific antigenic neutralization site, the BC loop in this region has been implicated with viral antigenicity, and substitutions resulting in conformational changes in this region are believed to play a role in host adaptation for HEVs [20,21].

In this study, to compare epidemic patterns of HFMD and herpangina, specimens from patients with HFMD and herpangina disease were collected and analyzed along with demographic data. Molecular detection by 5' NCR RT-PCR and sequencing of the VP1 region of HEVs were carried out.

Materials and methods

Diagnostic definitions

Herpangina was defined as the presence of oral ulcers on the anterior tonsillar pillars, soft palate, buccal mucosa, or uvula. Patients with HFMD had oral ulcers on the tongue or the buccal mucosa, and vesicular rashes over the palms, soles, knees, or buttocks [22]. This study was conducted in accordance with ethical principles as formulated in the World Medical Association Declaration of Helsinki and approved by the institutional review board (IRB No. 2012-48) of the Ethical Committee of Soonchunhyang University Cheonan Hospital. Additionally, informed consent was obtained from the parents on the patients' behalf who participated in the study and the parents of participants also gave their consent to publish the data.

Detection of enterovirus and molecular typing

Stool specimens from 29 HFMD and 32 herpangina patients were collected from collaborative JeongGene Pediatrics hospital in Chungnam province, Korea during 2009. The testing algorithm for detection and molecular typing of HEV has been previously described [12]. Viral RNA was extracted from the supernatant of infected cells using magnetic beads (Toyobo, Osaka, Japan). Extracted RNA was dissolved in 50 μ L of nuclease-free water and stored at -70°C until used for RT-PCR.

HEVs were assayed for in clinical samples using a AccuPower[®] Enterovirus Real-Time RT-PCR Kit (Bio-ner, Daejeon, Korea) based on 5' NCR of highly

conserved region in the HEV genome, according to the manufacturer's instructions [23]. If the sample was positive, it was performed semi-nested PCR in VP1 coding region for molecular typing. Semi-nested PCR condition and primer sequences amplifying the VP1 coding region were described previously [18]. In the initial PCR, a 50 μ L reaction mix containing 0.2 μ M of primers 224 and 222, 2 U of Taq DNA polymerase (Promega, Madison, WI), 100 μ M concentrations of mixture of dNTPs, and 2 μ M MgCl₂ was amplified by 40 cycles of 95°C for 30 sec, 42°C for 30 sec, and 60°C for 45 s. One microliter of the first PCR product was added to a second PCR for semi-nested amplification. Fifty microliters of a reaction mix containing 0.2 μ M of primers AN89 and AN88, 2.5 U of *Taq* DNA polymerase (Promega), 100 μ M concentrations of a mixture of dNTPs, and 2 μ M MgCl₂ was incubated at 95°C for 6 min prior to 40 amplification cycles of 95°C for 30 s, 60°C for 20 s, and 72°C for 15 s. PCR products were purified using the QIA quick PCR purification kit (Qiagen, Valencia, CA). Purified DNA was added in a reaction mixture containing 2 μ L of Big Dye terminator reaction mix (Applied Biosystems, Foster City, CA) and 2 pmoles of AN88 and AN89 primers. Sequencing reactions were subjected to initial denaturation at 96°C for 1 min and 25 cycles consisting of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min in a Gene Amp PCR system 2700 (Applied Biosystems, Foster City, CA). The products were purified by precipitation with 100% cold ethanol and 3 M sodium-acetate (pH 5.8), and then loaded on a model 3100 automated genetic analyzer (Applied Biosystems). The molecular type of each isolates was determined by the serotype of the highest scoring strain in Genbank using the Basic Local Alignment Search Tool (BLAST); that is, the sequence of the HEV strain that gave the highest nucleotide similarity value with the query sequence [24].

Sequence analysis

Sequence analysis was carried out for five strains of CVA 2, 16 strains of CVA 5, 18 strains of CVA 16, two

Table 1 Numbers of enterovirus serotype isolated from patients with HFMD and herpangina

Serotypes	HFMD		Herpangina	
	Isolate numbers	Percentage of subtotal	Isolate numbers	Percentage of subtotal
Coxsackievirus A2	0	-	5	20.0
Coxsackievirus A5	3	11.5	13	52.0
Coxsackievirus A16	16	61.5	2	8.0
Coxsackievirus B1	1	3.8	1	4.0
Enterovirus 71	4	15.4	2	8.0
Untypable	2	7.7	2	8.0
Total	26	100	25	100

strains of CVB 1, and six strains of EV71 (Table 1). Nucleotide and deduced amino acid sequences of all HEV isolates were compared with the reference strains using CLUSTAL W (version 1.81) and Megalign (DNASTAR) [25]. The phylogenetic relationships among the VP1 sequences of each virus isolate were inferred using MEGA software v. 5.05. Maximum Composite Likelihood was used as the substitution method, while the neighbor-joining method was used to reconstruct the phylogenetic tree [26]. The reliability of the phylogenetic tree was determined by bootstrap re-sampling of 1,000 replicates.

Nucleotide sequence accession numbers

The HEV sequences reported here were deposited in the GenBank sequence database under accession numbers JF773151 to JF773197.

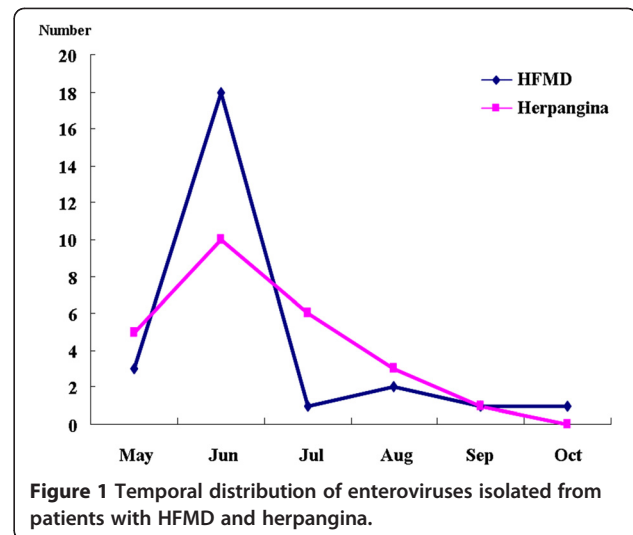
Results

Enterovirus detection and molecular typing

Among stool specimens from 29 HFMD and 32 herpangina patients, the approach detected 26 HEV isolates (89.7%) in HFMD and 25 HEV isolates (78.1%) in herpangina. The types in the 26 isolates from HFMD patients were mainly identified as CVA 16 (16 isolates, 61.5%), EV 71 (four isolates, 15.4%), CVA 5 (three isolates, 11.5%). The types in the 25 isolates from herpangina patients were mainly identified as CVA 5 (13 isolates, 52.0%), CVA 2 (five isolates, 20.0%) (Table 1).

Epidemiological features

In temporal distribution, HEV positive samples from HFMD patients comprised three isolates (11.5%) in May, 18 isolates (69.2%) in June, one isolate (3.8%) in July, two isolates (7.7%) in August, one isolate (3.8%) in September, and one isolate (3.8%) in October. The HEV positive samples from herpangina patients comprised five isolates (20.0%) in May, 10 isolates (40.0%) in June, six isolates (24.0%) in July, three isolates (12.0%) in August, and one isolate (4.0%) in September (Figure 1). Concerning the age of HFMD patients with enterovirus infection, two isolates (7.7%) were recovered from patients <1-year-of-age, six isolates (23.1%) at 1-year-of-age, four isolates (15.4%) at 2-years-of-age, four isolates (15.4%) at 3-years-of-age, five isolates (19.2%) at 4-years-of-age, and five isolates (19.2%) at >5-years-of-age. Concerning the age of herpangina patients with enterovirus infection, two isolates (8.0%) were recovered at <1-year-of-age, 12 isolates (48.0%) at 1-year-of-age, seven isolates (28.0%) at 2-years-of-age, three isolates (12.0%) at 3-years-of-age, and one isolate (4.0%) at 4-years-of-age (Figure 2).



Sequence analysis of enteroviruses

Based on the VP1 amino acid sequence comparison for all isolates, all sequences represented the same intra-serotype, except for the Kor09-CVA5-309cn (herpangina) isolate, regardless of syndrome (Figure 3). The pairwise VP1 nucleotide sequence alignment of all isolates was compared within the same serotypes; CVA2 isolates displayed 84.6–99.3% nucleotide identity within the intra-type. CVA5, 16, CVB1, and EV71 isolates showed 98.7–100%, 89.6–100%, 99.4%, and 98.9–100% identity, respectively. The phylogenetic relationships of Korean isolates and reference strains are shown in Figure 4.

Discussion

HFMD and herpangina were epidemic in Korea in 2009 [13,14] and are commonly prevalent illness in young children. They are similarly characterized by lesions on the skin and oral mucosa. Also, these two diseases are associated with various enterovirus serotypes. In HFMD, it has been reported that the major causative HEV types are CVA16 and EV71; these types were also more prevalent serotypes in Korea in 2009 [27,28]. Generally, CVA6 and CVA10 are the more prevalent serotypes in herpangina; however, CVA5 and CVA2 were the prevalent herpangina serotypes in Korea in 2009 [29]. In multiple comparison of partial VP1 gene in the intratypes, no notable variation in amino acid sequences by clinical syndrome was evident. The VP1 region containing the BC loop is associated with viral antigenicity and substitutions of amino acid sequences in this region are believed to be important in host infection of HEVs [20,21,30]. No significant clustered relationship was found according to the clinical syndromes. When the pairwise VP1 nucleotide sequence alignment of all isolates was compared within the same serotypes, CVA2

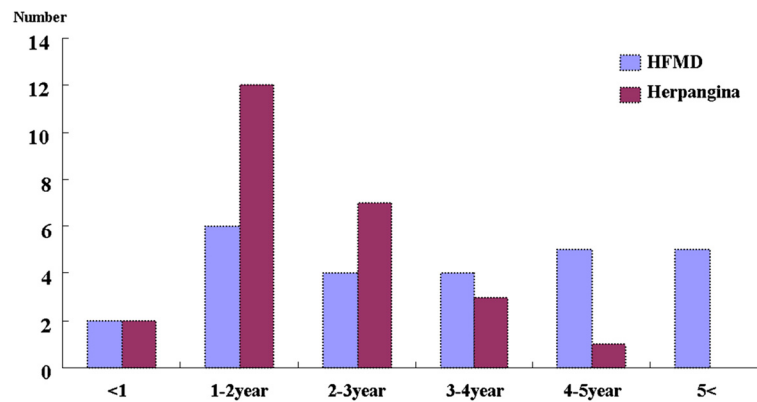


Figure 2 Age distribution of enteroviruses isolated from patients with HFMD and herpangina.

isolates showed an appreciable variation in nucleotide identity (84.6–99.3%) within the intra-type. Deduced amino acid sequences of all strains were in the same intra-serotype, except for one isolate, regardless of syndromes.

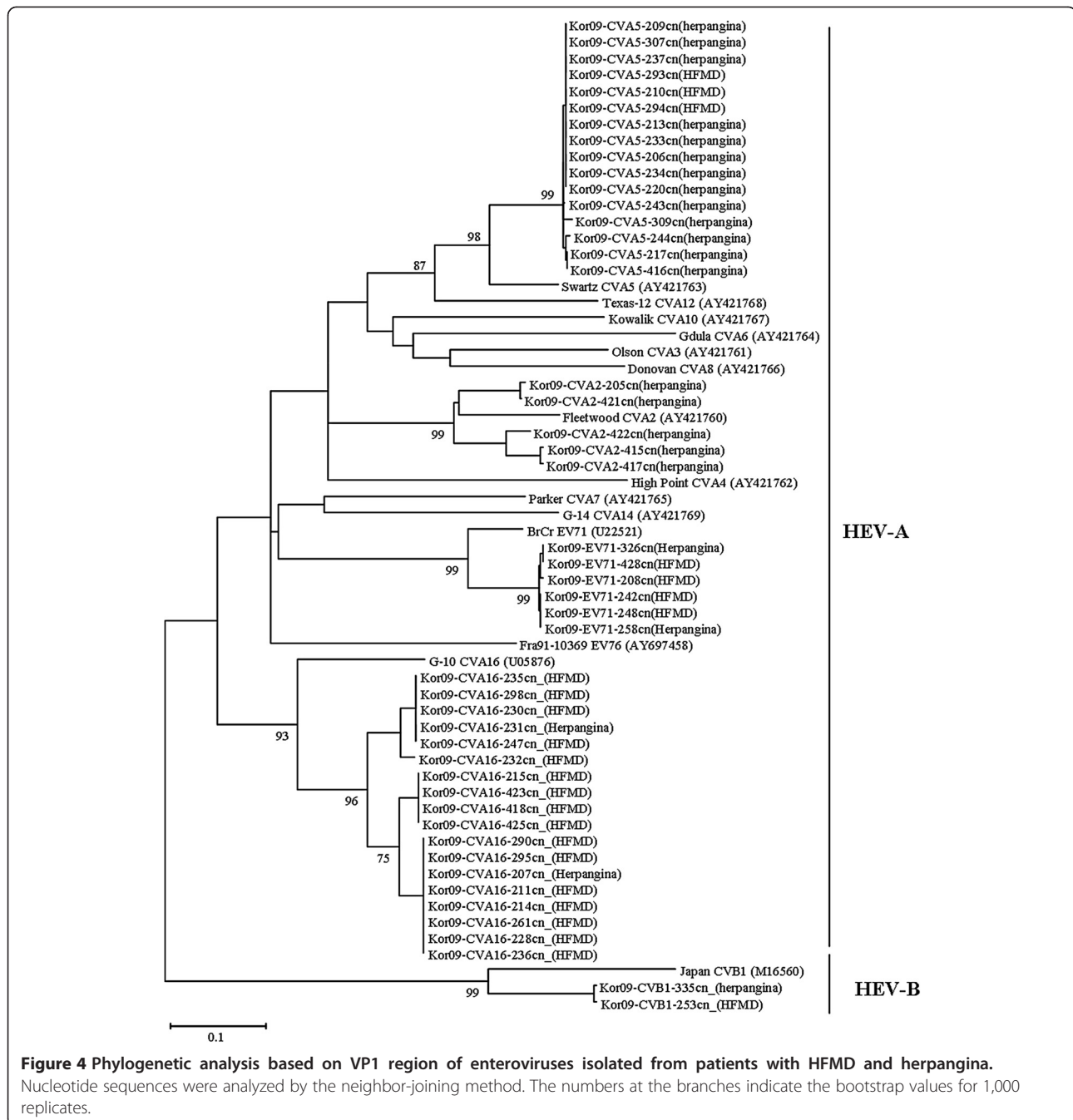
There was no significant difference in age distribution between herpangina (3.74 ± 2.48 years) and HFMD (3.54 ± 2.05 years) in a previous report [31]. But, in this study, age distribution of HEV positive in herpangina patients was relatively lower than in HFMD patients. In the 2009 Korean

epidemic, enterovirus infections were most prevalent in July [32]. However, temporal distribution of HEV positive in herpangina and HFMD peaked in June, with herpangina gradually decreased thereafter while HFMD decreased rapidly.

In conclusion, HFMD and herpangina showed differences in demographic data and serotypes of isolated HEVs, but there was no notable difference in amino acid sequences by clinical syndromes in multiple comparison of the partial VP1 gene sequence.

I ETRCVLNHHSTOETSI GHFFSRAGLVGI I TMPDGTGNT - GYANWDI DL MGYAOLRRKLELFT YMRFDAEFFTFVVAKPNGL										Majority													
10	20	30	40	50	60	70	80																
V	KN	VE	A	N	A	KVELN	A	FT	I	M	N	ATTRA	QV	Kor09-CVA2-205cn(herpangina)									
V	KN	VE	A	N	A	KVELN	A	FT	I	M	N	ATTRA	QV	Kor09-CVA2-415cn(herpangina)									
V	KN	VE	A	N	A	KVELN	A	FT	I	M	N	ATTRA	QV	Kor09-CVA2-417cn(herpangina)									
V	KN	VE	A	N	A	KVELN	A	FT	I	M	N	ATTRA	QV	Kor09-CVA2-421cn(herpangina)									
V	KN	VE	A	N	A	KVELN	A	FT	I	M	N	ATTRA	QV	Kor09-CVA2-422cn(herpangina)									
V	R	GVM	VE	S	A	LIIE	S	TK	T	E	V	FV	M	Kor09-CVA5-206cn(herpangina)									
V	R	GVM	VE	S	A	LIIE	S	TK	T	E	V	FV	M	Kor09-CVA5-209cn(herpangina)									
V	R	GVM	VE	S	A	LIIE	S	TK	T	E	V	FV	M	Kor09-CVA5-210cn(HFMD)									
V	R	GVM	VE	S	A	LIIE	S	TK	T	E	V	FV	M	Kor09-CVA5-213cn(herpangina)									
V	R	GVM	VE	S	A	LIIE	S	TK	T	E	V	FV	M	Kor09-CVA5-217cn(herpangina)									
V	R	GVM	VE	S	A	LIIE	S	TK	T	E	V	FV	M	Kor09-CVA5-220cn(herpangina)									
V	R	GVM	VE	S	A	LIIE	S	TK	T	E	V	FV	M	Kor09-CVA5-233cn(herpangina)									
V	R	GVM	VE	S	A	LIIE	S	TK	T	E	V	FV	M	Kor09-CVA5-234cn(herpangina)									
V	R	GVM	VE	S	A	LIIE	S	TK	T	E	V	FV	M	Kor09-CVA5-237cn(herpangina)									
V	R	GVM	VE	S	A	LIIE	S	TK	T	E	V	FV	M	Kor09-CVA5-243cn(herpangina)									
V	R	GVM	VE	S	A	LIIE	S	TK	T	E	V	FV	M	Kor09-CVA5-244cn(herpangina)									
V	R	GVM	VE	S	A	LIIE	S	TK	T	E	V	FV	M	Kor09-CVA5-293cn(HFMD)									
V	R	GVM	VE	S	A	LIIE	S	TK	T	E	V	FV	M	Kor09-CVA5-294cn(HFMD)									
V	R	GVM	VE	S	A	LIIE	S	TK	T	E	V	FV	M	Kor09-CVA5-307cn(herpangina)									
V	R	GVM	VE	S	A	LIIE	S	TK	T	E	V	FV	M	Kor09-CVA5-309cn(herpangina)									
V	R	GVM	VE	S	A	LIIE	S	TK	T	E	V	FV	M	Kor09-CVA5-416cn(herpangina)									
A	N	S	T	Q	D	V				C				Kor09-CVA16-207cn (Herpangina)									
A	N	S	T	Q	D	V				C				Kor09-CVA16-211cn (HFMD)									
A	N	S	T	Q	D	V				C				Kor09-CVA16-214cn (HFMD)									
A	N	S	T	Q	D	V				C				Kor09-CVA16-215cn (HFMD)									
A	N	S	T	Q	D	V				C				Kor09-CVA16-228cn (HFMD)									
A	N	S	T	Q	D	V				C				Kor09-CVA16-230cn (HFMD)									
A	N	S	T	Q	D	V				C				Kor09-CVA16-231cn (Herpangina)									
A	N	S	T	Q	D	V				C				Kor09-CVA16-232cn (HFMD)									
A	N	S	T	Q	D	V				C				Kor09-CVA16-235cn (HFMD)									
A	N	S	T	Q	D	V				C				Kor09-CVA16-236cn (HFMD)									
A	N	S	T	Q	D	V				C				Kor09-CVA16-247cn (HFMD)									
A	N	S	T	Q	D	V				C				Kor09-CVA16-261cn (HFMD)									
A	N	S	T	Q	D	V				C				Kor09-CVA16-290cn (HFMD)									
A	N	S	T	Q	D	V				C				Kor09-CVA16-295cn (HFMD)									
A	N	S	T	Q	D	V				C				Kor09-CVA16-298cn (HFMD)									
A	N	S	T	Q	D	V				C				Kor09-CVA16-418cn (HFMD)									
A	N	S	T	Q	D	V				C				Kor09-CVA16-423cn (HFMD)									
A	N	S	T	Q	D	V				C				Kor09-CVA16-425cn (HFMD)									
S	A	TLDS	E	DL	LE	T	PN			I	T	M	V	Kor09-EV71-208cn (HFMD)									
S	A	TLDS	E	DL	LE	T	PN			I	T	M	V	Kor09-EV71-242cn (HFMD)									
S	A	TLDS	E	DL	LE	T	PN			I	T	M	V	Kor09-EV71-248cn (HFMD)									
S	A	TLDS	E	DL	LE	T	PN			I	T	M	V	Kor09-EV71-258cn (Herpangina)									
S	A	TLDS	E	DL	LE	T	PN			I	T	M	V	Kor09-EV71-326cn (Herpangina)									
S	A	TLDS	E	DL	LE	T	PN			I	T	M	V	Kor09-EV71-428cn (HFMD)									
MQ	H	K	Y	RS	S	VEN	LC	SAC	YYA	Y	..	NNTEK	..	E	VVNTRQV	..	L	L	L	..	ITS	AQPS	Kor09-CVB1-253cn (HFMD)
MQ	H	K	Y	RS	S	VEN	LC	SAC	YYA	Y	..	NNTEK	..	E	VVNTRQV	..	L	L	L	..	ITS	AQPS	Kor09-CVB1-335cn (herpangina)

Figure 3 Comparison of the deduced amino acid sequences of VP1 region of 47 enteroviruses isolated from patients with HFMD and herpangina. The rectangular parts are BC loop previously reported.



Competing interests

The authors declare that they have no competing interests.

Authors' contributions

KSP, SGY, KAB, JWS, HKC and DSC performed molecular diagnosis and sequence analysis. BHL and JSP contributed to specimen collections and clinical diagnosis. KAY and YJC designed the study and critically revised the manuscript. All of the authors read and approved the final version of the manuscript.

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