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Original Article

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First Detection of Enterovirus D68 in Korean Children, September 2022

Ki Wook Yun [1,2], Bin Ahn [1,2], Sung Hwan Choi [2], Da Yeon Kang [2], Taek Soo Kim [3], Mi Kyung Lee [4], Kyoung Un Park [5], and Eun Hwa Choi [1,2]

[1]Department of Pediatrics, Seoul National University College of Medicine, Seoul, Korea
[2]Department of Pediatrics, Seoul National University Children's Hospital, Seoul, Korea
[3]Department of Laboratory Medicine, Seoul National University Hospital, Seoul, Korea
[4]Department of Laboratory Medicine, Chung-Ang University Hospital, Seoul, Korea
[5]Department of Laboratory Medicine, Seoul National University Bundang Hospital, Seongnam, Korea

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ABSTRACT

Background: Enterovirus D68 (EV-D68) is a re-emerging pathogen that is particularly common in children and may cause asthma-like respiratory infection and acute flaccid myelitis. However, in Korea, EV-D68 has never been reported thus far. This study aimed to identify EV-D68 from nasopharyngeal aspirates (NPAs) in Korean children with a respiratory tract infection.

Materials and Methods: The EV-D68 reference strain was purchased and blindly used to assess the detection ability of three commercial and one in-house mRT-PCR kit in 2018. Then, we selected children whose specimens were positive for human rhinovirus (HRV) and/or enterovirus (EV) by Allplex mRT-PCR (Seegene, Inc., Seoul, Korea) from April to December 2022. Total RNA was extracted from NPAs, and a partial 5′-UTR gene was amplified and sequenced for the identification of HRV/EV species. Additionally, PCR targeting the VP1 gene was performed to assess EV-D68-positive NPAs, followed by sequencing. Phylogenetic analysis and comparison of amino acid sequence alignments were performed using a partial VP1 gene of our and recent international EV-D68 strains.

Results: Among the mRT-PCR kits tested, only the in-house kit was able to detect EV-D68 in 2018. However, we detected three EV-D68 strains among children hospitalized with fever and/or respiratory symptoms in September - December 2022 who tested positive for EV by the Allplex kit. Two of them were healthy toddlers with lower respiratory infections accompanied by new-onset wheezing but no neurologic complications. Among 34 children with lower respiratory infection who tested positive for HRV during the same period, EV-D68 was not detected. Phylogenetic analysis revealed that the first Korean EV-D68 belonged to subclade B3. Amino acid sequence alignment of international subclade B3 EV-D68 strains also showed that our strain is genetically more related to those from Europe than those from Japan.

Conclusion: We first detected EV-D68 in three Korean children who had EV detected by the Allplex mRT-PCR kit in 2022. EV-D68 also circulated in Korea in fall 2022, but the prevalence and severity seemed to be lower than those in previous reports from other countries.

Keywords: Enterovirus D68; Children; Respiratory infection

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Corresponding Author:
Eun Hwa Choi, MD, PhD
Department of Pediatrics, Seoul National University Children's Hospital, 101 Daehak-ro, Jongno-gu, Seoul 03080, Korea.
Tel: +82-2-2072-3624, Fax: +82-2-743-3455, Email: eunchoi@snu.ac.kr

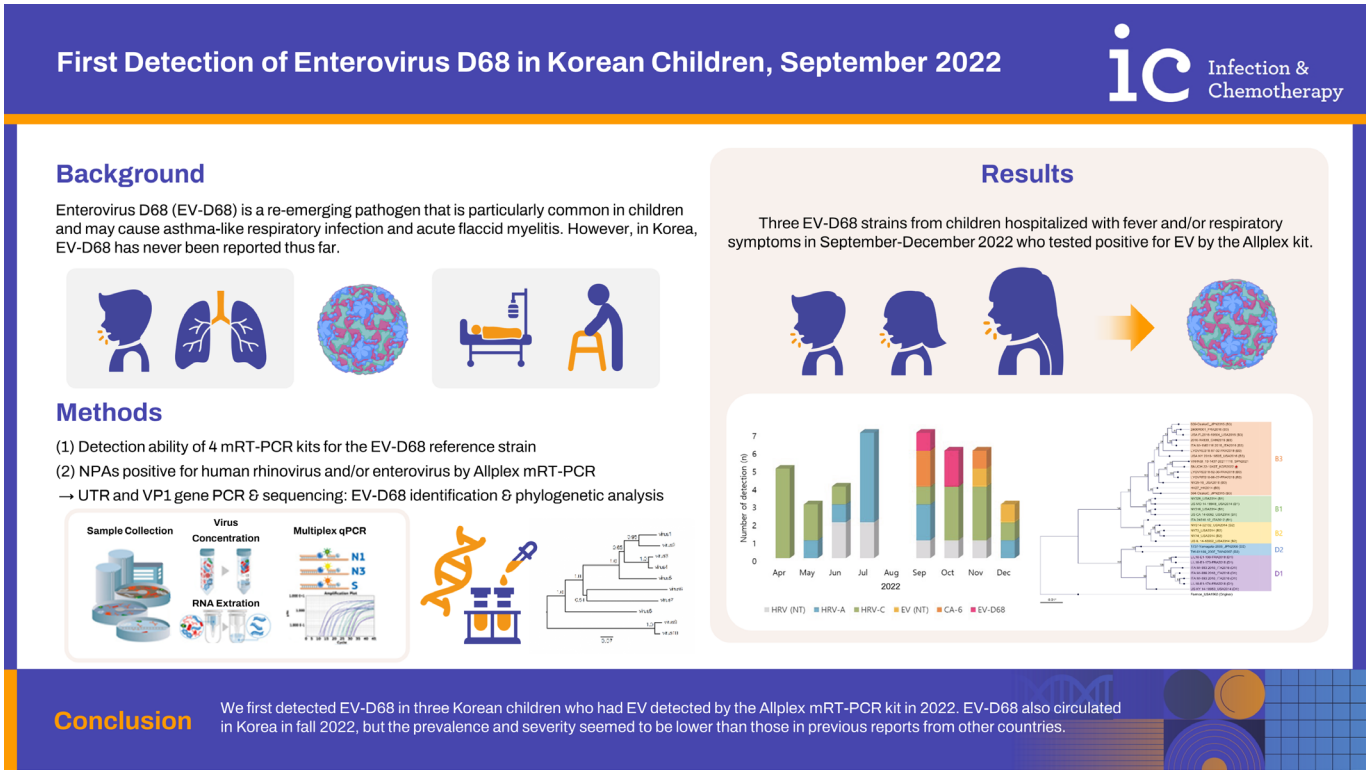
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GRAPHICAL ABSTRACT



INTRODUCTION

Enterovirus D68 (EV-D68) is a re-emerging respiratory pathogen that is particularly common in children and may cause asthma-like respiratory infection and acute flaccid myelitis (AFM) [1]. EV-D68 was first detected in the United States (US) in 1962, and then it rarely infected humans over the next 40 years. Since 2008, many countries have reported an EV-D68 epidemic, which usually occurs during late summer and fall and exhibits a biennial peak incidence [2-4]. However, in Korea, EV-D68 has never been reported thus far.

The reason why EV-D68 could be detected relatively early in the US epidemic was that EV-D68 showed a positive result corresponding to human rhinovirus or enterovirus (HRV/EV) in the multiplex real-time reverse-transcription polymerase chain reaction (mRT-PCR) kits. Thereafter, the EV-D68 epidemic could be confirmed through sequencing of viral RNA from clinical samples during a sudden increase in cases positive for HRV/EV [5-7]. Although most mRT-PCR kits were able to detect EV-D68, the most commonly used mRT-PCR kits in Korea could not, according to a previous study [8]. Thus, this study first aimed to confirm the EV-D68 detection ability of the mRT-PCR kits used in Korea and then to identify the circulation of EV-D68 among Korean children.

MATERIALS AND METHODS

1. Evaluation of EV-D68-detectability using mRT-PCR kits

In 2018, two mRT-PCR kits used for the detection of respiratory viral pathogens, including the Anyplex Respiratory Panel (Seegene, Inc., Seoul, Korea) and its advanced version, the Allplex Respiratory Panel (Seegene, Inc., Korea), comprised over 90% of kits marketed at university hospitals in Korea (personal communication). We selected these two kits and two additional kits, AmpliSens® ARVI-screen-FRT (Ecoli Dx, Nové Město, Czech Republic) and an in-house mRT-PCR kit, for the evaluation of EV-D68 detectability. These four kits were based on the same mRT-PCR platform, and the main differences might be the kind and number of viruses targeted and their primer sequences.

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First, we purchased the reference strains of EV-D68 (US/MO/1418947) from the American Type Culture Collection (Manassas, Virginia, US) and EV-71 (NCCP43165) and coxsackievirus 16 (CA-16; NCCP41207) from the Korean National Culture Collection for Pathogens (Seoul, Korea). In addition, we prepared one HRV-C clinical strain, which was collected from a nasopharyngeal aspirate (NPA) sample of a Korean child in 2018. From all these viral isolates, total RNA was extracted using a QIAamp® Viral RNA Kit (QIAGEN, Hilden, Germany) and converted to cDNA by using SuperScript II Reverse Transcriptase (Invitrogen, Waltham, MA, USA). We performed conventional PCR targeting the 5′-untranslated region (UTR) and subsequent sequencing with these cDNAs as described [9] and confirmed the integrity of each cDNA sample.

The four viral cDNAs were individually prepared at a concentration of 50 ng/ml, and cDNA from EV-D68 was additionally prepared at a concentration of 500 ng/ml. The samples were shipped for the blind test to the clinical laboratory department in Seoul National University Children's Hospital (SNUCH) by the Anyplex kit, Chung-Ang University Hospital (CAUH) by the Allplex kit, and Seoul National University Bundang Hospital (SNUBH) by both the AmpliSens and the in-house kits. In each hospital, the mRT-PCR test was conducted according to the recommendations of each kit and through the usual clinical pathway. Regarding the Anyplex and Allplex assays, the tests were duplicated with the separately prepared 2nd set of viral cDNA. All these tests were performed during September-October 2018.

2. Ethics statement

The study was conducted under approval by the Institutional Review Board (No. 1101-114-353, 2012-075-1181). The need for informed consent was waived.

3. Subjects

In December 2018, Allplex replaced the Anyplex kit in SNUCH, and several reports indicated that the Allplex kit used in Europe could be used to detect EV-D68 on an EV panel [10-12]. We assumed that the domestic Allplex kit might have been recently upgraded with new primers for EV, which can detect EV-D68. Because we could not exclude the cross-reactivity of EV-D68 on the HRV panel [7], we selected children whose specimens underwent Allplex mRT-PCR and were positive for HRV and/or EV in SNUCH from April to December 2022. Only HRV positivity in children with lower respiratory tract infection (LRTI) was considered, whereas EV positivity in children with any acute respiratory infection or even nonspecific febrile illness was considered unless the clinical diagnosis was hand-food-mouth disease or herpangina.

4. EV-D68-specific PCR & sequencing

Total RNA was extracted from NPAs, and partial 5′-UTR was amplified and sequenced for the species identification of HRV/EV, as described above. The sequenced region was analyzed with the Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov). Additionally, PCR targeting the viral protein (VP) 1 gene was performed with cDNA of the EV-D68-positive NPAs, followed by sequencing as described [13].

5. Phylogenetic analysis and sequence comparison

Phylogenetic analysis was performed on a partial VP1 gene sequence of 765 base pairs (bp) by the CLC genomic workbench (QIAGEN, Germany). We generated and compared multiple amino acid (aa) sequence alignments of the partial (164 aa) VP1 gene sequences from the recently identified international EV-D68 strains, which were selected from GenBank (https://www.ncbi.nlm.nih.gov/nuccore). The nucleotide sequences of all primers used and the information about EV-D68 reference strains for the phylogenetic analysis are presented in **Supplementary Table 1** and **2**, respectively.

RESULTS

1. HRV- and EV-detection ability of mRT-PCR kits in 2018

Anyplex failed to detect all EV isolates, and HRV-C was detected only in the first test. Allplex detected EV71, CA16, and HRV-C but not EV-D68. AmpliSens detected only EV71 and CA16 but not EV-D68 and HRV-C. However, the in-house mRT-PCR kit in SNUBH was able to detect all EVs, including EV-D68, but not HRV-C (**Table 1**).

2. HRV/EV epidemics and detection of EV-D68 in 2022

Between April and December 2022, we collected 41 NPAs from children hospitalized with fever and/or respiratory symptoms who tested positive for HRV (n = 27), EV (n = 7), or both (n = 7) by the Allplex mRT-PCR kit. The demographic and clinical features of children between the three groups were comparable (**Table 2**). Among the 27 HRV-only positive samples, 22 (75.9%) were amplified in the 5′-UTR gene PCR and typed as HRV-A (n = 6) or HRV-C (n = 16). Among the seven samples positive for both HRV

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Table 1. Comparison of the detection ability of various real-time reverse transcriptase polymerase chain reaction kits for enteroviruses and human rhinovirus type C

Tested viruses	Anyplex		Allplex		In house[a]	Amplisens
	1st	2nd	1st	2nd		
EV-D68	(−)	(−)	(−)	(−)	(+)	(−)
EV-D68 x10[b]	(−)	(−)	(−)	(−)	(+)	(−)
EV-71	(−)	(−)	EV (28.4)	EV (19.8)	(+)	(+)
CA-16	(−)	(−)	(−)	EV (32.9)	(+)	(+)
HRV-C	(+)	(−)	HRV (33.2)	HRV (36.4)	(−)	(−)

[a]In-house real-time reverse transcriptase polymerase chain reaction kit developed by the Department of Laboratory Medicine in Seoul National University Bundang Hospital.
[b]Ten times higher viral concentration.
Anyplex (Seegene, Inc., Seoul, Korea).
Allplex (Seegene, Inc., Seoul, Korea).
Amplisens (Ecoli Dx, Nové Město, Czech Republic).
Numbers in parentheses indicate the value of the cycle threshold of a positive reaction.
EV, enterovirus; CA, coxsackievirus; HRV, human rhinovirus.

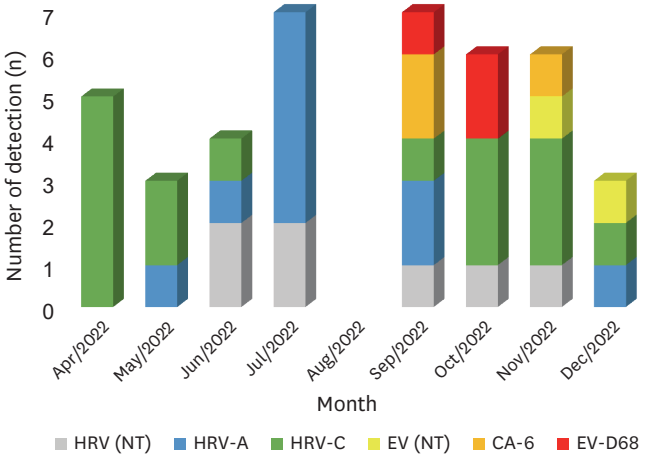


Figure 1. Detection frequency of human rhinovirus and enterovirus from children admitted to Seoul National University Children's Hospital with lower respiratory symptoms and/or fever during April-December 2022.
HRV, human rhinovirus; NT, not typed; EV, enterovirus; CA, coxsackievirus; EV-D68, enterovirus D68.

and EV, six (85.7%) were amplified and typed as HRV-A (n = 4), HRV-C (n = 1), and coxsackievirus A6 (CA6, n = 1). Among the seven EV-positive/HRV-negative samples, five (71.4%) were identified as CA6 (n = 2) and EV-D68 (n = 3).

During the study period, excluding August, HRV was persistently detected in children admitted with LRTI. HRV-C was prevalent in April and October - November, and HRV-A was prevalent in July. Enteroviruses, including CA-6 and EV-D68, were all detected from September to November (**Fig. 1**).

3. Clinical features of EV-D68 infection cases

Three EV-D68-positive cases were identified from two toddlers (2 and 3 years old, respectively) and one adolescent (16 years old). Two toddlers, who were both previously healthy and were not recurrent wheezers or asthma patients, had fever and cough as initial symptoms and exhibited wheezing on auscultation. The adolescent, who had relapsed acute leukemia as an underlying disease, showed only a brief febrile illness without any respiratory symptoms. Chest radiograph findings were normal, and EV-D68-related symptoms improved without any complications in all three patients (**Table 3**).

4. Sequence and phylogenetic analysis of EV-D68

The subsequent PCR for the VP1 gene was positive for the NPAs of patients 1 and 3, and sequencing was successful only from the amplicon of patient 3. We were able to retrieve a 770 base-pair (bp) sequence from the amplicon and then performed a phylogenetic analysis with several reference sequences obtained worldwide since 2010. The phylogenetic tree of the partial VP1 sequences (765 bp) revealed that the first Korean EV-D68 sequence belonged

Table 2. Demographic and clinical characteristics of the three patient groups

Demographic and clinical features	HRV-positive (n = 27)	HRV & EV-positive (n = 7)	EV-positive (n = 7)
Age (y), median (IQR)	2 (1 - 4)	1 (1 - 1.5)	2 (1.5 - 4.5)
Sex (male), n (%)	18 (66.7)	7 (100.0)	3 (42.9)
Diagnosis			
Pneumonia	17 (63.0)	1 (14.3)	1 (14.3)
Bronchiolitis	8 (29.6)	2 (28.6)	2 (28.6)
Others	2 (7.4)	4 (57.1)	4 (57.1)
Underlying disease, n (%)	18 (66.7)	5 (71.4)	4 (57.1)
Other respiratory virus codetected, n (%)	6 (22.2)	6 (85.7)	1 (14.3)
UTR PCR positivity, n (%)	22 (75.9)	6 (85.7)	5 (71.4)
EV-D68	0 (0.0)	0 (0.0)	3 (60.0)
CA6	0 (0.0)	1 (16.7)	2 (40.0)
HRV-A	6 (27.3)	4 (66.7)	0 (0.0)
HRV-C	16 (72.7)	1 (16.7)	0 (0.0)

HRV, human rhinovirus; EV, enterovirus; n, number; y, year; IQR, interquartile range; UTR, untranslated region; PCR, polymerase chain reaction; CA, coxsackievirus.

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Table 3. Clinical characteristics of the three children with EV-D68 detected in Korea

Clinical features	Patient 1	Patient 2	Patient 3
Sex	Female	Male	Female
Age (y)	2	3	16
NPA collection date	9/24/2022	10/4/2022	10/4/2022
Underlying Disease	None	None	ALL, relapsed
Initial symptom	Fever, cough, dyspnea	Fever, cough	Fever, headache
High fever >39℃	Yes	No	No
Rales	No	No	No
Wheezing	Yes	Yes	No
Dyspnea	Yes	No	No
Chest retraction	Yes	No	No
Lowest SpO_2 (%)	85	93	100
CXR findings	No active lesion	No active lesion	No active lesion
Diagnosis	Bronchiolitis	Bronchiolitis	Febrile illness
Respiratory support	Nasal prolong	Nasal prolong	None
Last F/U days from admission	68	60	104
Outcome at the last F/U	Cure	Cure	Cure

EV-D68, enterovirus D68; y, year; ALL, acute lymphoblastic leukemia; NPA, nasopharyngeal aspirate; CXR, chest X-ray; F/U, follow-up.

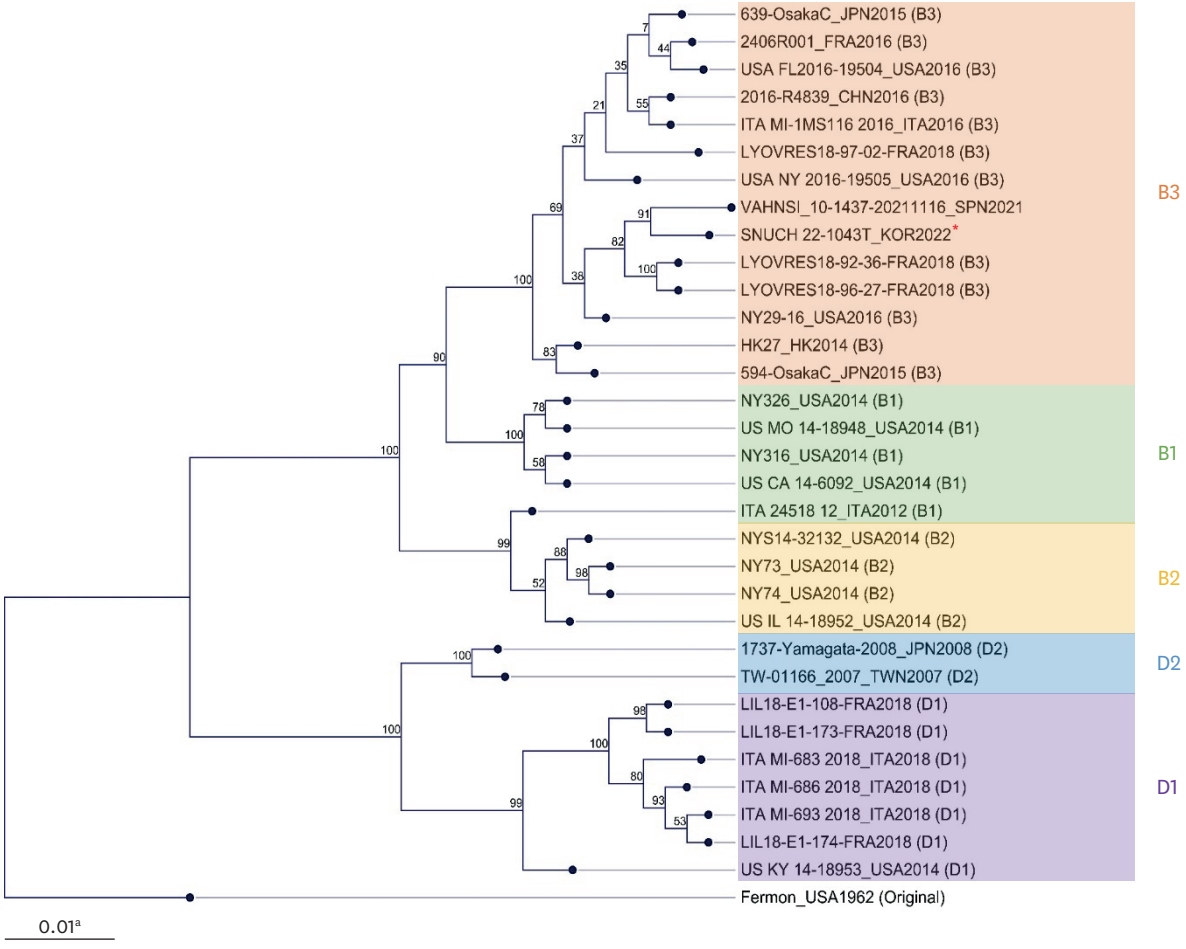


Figure 2. Phylogenetic tree of partial VP1 sequences for recently identified EV-D68 strains worldwide. The analysis was performed by using the maximum likelihood method with the neighbor-joining construction method and Jukes Cantor nucleotide substitution model, running 1000 bootstrap replicates on the partial VP1 region sequences for EV-D68 strains isolated since 2010 worldwide and available from GenBank. The sequences were represented as strain name_country and year isolated (subclade previously known). The Korean strain detected in this study is represented with a red star.
[a]Branches shorter than 0.0043 are shown as having a length of 0.0043.
VP, viral protein; EV-D68, enterovirus D68; JPN, Japan; FRA, France; USA, United States of America; CHN, China; ITA, Italy; SPN, Spain; KOR, Korea; HK, Hong Kong; TWN, Taiwan.

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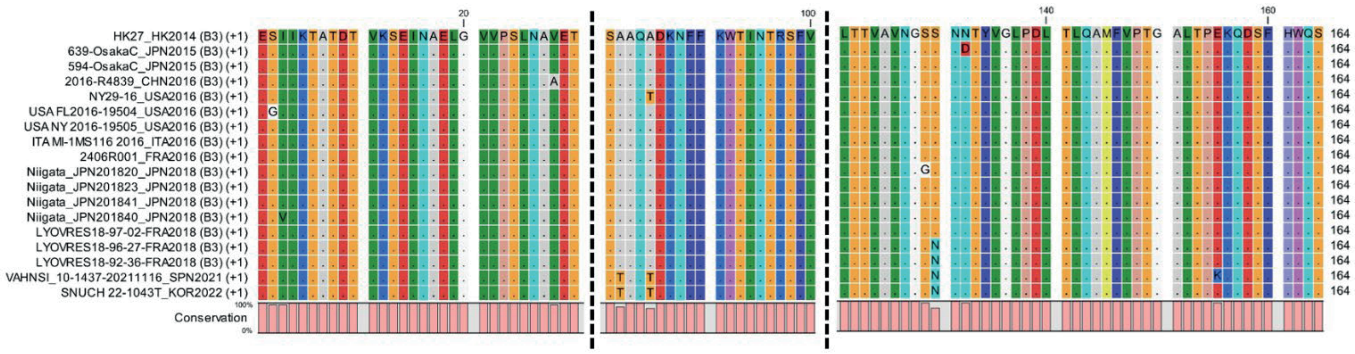


Figure 3. Amino acid sequence alignments of the partial VP1 gene of subclade B3 EV-D68.
In the hidden region, which is represented by the dotted vertical lines (amino acids 31-80 and 101-120), there was no sequence difference between the EV-D68 strains. Numbering of the amino acids here begins with the 14th position of the complete VP1 gene sequence.
VP, viral protein; EV-D68, enterovirus D68.

to subclade B3 (**Fig. 2**). On the additional phylogenetic analysis of the shorter (494 bp) VP1 sequences of subclade B3, including recent Japanese strains, our strain was the closest genetically to the Spanish strain identified in 2021 (**Supplementary Fig. 1**). Amino acid sequence alignment of subclade B3 also showed that our strain is genetically more related to those from Spain in 2021 and France in 2018 than to those from Japan in 2018 (**Fig. 3**). The partial 5′-UTR and VP1 gene sequences of the current EV-D68 strains have been deposited in the NCBI GenBank database (accession number: OQ450170-OQ450173).

DISCUSSION

In an earlier study in 2018, we confirmed that the commercial mRT-PCR kits that were commonly used in Korea (Anyplex and Allplex kits) could not detect EV-D68 using either the HRV or EV channel. Because we could not determine the suspected EV-D68-infected case by screening with an mRT-PCR kit, we decided not to perform the EV-D68 confirmatory test (5′-UTR sequencing) for the clinical specimens from Korean children. However, the latest version of the domestic Allplex kit could detect the first three Korean EV-D68 strains using the EV channel in September - October 2022. The clinical manifestation of EV-D68 infection in toddlers was comparable to those from previous reports, and the subclade of our EV-D68 isolate was B3.

A previous Canadian study reported that most commercial and laboratory-developed mRT-PCR tests could be used to detect EV-D68, but Seeplex and Anyplex kits, which are commercial mRT-PCR kits used most popularly in Korea, could not [8]. Moreover, the Allplex kit, which is the upgraded version of the Anyplex kit, failed to detect the EV-D68 reference genome at both standard and higher concentrations in the current study in 2018. However, in other studies conducted in Europe until 2018, Allplex seemed to be able to detect EV-D68 [10-12]. These differences in detectability between mRT-PCR kits might be mainly due to the difference in primer sequences used for the detection of the corresponding virus. Thus, we assumed that domestic and export-bound Allplex kits might differ in the composition of primers for EV, unless the results in the current study were false-negative even in the duplicated tests. If so, the domestic Allplex might have been upgraded for the detection of EV with new primers because we finally identified the EV-D68 genome from patients with positivity for EV using the Allplex kit in the current study. However, detailed information about the update history and nucleotide sequences of the primers is not available.

Due to the negative findings obtained using the Anyplex and domestic Allplex, EV-D68 could not have been detected in Korea even if EV-D68 had circulated. In this case, LRTI with unknown etiology might have presented with wheezing and dyspnea in Korean children, particularly in 2014, 2016 and 2018. Among the three children identified to be infected by EV-D68 in the current study, two were toddlers with LRTI accompanied by new-onset wheezing, which were compatible clinical features to the previous report [14]. However, no unusual increase in the number of these cases has recently been reported in Korea. In addition, there has been no recent nationwide increase in the incidence of AFM or asthma, which are the main clinical manifestations of EV-D68 [15, 16]. Moreover, EV-D68 has never been reported by the Korea Influenza and Respiratory Virus Surveillance System (http://www.

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cdc.go.kr/), which was established by the Korea Disease Control and Prevention Agency. This suggests that even considering that EV-D68 has not been easily recognized thus far due to the abovementioned limitations of the mRT-PCR kits in Korea, the EV-D68 epidemic may have been very small and mild in terms of clinical severity, unlike in other countries, including adjacent countries such as Japan and China [17, 18].

EV-D68 and HRV can cross-react on molecular diagnostic platforms because they are molecularly similar [7, 19]. Thus, we evaluated the patient with LRTI and positivity for HRV using the mRT-PCR kit. As a result, HRV-A and HRV-C were frequently detected as pathogens underlying LRTI in children, but EV-D68 was not detected as HRV using the Allplex kit. However, EV-D68 and HRV-C continued to evolve and cross-react with the primers for HRV/EV in the mRT-PCR kit. Thus, we should monitor LRTI presenting with wheezing and a positive HRV as well as EV in children, particularly during the autumn season.

Between 2008 and 2010, small outbreaks of EV-D68 were reported as severe LRTIs in young infants worldwide [5]. Since then, the incidence has decreased, but in 2014, outbreaks occurred in the US, Europe, and Asia [6]. Thereafter, biennial epidemics occurred in 2016 and 2018, but no increase in EV-D68 incidence was detected in 2020, probably due to the influence of the coronavirus disease 2019 (COVID-19) pandemic. However, with the end of the COVID-19 pandemic and the increase in international exchange and social activities, the prevalence of EV-D68 could be increasing during a global epidemic. The increased detection of EV-D68 in children and adolescents with acute respiratory illness and asthma/reactive airway disease during summer 2022 was recently reported from the US [3]. The two Korean toddlers with EV-D68 infection in the current study showed a comparable but milder clinical presentation and course than those from other countries. However, since the domestic epidemiology of EV-D68 might have been insignificant in Korea, herd immunity might be almost not formed, so when EV-D68 is circulating, a much larger epidemic causing severe LRTI in requiring hospitalization infants and toddlers and a serious complication, that is, AFM, can develop. Therefore, it is necessary to prepare a system to closely monitor the circulation of EV-D68 and respond rapidly.

Since 2016, the predominantly circulating EV-D68 was identified as subclade B3, which is the same genotype of the EV-D68 strain detected in the current study. In 2018, subclades B3 and D1 were detected worldwide [12, 20, 21]. Our strain was more similar to those from Europe than those from Japan. This may indicate that the emerging respiratory pathogen could be easily imported by international transport regardless of the distance between the countries.

In this study, we did not perform the test for EV-D68 detectability of the 2022 Korean domestic version of the Allplex kit with a reference strain that had been used in our earlier study. Thus, a direct comparison between the 2018 and 2022 versions of the mRT-PCR tests was not available, and our speculation for the primer change is not supported by the actual data. In addition, we detected only three EV-D68 strains and could perform sequencing analysis for only one strain, so we cannot generalize the clinical and molecular biologic features of Korean EV-D68 with the findings from this study. Additionally, because this study was conducted for clinical isolates obtained during April - December of one year, the findings may not adequately represent the actual epidemiology and seasonality of EV-D68 in Korea. Further studies including a full-year season for several years should be performed to verify the findings from the current study.

In conclusion, we confirmed EV-D68 circulation among Korean children in the 2022 season. The EV-D68 burden and clinical significance may be underestimated in countries where mRT-PCR does not detect EV-D68 or where mRT-PCR is not frequently used. It is necessary to monitor the EV-D68 epidemic, which is likely to start during the summer/autumn of 2024, and the resulting occurrence of severe LRTI and AFM. In particular, careful evaluation, including differentiation from asthma, is needed in LRTI accompanied by wheezing.

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ORCID iDs
Ki Wook Yun
https://orcid.org/0000-0002-0798-6779
Bin Ahn
https://orcid.org/0000-0002-3806-4275
Sung Hwan Choi
https://orcid.org/0000-0002-6340-8260
Da Yeon Kang
https://orcid.org/0000-0002-0314-9455

https://doi.org/10.3947/ic.2023.0036

Taek Soo Kim
https://orcid.org/0000-0002-2093-1721
Mi Kyung Lee
https://orcid.org/0000-0003-1824-476X
Kyoung Un Park
https://orcid.org/0000-0002-2402-7633
Eun Hwa Choi
https://orcid.org/0000-0002-5857-0749

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Conflict of Interest
KUP is associate editor of Infect Chemother; however, he did not involve in the peer reviewer selection, evaluation, and decision process of this article. Otherwise, no potential conflicts of interest relevant to this article was reported.

Author Contributions
Conceptualization: KWY, MKL, KUP, EHC. Data curation: KWY, TSK, BA, SHC, DK. Formal analysis: KWY. Funding acquisition: KWY. Investigation: KWY, TSK, MKL, KUP. Methodology: KWY, TSK, MKL, KUP, EHC. Project administration: KWY, EHC. Resources: KWY, TSK, EHC. Software: KWY. Supervision: TSK, MKL, KUP, EHC. Validation: TSK, MKL, KUP, EHC. Visualization: KWY, BA, SHC, DK. Writing - original draft: KWY, BA, EHC. Writing - review & editing: BA, SHC, DK, TSK, MKL, KUP, EHC.

SUPPLEMENTARY MATERIALS

Supplementary Table 1
Nucleotide sequences of all primers used in this study

Click here to view

Supplementary Table 2
Reference strains used in the phylogenetic analysis

Click here to view

Supplementary Figure 1
Phylogenetic tree with the partial VP1 gene sequences of the subclade B3 EV-D68.

Click here to view

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