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Original Article

Detection of enterovirus D68 among children with severe acute respiratory infection in Myanmar

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Abstract *Background:* Enterovirus D68 (EV-D68) is an important reemerging pathogen that causes severe acute respiratory infection and acute flaccid paralysis, mainly in children. Since 2014, EV-D68 outbreaks have been reported in the United States, Europe, and east Asia; however, no outbreaks have been reported in southeast Asian countries, including Myanmar, during the previous 10 years.
Methods: EV-D68 was detected in nasopharyngeal swabs from children with acute lower respiratory infections in Myanmar. The samples were previously collected from children aged 1 month to 12 years who had been admitted to the Yankin Children Hospital in Yangon, Myanmar, between May 2017 and January 2019. EV-D68 was detected with a newly developed EV-D68

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—specific real-time PCR assay. The clade was identified by using a phylogenetic tree created with the Bayesian Markov chain Monte Carlo method.
Results: During the study period, nasopharyngeal samples were collected from 570 patients. EV-D68 was detected in 42 samples (7.4 %)—11 samples from 2017 to 31 samples from 2018. The phylogenetic tree revealed that all strains belonged to clade B3, which has been the dominant clade worldwide since 2014. We estimate that ancestors of currently circulating genotypes emerged during the period 1980—2004.
Conclusions: To our knowledge, this is the first report of EV-D68 detection in children with acute lower respiratory infections in Yangon, Myanmar, in 2017—2018. Detection and detailed virologic analyses of EV-D68 in southeast Asia is an important aspect of worldwide surveillance and will likely be useful in better understanding the worldwide epidemiologic profile of EV-D68 infection.

Introduction

Enterovirus D68 (EV-D68) is an important re-emerging pathogen that causes severe acute respiratory infection and acute flaccid paralysis (AFP), mainly in children.[1] EV-D68 was first isolated in 1962 in the United States[1] and has been detected sporadically since then.[2] A large, worldwide outbreak was reported in 2014. Periodic outbreaks have been reported around the world since then, and EV-D68 is now regarded as an important re-emerging infection in many countries.[3]

During EV-D68 outbreaks, prominent increases in asthma-like respiratory illness have been reported in children,[1,3,4] who typically present with severe disease that responds poorly to standard asthma treatments.[1] EV-D68 also causes acute lower respiratory infection (ALRI).[5—7] In a year when EV-D68 was circulating, it was detected in 2—3 % of children hospitalized for community-acquired pneumonia, which presents with symptoms that are more severe than those caused by other viruses.[5,8] Additionally, 2 studies in Taiwan indicated that EV-D68 spread widely in children: the seroconversion rate for EV-D68 among kindergarteners was 73 %,[9] and the seropositive rate before graduation from elementary school was greater than 98 %.[10]

Continuous surveillance targeting EVs, including EV-D68, has been conducted and reported in the United States and European countries.[11,12] However, epidemiologic studies have been sporadic in southeast Asian countries, including the Philippines, Thailand, and Cambodia.[13] Annual surveillance for EV-D68 has not been performed, and no reports from these regions have been published, during the previous 10 years. In Myanmar, the current epidemiologic profile of EV-D68 is unknown.

To better understand the worldwide distribution and regional links of EV-D68, genetic data for EV-D68 must be collected and analyzed. EV-D68 is classified phylogenetically into 4 clades (A to D). Until 2014, multiple EV-D68 clades had circulated; however, clade B3 has been predominant since 2014.[2,3] Phylogenetic analyses of EV-D68 that use a method to create a chronological phylogenetic tree—like the Bayesian Markov chain Monte Carlo (MCMC) method[14,15]—can clarify the regional links and genetic diversity of EV-D68.

We previously investigated viral causes of severe ALRIs in children in Yangon, Myanmar, during the period 2017—2019.[16] Although we used an EV-specific PCR assay for detecting EV, we did not specifically evaluate EV-D68 in the analyses. Of note, in 2018, EV-D68 outbreaks were reported in the United States, Europe, and Japan.[3] Evaluating EV-D68 circulation in Myanmar during 2017—2019 may be important in clarifying EV-D68 spread worldwide. An EV-D68—specific real-time PCR assay has been widely used to detect EV-D68;[17] however, its sensitivity is decreasing because of genetic changes in recent EV-D68 strains.[18] To adapt to these genetic changes, we developed a new real-time PCR assay with better sensitivity for current EV-D68 strains.[18]

The objectives of this study were (1) to determine if EV-D68 was detectable in samples from children with severe ALRIs in Yangon, Myanmar, during 2017—2019, (2) to validate a newly developed EV-D68—specific PCR assay, if such cases were detected, and (3) to identify the EV-D68 clade and compare it with clades reported in outbreaks around the world.

Materials and methods

Patients

We used nasopharyngeal swabs that were previously collected for a study of severe ALRI in children in Myanmar.[16] The samples were collected from children aged 1 month to 12 years who were admitted to the Yankin Children Hospital (YKCH) in Yangon, Myanmar, for severe ALRIs during the period from May 2017 through January 2019. We divided the study period into 2 periods—from May 2017 through March 2018 and from April 2018 through January 2019—because these periods include both the rainy season (June to October) and dry season (November to March) in Myanmar. In accordance with the World Health Organization (WHO) definition,[19] ALRI was defined as (1) a recent history of or current fever of $\geq$38 °C, (2) cough, (3) disease onset during the previous 10 days, and (4) need for hospitalization. Written informed consent was obtained from the patients' caregivers. This study was approved by the ethics committees of the Department of Medical

Research, Ministry of Health and Sports, Myanmar (016, 616), and Niigata University, Japan (2547).

Clinical data

We used a dataset from a previous study,[16] which included information on age, gender, clinical symptoms and signs, management, and outcomes.

EV-D68 detection

RNA was extracted from samples with a QIAamp MinElute Virus Spin Kit (Qiagen, Valencia, CA, USA), in accordance with the manufacturer's instructions. To compare positive rates and Ct values, we used 2 EV-D68–specific PCR assays targeting the viral protein (VP) 1 region, namely, our new assay (NU assay)[18] and the standard assay (WashU assay)[17] (Supplementary Table 1). Ct values > 40 were considered a "negative" result. If Ct values were >35, we checked the size of the PCR product by gel electrophoresis. If the size was appropriate (appropriate size: 94 bp), the PCR result was considered positive; if the size was inappropriate, the PCR result was considered negative. The sensitivity and specificity of the NU assay were 87.9–90.7 % and 100 %, respectively. In contrast, the respective values were 50.8–86.1 % and 100 % for the WashU assay using *in silico* analysis.[17,18]

Genotyping of EV-D68

After viral RNA was converted to cDNA by using SuperScript VILO MasterMix (Invitrogen, Carlsbad, CA, USA), PCR for the partial VP1 region using N-Set, an EV-D68–specific PCR assay, was performed for EV-D68–positive samples, as previously described (Supplementary Table 1).[18] The samples were analyzed by sequencing and genotyping with BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Data on respiratory viruses other than EV-D68

We collected the results of viral analyses from the dataset.[16] The evaluated viruses included EV, influenza type A (Flu A), Flu B, respiratory syncytial virus subgroup A (RSV A), RSV B, human rhinovirus (HRV), human metapneumovirus, human coronavirus (NL63, OC43, 229E, and HKU), adenovirus, and human bocavirus (HBoV). These viruses were tested by real-time PCR, as previously described.[16] If multiple viruses were detected from one sample, it was considered a co-infection.

Molecular and phylogenetic analyses

For phylogenetic analysis with the MCMC method, we utilized partial VP1 region sequences for EV-D68 strains that were used in previously reported phylogenetic analyses that described EV-D68 outbreaks during 2014–2021.[2,3,11,13,14,20–29] The partial VP1 regions of the 25 current EV-D68 strains have been deposited in the NCBI GenBank database (accession number: LC746308–LC746323). Using Molecular Evolutionary Genetics Analysis software version 6 (MEGA6), after multiple alignments with the Clustal W program, we selected the Hasegawa-Kishino-Yano model with gamma distribution and invariant sites as the best substitution model. The software package BEAST version 2.6.7 was used to obtain estimated evolutionary rates and time to the most recent common ancestor (TMRCA) of EV-D68 clades A to D.

Statistical analyses

Descriptive statistics are reported as medians with interquartile ranges or as percentages, as appropriate. We compared clinical data for variables such as gender, age, symptoms, clinical signs, and outcomes for patients with positive and negative results for the EV-D68 RT-PCR assay (NU assay) by using the chi-square test or Mann–Whitney U test, in accordance with the data distribution. To compare the Ct values for the 2 assays and Ct values for the NU assay with those for WashU assay–positive and WashU assay–negative patients, we used the Wilcoxon signed rank test and Mann–Whitney U test, respectively. Statistical analyses were performed with STATA software version 16.1 (Stata Corp., College Station, TX). A P value of <0.05 was considered to indicate statistical significance.

Results

Detection of EV-D68 in children with ALRIs in Myanmar

During the period from May 2017 through January 2019, nasopharyngeal samples were collected from 570 hospitalized patients with ALRIs at YKCH in Yangon, Myanmar (289 samples [50.7 %] for May 2017 through March 2018, and 281 samples [49.3 %] for April 2018 through January 2019). EV-D68 was detected in 42 samples (7.4 %)—in 11 samples from the earlier period and 31 samples from the later period. EV-D68 was most frequently detected in samples collected during September through October 2018 (Table 1).

Characteristics of EV-D68–infected cases

Among 42 EV-D68–positive patients, 18 (43 %) were male and median age was 8.5 months. Frequently observed symptoms and signs included cough (100 %), difficulty breathing (74 %), retraction (69 %), and rhonchi (74 %). Four patients (10 %) received care in an intensive care unit and 2 patients (5 %) died in the hospital: 1 of the 4 ICU patients was infected with EV-D68 only, 2 were co-infected with RSV, and 1 was co-infected with Flu A. Four patients with severe disease had radiographically confirmed pneumonia and required treatment with antibiotics.

A comparison of the characteristics of patients with and without EV-D68 infection showed no significant difference between these groups (Table 2). Among the 42 EV-D68–positive patients, 8 (19 %) were positive for EV-D68 only, while the remaining 34 (81 %) patients had multiple viruses, including RSV A (53 %, 18/34), RSV B (21 %, 7/34), HRV (29 %, 10/34), adenovirus (15 %, 5/34), Flu A (9 %, 3/34), Flu B (1 %, 1/34), and HBoV (1 %, 1/34).

Table 1 Rates of enterovirus D68–positive samples, by month, from April 2017 through January 2019.

Year	Month	Evaluated samples (N = 570)	EV-D68–positive samples (N = 42)	EV-D68–negative sample (N = 528)	EV-D68 detection rate
2017	April	1	0	1	0.0 %
	May	9	0	9	0.0 %
	June	24	1	23	4.2 %
	July	32	2	30	6.3 %
	August	121	4	117	3.3 %
	September	61	3	58	4.9 %
	October	18	1	17	5.6 %
	November	5	0	5	0.0 %
	December	4	0	4	0.0 %
2018	January	5	0	5	0.0 %
	February	6	0	6	0.0 %
	March	3	0	3	0.0 %
	April	3	0	3	0.0 %
	May	1	0	1	0.0 %
	June	4	0	4	0.0 %
	July	12	0	12	0.0 %
	August	56	5	51	8.9 %
	September	88	11	77	12.5 %
	October	63	8	55	12.7 %
	November	44	6	38	13.6 %
	December	6	1	5	16.7 %
2019	January	4	0	4	0.0 %

Abbreviations: EV-D68, Enterovirus D68.

Table 2 Comparison of clinical characteristics of patients with positive and negative results for enterovirus D68.

	EV-D68 positive (N = 42)	EV-D68 negative (N = 528)	P-Value
Age, median months (IQR)	8.5 (4–12)	8 (4–16)	0.73
Male (%)	18 (43)	303 (57)	0.07
Symptoms			
Cough (%)	42 (100)	492 (94)*	0.11
Difficulty breathing (%)	31 (74)	384 (73)*	0.96
Rhinorrhea (%)	19 (45)	228 (44)*	0.84
Fever (>38.0 °C) (%)	19 (45)	204 (39)	0.40
Clinical signs			
Retraction (%)	29 (69)	354 (67)	0.87
Coarse Crackles (%)	22 (52)	269 (51)*	0.91
Wheezing (%)	16 (38)	196 (37)*	0.94
Rhonchi (%)	31 (74)	326 (62)	0.14
Management			
ICU management (%)	4 (10)	47 (9)	0.89
CPAP (%)	2 (5)	12 (2)	0.32
Mechanical ventilation (%)	4 (10)	35 (7)	0.47
Outcomes			
Length of stay, median days (IQR)	5 (3–8)**	4 (3–6)***	0.13
In-hospital mortality (%)	3 (7)	33 (6)	0.82

Abbreviations: EV-D68, Enterovirus D68; IQR, interquartile range; ICU, intensive care unit; CPAP, continuous positive airway pressure. The data were incomplete for some variables (*N = 523, **N = 40, ***N = 512).

Validation of EV-D68–specific real-time PCR assays

Among the 570 samples, the NU assay was positive for 42 patients (7.4 %) and the WashU assay was positive for 15 patients (2.6 %). No sample was positive for the WashU assay and negative for the NU assay (Supplementary Table 2). Median Ct was significantly lower for the NU assay (31.63) than for the WashU assay (36.77) ($P < 0.01$). Additionally, among patients with a positive NU assay result (n = 42), median Ct was significantly lower for WashU-

positive patients (31.63, n = 15) than for WashU-negative patients (36.96, n = 27) (P = 0.03). Real-time PCR analysis targeting all EV types was also performed, and 69 patients (12 %) were positive for EV on real-time PCR[16]; however, the NU assay yielded a positive result for 25 patients who had a negative result for EV on real-time PCR (n = 501) (Supplementary Table 2).

Analyses of mismatch of the NU and WashU assays

The sequences obtained by genotyping were used to count mismatches in the regions where primers and probes of the WashU and NU assays attached, respectively. We were able to obtain 25 sequences from among 42 EV-D68—positive samples; 13 samples were negative on the WashU assay. There was 1 mismatch at the binding region of the WashU probe in 62 % (8/13) of WashU-negative samples and 8 % (1/12) of WashU-positive samples (Supplementary Table 3). There was no mismatch for the probe of the NU assay in the 25 samples.

Molecular and phylogenetic analyses

VP1 sequences were available for 60 % (25/42) of EV-D68 samples. We confirmed that all sequences belonged to the clade B3 branch of the phylogenetic tree (Fig. 1). The clade

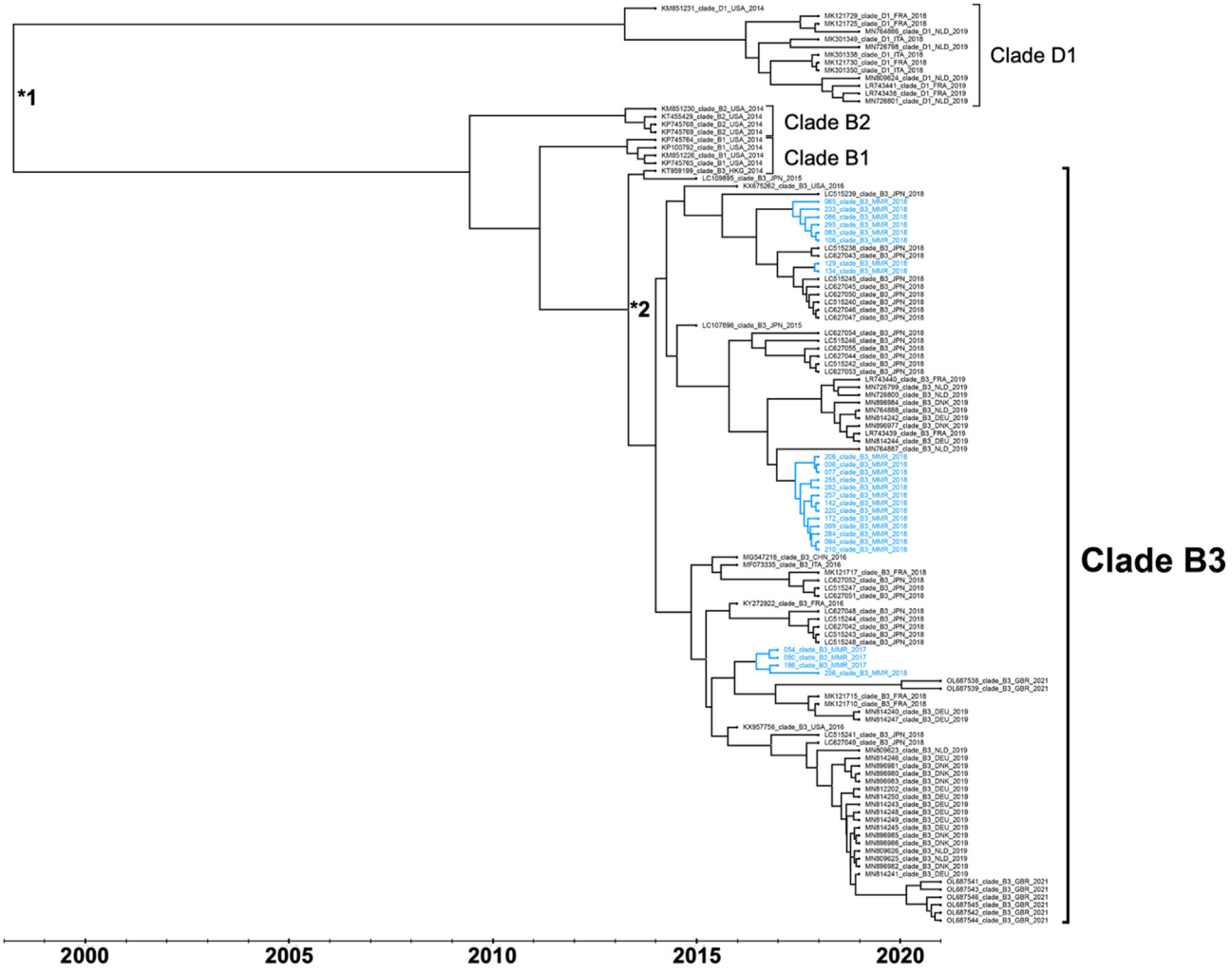

Figure 1. Phylogenetic Tree of Enterovirus D68, 2014—2021

Phylogenetic analysis with the Bayesian Markov chain Monte Carlo method was performed by using the partial VP1 regions of enterovirus D68 (EV-D68) detected in Myanmar and other countries. The phylogenetic tree was created with the software package BEAST version 2.6.7. A lognormal relaxed molecular clock and constant population size coalescent model with a chain length of 30 million were used for the analysis. The effective sample size of each estimate was confirmed to be greater than 200 (minimum, 371) by using Tracer version 1.7.2. A maximum clade credibility tree was constructed with TreeAnnotator version 2.6.6 after a 10 % burn-in. The tree was rendered with FigTree version 1.4.4. The year of each virus strain corresponded to the date of sample collection. The 25 sequences detected in the current study are listed in blue letters in the tree. Each strain was described as "accession number_clade_detected region_detected year". The mean substitution rate was 8.25×10^{-3} (95 % highest probability density [HPD], 6.19×10^{-3} to 10.5×10^{-3}) per site per year. The mean time to the most recent common ancestor (TMRCA) in the current dataset was estimated to be 1998 (95 % HPD, 1980—2004) (*1). The mean TMRCA of the diverse clade B3 was estimated to be 2013 (95 % HPD, 2012—2013) (*2).

B3 branch included other strains identified in the United States[4] and China[2] in 2014, in Japan[29] in 2015, in the United States[2] and Europe[14,25] in 2016, in the United States[11] and France[20] in 2018, in Europe in 2019,[28] and in the United Kingdom in 2021.[27] The strains detected in 2017 in Myanmar belonged to the same branch as 1 sequence from 2018 in Myanmar and sequences from 2018 in France, 2019 in Germany, and 2021 in the United Kingdom. The others detected in 2018 in Myanmar belonged to 2 different branches; 1 branch included some sequences identified in 2018 in Japan, and the other branch included sequences identified in 2018 in Japan and in 2019 in Europe. The mean substitution rate was 8.25×10^{-3} (95 % highest probability density [HPD], 6.19×10^{-3} to 10.5×10^{-3}) per site per year. The mean TMRCA in the current dataset was estimated to be 1998 (95 % HPD, 1980–2004) (*1 in Fig. 1). In addition, the mean TMRCA of the diverse clade B3 was estimated to be 2013 (95 % HPD, 2012–2013) (*2 in Fig. 1).

Discussion

In this study, we were able to detect EV-D68 in children with severe ALRIs in Yangon, Myanmar, during 2017–2018. To our knowledge, this is the first report to show circulation of EV-D68 clade B3 in Myanmar. Information regarding the epidemiology of EV-D68 in southeast Asia is extremely rare. This report confirms circulation of EV-D68 clade B3 in a southeast Asian country, Myanmar, and adds important information missing in the literature.

EV-D68 infection causes ALRI, asthma-like illness, and AFP in children. Sporadic reports have suggested possible candidates for treatment of EV-D68 infection; however, no specific treatments are currently available.[30,31] Children with asthma-like illness due to EV-D68 infection sometimes require treatment with systemic corticosteroids, magnesium, or β-stimulant inhalation, which are the recommended treatments for asthma exacerbation.[3,32,33] Intravenous immunoglobulin, corticosteroids, and plasmapheresis have been used to treat AFP due to EV-D68 infection; however, these are used for general treatment of AFP caused by other viruses and are not specific to EV-D68.[34]

EV-D68 outbreaks may cause an increase in severe respiratory illness in children, which could lead to a strain on medical resources, including drugs, hospital beds, and healthcare workers. Thus, EV genotyping for surveillance targeting EV-D68 is important for detecting the start of an outbreak and preparing medical resources for an increase in EV-D68–infected children. In addition, an increase in EV-D68 infections could lead to sporadic AFP cases. AFP due to poliovirus has been eradicated by distribution of poliovirus vaccines in Myanmar. Therefore, EV-D68 is important in the pathogenesis of AFP in the post–polio vaccine era, and surveillance of EV-D68 is a necessary aspect of AFP surveillance in Myanmar.

Phylogenetic analysis of EV-D68 is important in understanding the global distribution of EV-D68. Phylogenetic analysis frequently uses the Maximum Likelihood (ML) method,[3,23] as it is relatively uncomplicated and reveals the diversity of EV-D68 in a short period of time. In contrast to the ML method, phylogenetic analysis with the MCMC method requires more-complicated analysis but clearly reveals genetic diversity in chronological order[14,15] MCMC methods can be used to estimate the timing of phylogenetic branching and evolutionary rates. For EV-D68, it is important to understand the timing of the emergence of clades and the now-dominant clade B3 variations. The present study (*1 in Fig. 1) and previous reports showed that EV-D68 became genetically diverse in the late 1990s and that the phylogenetic trees began to branch into 4 clades.[14,15] The mean TMRCA of the diverse clade B3 was estimated to be 2013 (95 % HPD, 2012–2013) (*2 in Fig. 1). This is the first analysis of the mean TMRCA of the diverse clade B3, and the diversity of this clade may affect subsequent outbreaks worldwide.

Recent analyses of EV-D68 sequences circulating during 2010–2018 suggest that clade B3 is predominant.[14,35] The present study showed that sequences during 2021 showed persistent predominance of clade B3 and that 2017–2018 sequences in Myanmar may be related to later circulation in Europe. A previous phylogenetic analysis of EV-D68 revealed global migration between countries/regions.[35] In the present study, some 2017–2018 strains in Myanmar were positioned close to branches of 2019–2021 strains in European countries (Fig. 1). Therefore, because EV-D68 circulation in southeast Asia, including Myanmar, may affect subsequent outbreaks in surrounding parts of the world, continuous surveillance in southeast Asia is needed to characterize global migration of EV-D68.

WHO guidance specifies 2 methods for EV-D68 detection: direct detection by EV-D68–specific real-time PCR (including the WashU assay) and detection of EV by pan-EV real-time PCR followed by sequencing.[17,36] In the current study, sensitivity was higher for the NU assay than for the WashU assay (Supplementary Table 2). This result is consistent with the findings of a past study reporting similar mismatches at the probe binding site,[18] which validates the NU assay and indicates that it is valuable for surveillance targeting the current EV-D68 strain in samples from different regions of the world. To detect EV-D68, PCR assays should be adjusted to match genetic changes in recently circulating EV-D68 strains.

This study has limitations that warrant mention. First, we were unable to characterize the clinical characteristics of patients infected with EV-D68 because multiple viruses were detected in 81 % of the patients. Second, we did not collect samples directly from the lower respiratory tract; rather, we made estimates based on microorganisms detected from the upper respiratory tract. EV-D68 is detected less frequently in the lower respiratory tract than in the upper respiratory tract because of the nature of EV-D68.[30] Thus, collecting samples from the upper respiratory tract is reasonable because of the higher sensitivity for detection of EV-D68. Third, we were unable to focus on AFP because long-term follow-up of EV-D68–infected patients for development of AFP after ALRIs was not possible.

In conclusion, we detected EV-D68 clade B3 in samples from children with ALRI in Yangon, Myanmar, in 2017–2018. Detection and detailed virologic analyses of EV-D68 in southeast Asia are important for worldwide surveillance and could be useful in understanding the epidemiologic characteristics of EV-D68 infection worldwide.

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Declaration of competing interest

None declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2024.01.001.