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ORIGINAL ARTICLE

Molecular and epidemiological study of enterovirus D68 in Taiwan

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KEYWORDS
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Abstract *Background/purpose:* As an immunofluorescence assay for enterovirus D68 (EV-D68) is not available in the enteroviruses surveillance network in Taiwan, EV-D68 may be the actual pathogen of untypeable enterovirus-suspected isolates.
Methods: The untypeable isolates collected from 2007 through 2014 were identified by nucleic acid amplification-based methods and sequencing of the VP1 region to analyze the phylogeny and epidemiology of EV-D68 in Taiwan.
Results: Twenty-nine EV-D68 isolates were sequenced, including 15 Cluster 3 and 14 Cluster 1 viruses. Approximately 41% of the patients were children under 5 years of age and their infections peaked in August. The ratio of male to female patients was 1.5 and 3.67 for Cluster 3 and Cluster 1, respectively. Fever and respiratory symptoms were commonly reported in EV-D68-infected patients. The results of phylogenetic analyses showed that EV-D68 isolates between 2007 and 2014 belonged to different clusters and existed for years, indicating that endemic circulation of EV-D68 existed in Taiwan.
Conclusion: This study showed that EV-D68 has been endemic in Taiwan for some years despite a small number of positive cases. The continuous monitoring and efforts towards the improvement of diagnostic techniques are required to complete the surveillance system. This study provided the genetic and epidemiological information which could contribute to understanding the etiology and epidemiology of EV-D68.

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Introduction

Enterovirus D68 (EV-D68) contains a positive-sense, single-stranded RNA genome, and belongs to the family of *Picornaviridae*, the genus of *Enterovirus*, and the species of *Enterovirus D*. There are five known serotypes in the species of *Enterovirus D*, namely EV-D68, EV-D70, EV-D94, EV-D111, and EV-D120.[1] EV-D68 was first isolated from samples obtained in California in 1962.[2] Unlike other enteroviruses, EV-D68 is associated with respiratory illness, and shares important biological and molecular properties with both the enteroviruses and the rhinoviruses.[3] However, the full spectrum of clinical diseases caused by EV-D68 is still unclear.

EV-D68 infections have been reported in Japan (2005–2010), China (2006–2012), Thailand (2006–2011), Italy (2008–2009), the United Kingdom (2008–2010), Kenya (2008–2011), France (2009), Philippines (2009–2011), the United States of America (USA; 2009–2010), New Zealand (2010), and The Netherlands (2010).[4–14] The majority of previously reported EV-D68 cases were associated with acute respiratory infections, and were implicated in some rare cases of fatal infections.[15] In the USA, between 1997 and 2005, the most common age group associated with EV-D68 was children aged between 1 year and 4 years, but approximately one fourth of all infections were reported in adults.[16]

EV-D68 was reported rarely in the USA in the past, and only small clusters of EV-D68 associated with respiratory illness were reported between 2009 and 2010.[4] Abnormally, an increase in EV-D68-confirmed patients with severe respiratory illness was noted after August 2014.[17] As of January 15, 2015, a total of 1153 laboratory-confirmed cases were identified.[18]

For disease surveillance, the Taiwan Virology Reference Laboratory Network, covering all four regions in Taiwan, has been set up by Taiwan Centers for Disease Control (Taiwan CDC) since 1999 in cooperation with virological laboratories and physicians.[19] The virus isolation combined with immunofluorescence assay (IFA) and molecular methods were used for the detection of pathogens in this network. However, EV-D68 could not be confirmed by using commercial IFA kits, and some isolates were reported as untypeable enteroviruses that may be identifiable as EV-D68. Only molecular methods and sequence analysis could be used for the confirmation and molecular typing for EV-D68. In a previous study, some enteroviruses of the species *enterovirus D* were detected in Taiwan from 2007 through 2012.[20] However, no genetic or epidemiological analyses were available. In this study, the untypeable isolates were reexamined by reverse transcription polymerase chain reaction (RT-PCR)-based methods and sequence analysis to survey the epidemiology of EV-D68 in Taiwan.

Methods

Ethical statement

The project was reviewed and approved by the Institutional Review Board (IRB) of the Taiwan CDC (IRB No. 101011, 102017, and 103305). Based on Taiwan's Communicable Disease Control Act, informed consent is not necessary for collecting clinical specimens from patients with suspected notifiable communicable diseases. If these samples were used for research purposes, they must be de-identified prior to publication. In this study, all EV-D68 strains isolated from patients were de-identified according to the IRB approved protocol of Taiwan CDC to meet the patient confidentiality guidelines.

Specimen collection and selection criteria of EV-D68 for analysis

Throat swabs, rectal swabs, stools, sera, or cerebrospinal fluid specimens from patients with suspected enterovirus infections were collected, and the viruses were isolated following the standard protocols for the enterovirus surveillance system conducted by Taiwan CDC as previously described.[21] The virus isolates were then identified by IFA using commercial antibodies against enterovirus (Light Diagnostics, Millipore Corporation, Billerica, MA, USA) according to the manufacturer's protocol. IFA-untypeable isolates were then sent to Taiwan CDC for EV-D68 identification.[22] The selection of EV-D68 viruses for analysis in the study was based on the following criteria: (1) strains isolated in each year were included; (2) for the yearly number less than five, two isolates were randomly selected for each year; (3) if there was more than six isolates, approximately one third of them were randomly selected; and (4) all isolates collected in 2014 were used to compare with those in the 2014 outbreak in the USA.

RNA extraction and RT-PCR amplification

QIAamp Viral RNA Mini Kit (Qiagen, Santa Clara, CA, USA) was used for RNA extraction according to the manufacturer's instructions. At first, all the IFA-untypeable enteroviruses were tested by traditional RT-PCR and sequencing.[22] Then, an enterovirus COnsensus-DEgenerate Hybrid Oligonucleotide Primers (CODEHOP) method was used to identify untypeable enteroviruses.[23] The VP1 region was amplified by RT-PCR with primer sets 484/222 and 292/485 as previously described.[3] The Basic Local Alignment Search Tool (BLAST) was used for virus classification and genotyping using the reference sequence database in GenBank.

Phylogenetic analysis

The nucleotide and deduced amino acid sequences of the VP1 region were aligned using BioEdit program, and the phylogenetic tree was constructed by MEGA program (version 5, http://www.megasoftware.net) using the neighbor-joining method with a bootstrap value of 1000.[24] An amino acid substitution map was performed based on the entropy values of VP1 sequences. Different amino acid residues were indicated by different colors with single-letter abbreviations. Meanwhile, only the amino acids with entropy values over 0.5 were shown.

Clinical data

Clinical data were obtained from medical records in the surveillance system, including the records of patients' age,

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sex, and illnesses. The patient's clinical symptoms were scored as the following characteristics: fever, respiratory tract symptoms (cough, rhinorrhea, sore throat, or respiratory infections), and gastrointestinal tract symptoms (diarrhea, vomiting, or abdominal distention). Clinical symptoms were compared using Fisher's exact test. A p value < 0.05 was considered statistically significant.

Nucleotide sequence accession numbers

Twenty-nine EV-D68 sequences generated in this study have been deposited into GeneBank. The accession numbers of these isolates are KP657701—KP657729.

Results

EV-D68 identification

Between 2007 and 2014, a total of 575 untypeable enteroviruses-suspected isolates were tested by CODEHOP-based PCR, in which 65 EV-D68 strains were confirmed by sequencing. The yearly numbers of EV-D68 isolates from 2007 to 2014 were 15, three, four, 18, 11, two, four, and eight, respectively. Based on the selection criteria, 29 strains were selected for phylogenetic analysis in the study, including 21 strains isolated from 2007 to 2013 (4, 2, 2, 6, 3, 2, and 2, respectively) and eight strains from 2014.[20] The yearly distribution of different clusters of enterovirus D68 between 2007 and 2014 in Taiwan is shown in Figure 1.

Phylogenetic analysis of EV-D68

The major phylogenetic clusters (1, 2, and 3) of EV-D68 have been described previously.[25,26] As shown in Figure 2, 29 partial VP1 genomic region sequences of enterovirus D68 isolated in Taiwan between 2007 and 2014 were used for phylogenetic analysis, in which 15 isolates were identified as Cluster 3, and 14 isolates as Cluster 1 viruses. The Cluster 3 viruses with sequence similarity ranged from 85% to 88% when compared to the prototype strain Fermon. However, they clustered closely with recently genotyped EV-D68 sequences including the strains in the USA, China, Japan, The Netherlands, Spain, and Gambia. Among them, the Cluster 3 viruses in Taiwan were grouped into two major lineages. Similar to Cluster 3 viruses, the Cluster 1 isolates in Taiwan ($n = 14$), including all eight isolates in 2014, were 86—88% homologous to the prototype strain Fermon. Among them, the isolates in 2014 were closely related to the Taiwan isolates in 2011—2012 (Figure 2). Interestingly, some unique amino acid substitutions (residue 90 N→D, residue 243 I→V) were observed in Cluster 1 (Figure 3). The American isolates in 2014 were also grouped in Cluster 1 and Cluster 3. However, these American isolates were genetically different from the Taiwan isolates in 2014 according to the phylogenetic analysis.

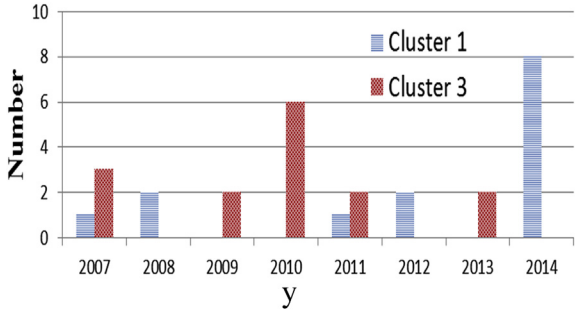

Figure 1. Yearly distribution of different clusters of enterovirus D68 between 2007 and 2014 in Taiwan. Based on molecular detection methods and the Basic Local Alignment Search Tool (BLAST), genotyping results of the 29 enterovirus D68 isolates collected from 2007 through 2014 in Taiwan are shown. Cluster 1 is indicated in blue, while Cluster 3 is indicated in red.

Epidemiology and clinical data

Cluster 3 viruses were detected in 2007, 2009, 2010, 2011, and 2013, while Cluster 1 viruses were detected in 2007, 2008, 2011, 2012, and 2014. Both Cluster 3 and Cluster 1 viruses were found in 2007 and 2011, but no Cluster 2 viruses were found (Figure 1).

The ratio of male to female patients infected with Cluster 3 and Cluster 1 was 1.5 and 3.67, respectively (Table 1). In addition, Cluster 3 viruses were nearly seen throughout the year, while Cluster 1 viruses were more likely to be detected in the typical enterovirus season (Figure 4).

Clinical symptoms of EV-D68 infected patients are summarized in Table 1. No fatalities were reported. In general, only mild symptoms were observed. Fever was the most frequent clinical symptom in both Cluster 3 and Cluster 1 EV-D68-detected patients, followed by respiratory symptoms (cough, rhinorrhea, and sore throat). No significant differences of respiratory tract or gastrointestinal tract symptoms were shown between Cluster 3-infected patients and Cluster 1-infected patients ($p > 0.05$), but Cluster 1 EV-D68-infected patients had significantly more fever when compared to Cluster 3-infected patients ($p < 0.05$; Table 1).

Discussion

EV-D68 deserves more attention in recent years, especially in 2014 due to the nationwide outbreak associated with severe respiratory illness in the USA. About 2600 specimens were tested by the US-CDC during 2014, and ~36% of those tested positive for EV-D68. Almost all the confirmed cases of EV-D68 infection were children. Fourteen people infected with EV-D68 have died, but it is still unknown what role the virus played in their deaths.[18] Furthermore, the viruses identified in 2014 were genetically related to strains of EV-D68 that were detected in previous years in the USA, Europe, and Asia.

To the best of our knowledge, this is the first reported characterization of EV-D68 clinical isolates in Taiwan. This study has confirmed that a number of untypeable enteroviruses collected during enterovirus surveillance by Taiwan Virology Reference Laboratory Network from 2007 to 2014 were EV-D68 cases. These cases were tested by sensitive

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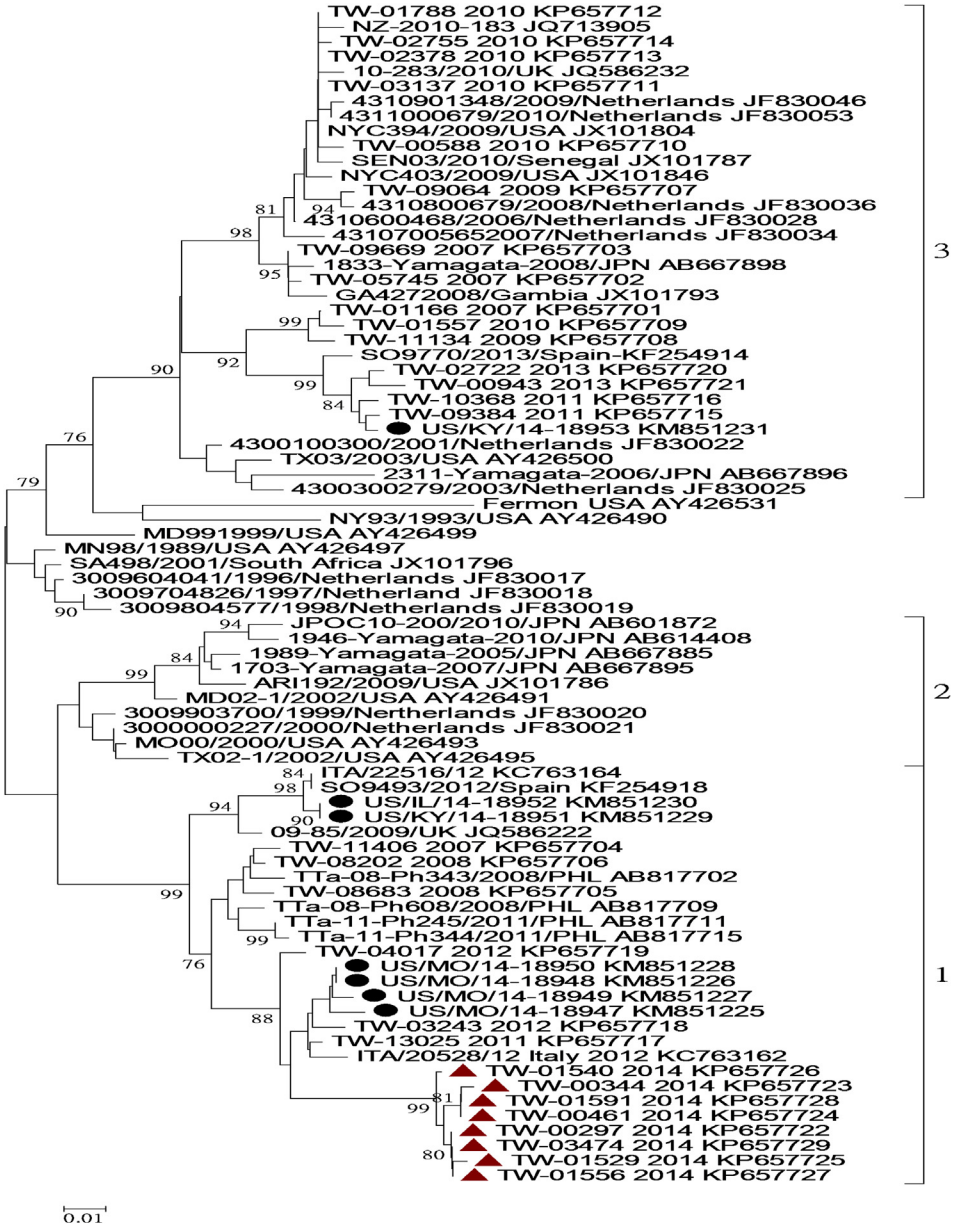

Figure 2. Phylogenetic analysis of partial VP1 gene sequence (nucleotides 132—471) of enterovirus D68 isolated from 2007 to 2014 in Taiwan. The Taiwan EV-D68 isolates in 2014 are indicated by triangles, while the American EV-D68 isolates in 2014 are indicated by circles.

PCR assays, and the VP1 region sequences were used for phylogenetic analysis. About 52% of the EV-D68 cases belonged to Cluster 3, and the others belonged to Cluster 1. Of them, two cases in Taiwan in 2014 are grouped in Cluster 1 and showed 94—96% similarity of VP1 region with the Cluster 1 viruses isolated in the USA in 2014. The presence of these EV-D68-positive isolates revealed the diagnostic deficit in enterovirus surveillance, since there were no standard EV-D68-specific diagnostic methods. In addition, the true number and incidence rate of EV-D68 is probably underestimated because cases with mild symptoms may not be reported, or might be misidentified as rhinovirus.[4] A standard diagnostic technique for EV-D68 is needed. In this study, EV-D68 isolates were identified and confirmed from the untypeable enteroviruses by nucleic acid amplification methods and sequence analysis.

Both Cluster 1 and Cluster 3 viruses of EV-D68 were detected in this study and peaked in August, but no apparent correlation with seasonal distribution was observed in Taiwan. A further investigation is needed since the number of cases in each cluster is low. However, Ikeda et al[14] reported that EV-D68 infections have a clear seasonality, as described in another surveillance report in The Netherlands.[25] In addition, most EV-D68 infections were detected during the rainy season in Thailand,[6] while the peak of EV-D68 detection in China was from August to October.[8] By contrast, ~41% of the EV-D68-infected patients in this study were aged $\leq$ 5 years, which was in line

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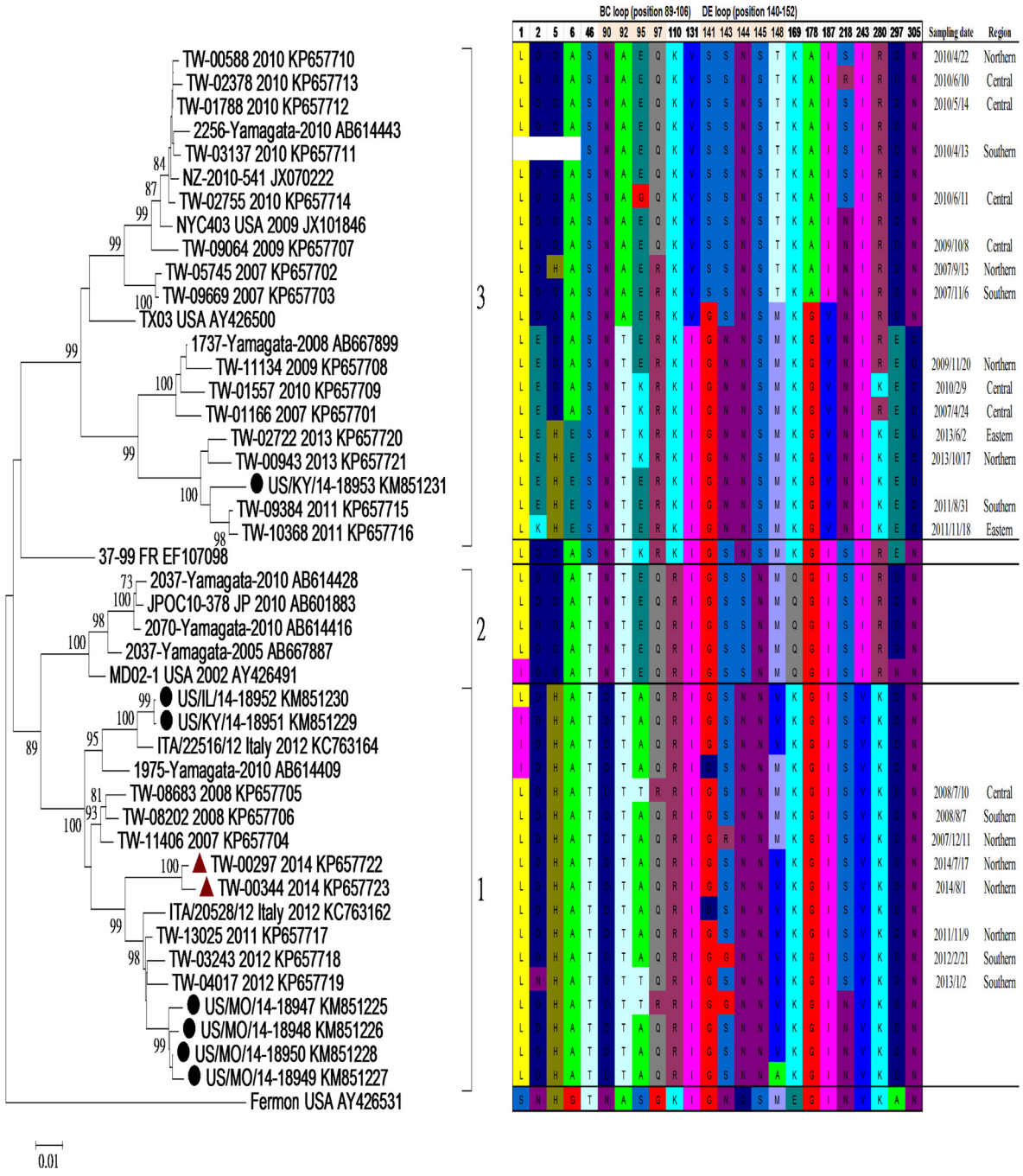

Figure 3. Phylogenetic analysis and amino acid substitutions of enterovirus D68 in Taiwan based on the VP1 gene sequence. Amino acid substitutions were labeled by different colors through a proteotyping map. Only entropy values > 0.5 were shown, and amino acid position were indicated in each column. The Taiwan EV-D68 isolates in 2014 are indicated by triangles, while the American EV-D68 isolates in 2014 are indicated by circles.

with the previous study.[16] However, the children older than 5 years and the adults are also targeted by EV-D68.[4,6]

Previous studies demonstrated that EV-D68 was associated with respiratory diseases,[14] and the phenomenon was also found in this study. Fever and respiratory illness were the most common symptoms among these EV-D68-infected patients in Taiwan. The Taiwan EV-D68 viruses showed high similarity (94—96%) in VP1 region with the viruses in the USA in 2014. The description of the nationwide outbreak of EV-D68 in the USA specifically focused on more severe or hospitalized cases, but there were most likely more milder cases in the USA than reported. By contrast, only mild EV-D68-associated symptoms were observed in Taiwan. However, in this study, the clinical information collected by the physicians and reference laboratories at the very beginning was limited, and no more details were available in such a retrospective study. More samples, complete case histories, and studies are needed to clarify

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Table 1 Characteristics and clinical symptoms of cases infected with different clusters of enterovirus D68 from 2007 to 2014.

Characteristics and clinical symptoms	Patients, *n* (%)		*p*
	Cluster 3	Cluster 1	
Sex			0.4270
Female	6 (40)	3 (21)	
Male	9 (60)	11 (79)	
Age (y)			0.0837
≤5	5 (33)	7 (50)	
6–17	2 (13)	5 (36)	
≥18	8 (53)	2 (14)	
Fever	7 (47)	13 (93)	0.0142*
Respiratory tract symptoms	11 (73)	9 (64)	0.6999
Gastrointestinal tract symptoms	1 (7)	1 (7)	> 0.99
Total	15	14	

* Statistically significant.

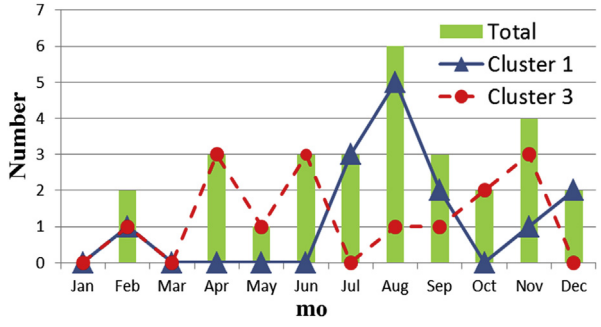

Figure 4. Seasonal distribution of enterovirus D68 in Taiwan. Seasonal distribution of different clusters of the 29 enterovirus D68 isolates collected from 2007 to 2014 in Taiwan are shown. Cluster 1 is indicated in blue, while Cluster 3 is indicated in red.

the impact of EV-D68 on the severity of infection, clinical presentation, and seasonal distribution as well.

A large increase in respiratory illness associated with EV-D68 has been reported in Africa, Asia, Europe and North America in recent years.[6,7,12,17,27] Genetic differences of several clusters were identified among these circulating strains. The EV-D68 isolates in Taiwan from 2007 to 2014 belonged to Cluster 1 and Cluster 3, indicating the existence of endemic circulation of EV-D68 in Taiwan. Based on the phylogenetic analysis using VP1 sequences, the EV-D68 viruses could be divided into three main clusters, despite different grouping names.[5,6,14,25,27,28] Some amino acid substitutions, especially in the BC and DE loop, were found in specific clusters, which may change the antigenicities. It is suggested that the emergence of strains with different antigenicities was the possible reason for the increased detection of EV-D68 in recent years.[29] In addition, one specific amino acid exchange in the BC loop of VP1 region of coxsackievirus B4 could lead to the loss of reactivity with specific antibodies.[30] The EV-D68 isolates in Taiwan in 2014 were grouped in the same cluster and were genetically similar to each other. These Taiwan EV-D68 viruses were isolated from the patients since July, and did not cause severe illness, unlike the ones identified in the USA in 2014. Nonetheless, it is still unclear why the EV-D68 viruses caused different degree of illness in spite of the high similarity between the viruses in Taiwan and the USA.

In conclusion, firstly, this is the first report of EV-D68 infections in Taiwan, and the results showed that EV-D68 has been circulating for years in this country. Secondly, continuous monitoring and efforts towards the improvement of diagnostic techniques are required to complete the surveillance system. Thirdly, our study provided the genetic and epidemiological information which could contribute to understanding the etiology and epidemiology of EV-D68.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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