

R E V I E W



Global reemergence of enterovirus D68 as an important pathogen for acute respiratory infections

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SUMMARY

We previously detected enterovirus D68 (EV-D68) in children with severe acute respiratory infections in the Philippines in 2008–2009. Since then, the detection frequency of EV-D68 has increased in different parts of the world, and EV-D68 is now recognized as a reemerging pathogen. However, the epidemiological profile and clinical significance of EV-D68 is yet to be defined, and the virological characteristics of EV-D68 are not fully understood. Recent studies have revealed that EV-D68 is detected among patients with acute respiratory infections of differing severities ranging from mild upper respiratory tract infections to severe pneumonia including fatal cases in pediatric and adult patients. In some study sites, the EV-D68 detection rate was higher among patients with lower respiratory tract infections than among those with upper respiratory tract infections, suggesting that EV-D68 infections are more likely to be associated with severe respiratory illnesses. EV-D68 strains circulating in recent years have been divided into three distinct genetic lineages with different antigenicity. However, the association between genetic differences and disease severity, as well as the occurrence of large-scale outbreaks, remains elusive. Previous studies have revealed that EV-D68 is acid sensitive and has an optimal growth temperature of 33 °C. EV-D68 binds to α 2,6-linked sialic acids; hence, it is assumed that it has an affinity for the upper respiratory track where these glycans are present. However, the lack of suitable animal model constrains comprehensive understanding of the pathogenesis of EV-D68. © 2014 The Authors. *Reviews in Medical Virology* published by John Wiley & Sons Ltd.

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INTRODUCTION

Human enterovirus D68 (EV-D68) is a member of species enterovirus D (EV-D), which belongs to the genus *Enterovirus* and the family of *Picornaviridae*. Only five EV-D serotypes have been identified thus far: enterovirus D70 (which is associated with acute hemorrhagic conjunctivitis) [1], enterovirus D94 (which is a causative agent of acute flaccid paralysis) [2], enteroviruses D111 and D120 (which were identified in non-human primates) [3], and EV-D68.

Enterovirus D68 was first isolated from four hospitalized pediatric patients with lower respiratory tract infections in California, USA, in 1962 [4]. Oropharyngeal specimens collected from the four patients were inoculated onto primary rhesus monkey kidney cells, leading to the isolation of the following four strains, Fermon, Franklin, Robinson,

and Rhyne, with typical enterovirus-like cytopathic effects [4]. The four isolates were tested for serological identification by using neutralization (NT) tests with immune sera specific for a panel of viruses. The test revealed that the isolates were antigenically distinct from other any known enteroviruses [4]. Therefore, the four isolates were proposed to be members of a new serotype of the *Picornaviridae* family. The Fermon strain was selected as a representative strain of this new serotype, because the four strains had identical antigenic properties [4].

After the initial identification of EV-D68 in 1962, detection of this virus was rarely reported until the early 2000s. However, we detected EV-D68 in children hospitalized with severe acute respiratory infections in the Philippines in 2008–2009, which was followed by a number of similar reports from different parts of the world [5]. Most EV-D68 viruses have been detected in patients with acute respiratory infections. A considerable number of these cases were severe, and some were fatal [6–8].

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However, the mechanisms underlying the recent global increase in EV-D68 detections are still not fully understood. Moreover, there is limited information about the virological characteristics of EV-D68, despite the increasing epidemiological and clinical significance of this virus.

In this review, we summarize current knowledge about EV-D68, by reviewing published articles and analyzing the EV-D68 sequence data deposited in GenBank.

GENOME STRUCTURE

Enterovirus is a non-enveloped virus containing a single-stranded RNA genome with positive polarity [9]. The viral RNA encodes four structural proteins, namely, VP1, VP2, VP3, and VP4. Structural proteins VP1, VP2, and VP3 comprise the outer surface of the virion, whereas VP4 resides inside the protein shell of the virion [9].

The VP1 gene is generally considered the most variable region of the enterovirus genome. Therefore, it has been used for classifying viruses into different serotypes and genotypes [9]. This is why the VP1 sequences of enteroviruses, including those of EV-D68, have been extensively studied. A number of EV-D68 VP1 sequences from different

parts of the world including Asia [6,10–15], Africa [7], Europe [8,16–21], Oceania [22], and the USA [7,23], have been deposited in GenBank. The EV-D68 strains detected in recent years are classified into three genetic groups based on the phylogenetic tree generated for VP1 nucleotide sequences (Figure 1) [8,11]. These genetic groups are designated as lineages 1, 2, and 3 in this review, although there are variations in the names given to the three genetic groups in other reports: clades A–C [7,18], clusters 1–3 [13], and lineage 1, which contains sub-lineages 1 and 2, and lineage 2 [21]. Mutations have accumulated in two specific regions of the VP1 sequences: the BC and DE loops [8]. Despite the variety of geographical sources for EV-D68, the strains detected in recent years have similar VP1 sequences as long as they belong to the same genetic lineage [24]. All strains classified as lineage 3 share a unique sequence characteristic, nucleotide deletions at positions 2806–2808 in the VP1 region compared with the Fermon strain, which results in an amino acid deletion at this position (Figure 2b) [8,10]. This unique genetic signature suggests a common origin for this genetic lineage. It is known that VP1 of enteroviruses has β -barrel structures containing eight β -strands, B,

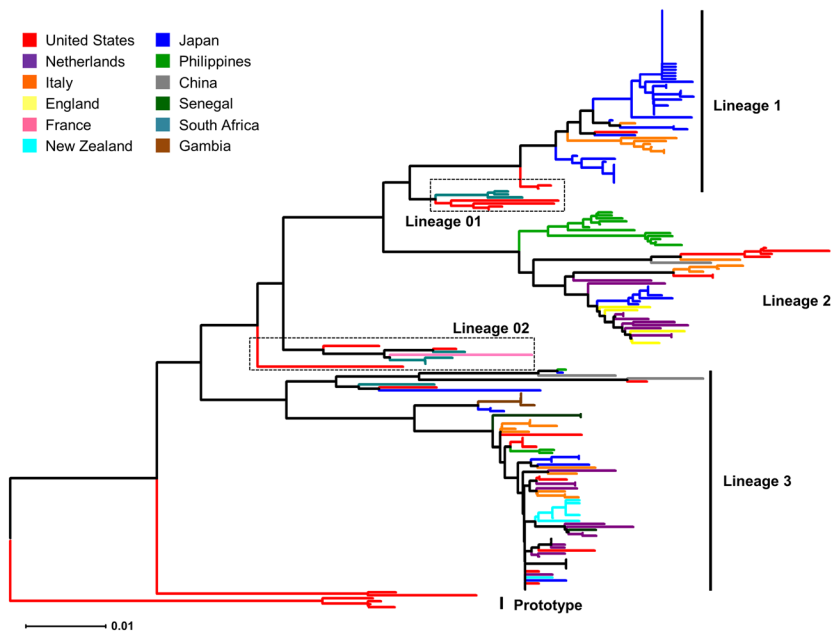


Figure 1. Phylogenetic relationships among enterovirus D68 strains detected in different countries. The phylogenetic tree for the VP1 region was generated using the neighbor-joining method, as implemented in MEGA software (<http://www.megasoftware.net/>). The partial VP1 sequences at nucleotide positions 2446–3033 corresponding to the Fermon strain (GenBank accession number: AY426531) were obtained from GenBank

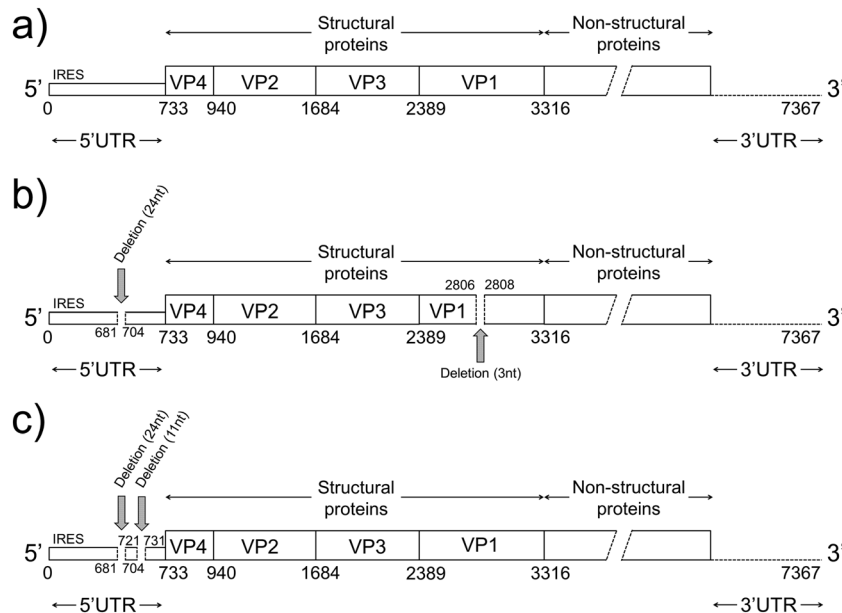


Figure 2. Genome structure of enterovirus D68 (EV-D68). The genome structures of the Fermon strain and lineage 2 (a), lineage 3 (b), and lineage 1 (c) of EV-D68 are depicted. Each genome region is indicated with a bar, and the nucleotide positions of these regions are annotated with numbers below the bars

C, D, E, F, G, H, and I, and that these strands are connected by seven loop structures: the BC, CD, DE, EF, FG, GH, and HI loops [9]. The loop structures, including the BC and DE loops, are generally located on the viral surface and are associated with antigenic epitopes [25–27]. Therefore, it has been suggested that unique sequence variations in the BC and DE loops might cause altered antigenicity in these viruses [8,13]. Subsequently, we confirmed that there are antigenic differences between the recently detected EV-D68 strains and the Fermon strain, as well as among the lineages of recently detected strains [24].

Selection analyses conducted on the VP1 amino acid sequences in several studies have revealed that most of the positively selected codons are found in the BC and DE loop regions [13,24]. These findings suggest that antigenic epitopes might be located in the BC and DE loop regions. However, more detailed studies are required to determine the antigenic epitopes of the virus.

Recent studies using the Bayesian Markov chain Monte Carlo approach have estimated that genetic diversity in the VP1 region increased after the late 1990s, which may have resulted in the emergence of the three lineages [8]. The emergence of genetically variant EV-D68 strains might be associated with the increasing trend of EV-D68 detections in

recent years. However, considering that most of the VP1 sequences available in GenBank are from strains detected in recent years, the rapid increase in VP1 sequence variation might simply be a result of sampling greater numbers of viruses from different geographical locations. Careful observations are required to draw any conclusions from these data.

Compared with the VP1 region, VP2, VP3, and VP4 have been less intensively studied, and only a limited number of sequences have been deposited in GenBank. We previously reported an analysis of VP2 and VP3 amino acid sequences from EV-D68 [24]. Unique amino acid mutations in the VP2 and VP3 regions that can differentiate recently detected strains from the Fermon strain, as well as recent strains of one genetic lineage from another, have been identified; however, such mutations were found less frequently than those in the VP1 region [24].

The 5'UTR is generally the most conserved genomic region in enteroviruses [9] and is commonly used as a target region for RT-PCR and sequencing for screening purposes [5]. The majority of the EV-D68 detections reported in recent years were based on positive results from RT-PCR and sequencing targeting the 5'UTR of EV-D68. The 5'UTR sequences of EV-D68 are known to be similar to those

of human rhinoviruses (HRV) [28]; therefore, some of the recent EV-D68 strains were detected by PCR and sequencing using primers designed against the 5'UTR of HRV [6,19]. As the most conserved region, it was reported that the 5'UTR sequences were similar among the strains detected during the same outbreaks [6,19]. However, some genetic variations have been reported at the 3' end of the 5'UTR, the spacer region between the internal ribosome entry sites and the open reading frame of the VP4 region [7,12]. Some strains were reported to have a 24-nucleotide deletion at positions 681–704 of the Fermon strain (Figure 2b), and some of them had an additional 11-nucleotide deletion at positions 721–731 (Figure 2c) [7,12]. Strains of lineage 1 were likely to have deletions at both positions 681–704 and 721–731. In contrast, lineage 3 strains had deletions only at positions 624–704, whereas those of lineage 2 had no deletions in the 5'UTR. The role of the spacer region in the pathogenesis of EV-D68 is still unresolved. Therefore, the significance of these deletions remains elusive.

BIOLOGICAL FEATURES

Acid liability

In the report of the initial identification of EV-D68 in 1962, Schieble *et al.* demonstrated that the Fermon strain was acid stable [4]. In contrast, Blomqvist *et al.* reported in 2002 that two sublines of the Fermon strain (VR-561 and VR-1076) were acid sensitive, which was confirmed by similar experiments [29]. The report by Oberste *et al.* in 2004 concurred with that of Bloqvist *et al.* concerning the acid sensitivity of the Fermon strain and additionally showed that five other clinical isolates including MN89 (Minnesota, 1989), MN98 (Minnesota, 1989), MD02-1 (Maryland, 2002), TX01 (Texas, 2001), and TX02 (Texas, 2002) were also acid sensitive [28]. The variation observed in the preceding results might be caused by the use of different methods, including cell lines or strains used in each experiment. Oberste *et al.* also demonstrated that EV-D68 proliferated more efficiently at 33°C than at 37°C [28]. The acid sensitivity profile and optimal growth temperature indicate that EV-D68 strains share similar biological characteristics with HRV. These shared similarities might underlie the mechanisms for their respiratory tract tropism.

Receptor specificity

It was previously reported that EV-D68 strongly agglutinates guinea pig erythrocytes [4]. We additionally confirmed that EV-D68 agglutinates those from turkey, but not chicken or sheep (Imamura *et al.*, unpublished data). However, the mechanisms controlling the agglutination activity of EV-D68 have not been documented. In 1991, neuraminidase treatment of HeLa cell monolayers was shown to result in an approximately 90% reduction of EV-D68 attachment and replication, suggesting that sialic acids (SAs) were a possible receptor for EV-D68 [30]. We investigated further the SA binding specificities of EV-D68 using glycan array analysis as well as enzymatically modified erythrocytes and found that EV-D68 had a stronger affinity with α 2,6-linked SAs (NeuAca2, 6Gal) than α 2,3-linked SAs (NeuAca2, 3Gal) [24]. It is known that α 2,6-linked SAs are dominantly expressed in the upper respiratory tract whereas α 2,3-linked SAs are expressed in the lower respiratory tract of human airways [31]. These results suggest that EV-D68 may have an affinity for the upper respiratory tract, compared with the lower respiratory tract. However, several epidemiological studies have shown that the EV-D68 detection rate was significantly higher among patients with lower respiratory tract infections than among those with upper respiratory tract infections [10,13]. Hence, mechanisms other than the distribution of potential receptors might be responsible for severe respiratory infections. It was previously reported that EV-D68 is associated with asthma exacerbations in children [32]. Therefore, enhanced immune responses in the lower respiratory tract might cause severe respiratory illnesses observed in EV-D68 infections.

It is also worth mentioning that EV-D68 has affinity for glycans with *N*-glycolyl neuraminic acid termini, which are commonly found in non-human species [24,33,34]. Thus, it might be possible that animals other than human are susceptible to EV-D68 infection. However, to date, EV-D68 has only been detected in humans.

Enterovirus 70 (EV70), which is another EV-D species member, is known to utilize α 2,3-linked SAs for attachment and infection in cell culture [35]. The amino acid sequences of the capsid regions (VP4, VP2, VP3, and VP1) of EV70 share >76% similarity with those of EV-D68 [28]. Despite their sequence similarity, the pathogenesis of EV-

D68 and EV70 in human appears to vary. The receptor specificities might underlie the mechanism controlling such clearly distinct tissue tropisms for these two enterovirus serotypes.

Antigenicity

We have shown by hemagglutination inhibition (HI) and NT tests that the Fermon strain has only limited cross-reactivity against the antisera generated for the EV-D68 strains detected in recent years [24]. This has led to the suggestion that the Fermon strain differs significantly from the EV-D68 strains that have been circulating in recent years. This result is in line with a previous study that demonstrated that the NT titer of sera collected from pregnant Finnish women against the Fermon strain decreased as the collection year progressed [36]. We found that antigenic differences were also present among the EV-D68 strains circulating in recent years: the HI and NT titer differed significantly among the strains from three different genetic lineages. Emergence of antigenically variant EV-D68 strains may be one of the reasons underlying the worldwide increase in detection of this virus in recent years.

Pathogenesis in animal models

Schieble *et al.* examined the pathogenesis of EV-D68 in suckling mice by inoculating them with four strains of EV-D68 (Fermon, Franklin, Robinson, and Rhyne) via the intracerebral and intraperitoneal routes [4]. The mice were observed for 2 weeks post-inoculation, after which the muscle and brain tissue subsequently harvested were passaged to new mice. No evidence of pathogenicity was observed for the Fermon, Franklin, or Robinson strain during passages of up to three times. However, mice inoculated with the Rhyne strain, which had been passaged twice in mice, developed limb tremor and weakness. Notably, mice developed paralysis and died after inoculation with the Rhyne strain that had been passaged three times [4]. This demonstrates the neuro-virulence of EV-D68 in mice, which is consistent with a case report describing the detection of EV-D68 in CSF collected from a fatal case of meningomyeloencephalitis [37]. However, a study by Schieble *et al.* did not document any pathogenic effects of EV-D68 in the respiratory systems of suckling mice. Therefore, despite the

etiological importance of EV-D68 among patients with acute respiratory tract infections, animal models for EV-D68 respiratory infections have still not been established.

EPIDEMIOLOGY

After its initial identification in 1962, detection of EV-D68 was reported only rarely until the early 2000s [38]. In fact, enterovirus surveillance conducted in the USA between 1970 and 2005 identified only 26 EV-D68 cases [38]. Epidemiological data on EV-D68 have also been limited. The largest case series was seven EV-D68-positive cases among military recruits in the USA between 2004 and 2005 [39]. However, increased detection of EV-D68 has been reported from different parts of the world [5], including Asia [6,10–15], Africa [7], Europe [8,16–21], Oceania [22], and the USA [7,23] since the late 2000s (Table 1). Initially, it was pointed out that the increasing trend of EV-D68 detections in recent years might be due to sampling bias or the introduction of highly sensitive detection methods such as PCR. However, retrospective testing using stored respiratory samples from Yamagata, Japan, and the Netherlands revealed that EV-D68 detection had actually increased in recent years [8,11]. In those studies, EV-D68 had been detected with no more than 10 cases per year until 2009, however, a significantly increased number of positive cases were reported in 2010: 40 in Yamagata and 24 in the Netherlands.

It is not fully understood whether EV-D68 has cyclic patterns of increased detections. The retrospective studies in Yamagata and the Netherlands suggested that EV-D68 was circulating every year, although the number of cases was significantly larger in 2010 for unknown reasons. In the Philippines, we identified EV-D68 three times: first in 2008–2009 [6], second in 2011 [10], and third in 2013–2014 (Furuse and Chaimongkol *et al.*, unpublished data). It is possible that EV-D68 outbreaks occur in cyclic patterns at 2-year intervals in the Philippines, suggesting that the frequency of outbreaks may vary between countries.

Increased detection of EV-D68 has recently been reported in the USA [40]. From mid-August to 10 October 2014, a total of 691 cases have been reported in 46 states. Most of the cases occurred among children with severe respiratory illnesses, and some cases were fatal [40]. Detection of EV-D68 in the USA was previously

Table 1. Incidence of worldwide EV-D68 infections in recent years

Location [references]	Period of EV-D68 detection	Study period	Study population	Number of EV-D68-positive cases		Age of EV-D68-positive cases	Diagnosis
				(overall detection rate, %)	Number of fatal cases		
The Netherlands [8]	1996, 1997, 1998, 1999, 2000, 2001, 2003, 2006, 2007, 2008, 2009, 2010	1994–2010	ILI surveillance, children cohorts for wheezing illness, cystic fibrosis, and amniotic fluid study	70 (70/12,743, 0.55%)	0	0.8 months–80s	ILI, ARI
South Africa [7]	May 2000–May 2001	ND	Hospitalized children	8	0	5 months–1 year 11 months	Respiratory illness (hospitalized) URTI, LRTI, others (e.g., asthma)
Japan (Yamagata) [11]	Sep–Oct 2005, Sep 2006, Aug–Oct 2007, Sep 2009, Aug–Oct 2010	2005–2010	Pediatric outpatients with ARI	55 (55/6307, 0.87%)	0	5 months–15 years (mean: 5.2 years)	Mild ARI
China (Beijing) [14]	Aug–Oct 2006, Aug 2008	Aug 2006–Apr 2010	Adult patients admitted to outpatient clinics and hospitals	13 (13/6942, 0.18%)	0	18–67 years (median: 34 years)	Respiratory illness (hospitalized) Pneumonia, ILI
The Gambia [7]	Jun 2008	ND	Hospitalized children	5	0	<2 years	Respiratory illness (hospitalized)
Philippines (Leyte) [6,10]	Oct 2008–Feb 2009, Jun–Aug 2011	May 2008–Dec 2011	Hospitalized pneumonia patients, outpatients with ILI	33 (33/6056, 0.54%)	4	1 month–76 years (median: 1 year 8 months)	URT, LRTI
Italy (Pavia) [16,17]	Oct 2008–Apr 2009	Oct 2008–Sep 2009	Pediatric and adult patients hospitalized with ARI	12 (12/1500, 0.80%)	0	1 month–57 years	URT, LRTI

(Continues)

Table 1. (Continued)

Location [References]	Period of EV-D68 detection	Study period	Study population	Number of EV-D68-positive cases		Age of EV-D68-positive cases	Diagnosis
				D68-positive cases (overall detection rate, %)	Number of fatal cases		
USA (New Hampshire) [37]	Autumn 2008	CR	CR	1	1	5 years	Meningo-yeoencephalitis
USA (Arizona) [7]	Oct 2009	ND	ND	1	0	1 year 11 months	Fever, cough, and rhinitis
The Gambia [7]	Jun 2008	ND	Hospitalized children	5	0	<2 years	Respiratory illness (hospitalized)
Thailand [13]	Jun–Sep 2009, Feb–Oct 2010, Jun–Sep 2011	Feb 2006–Nov 2011	Children with ARTI	25 (25/1810, 1.38%)	0	7 months–15 years (mean: 7.6 years)	Pneumonia, ILI
China (Chongqing, Beijing, Tianjin) [15]	Jun 2009–Jun 2012	Sep 2010–Aug 2011	Hospitalized children and adult outpatients with ARTI	9 (9/2150, 0.42%)	0	7 children, 2 adults	ARTI (e.g., pneumonia, URTI, and asthma)
USA (New York) [7]	Aug–Oct 2009	ND	Outpatients with respiratory illnesses	16	0	14–47 years	Respiratory illness (outpatients)
France (Champagne-Ardenne) [19]	Sep–Nov 2009	Sep 2009–Jun 2010	Pediatric patients admitted to a hospital with acute airway diseases	10 (10/651, 1.53%)	0	6 months–10 years (median: 3.8 years)	Asthma, bronchiolitis
USA (Arizona) [7]	Oct 2009	ND	ND	1	0	1 year 11 months	Fever, cough, and rhinitis
England (London) [21]	Nov–Dec 2009, Sep–Dec 2010	Nov 2009–Dec 2010	Patients with respiratory symptoms attending primary care and hospitals	17	1	7 weeks–45 years (mean: 12.2 years, median: 5.8 years)	Respiratory symptoms
Italy (Pavia) [18]	Jan 2010–Dec 2012	2010–2012	Patients who stayed or visited a hospital with	9 (9/3736, 0.24%)	0	7 pediatrics (4 months–6 years, median)	URT, LRTI

Senegal [7]	Feb–Mar 2010	ND	respiratory tract infections	3	0	15 months), 2 adults	Respiratory illness (hospitalized)
New Zealand [22]	Mar–Aug 2010	ND	Hospitalized children	15	0	1 months–48 years (median: 11 years)	Bronchiolitis, asthma, cough, coryza, wheeze, strider, pertussis, sepsis, heart failure, burns
Japan (Osaka) [12]	Jul–Sep 2010	Oct 2009–Oct 2010	Children with RTI	15 (15/448, 3.35%)	0	3 months–5 years (mean: 2 years 10 months)	Pneumonia, bronchopneumonia, bronchitis, LRTI, asthma, pharyngitis, febrile convulsion
Japan (Yamaguchi) [32]	Jul–Sep 2010	Jul–Sep 2010	Hospitalized children with history of asthma	26 (26/35, 74.3%)	0	Mean: 4 years	Asthma attack
Netherlands [20]	Aug–Nov 2010	2009–Jan 2011	Children hospitalized with respiratory infections	24 (24/252, 9.52%)	0	1 months–72 years (median: 14 years)	Respiratory infections (hospitalized)

Summary information for EV-D68 incidence. The data include the study sites, year of detection, case numbers, age distribution, and diagnosis. The numbers of reported cases of EV-D68-positive respiratory illnesses in the USA in 2014 have not been included in this table because of the limited information at present.
 ILLI, influenza-like illness; RTI, respiratory tract infections; URTI, upper respiratory tract infections; LRTI, lower respiratory infections; ARTI, acute respiratory tract infections; ARI, acute respiratory infections; ND, unknown; CR, case report.

reported in New York and Arizona in 2009 [7]. It is still unclear whether the present outbreak is associated with any changes that may have occurred in EV-D68 viruses that have been circulating in this country.

MOLECULAR EPIDEMIOLOGY

The EV-D68 strains circulating in recent years are classified into three genetic groups as mentioned earlier [8,11]. For the VP1 region, strains belonging to lineages 1 and 2 are likely to form clusters containing viruses from the same geographical origins on the phylogenetic tree. Strains from Yamagata, Japan (blue lines), form a cluster in lineage 1, those from the Philippines form a cluster in lineage 2 (green lines), and those from Italy form a cluster in lineages 1 and 2 (orange lines; Figure 1). In contrast, strains belonging to lineage 3 are less likely to show geographical clustering patterns (Figure 1). These data suggest that transmission at the global level might be more common for lineage 3 than for lineages 1 and 2.

The EV-D68 strains identified in each geographical area are likely to consist of strains of multiple

genetic lineages. In Yamagata, Japan, strains belonging to all three lineages were detected during a period of increased detection in 2010 (Figure 3). In Italy, England, Thailand, and the Philippines, co-circulation of lineages 2 and 3 strains was reported (Figure 3). In contrast, strains of only one lineage were detected each year in some of the study sites, including New Zealand (lineage 3 only); New York, USA (lineage 3); China (lineage 3); and Osaka, Japan (lineage 1; Figure 3). Enterovirus 71 (EV71), which also belongs to the genus *Enterovirus*, is known to have three genogroups and several genotypes [41,42]. None of the specific genogroups or genotypes has been reported to be associated with an increased risk of severe illness. However, it is likely that genotype replacement is associated with the occurrence of large outbreaks [43]. Nevertheless, such a replacement of EV-D68 lineages has not been observed in any of the previous studies in which multiple EV-D68 lineages were detected. In Yamagata, Japan, the majority of the EV-D68 strains detected during the increased detections in 2010 belonged to lineage 1, which was the same genetic lineage as the strains detected in

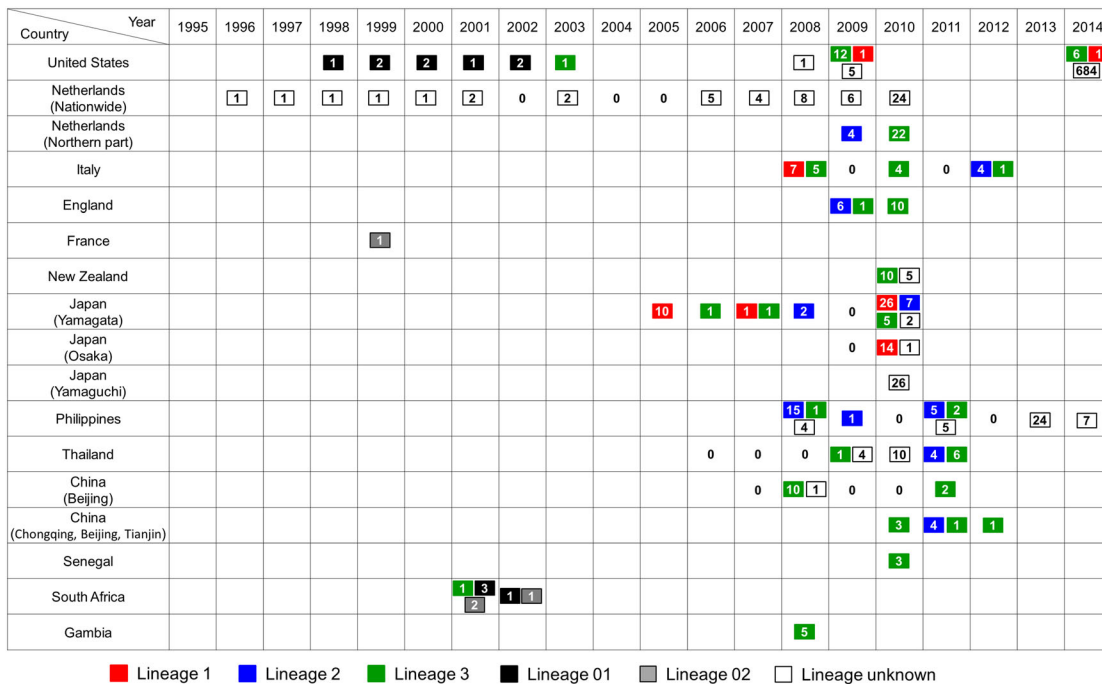


Figure 3. Circulation of different genetic lineages of enterovirus D68 (EV-D68) strains in recent years. The number of EV-D68 cases detected in a year at each study site is indicated in the box. Each year for which EV-D68 testing was conducted and no EV-D68-positive cases were found is shown as "0," without a box in the column. Each year when no EV-D68 testing was reported is left blank

the same area before 2010 [11]. The mechanisms responsible for the increase in detections of EV-D68 might differ from those of viruses of the same genus, such as EV71.

CLINICAL SIGNIFICANCE

Detection of EV-D68 has been exclusively reported in studies where respiratory samples from patients with acute respiratory infections or asthma were tested, indicating that EV-D68 might only be associated with respiratory illnesses (Table 1). The importance of including healthy controls in viral etiological studies has been recently recognized, as some viral pathogens have also been detected in healthy individuals [44]. In the sentinel general practice (GP) network surveillance for influenza-like illness (ILI) conducted in the Netherlands, EV-D68 was detected in 57 out of 9979 patients (0.57%) with respiratory symptoms, whereas only one out of 567 healthy individuals (0.17%) was positive for EV-D68 [8]. The EV-D68 detection rate was higher among patients than healthy controls, suggesting that EV-D68 is more likely to be associated with respiratory diseases in humans.

Several study groups have compared EV-D68 detection rates in patients with different disease severities. In the epidemiological study for acute respiratory infections in the Philippines, the EV-D68 detection rate was significantly higher among patients hospitalized with pneumonia (9/1187, 0.76% of pediatric cases, and 2/456, 0.44% of adult cases) than among outpatients diagnosed as ILI (1/3597, 0.028%; $p < 0.0001$) [10]. Similarly, in a population-based study among children with acute respiratory tract illnesses in Thailand, the EV-D68 detection rate was higher in the hospitalized cases (1.5%, 9/597) than it was in the outpatients (1.3%, 16/1213), but the data lacked statistical significance [13]. In the Netherlands, respiratory samples were collected in the sentinel GP network surveillance for ILI, and EV-D68-positive acute respiratory infections were more commonly accompanied with severe respiratory symptoms, such as dyspnea (11/57, 36.67%, vs 22/833, 10.95%; $p = 0.0002$) and bronchitis (13/57, 23.21%, vs 46/833, 5.67%; $p < 0.0001$), than were EV-D68-negative cases [8]. These studies indicate that EV-D68 infections are more likely to be associated with severe respiratory illnesses. Clinical manifestations of the pediatric patients were reported in the recent outbreaks in the USA, including the clusters in Kansas City

and Chicago [45,46]. Many of these patients had clinical characteristics indicative of severe illness, including difficulty of breathing, hypoxia, admission to an intensive care unit, and the requirement for mechanical ventilation. Such reports from the USA are consistent with previous studies indicating that EV-D68 infections are associated with severe respiratory illnesses. It is noteworthy that, of 30 pediatric patients whose respiratory samples were positive for EV-D68 in the USA, 21 (70%) had a previous history of asthma or wheezing [46]. An association between EV-D68 infections and asthma was also reported in Japan [32]. It might be possible that children with a history of asthma or wheezing illnesses are more likely to develop severe EV-D68 infections.

So far, a total of six fatal cases involving EV-D68 infections have been reported: three pediatric patients and one adult patient with pneumonia in the Philippines [6,10], one adult patient with severe respiratory symptoms in London, UK [21], and one pediatric patient admitted to the emergency department with meningomyeloencephalitis in New York, USA [37]. These reports indicate that EV-D68 infection can result in a fatal outcome. It should be mentioned that the two adult patients who died in the Philippines and London had comorbidities of hepatic cirrhosis and HIV infection, respectively [10,21]. These reports suggest that EV-D68 is also capable of causing fatal illness in adults with underlying diseases.

Susceptibility to EV-D68 infection per age group has not been fully defined, because most detected cases of EV-D68 have been reported from studies targeting pediatric patients. Only a few studies compared the EV-D68 detection rates among different age groups. In the ILI study conducted in the Netherlands, EV-D68 cases were most commonly identified in 50- to 59-year-olds, and the EV-D68-positive rate was lowest in children <10 years of age [8]. We previously reported that the EV-D68 detection rate was higher among pediatric pneumonia patients compared with adult pneumonia patients in the Philippines, but the data lacked statistical significance [10]. Because the disease severity of the study subjects differed, it is difficult to compare these two results. Further studies are needed to define the prevalence of EV-D68 infections in different age groups.

In several studies, co-detection of other viral pathogens in respiratory samples was reported for

some of the EV-D68-positive cases [7,13]. In a study conducted in Thailand, 9 out of 13 EV-D68-positive cases had co-infections: one had RSV group A (RSV-A), three had influenza B virus, and five had influenza A(H1N1)pdm [13]. In another study, two patients in South Africa were co-infected with RSV-A, and one patient in the Gambia was co-infected with human parainfluenza virus type 1 [7]. In both studies, a possible association between disease severity and co-infections was not discussed, presumably because of the limited number of cases in which more than one virus was detected.

The etiological association between EV-D68 and other diseases also remains elusive. To the best of our knowledge, only two studies have identified EV-D68 in non-respiratory samples: one was a case report of a pediatric fatality from meningomyeloencephalitis (with radiological evidence of pneumonia) who was positive for EV-D68 in CSF [37], and the other was where EV-D68 was detected in serum collected from pneumonia patients who were positive for EV-D68 in respiratory samples [47]. Any association between the presence of EV-D68 in serum and disease severity has not been defined. However, that EV-D68 was detected in the CSF of a patient raises the possibility that EV-D68 might be capable of causing systemic spread through viremia. EV-D68 infections can result in very severe outcomes once the virus is spread to the CNS from the respiratory tract. It was recently reported that EV-D68 was detected in nasopharyngeal specimens collected from pediatric patients with polio-like illnesses in the USA: two patients in California in 2012–2013 and four in Colorado in 2014 [48,49]. Preceding respiratory illnesses were reported in those patients, by which systemic spread of EV-D68 from the respiratory tract was suggested as the underlying mechanism for those cases, as was also suggested for the fatal case of EV-D68-positive meningomyeloencephalitis in New York [37]. It was previously shown that one of the four EV-D68 strains detected in 1962 (the Rhyne strain) replicated in the brains and muscles of suckling mice and caused paralysis in those mice [4]. It might be possible that EV-D68 has an affinity for the CNS, as do most other enteroviruses [9].

CONCLUSIONS

The number of reported cases of EV-D68 greatly increased in the late 2000s, and the etiological

significance of EV-D68 in acute respiratory infection has been strongly suggested. The virological properties of EV-D68 have also been studied, revealing that it is acid sensitive, binds to $\alpha 2,6$ SAs, and has a unique antigenicity corresponding to its genetic lineages. However, further studies are required to fully define the pathogenesis and clinical significance of EV-D68 in humans.

FUTURE PERSPECTIVES

The clinical and epidemiological significance of EV-D68 infections has been suggested after the worldwide increase in EV-D68 detections in recent years. However, there are still a number of unsolved questions, including those related to the virological characteristics and pathogenesis of EV-D68. For example, it is still not fully understood why EV-D68 infections occasionally develop into severe lower respiratory tract infections, whereas EV-D68 recognizes $\alpha 2,6$ SAs, which are more common in the upper respiratory tract. It is known that patients infected with influenza A(H1N1)pdm 2009, which recognizes $\alpha 2,6$ SAs in the upper respiratory tract, occasionally develop severe lower respiratory tract illnesses such as acute respiratory distress syndrome [50]. Enhanced immune response induced in the airway could be the possible mechanisms also for severe EV-D68 infections. However, details of the immune responses induced by infection with EV-D68 have not been documented *in vivo* or *in vitro*. The functions of the non-structural proteins of EV-D68 have also been poorly documented. Indeed, only two previous studies have analyzed the role of the 3C protease of EV-D68 in antiviral development [51,52]. A more comprehensive understanding of the pathogenesis of EV-D68 is currently difficult to obtain because of the lack of suitable animal models for EV-D68 respiratory infections. Therefore, further studies are required to fully define the pathogenesis of EV-D68.

CONFLICT OF INTEREST

The authors have no competing interest.

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