

A novel outbreak enterovirus D68 strain associated with acute flaccid myelitis cases in the USA (2012–14): a retrospective cohort study



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Summary

Background Enterovirus D68 was implicated in a widespread outbreak of severe respiratory illness across the USA in 2014 and has also been reported sporadically in patients with acute flaccid myelitis. We aimed to investigate the association between enterovirus D68 infection and acute flaccid myelitis during the 2014 enterovirus D68 respiratory outbreak in the USA.

Methods Patients with acute flaccid myelitis who presented to two hospitals in Colorado and California, USA, between Nov 24, 2013, and Oct 11, 2014, were included in the study. Additional cases identified from Jan 1, 2012, to Oct 4, 2014, via statewide surveillance were provided by the California Department of Public Health. We investigated the cause of these cases by metagenomic next-generation sequencing, viral genome recovery, and enterovirus D68 phylogenetic analysis. We compared patients with acute flaccid myelitis who were positive for enterovirus D68 with those with acute flaccid myelitis but negative for enterovirus D68 using the two-tailed Fisher's exact test, two-sample unpaired *t* test, and Mann-Whitney *U* test.

Findings 48 patients were included: 25 with acute flaccid myelitis, two with enterovirus-associated encephalitis, five with enterovirus-D68-associated upper respiratory illness, and 16 with aseptic meningitis or encephalitis who tested positive for enterovirus. Enterovirus D68 was detected in respiratory secretions from seven (64%) of 11 patients comprising two temporally and geographically linked acute flaccid myelitis clusters at the height of the 2014 outbreak, and from 12 (48%) of 25 patients with acute flaccid myelitis overall. Phylogenetic analysis revealed that all enterovirus D68 sequences associated with acute flaccid myelitis grouped into a clade B1 strain that emerged in 2010. Of six coding polymorphisms in the clade B1 enterovirus D68 polyprotein, five were present in neuropathogenic poliovirus or enterovirus D70, or both. One child with acute flaccid myelitis and a sibling with only upper respiratory illness were both infected by identical enterovirus D68 strains. Enterovirus D68 viraemia was identified in a child experiencing acute neurological progression of his paralytic illness. Deep metagenomic sequencing of cerebrospinal fluid from 14 patients with acute flaccid myelitis did not reveal evidence of an alternative infectious cause to enterovirus D68.

Interpretation These findings strengthen the putative association between enterovirus D68 and acute flaccid myelitis and the contention that acute flaccid myelitis is a rare yet severe clinical manifestation of enterovirus D68 infection in susceptible hosts.

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Introduction

Enteroviruses cause a broad spectrum of clinical illnesses, including acute respiratory infection, febrile rash, hand-foot-and-mouth disease, meningitis, encephalitis, and, rarely, acute flaccid paralysis. Enterovirus D68, which was first identified in 1962, causes respiratory illness,¹ but has also been sporadically detected in patients with acute flaccid paralysis.^{2–7} In 2014, in the USA, a nationwide outbreak of enterovirus D68 occurred in association with severe respiratory illness,⁸ with more than 1150 confirmed cases. This enterovirus D68 outbreak coincided with an apparent increase in incidence of reported cases of acute flaccid paralysis, including a temporally associated cluster

in Colorado.^{2,7} To more specifically describe this syndrome, the Centers for Disease Control and Prevention and the California Department of Public Health (CDPH) have proposed the term acute flaccid myelitis to include the subset of acute flaccid paralysis cases with myelitis primarily involving the grey matter.⁹ However, whether enterovirus D68 is an incidental finding in these patients or a newly emerging cause of acute flaccid myelitis remains uncertain.

Metagenomic next-generation sequencing (NGS) is a promising approach for the detection and discovery of pathogens in diseases that remain challenging to diagnose, such as encephalitis,^{10–12} and for investigation

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Research in context

Evidence before this study

A nationwide outbreak of enterovirus D68 associated with severe cases of respiratory illness occurred in 2014. In addition to respiratory symptoms, a few patients infected with enterovirus D68, nearly all of whom were children, presented with acute flaccid myelitis. However, evidence addressing whether enterovirus D68 is an incidental finding in these patients or a newly emerging cause of acute flaccid myelitis is limited. Also, several studies have reported detection of enterovirus D68 in individuals with acute flaccid myelitis or in patients with respiratory illnesses, but genomic and metagenomic analyses of enterovirus D68 from clusters of patients presenting with acute flaccid myelitis have not been described.

Added value of this study

In this study, we report new and extensive genomic data that strengthen the association between enterovirus D68 infection and acute flaccid paralysis. A novel B1 strain of enterovirus D68 was detected in two temporally and geographically linked clusters of acute flaccid myelitis that occurred during the height of the 2014 respiratory outbreak, and was also detected for the first time in blood from a child with acute flaccid myelitis during

the progressive phase of his paralytic illness. Although enterovirus D68 was not detected in cerebrospinal fluid from patients with acute flaccid myelitis, no other candidate infectious cause was identified in cerebrospinal fluid by metagenomic deep sequencing.

Implications of all the available evidence

The collective evidence provided in this study implicates the virus as a trigger of acute flaccid myelitis, and the identification of enterovirus D68 viraemia shows its capacity to cause systemic infection. Thus, this study underscores the importance of ongoing clinical and public health surveillance for enterovirus D68 and the need for an effective treatment or vaccine for enterovirus-D68-associated illness. The finding of one child with acute flaccid myelitis and her sibling with only upper respiratory illness shows that the clinical manifestations of enterovirus D68 infection can be variable and suggests a potential role for host-specific factors, such as differential immunological responses, in the pathogenesis of enterovirus D68. Although no one enterovirus D68 polymorphism was specific to patients with acute flaccid myelitis, the identified sequence homologies at several amino acid sites with neuropathogenic enteroviruses merits further investigation.

of outbreaks.^{13,14} Herein, we combined pathogen identification by metagenomic NGS, viral genome recovery, and enterovirus D68 phylogenetic analysis to investigate cases of acute flaccid myelitis in California and Colorado from 2012 to 2014, most of which occurred during the height of the 2014 enterovirus D68 respiratory outbreak in the USA.

Methods

Study design and patients

Patients with acute flaccid myelitis who presented to Children's Hospital Colorado (CHCO; Aurora, CA, USA) or Children's Hospital Los Angeles (CHLA; Los Angeles, CA, USA) from Nov 24, 2013, to Oct 11, 2014, or who were identified via statewide surveillance by the CDPH from Jan 1, 2012, to Oct 4, 2014, were included in the study. All study patients met the clinical case definition of acute flaccid myelitis, and there were no age limits or other inclusion criteria. Acute flaccid myelitis cases were defined as patients with acute flaccid weakness; with radiological (MRI) or neurophysiological (electromyogram [EMG]) evidence of acute spinal motor neuron injury; and who did not meet criteria for Guillain-Barré syndrome, West Nile virus infection, stroke, transverse myelitis, myasthenia gravis, botulism, or other known causes of acute flaccid paralysis.⁹ The clinical features of these patients have been summarised previously.^{2,5,7}

Local institutional review board approval was obtained from CHCO and CHLA for collection of clinical samples

and medical chart review, and from University of California, San Francisco (UCSF; San Francisco, CA, USA) for genomic and metagenomic analyses for masked patient samples. Patients or their surrogates (eg, parents on behalf of their children) provided written informed consent for enrolment in the study. Institutional review board approval was not required for samples from the CDPH because they were collected and analysed under a statewide mandate for the purpose of public health surveillance.

Procedures

Nasopharyngeal swab or wash, oropharyngeal swab or wash, serum, whole blood, rectal swab, stool, and cerebrospinal fluid (CSF) samples from patients were taken at various timepoints during hospital admission and were analysed by PCR or metagenomic NGS, or both (appendix A).

Clinical samples that had already undergone routine laboratory testing at CDPH, CHCO, and CHLA were screened for enterovirus D68 using a pan-rhinovirus and enterovirus RT-PCR targeting the 5'-untranslated region (5'-UTR),¹⁵ followed by heminested PCR to increase sensitivity (appendix A). Samples positive for enterovirus D68 were further screened using a VP1 heminested RT-PCR assay developed at UCSF (appendix A) and a 5'-UTR SYBR Green quantitative RT-PCR assay¹⁶ to obtain viral copy numbers by standard curve analysis.

Metagenomic NGS for pathogen detection is shotgun (ie, random) sequencing of the genomic nucleic acid

See Online for appendix A

(RNA and DNA) of a clinical sample, followed by microbial identification by comparison with comprehensive reference databases such as the National Institutes of Health GenBank.¹⁷ We did pathogen identification from metagenomic NGS data using the SURPI computational pipeline (appendix A).¹⁸

Full VP1 sequences were obtained using an enterovirus-D68-specific heminested RT-PCR assay targeting the VP1 region (appendix A), followed by Sanger sequencing. Consensus genomes were recovered by mapping reads and de novo assembled contigs (ie, contiguous sequences) obtained by metagenomic NGS (with or without viral probe enrichment) or Sanger sequencing to the reference enterovirus D68 Fermon strain (AF081348). We did phylogenetic and molecular clock analyses by alignment of recovered enterovirus D68 VP1 or complete genome sequences with all corresponding available sequences in GenBank. Full details, including accession numbers for the genomic and metagenomic sequencing data, are given in appendix A.

Statistical analysis

p values comparing patients with enterovirus-D68-positive and enterovirus-D68-negative acute flaccid myelitis were calculated using the two-tailed Fisher's exact test for categorical variables, two-sample unpaired *t* test for means, and Mann-Whitney *U* test for medians.

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

48 patients were included: 25 with acute flaccid myelitis, two with enterovirus-associated encephalitis, five with enterovirus-D68-associated upper respiratory illness (URI), and 16 consecutive paediatric patients with aseptic meningitis or encephalitis who tested positive at CHLA for enterovirus between August and October, 2014, and were subsequently screened for enterovirus D68 (figure 1). Among the 25 patients with acute flaccid myelitis, 16 originated from California and nine from Colorado. In California, formal statewide surveillance for cases of acute flaccid myelitis was initiated by the CDPH in June, 2012. As of October, 2014, 37 patients with acute flaccid myelitis had been tested at the CDPH, one before (in January, 2012, with hospital admission beginning in December, 2011) and 36 during the surveillance period. At the CHCO, 16 paediatric patients admitted to hospital met the case definition of acute flaccid myelitis from January, 2012, to October, 2014. Three of the patients from California were part of a geographically and temporally linked cluster at CHLA from August to October, 2014. From Colorado, we analysed samples collected from eight

of ten patients with acute flaccid myelitis corresponding to a previously reported cluster in August to October, 2014, at CHCO (consent was not available for the remaining two patients),²⁷ and one additional patient diagnosed in November, 2013. Thus, 11 of 25 patients with acute flaccid myelitis who were analysed in this study comprised part of a cluster, whereas the remaining 14 cases were sporadic.

All 25 cases of acute flaccid paralysis included in this study met the case definition for diagnosis of acute flaccid myelitis and presented with acute flaccid weakness, anterior horn cell grey matter involvement on MRI or EMG, and no identified cause (table 1). Patients with acute flaccid myelitis were predominantly children, with a median age of 7.0 years (range 0.3–73.0) and 60% were male. 20 (80%) of 25 patients reported URI prodrome, a mean of 5.6 days (SD 3.2) before the appearance of acute flaccid myelitis symptoms, 8.0 days (3.7) before CSF collection, and 11.6 days (7.2) before nasopharyngeal and oropharyngeal collection. Patients with acute flaccid myelitis with or without enterovirus D68 were largely comparable, with the exceptions of fewer days between URI onset and nasopharyngeal and oropharyngeal collection (7.8 vs 15.1 days; *p*=0.012) and decreased likelihood of corticosteroid treatment after onset of acute flaccid myelitis (33% vs 77%; *p*=0.047) in enterovirus-D68-positive patients. Clinical outcomes at 30 days of follow-up were poor, with persistent neurological deficits in all of 22 patients for whom data were available and no or minimal improvement in flaccid paralysis in 17 patients (77%).

12 (48%) of 25 nasopharyngeal or oropharyngeal samples from patients with acute flaccid myelitis were positive for enterovirus D68 by screening and confirmatory sequencing with two independent RT-PCR assays. Among the 11 patients comprising two clusters in California and Colorado, seven (64%) tested positive for enterovirus D68 (figure 1). Of the patients with acute flaccid myelitis, none of 25 assessed by clinical assays and none of 19 assessed by enterovirus D68 5'-UTR heminested RT-PCR tested positive for enterovirus from CSF (table 2).¹⁵ However, enterovirus D68 was detected in whole blood, stool, and nasopharyngeal and oropharyngeal samples from a 6-year-old child (US/CA/14-6070) in the CHLA acute flaccid myelitis cluster. This was the only patient in whom enterovirus D68 was detected in whole blood or stool, and viral titres were lower than in nasopharyngeal and oropharyngeal samples (appendix A). Enterovirus D68 viraemia was identified more than 1 week after URI onset during the child's hospital stay for progressive paralysis.

To search more broadly for potential neuroinvasive infections from enterovirus D68, we also analysed enterovirus-positive CSF from 16 consecutive paediatric patients from CHLA who presented with aseptic meningitis or encephalitis in August, 2014 (figure 1). These patients were part of the same paediatric population from which the three patients with acute

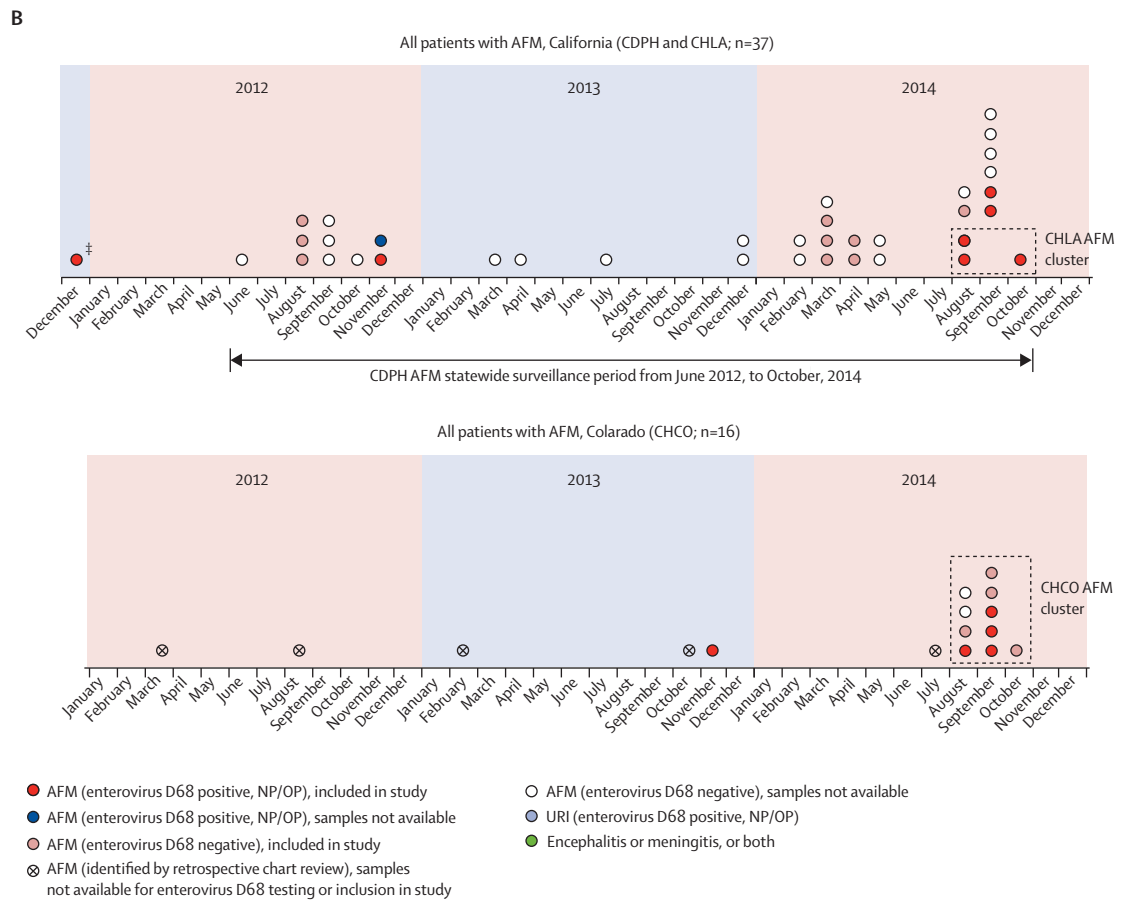
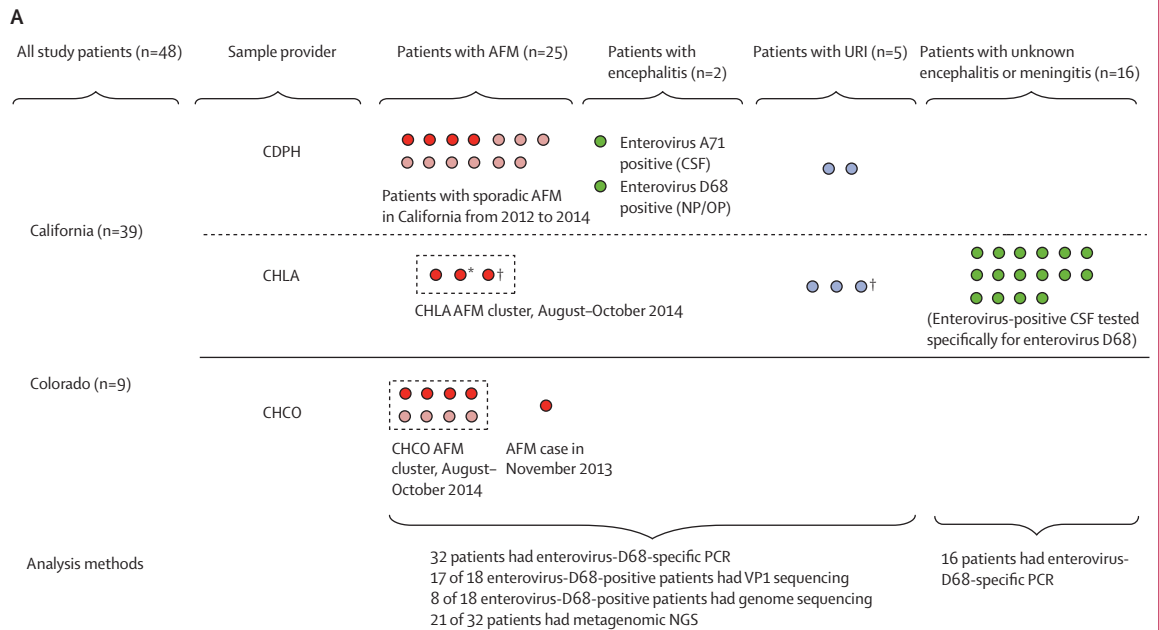


Figure 1: Description of study patients

(A) Flow chart of patients whose samples were analysed in this study, including the sample provider, number of cases, and methods used for analysis. (B) Timeline of patients investigated by the CDPH and CHLA (California) and CHCO (Colorado) who met the case definition of AFM. The dashed boxes outline temporally linked clusters of patients with AFM. AFM=acute flaccid myelitis. CDPH=California Department of Public Health. CHCO=Children’s Hospital Colorado. CHLA=Children’s Hospital Los Angeles. CSF=cerebrospinal fluid. NGS=next-generation sequencing. NP/OP=nasopharyngeal and oropharyngeal swab testing. URI=upper respiratory illness. *Enterovirus D68 positive from blood and stool. †Sibling pair. ‡Patient admitted to hospital on Dec 30, 2011, and reported to the CDPH in January, 2012.

	All patients (n=25)	Enterovirus D68 positive (n=12)	Enterovirus D68 negative (n=13)	p value
Part of an AFM cluster in California (n=3) or Colorado (n=8)	11/25 (44%)	7/11 (64%)	4/11 (36%)	..
Age, years	Median 7.0 (6.0, 0.3-73.0)	Median 4.5 (3.0, 0.5-18.0)	Median 13.0 (6.0, 0.3-73.0)	0.068
Sex				
Male	15 (60%)	7 (58%)	8 (62%)	1.00
Female	10 (40%)	5 (42%)	5 (38%)	..
Clinical presentation				
Fever	21 (84%)	11 (92%)	10 (77%)	0.22
URI prodrome (fever, rhinorrhoea, and cough)	20 (80%)	11 (92%)	9 (69%)	0.10
URI onset (number of days before AFM onset)	5.6 (3.2, 0.0-13.0)	5.1 (1.8, 2.0-8.0)	6.1 (4.2, 0.0-13.0)	0.47
URI onset (number of days before CSF collection)	8.0 (3.7, 0.0-17.0)	7.5 (2.1, 3.0-11.0)	8.4 (4.8, 0.0-17.0)	0.59
URI onset (number of days before NP/OP collection)	11.6 (7.2, 3.0-33.0)	7.8 (2.4, 3.0-11.0)	15.1 (8.4, 6.0-33.0)	0.012
AFM onset (number of days before CSF collection)	2.8 (3.5, 0.0-16.0)	2.5 (2.1, 0.0-6.0)	3.2 (4.4, 0.0-16.0)	0.63
Weakness or paralysis in one or more limbs	25 (100%)	12 (100%)	13 (100%)	1.00
Ventilatory or feeding support needed	7 (28%)	4 (33%)	3 (23%)	0.67
Bowel or bladder dysfunction	7 (28%)	1 (8%)	6 (46%)	0.073
Grey matter spinal cord injury on MRI or electromyogram	25 (100%)	12 (100%)	13 (100%)	1.00
Final clinical diagnosis of AFM	25 (100%)	12 (100%)	13 (100%)	1.00
CSF profile				
Leucocyte count ($\times 10^9$ cells per mm^3)	91 (104, 0-396)	63 (43, 3-155)	100 (141, 0-396)	0.39
Pleocytosis (leucocyte count $> 10 \times 10^9$ cells per μL)	18 (72%)	11 (92%)	7 (54%)	0.073
Neutrophilic predominance	4 (16%)	2 (17%)	2 (15%)	1.00
Protein (normal 0.20-0.45 g/L)	0.58 (0.51, 0.16-2.34)	0.45 (0.25, 0.17-0.92)	0.70 (0.64, 0.16-2.34)	0.24
Glucose (normal 0.50-0.80 g/L)	0.58 (0.20, 0.28-1.24)	0.57 (0.7, 0.47-0.68)	0.58 (0.27, 0.28-1.24)	0.88
Treatment after onset of AFM				
Experimental antiviral (pocapavir)	4 (16%)	3 (25%)	1 (8%)	0.32
Systemic corticosteroids	14 (56%)	4 (33%)	10 (77%)	0.047
Intravenous immunoglobulin	19 (76%)	11 (92%)	8 (62%)	0.16
Clinical follow-up of AFM at 30 days*				
No or limited improvement in flaccid paralysis	17/22 (77%)	7/10 (70%)	10/12 (83%)	0.62
Partial recovery with residual deficits	5/22 (23%)	3/10 (30%)	2/12 (17%)	0.62

Data are n/N (%), mean (SD, range), or number (%), unless otherwise specified. AFM=acute flaccid myelitis. CSF=cerebrospinal fluid. NP/OP=nasopharyngeal and oropharyngeal swab testing. URI=upper respiratory illness. *Data unavailable for three patients.

Table 1: Clinical characteristics of patients positive or negative for enterovirus D68

flaccid paralysis in the CHLA outbreak were derived. None of the CSF samples were positive for enterovirus D68 by VP1 heminested RT-PCR screening followed by confirmatory sequencing (table 2).

To further characterise enterovirus D68 strains associated with patients with acute flaccid myelitis or non-acute flaccid myelitis (defined as URI or encephalitis without acute flaccid myelitis), we recovered 17 new full-length enterovirus D68 VP1 sequences and eight full-length or near-full-length genomes. Because of low concentrations of enterovirus D68 in several nasopharyngeal and oropharyngeal samples (appendix A), a combination of metagenomic NGS, probe-based enterovirus D68 target enrichment, and PCR with Sanger sequencing was needed for genome sequence recovery (appendix A). Alignment and phylogenetic analysis of the new VP1 sequences in comparison to all published VP1 sequences from 1962 to 2014 confirmed that the original 1962 Fermon strain had

diverged into three distinct clades (figure 2), as previously reported.²¹ All 11 of the acute-flaccid-myelitis-associated enterovirus D68 viruses belonged to an evolutionarily recent cluster from 2012-14 (clade B1), which also included 17 VP1 sequences from non-acute flaccid myelitis 2014 respiratory outbreak isolates.^{8,22,23} A 2011 enterovirus D68 isolate from a patient with non-acute flaccid myelitis encephalitis (US/CA/11-1767) was positioned just outside clade B1, whereas two strains from patients with non-acute flaccid myelitis URI (US/CA/09-871 and US/CA/10-786) were phylogenetically distant. The two initial patients with enterovirus-D68-positive acute flaccid myelitis, reported to the CDPH in January, 2012 (US/CA/12-5641, with hospital admission beginning in December, 2011), and November, 2012 (US/CA/12-5837),⁵ were positioned near the root of clade B1, which is consistent with the emergence of this clade about 4.5 years ago by molecular clock analysis (appendix A).

Phylogenetic analysis of all 17 enterovirus D68 genomes available as of Dec 1, 2014 (nine genomes from GenBank and eight newly sequenced enterovirus D68 genomes), recapitulated the same phylogenetic relationships noted in the VP1 tree, and all acute flaccid myelitis-associated enterovirus D68 genomes belonged to clade B1 (figure 3). Clade B1 genomes were 87% identical to the ancestral

1962 Fermon strain, 87–97% identical to other enterovirus D68 strains outside of clade B1, and 97–100% identical to each other by pairwise alignment, with no evidence of recombination (figure 3).

The 5'-UTR internal ribosome entry site of enteroviruses has been associated with neurovirulence.²⁴ The sole nucleotide difference in the 5'-UTR of the clade B1

	Clinical laboratory PCR testing*		Enterovirus PCR screening		Enterovirus D68 sequencing	Metagenomic NGS	
	CSF	NP/OP (Ct)	CSF	NP/OP	NP/OP (% genome recovery)	CSF	NP/OP (number of NGS reads)
AFM, enterovirus D68 positive							
US/CA/12-5641	Negative	Positive for enterovirus D68	NA	Positive for enterovirus D68	VP1 and genome (93.8%)	NA	Enterovirus D68 (1702); anelloviridae† (7)
US/CA/12-5837‡§	Negative	Positive for enterovirus¶	NA	Positive for enterovirus D68	VP1	NA	Enterovirus D68 (2790); human papillomavirus 136† (10)
US/CA/14-6067‡	Negative	Positive for enterovirus¶	Negative	Positive for enterovirus D68	VP1	NONE	NA
US/CA/14-6070‡**	Negative	Positive for enterovirus††	Negative	Positive for enterovirus D68	VP1	Alphapapillomavirus-10†	NA
US/CA/14-6089	Negative	Positive for enterovirus D68	NA	Positive for enterovirus D68	VP1	NA	NA
US/CA/14-6092	Negative	Positive for enterovirus¶	NA	Positive for enterovirus D68	VP1 and genome (95.9%)	NA	NA
US/CA/14-6100‡‡	Negative	Positive for enterovirus††	Negative	Positive for enterovirus D68	VP1 and genome (96.1%)	NA	NA
US/CO/13-60	Negative	Positive for enterovirus D68	Negative	Positive for enterovirus D68	VP1 and genome (100%)	NONE	NA
US/CO/14-93§§	Negative	Positive for enterovirus D68	Negative	Positive for enterovirus D68	VP1 and genome (99.1%)	NONE	NA
US/CO/14-94§§	Negative	Positive for enterovirus D68	Negative	Positive for enterovirus D68	VP1 and partial genome (84.5%)	NONE	NA
US/CO/14-98§§	Negative	Negative	NA	Positive for enterovirus D68	Unable to recover	NA	NA
US/CO/14-86§§	Negative	Positive for enterovirus D68	Negative	Positive for enterovirus D68	VP1	Anelloviridae†	NA
AFM, enterovirus D68 negative							
US/CA/12-5806	Negative	Negative	Negative	Negative	NA	NONE	Human adenovirus C (1)
US/CA/12-5807	Negative	Negative	Negative	Negative	NA	NONE	HHV6A (5); gammapapillomavirus-1† (10)
US/CA/14-5809	Negative	Negative	Negative	Negative	NA	NONE	HSV1 (5)
US/CA/14-5999	Negative	Negative	Negative	Negative	NA	NONE	Human metapneumovirus (521)
US/CA/14-6000	Negative	Negative	Negative	Negative	NA	NONE	Betapapillomavirus† (2)
US/CA/14-6007¶ ¶	Negative	Negative	Negative	Positive for HRVA	NA	Anelloviridae†	
US/CA/14-6010	Negative	NA	Negative	Positive for HRVA	NA	NONE	HRVA65 (157 000)
US/CA/14-6013	Negative	Negative	Negative	Negative	NA	Bovine parvovirus	NONE
US/CA/14-6072‡‡	Negative	Positive for enterovirus	NA	Positive for enterovirus B (not subtyped)	NA	NA	NA
US/CO/14-84§§	Negative	Positive for HRVB¶	Negative	Positive for HRV-B	NA	NA	NA
US/CO/14-88§§	Negative	NA	Negative	Negative	NA	NA	Alphapapillomavirus-2† (60)
US/CO/14-95§§	Negative	Positive for adenovirus, HRVA24	Negative	Negative	NA	NA	HRVA24 (18 million); human bocavirus (8)
US/CO/14-101§§	Negative	Negative	Negative	Negative	NA	NA	NA
Encephalitis, enterovirus D68 positive							
US/CA/11-1767	Negative	Negative	NA	Positive for enterovirus D68	VP1 and genome (100%)	NA	Enterovirus D68 (3 million)

(Table 2 continues on next page)

	Clinical laboratory PCR testing*		Enterovirus PCR screening		Enterovirus D68 sequencing	Metagenomic NGS	
	CSF	NP/OP (Ct)	CSF	NP/OP	NP/OP (% genome recovery)	CSF	NP/OP (number of NGS reads)
(Continued from previous page)							
Upper respiratory illness, enterovirus D68 positive							
US/CA/09-871	NA	Negative	NA	Positive for enterovirus D68	VP1	NA	Enterovirus D68 (3027)
US/CA/10-786	NA	Negative	NA	Positive for enterovirus D68	VP1 and genome (100%)	NA	Enterovirus D68 (46 million); bovine diarrheal virus (257)
US/CA/14-R1	NA	Positive for enterovirus†† (28-6)	NA	Positive for enterovirus D68	VP1	NA	NA
US/CA/14-R2	Negative	Positive for enterovirus†† (36-3)	NA	Positive for enterovirus D68	VP1	NA	NA
US/CA/14-6103SIB‡‡	Negative	Positive for enterovirus†† (32-1)	NA	Positive for enterovirus D68	VP1 and genome (100%)	NA	NA

AFM=acute flaccid myelitis. CSF=cerebrospinal fluid. Ct=cycle threshold. HHV=human herpesvirus. HRV=human rhinovirus. HSV=herpes simplex virus. NA=not applicable or sample not tested because of no availability. NGS=next-generation sequencing. NONE=no reads from credible pathogens found in NGS data. NP/OP=nasopharyngeal and oropharyngeal swab testing. *Routine laboratory testing includes viral, bacterial, mycobacterial, and fungal culture; and respiratory virus, enterovirus, HSV1/2, Epstein-Barr virus, cytomegalovirus, and varicella-zoster virus PCR. For samples from Colorado, anti-neuromyelitis optica antibody testing was also done for cases with bulbar involvement. For samples from California, additional specific parechovirus PCR, HHV6 PCR, anti-N-methyl-D-aspartate receptor antibody, and *Mycoplasma pneumoniae* IgM and IgG antibody testing was done. †Judged to be part of normal microbial flora of the blood or skin. ‡Part of a three-person AFM cluster in Los Angeles (CA, USA), from August to October, 2014. §Serum sample from the patient was positive for mycoplasma IgM (not shown in table). ¶Quantitative pan-rhinovirus and enterovirus RT-PCR assay with confirmatory Sanger sequencing.¹⁶ ||Stool sample tested positive for coxsackievirus A16 (Ct 30-5; not shown in table). **Stool sample tested positive for enterovirus D68 (Ct 36-6; not shown in table). ††Quantitative pan-enterovirus RT-PCR FOCUS Simplexa assay. ‡‡Stool sample tested positive for enterovirus B (5'-untranslated region sequencing of PCR amplicon insufficient for subtyping; not shown in table). §§Part of an eight-person AFM cluster in Aurora (CO, USA), from August to October, 2014. ¶¶Serum sample tested positive for West Nile virus IgG antibody; West Nile virus IgM antibody was negative (not shown in table). ||||Common viral laboratory contaminant of fetal bovine serum.

Table 2: Microbiological testing of patients with acute flaccid myelitis and patients with encephalitis or upper respiratory illness who were positive for enterovirus D68

strain was U339 compared with C339 in other enterovirus D68 strains. Among the three 5'-UTR nucleotides associated with neurovirulence in enterovirus 71 (U448, A/U700, and G272),²⁴ only G272 was present in enterovirus D68, and this nucleotide was common to all sequenced enterovirus D68 genomes, regardless of clade. However, analysis of the enterovirus D68 polyprotein revealed five coding single nucleotide polymorphisms specific to clade B1 (figure 3). An additional coding polymorphism G1108 (figure 3), also noted in strains positioned just outside of clade B1, was situated at the 2B-2C proteolytic junction. Five of the six coding single nucleotide polymorphisms matched with poliovirus or enterovirus D70, or both.

One patient with acute flaccid myelitis in the present study (US/CA/14-6100) developed acute flaccid myelitis after a URI prodrome, whereas her sibling (US/CA/14-6100SIB) only exhibited URI symptoms (figure 1; table 2). Nasopharyngeal and oropharyngeal samples from the two children, both collected 9 days after onset of URI symptoms, were positive for enterovirus D68. Full genome sequencing of enterovirus D68 strains from the two siblings revealed no nucleotide differences. Despite being infected with a clade B1 enterovirus D68 strain (table 2), neither the unaffected sibling nor two additional patients with URI developed acute flaccid myelitis within 60 days of follow-up.

To search for other pathogens that might be associated with acute flaccid myelitis, we did metagenomic NGS of 15 CSF samples, including 14 from patients with acute

flaccid myelitis (six enterovirus D68 positive and eight enterovirus D68 negative) and one from a patient with non-acute flaccid myelitis enterovirus A71 encephalitis as a positive control (appendix B). 14 of 25 acute flaccid myelitis CSF samples were analysed because of limited sample availability and to maximise the depth of sequencing achieved. Between 13 million and 117 million sequence reads were generated per CSF sample (appendix B). No sequence reads corresponding to putative neuropathogenic viruses, bacteria, fungi, or parasites were recovered from CSF samples collected from patients with acute flaccid myelitis (appendices A and B). By contrast, the positive control CSF sample revealed 4600 of about 39 million sequenced reads aligned to enterovirus A71, with recovery of 92% of the genome (appendices A and B).

To aid in the recovery of enterovirus D68 genome sequences and detect potential co-infections from other viruses, we also did metagenomic NGS on 13 nasopharyngeal and oropharyngeal samples (two enterovirus-D68-positive acute flaccid myelitis, nine enterovirus-D68-negative acute flaccid myelitis, one enterovirus-D68-positive non-acute flaccid myelitis encephalitis, and one enterovirus-D68-positive URI), with 32–402 million reads generated per sample. Metagenomic NGS of nasopharyngeal and oropharyngeal samples from the two enterovirus-D68-positive acute flaccid myelitis cases—US/CA/12-5641 and US/CA/12-5837—revealed 1702 of 381 million and 2790 of 402 million reads corresponding to enterovirus D68 (appendices A and B),

See Online for appendix B

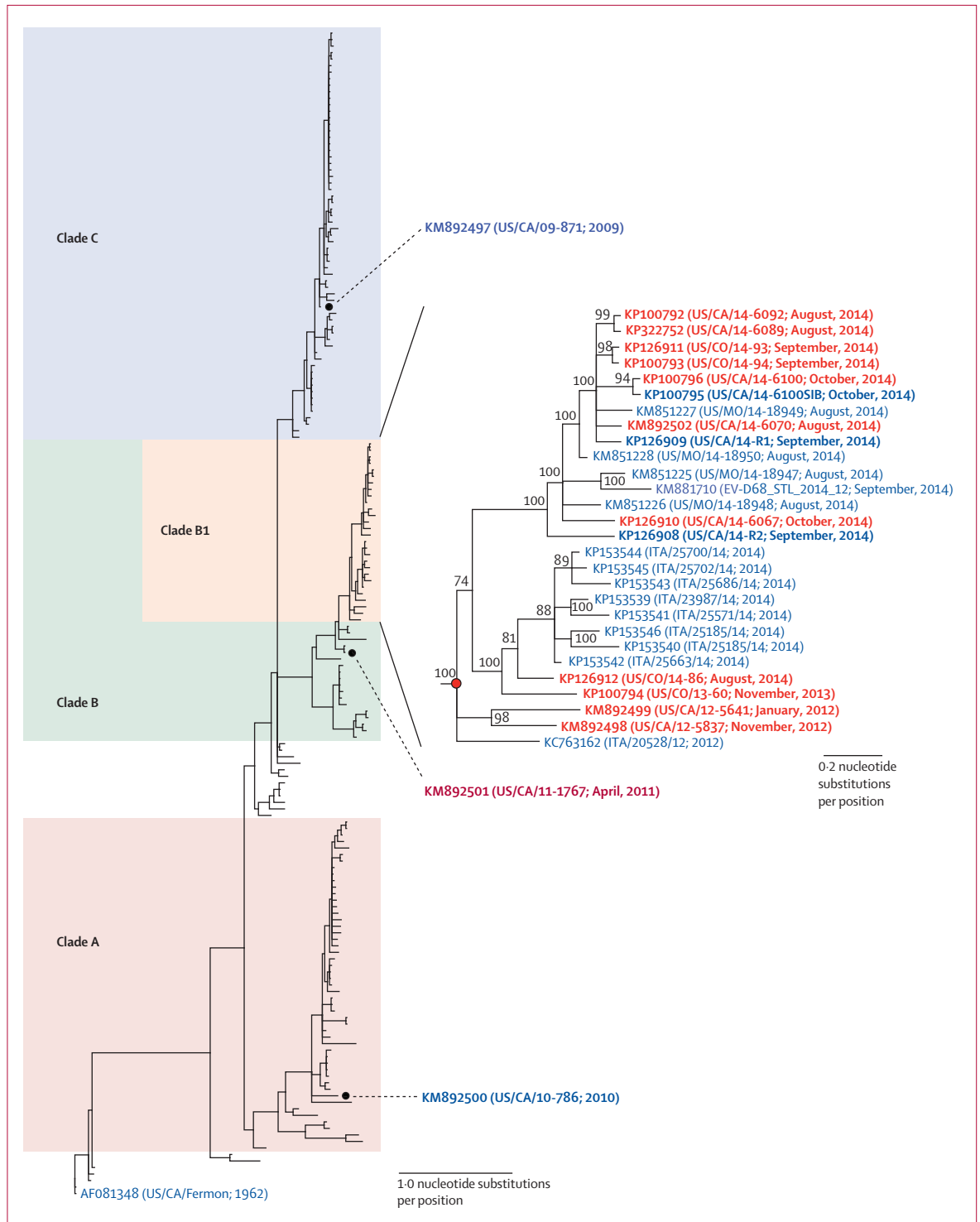


Figure 2: Phylogeny of enterovirus D68 by VP1 gene sequence

All 180 complete enterovirus D68 VP1 sequences available in GenBank as of Dec 1, 2014, including the 17 new enterovirus D68 VP1 gene sequences identified in this study (in bold), were aligned using MUSCLE,¹⁹ and phylogenetic trees were constructed using the MrBayes algorithm.²⁰ Enterovirus D68 strains from patients with acute flaccid myelitis were grouped together in a novel clade (clade B1) and included sequences from patients with severe respiratory illness from the 2014 outbreak. Patients with acute flaccid myelitis are in red, encephalitis in dark red, and respiratory illness only in blue. Branch lengths are drawn proportionally to the number of nucleotide substitutions per position, and support values are shown for each node.

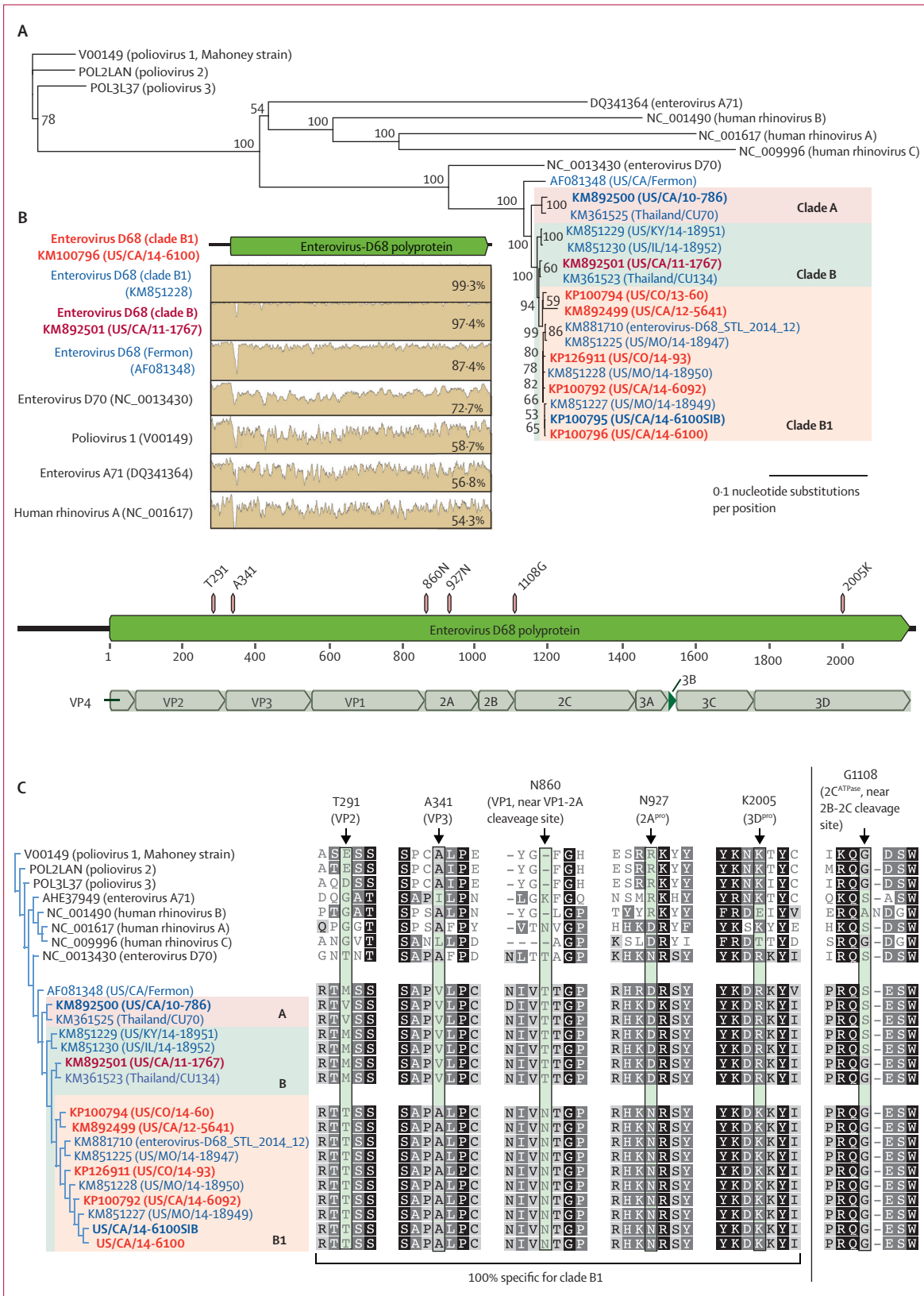


Figure 3: Coding polymorphisms associated with enterovirus D68 clade B1

(A) Eight enterovirus D68 genomes were recovered from clinical nasopharyngeal and oropharyngeal samples harbouring enterovirus D68 at concentrations sufficient for genome recovery (bold). Translated polyproteins were aligned using MUSCLE¹⁹ and phylogenetically clustered using MrBayes²⁰ with the nine other complete enterovirus D68 genomes in GenBank as of Dec 1, 2014, and the genomes of other representative enteroviruses. Patients with acute flaccid myelitis are in red, encephalitis in dark red, and respiratory illness only in blue. Branch lengths are drawn proportionally to the number of nucleotide substitutions per position, and support values are shown for each node.

(B) Pairwise identity plots of a clade B1 enterovirus D68 genome (US/CA/14-6100) versus other enteroviruses.

(C) Coding polymorphisms associated with the enterovirus D68 clade B1 polyprotein compared with non-clade B1 enterovirus D68 and other representative enteroviruses.

consistent with the low calculated enterovirus D68 titres of 53 575 copies per mL and 11 141 copies per mL, respectively (appendix B). A virus other than enterovirus D68 was identified from four of nine patients with enterovirus-D68-negative acute flaccid myelitis by metagenomic sequencing, including human adenovirus C (one read), human metapneumovirus (554 reads), a co-infection with human rhinovirus A24 (HRVA24; about 18 million reads) and human bocavirus (eight reads), and HRVA65 (157 000 reads; table 2; appendices A and B). By RT-PCR testing for enteroviruses¹⁵ and confirmatory Sanger sequencing of all 13 patients with enterovirus-D68-negative acute flaccid myelitis (table 2), three additional viruses—HRVA, HRVB, and enterovirus B—were detected. Nasopharyngeal and oropharyngeal swabs from seven (54%) of 13 patients with enterovirus-D68-negative acute flaccid myelitis harboured a virus different from enterovirus D68.

Discussion

In this study, we detected enterovirus D68 in respiratory samples in over a third of patients with sporadic acute flaccid myelitis and two-thirds of those with acute flaccid myelitis from two temporally linked clusters, coincident with the widespread 2014 respiratory outbreak across the USA. The two clusters were also geographically linked to patients within the regions covered by CHCO and CHLA hospitals. By phylogenetic analysis, enterovirus D68 strains associated with acute flaccid myelitis grouped into a distinct clade B1 that emerged 4–5 years ago, as did most of the respiratory outbreak strains from 2014 sequenced so far. The two initial patients with enterovirus-D68-positive acute flaccid myelitis, reported from California in 2012,⁵ were situated near the root of the clade. Coding polymorphisms associated with the clade B1 enterovirus D68 polyprotein shared homology with poliovirus and enterovirus D70. Enterovirus D68 viraemia was also detected for the first time in a child with acute flaccid myelitis.

Our data strengthen the putative association between enterovirus D68 respiratory infection and acute flaccid myelitis and suggest that detection of enterovirus D68 in respiratory secretions from patients with acute flaccid myelitis is unlikely to be incidental. The timing of the enterovirus-D68-associated outbreak of severe respiratory illness across the USA in 2014^{8,22,23} coincided with an apparent increased incidence of acute flaccid myelitis.^{2,5,7} More than 80% of patients with acute flaccid myelitis reported fever or a viral URI prodrome, or both, which is consistent with antecedent enterovirus D68 respiratory infection. Enterovirus D68 was also the most common virus detected in nasopharyngeal and oropharyngeal samples from patients with acute flaccid myelitis, and none of the neurotropic enteroviruses typically associated with acute flaccid myelitis (enterovirus 70, enterovirus 71, and poliovirus) were detected. Other respiratory viruses found in

nasopharyngeal and oropharyngeal samples from patients with acute flaccid myelitis—including four strains of rhinovirus, enterovirus B, bocavirus, and metapneumovirus, and coxsackievirus A16 and enterovirus B in the stool of two patients with enterovirus-D68-positive acute flaccid myelitis—were detected only as individual cases. Finally, deep metagenomic sequencing of CSF samples from 14 patients with acute flaccid myelitis did not reveal evidence of an alternative infectious cause. Taken together, these findings suggest that enterovirus D68 can be considered the most likely causative candidate for acute flaccid myelitis.

The anterior horn grey matter involvement noted in patients with acute flaccid myelitis is consistent with spinal motor neuron injury from direct viral invasion of tissue, which is characteristic of poliovirus and enterovirus A71 infections.^{25,26} We speculate that the coding polymorphisms noted in the clade B1 polyprotein might have conferred on enterovirus D68 an increased propensity for neurovirulence. The N860 polymorphism in the VP1 protein and the G1108 polymorphism at the proteolytic 2B/2C junction are particularly intriguing because the VP1 capsid change is specific to clade B1 enterovirus D68 and protease profiling of enterovirus 3C proteins has shown enhanced cleavage of Q/G compared with Q/S peptides.²⁷ However, because the functional consequences of these polymorphisms are unknown, further investigation is needed to identify their clinical relevance, if any, with regard to acute flaccid myelitis.

So far, infections by clade B1 viruses have been reported in several patients with respiratory illness but without acute flaccid myelitis or other neurological disease.^{8,22,23} Additionally, we identified a sibling pair infected with identical clade B1 strains: one with a URI prodrome progressing to acute flaccid myelitis and the other with a self-limited URI. These two findings suggest that the potential clinical manifestations and severity of enterovirus D68 infection are broad, and that host-specific—perhaps immunological—or environmental factors might have a role in differential responses to infection.

Collection of clinical samples usually occurred more than 7 days after URI onset, which probably reduced overall titres and yield of enterovirus D68. Enterovirus D68 titres in the respiratory tract of several patients with acute flaccid myelitis were low, in some cases needing two rounds of RT-PCR for detection (appendix B). Patients with acute flaccid myelitis who tested negative for enterovirus D68 had nasopharyngeal and oropharyngeal samples collected on average about 7 days later relative to URI onset than those who were positive for enterovirus D68 acute flaccid myelitis ($p=0.012$). The comparable clinical findings between patients positive and negative for enterovirus D68 also suggest that the identification of enterovirus D68 in only a subset of nasopharyngeal and oropharyngeal

samples might be because of insufficient detection sensitivity caused by delayed sample collection.

The inability to detect enterovirus D68 in CSF is not surprising since reported rates of CSF detection for known neurotropic enteroviruses such as polioviruses and enterovirus A71 are as low as 0–5%.^{25,28,29} Brain or spinal cord tissue from affected patients, which can assist in the diagnosis of viral encephalitis for which parallel CSF testing is negative,¹⁰ was not available for testing. Another possible reason for the inability to detect enterovirus D68 in CSF is that the pathogenesis of acute flaccid myelitis is related to an aberrant immune response to recent enterovirus D68 infection and is not caused by direct neuroinvasion by the virus.

Two published cases suggest that enterovirus D68 is probably neurotropic. Enterovirus D68 was detected in the CSF of a young adult with acute flaccid paralysis in 2005,⁴ and in the CSF and brain at autopsy in a 5-year-old boy with fulminant encephalitis in 2008.³ The emergence in 2014 of rare cases of acute flaccid myelitis occurring concomitantly with a widespread enterovirus D68 respiratory outbreak in the USA^{2,5,7} and worldwide⁶ is reminiscent of the large-scale outbreaks of acute haemorrhagic conjunctivitis accompanied by acute flaccid paralysis in about one in 10 000 patients from enterovirus D70 during the 1970s³⁰ and, more recently, sporadic outbreaks of enterovirus-A71-associated acute flaccid paralysis throughout the Asia-Pacific region from the 1990s to the present.²⁵ We detected enterovirus D68 in whole blood, nasopharyngeal and oropharyngeal swab, and stool samples from a 6-year-old child in California with acute flaccid myelitis more than 1 week after URI onset and during the progressive period of his paralytic illness. Prolonged viraemia with enterovirus D68 might directly facilitate the development of neuroinvasive disease, akin to that noted in poliomyelitis.²⁶ Since all patients with acute flaccid myelitis in the present study continue to have residual limb weakness or other neurological deficits, further investigation of the neuropathogenic potential of enterovirus D68, especially clade B1 strains, is needed.

Contributors

ALG, SNN, KM, SRD, CG, GA, and CYC conceived of and designed the study. ALG, SNN, GY, SS, CA, and SY did the experiments. KM, AC, SM, DW, DX, JPW, SRD, CG, and GA contributed clinical samples. KM, AC, JPW, KVH, SRD, CG, GA, and CYC analysed the clinical and epidemiological data. ALG, SNN, GY, SS, and CYC analysed the sequencing data. SNN, SF, DS, and CYC developed and contributed software analysis tools. ALG, SNN, and CYC wrote the paper. All authors read the final manuscript and approved submission.

Declaration of interests

CYC is the director of the UCSF-Abbott Viral Diagnostics and Discovery Center (VDDC). All other authors declare no competing interests.

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