

Multirecombinant Enterovirus A71 Subgenogroup C1 Isolates Associated with Neurologic Disease, France, 2016–2017

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In 2016, an upsurge of neurologic disease associated with infection with multirecombinant enterovirus A71 subgenogroup C1 lineage viruses was reported in France. These viruses emerged in the 2000s; 1 recombinant is widespread. This virus lineage has the potential to be associated with a long-term risk for severe disease among children.

Enterovirus A71 (EV-A71) comprises 7 genogroups (A–G) and various subgenogroups (e.g., B0–B5, C1–C5) (1). B4, B5, and C4 viruses circulate mainly in Asia, and C1 and C2 viruses have been detected in Europe (2). In

2016, an upsurge in neurologic manifestations of enterovirus infection was reported in France (3). These cases were associated with an emerging lineage of subgenogroup C1 enteroviruses first reported in 2015 in Germany and later in Spain and 4 other countries (Figure 1, panel A) (4–8). Our aim was to obtain the full genomes of the viruses from the specimens collected in France and track down the origin of this emerging lineage, hereafter referred to as C1v2015.

The Study

According to consolidated data recorded from the French Enterovirus Surveillance Network, 77 laboratory-confirmed cases of C1v2015 infection occurred during March–October 2016; in comparison, 136 EV-A71 infections of all genogroups combined were recorded during 2010–2015. The C1v2015 cases were widespread throughout France and associated with various clinical manifestations, including meningitis, cerebellitis, encephalitis, and myelitis, as well as hand, foot and mouth disease (HFMD) (Figure 1, panels B, C). One fatal case resulted from HFMD and cardiorespiratory failure. We analyzed 32 clinical specimens available from 25 patients reported as having a C1v2015 infection in 2016 and 2017 (Table 1; Appendix Table 1, <https://wwwnc.cdc.gov/EID/article/25/6/18-1460-App1.pdf>). Specimens and clinical data were collected during routine clinical work-up and epidemiologic surveillance, and patient data were deidentified before this study was conducted. The study was approved by the review board Comité de Protection des Personnes Sud-Est VI (no. 2018/CE44) in Clermont-Ferrand, France. The study population comprised 16 hospitalized children (median age 0.1 years), 4 children seen via ambulatory care (median age 1.8 years), and 5 children with asymptomatic infection (median age 1.4 years) in a childcare facility placed under community surveillance. We obtained the complete genomes, including the full 5' and 3' untranslated regions (UTRs), of 18 of 20 specimens and partial genomes of 2 of 20 specimens (2,893-nt and 4,380-nt long) acquired from 18 children (Appendix 2). We also determined the genomes of 12 isolates recovered during routine enterovirus surveillance to investigate their genetic relationships with C1v2015 (Appendix Table 2); we selected these viruses on the basis of previous exploratory investigations of their partial sequences (2,9,10).

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DOI: <https://doi.org/10.3201/eid2506.181460>

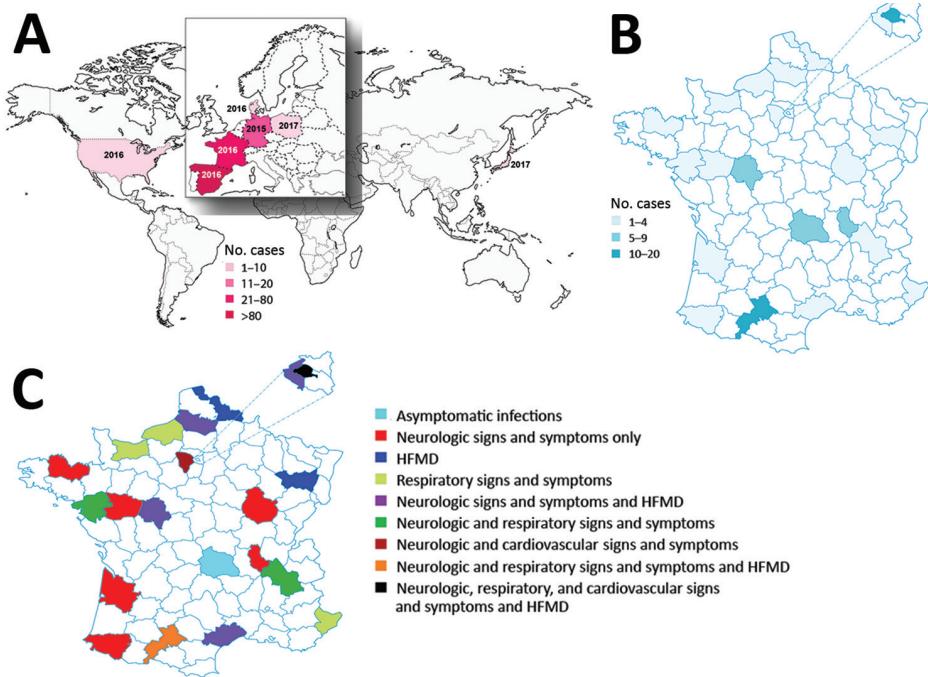


Figure 1. Geographic locations and numbers of enterovirus A71 (EV-A71) subgenogroup C1v2015 infections reported during 2015–2017. A) Countries in which EV-A71 C1v2015 was reported. The year the virus was first reported is indicated. The size of Europe is increased for easier visualization. B) Geographic distribution and number of cases of EV-A71 C1v2015 infection reported in hospitals, by department, France, 2016–2017. C) Geographic distribution of clinical manifestations associated with EV-A71 C1v2015 infection reported in hospitals, by department, France, 2016–2017. The size of a select set of departments is enlarged for easier visualization. HFMD, hand, foot and mouth disease.

Table 1. Characteristics of patients with EV-A71 subgenogroup C1v2015 infection, France, 2016–2017*

Patient no.	Specimen no.	Care setting (City)	Clinical diagnosis	Specimen material	Collection date	C _t
01	01†	Hospital (Toulouse)	Acute meningitis	Throat swab	2016 May 3	25
02	02	Hospital (Toulouse)	Fever	Nasopharyngeal aspirate	2016 May 19	30
02	03	Hospital (Toulouse)	Fever	Feces	2016 May 19	31
03	04	Hospital (Paris)‡	Fever	Plasma	2016 Jun 7	37
04	05	Hospital (Paris)‡	Fever	Plasma	2016 Jun 10	32
05	06	Hospital (Bayonne)	Encephalitis	Cerebrospinal fluid	2016 Jun 12	35
05	07†	Hospital (Bayonne)	Encephalitis	Throat swab	2016 Jun 24	35
05	08	Hospital (Bayonne)	Encephalitis	Rectal swab	2016 Jun 24	35
06	09	Hospital (Toulouse)	Infant fever	Feces	2016 Jul 10	31
07	10†	Hospital (Toulouse)	Sepsis-like disease	Throat swab	2016 Aug 10	24
07	11†	Hospital (Toulouse)	Sepsis-like disease	Nasopharyngeal aspirate	2016 Aug 10	NR
08	12†	Hospital (Paris)‡	Convulsions	Nasopharyngeal aspirate	2016 Aug 11	28
09	13§	Ambulatory (Mirecourt)	HFMD	Mouth swab	2016 Aug 30	32
10	14†	Ambulatory (Mirecourt)	HFMD	Throat swab	2016 Aug 30	29
11	15†	Hospital (Paris)‡	Fever, hypotonia	Blood	2016 Sep 5	30
12	16§	Hospital (Toulouse)	Acute meningitis, HFMD	Throat swab	2016 Sep 7	33
13	17†	Ambulatory (Toulouse)	HFMD	Mouth swab	2016 Sep 14	29
14	18†	Hospital (Paris)‡	Fever	Feces	2016 Sep 27	20
15¶	19†	Daycare (Volvic)	NR	Feces	2016 Oct 4	29
16¶	20†	Daycare (Volvic)	NR	Feces	2016 Oct 4	31
17¶	21†	Daycare (Volvic)	NR	Feces	2016 Oct 4	31
18¶	22†	Daycare (Volvic)	NR	Feces	2016 Oct 4	29
19¶	23†	Daycare (Volvic)	NR	Feces	2016 Oct 4	31
20	24	Hospital (Toulouse)	Sepsis-like disease	Throat swab	2016 Oct 5	33
21	25	Hospital (Versailles)	Diarrhea	Cerebrospinal fluid	2016 Oct 9	35
22	26	Hospital (Toulouse)	Acute meningitis, cerebellitis	Throat swab	2016 Oct 10	36
22	27	Hospital (Toulouse)	Acute meningitis, cerebellitis	Feces	2016 Oct 10	30
23	28†	Hospital (Toulouse)	Fever	Throat swab	2016 Oct 11	27
23	29	Hospital (Toulouse)	Fever	Feces	2016 Oct 12	29
24	30†	Hospital (Amiens)	Myelitis	Nasopharyngeal swab	2016 Oct 18	30
24	31†	Hospital (Amiens)	Myelitis	Feces	2016 Oct 20	33
25	32†	Ambulatory (Montesson)	Atypical HFMD, herpangina	Throat swab	2017 Jul 3	22

*See Appendix Table 1 (<https://wwwnc.cdc.gov/EID/article/25/6/18-1460-App1.pdf>) for extended data, including GenBank accession nos. C_t, cycle threshold; EV-A71, enterovirus A71; HFMD, hand, foot and mouth disease; NR, not reported.

†Specimens for which the complete viral genomes (including the full 5' and 3' untranslated regions) were obtained.

‡Assistance Publique-Hôpitaux de Paris Cochin, Paris, France.

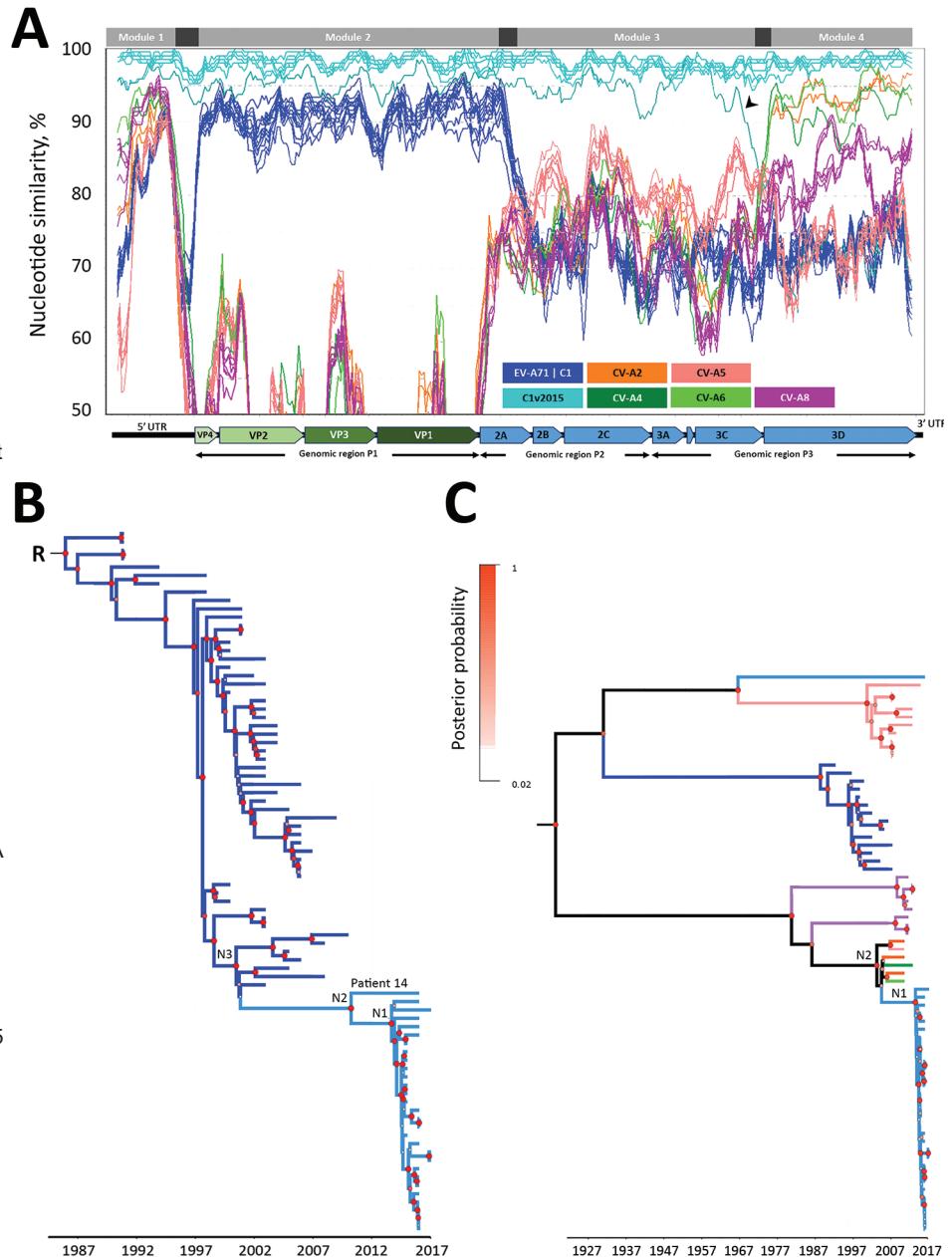
§Specimens for which partial genomes were obtained.

¶Patients 15–19 were children in the same daycare facility who had no evidence of clinical disease.

We performed whole-genome sequence analyses as previously described (11) to identify which viruses were the closest relatives of C1v2015. The C1v2015 genome appears to be a mosaic comprising 4 modules defined by distinct patterns of similarity possibly arising through

recombination (Figure 2, panel A). The nucleotide similarity patterns for module 2 (genomic region P1 comprising 4 capsid protein genes) suggest this region was inherited en bloc from an earlier subgenogroup C1 lineage. We used genomic region P1 to determine the evolutionary

Figure 2. Nucleotide similarity and phylogenetic analyses of EV-A71 subgenogroup C1v2015 isolates, France, 2016–2017, constructed to determine temporal origin of C1v2015 lineage. A) Nucleotide similarity patterns between EV-A71 C1v2015 and other EV-A lineages indicate the C1v2015 genome has a mosaic structure. A schematic diagram of the enterovirus genome is shown at the bottom of the panel. Four genomic modules (labeled at top of panel) with different genetic origins are identified; genomic modules are indicated in dark gray. The arrowhead indicates a previously undescribed recombinant lineage of C1v2015 (Appendix Figure 2, <https://wwwnc.cdc.gov/EID/article/25/6/18-1460-App1.pdf>). B) Phylogenetic tree constructed by using genomic region P1, encoding capsid proteins VP1–VP4, and methods described earlier (11). We performed this analysis with 85 sequences assigned to the EV-A71 C1 and C1v2015 lineages. Tree shows the temporal distribution of lineages, including the emergence of lineage C1v2015. C) Phylogenetic tree constructed by using 3Dpol, encoding the viral RNA polymerase common to C1v2015 and several CV-A strains. The dataset comprised 70 sequences: 24 CV-A (including 5 from this study), 14 EV-A71 C1 (including 6 from this study), 12 publicly available C1v2015, and 20 C1v2015 from this study. Recombination analyses provided no evidence of internal breakpoints within the sequences. N1 represents the time to most recent common ancestor (MRCA) of all included EV-A71 C1v2015 isolates except the virus from patient 14; N2 in panel B represents the MRCA of all EV-A71 C1v2015 isolates, including the virus from patient 14; N2 in panel C represents the MRCA of EV-A71 C1v2015 and its parent C1 lineage; and N3 represents the MRCA of EV-A71 C1v2015 and its parent C1 lineage (see Table 2). Diameters of circles at nodes reflect posterior probability. Branches of trees are color coded according to virus lineage as indicated in panel A. C1v2015, enterovirus subgenogroup C1 strain discovered in 2015; CV-A, coxsackievirus genogroup A; EV-A71C1, enterovirus A71 subgenogroup C1; N, node; UTR, untranslated region; VP, viral protein. An expanded version of this figure and legend describing complete methods and providing details of the trees in panels B and C is available online (<https://wwwnc.cdc.gov/EID/article/25/6/18-1460-F2.htm>).



relatedness between C1v2015 and earlier C1 viruses and to date when the upsurge of C1v2015 infections began in Europe (Figure 2, panel B). All C1v2015 viruses clustered in a lineage distinct from that comprising the C1 viruses reported during 1991–2010. The nucleotide substitution rate of C1v2015 (5.2238 [95% highest probability density HPD interval 4.124 – 6.3737] $\times 10^{-3}$ nt substitutions/y) and earlier C1 lineages (4.6302 [95% HPD interval 4.1769 – 5.1353] $\times 10^{-3}$ nt substitutions/y) was similar. All of the P1 sequences from these viruses, except that of the virus from patient 14, had a maximum nucleotide sequence difference from each other of 2%; the P1 sequence of the virus from patient 14 differed from that of other C1v2015 viruses by 4.8%. The close genetic relatedness between the C1v2015 sequences reported during 2015–2017 in France, Germany, Japan, and the United States was indicative of rapid widespread transmission. We estimated that interpersonal transmission of this lineage began during 2009–2011 (Table 2; Figure 2, panel B) and that its spread was sustained during 2013–2014, just 1–2 years before C1v2015 was first reported. The most recent common ancestor between C1v2015 and earlier C1 viruses was dated to 2000–2002. Seven EV-A71 subgenogroup C1 viruses from Africa and Europe were located at the base of the C1v2015 lineage (Figure 2, panel B), suggesting that the C1 strain involved in the emergence of C1v2015 was circulating in this region during the 2000s.

The C1v2015 genomic module 4 comprises the entire 3Dpol gene and has a 90%–95% nucleotide similarity with 4 distinct EV-A genomes: coxsackievirus A2 (CV-A2) and CV-A5 from Russia, CV-A4 from China, and CV-A6 from Turkmenistan (Figure 2, panel A). We performed another phylogenetic analysis to assess the temporal origin of C1v2015 using this module. With the 3Dpol phylogenetic analysis, we estimated that C1v2015 began spreading in 2010–2014 (Table 2; Figure 2, panel C), an estimate similar to that calculated with the P1 phylogeny. The nucleotide substitution rates with this analysis were also similar (C1v2015 3.7689 [95% HPD interval 1.3003 – 6.5838] $\times 10^{-3}$ nt substitutions/y and C1 3.6318 [95% HPD interval 1.6064 – 6.2072] $\times 10^{-3}$ nt substitutions/y). Whole-genome sequencing analysis showed that the isolate from patient 14 (14|COC286037|FRA|2016) shared distinct 3Dpol genes with other C1v2015 viruses (Appendix Figure 1). Overall, data indicate that the virus from patient 14 was an early recombinant of the C1v2015 lineage (Appendix Figure 2).

Within genomic module 1 (5' UTR, first 600 nt), we found areas of moderate nucleotide similarity (90%–95%) between the C1v2015 genome and the CV-A6 and CV-A8 genomes and lower similarity (<88%) with the EV-A71 subgenogroup C1 genomes (Figure 2, panel A). The C1v2015 5' UTR was therefore inherited from an EV-A

Table 2. Estimation of year of MRCA of EV-A71 subgenogroup C1v2015 lineage by using different enteroviruses*

Node†	Year of MRCA (95% HPD interval)	
	Genomic region P1	3Dpol gene
1‡	2013.6 (2013.2–2014.1)	2013.6 (2012.9–2014.3)
2§	2010.2 (2009–2011.3)	2004.1 (2001.7–2006.2)
3¶	2000.5 (2000.1–2001.6)	ND
Root	1986 (1984.7–1987.3)	ND

*EV-A71, enterovirus A71; HPD, highest probability density; MRCA, most recent common ancestor; ND, not done.
†MRCAs were determined for nodes and root in Figure 2.
‡Node 1 represents the MRCA of all included EV-A71 C1v2015 isolates except the virus from patient 14.
§For genomic region P1, node 2 represents the MRCA of all EV-A71 C1v2015 isolates, including the virus from patient 14. For 3Dpol gene, node 2 represents the MRCA of EV-A71 C1v2015 and its parent C1 lineage.
¶Node 3 represents the MRCA of EV-A71 C1v2015 and its parent C1 lineage.

lineage virus but not from the C1 ancestors that provided the capsid region. The pattern of sequence variation in the 5' UTR precludes the possibility of analysis with a molecular clock.

The genomic module 3 of C1v2015 had low similarity with all the publicly available EV-A genomes; thus, the precise origin remains unknown (Figure 2, panel A). The highest nucleotide similarity scores (<90% with CV-A5 genomes) indicate only a distant genetic relationship. We conclude that genes 2A (except the 5' terminus), 2B, 2C, and 3A–3C were transferred into the C1v2015 genome from a previously unreported lineage.

Conclusions

Thirty years after the outbreaks in central Europe (12,13), the 2016 upsurge of infections is a reminder that EV-A71 is of growing public health concern. After the B5 and C4 subgenogroup upsurges, C1v2015 is the latest example of an emerging recombinant EV-A71 associated with neurologic manifestations. Recombination, which frequently occurs in enteroviruses, is considered a factor driving this viral emergence (14,15). Compared with earlier circulating lineages of EV-A71, C1v2015 is a multirecombinant that arose through complete shuffling of all nonstructural genomic regions, although the capsid genes are phylogenetically typical of C1 viruses. Shuffling involved ≥ 2 recombination events with EV-A genomes before the emergence of C1v2015 as a life-threatening pathogen (Appendix Figure 2). From a public health perspective, the spread of C1v2015 could have resulted from acquired genomic features, notably a unique combination of the 5' UTR and 3Dpol gene, because recombination events clearly preceded the extensive circulation of C1v2015. The mosaic structure of the genome indicates that C1v2015 is an integral part of a large recombination network including multiple EV-A viruses transmitted in Eurasia. Given the propensity of enteroviruses to recombine their genomes and spread rapidly across distant countries (2,11) and that C1v2015 circulation continued throughout

2017 and 2018 in France, we need to determine if this virus is associated with a long-term recurrent risk for severe disease in the pediatric population through sharing data from global surveillance.

Acknowledgments

The authors are indebted to Patrice Bouissou, Annick Givois, and Martine Wagner-Vaucard, who obtained samples in children with hand, foot and mouth disease. We acknowledge the technical contribution of Jeroen Cremer, Adeline Duard, Nathalie Rodde, and Isabelle Simon for helpful assistance with molecular typing and sequencing. We thank Jeffrey Watts for help in preparing the manuscript in English.

Financial support was provided by the University Clermont Auvergne, France.

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Appendix

Materials and Methods

Amplification of Gene Sequences and Nucleotide Sequencing of PCR Products

Viral RNA was extracted from patient specimens or cell culture supernatants with the NucliSENS EasyMAG semi-automatic extractor (BioMérieux, <https://www.biomerieux.com>) using specific B protocol. The complete genomes were amplified into 2 amplicons (A, 5' untranslated region [UTR] – 2C; no. 4,500 bp) and (B, 2C – 3' UTR; no. 3,000 bp). cDNA synthesis was performed with SuperScript III reverse transcription (Invitrogen, <https://www.thermofisher.com/us/en/home/brands/invitrogen.html>) using a single primer located within the 2C and the 3' UTR. PCR was performed with Invitrogen Platinum SuperFi PCR Master Mix in 20 µL total volume with 2 µL of cDNA, 10 µL of PCR Master Mix, 6 µL of H₂O, and 1 µL of each primer (Appendix Table 3). PCR primers are indicated for both amplicons in Appendix Table 3. Gene amplification reactions to obtain the 5' UTR-2C amplicon were performed under the following conditions: 98°C for 30 s, followed by 41 cycles of 98°C for 5 s, 66°C for 10 s, and 72°C for 2 min 20 s, with a final extension step at 72°C for 5 min. To obtain the 2C-3' UTR amplicon, the reaction conditions were 98°C for 30 s, followed by 41 cycles of 98°C for 5 s, 70°C for 10 s, and 72°C for 2 min 25 s, with a final extension step at 72°C for 5 min. PCR products were visualized by 1% agarose gel electrophoresis. Sequencing was performed as previously described (*1*) with the Big Dye Cycle Sequencing Kit version 3.0 and an ABI 3500Dx automated DNA sequencer (Applied Biosystems, <https://www.thermofisher.com/us/en/home/brands/applied-biosystems.html>).

Bayesian Coalescent Analyzes

Two parameters, evolutionary rate and divergence times, were inferred from the P1 genomic region encoding the 4 capsid proteins and 3D polymerase gene of enterovirus A71 (EV-A71) and EV-A. The sequence data sets were analyzed with a Bayesian Markov chain Monte Carlo algorithm allowing estimation of the posterior distribution of parameters, which is implemented in the BEAST v1.8.4 program (<http://beast.community>) (2). The nucleotide substitution model used was the general time reversible model with 4 gamma rate categories, invariant sites, and partition of the 3 codon positions. A relaxed molecular clock assuming uncorrelated lognormal prior distributions of substitution rates among lineages was used with the Bayesian skyline, which does not assume a specified model of demography (3,4). The analyzes were run for 50 million generations, sampling a tree every 5,000 steps and discarding the first 10% as burn-in. Markov chain Monte Carlo convergence and effective sample sizes were checked using the TRACER v1.6 program (<http://tree.bio.ed.ac.uk/software/tracer>). Analyses were considered to have converged and reached stability after the burn-in period when effective sample sizes were >200. Uncertainty in the estimates was indicated by the 95% highest probability density values. The TreeAnnotator v1.5.4 program (<http://beast.community/>) computed the maximum clade credibility tree from all plausible trees created during the BEAST run, with the first 10% of trees removed as burn-in. The tree was annotated by using FigTree v1.4.3 (<http://tree.bio.ed.ac.uk/software/figtree>).

Recombination Detection in Complete Genomes

The EV-A71 C1v2015 genome sequences were compared with 12 EV-A determined in the present study and 31 genomes selected among those available in international databases. The nucleotide similarity patterns were determined with the SimPlot v3.5.1 program (5) with a sliding window of 200 nt moving in steps of 20 nt. Other methods implemented in RDP v.4.33 (6) (<http://web.cbio.uct.ac.za/~darren/rdp.html>) were used to locate the positions of possible recombination events. The default methods, RDP (7), Geneconv (8), MaxChi (9), Chimaera (10), Bootscan (11), and SiScan (12) were used to survey the sequences. Only putative recombinant signals detected with 5 methods were considered to exclude the possibility of false-positive detection.

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Table 1. Characteristics of patients with EV-A71 C1v2015 infection included in the study*

Patient no.†	Specimen no.	Age, y/sex	Care setting (City)	Clinical diagnosis	Specimen material	Collection date	C _t	Sequence designation	GenBank accession no.‡	
01	01§	2.6/F	Hospital (Toulouse)	Acute meningitis	Throat swab	2016 May 3	25	01 TOU148153 FRA 2016	LR027527	
02	02	0.09/M	Hospital (Toulouse)	Fever	Nasopharyngeal aspirate	2016 May 19	30	02 TOU148165 FRA 2016		
02	03	0.09/M	Hospital (Toulouse)	Fever	Feces	2016 May 19	31	02 TOU148166 FRA 2016		
03	04	0.1/M	Hospital Cochin (Paris)	Fever	Plasma	2016 Jun 7	37	03 COC166142 FRA 2016		
04	05	0.11/F	Hospital Cochin (Paris)	Fever	Plasma	2016 Jun 10	32	04 COC166146 FRA 2016		
05	06	15.6/F	Hospital (Bayonne)	Encephalitis	Cerebrospinal fluid	2016 Jun 12	35	05 BAY180005 FRA 2016		
05	07§	15.6/F	Hospital (Bayonne)	Encephalitis	Throat swab	2016 Jun 24	35	05 BAY180009 FRA 2016		LR027529
05	08	15.6/F	Hospital (Bayonne)	Encephalitis	Rectal swab	2016 Jun 24	35	05 BAY180095 FRA 2016		
06	09	0.08/M	Hospital (Toulouse)	Fever	Feces	2016 Jul 10	31	06 TOU207118 FRA 2016		
07	10§	0.02/M	Hospital (Toulouse)	Sepsis-like disease	Throat swab	2016 Aug 10	24	07 TOU307016 FRA 2016		LR027521
07	11§	0.02/M	Hospital (Toulouse)	Sepsis-like disease	Nasopharyngeal aspirate	2016 Aug 10	NR	07 TOU307017 FRA 2016	LR027522	
08	12§	7.6/F	Hospital Cochin (Paris)	Convulsions	Nasopharyngeal aspirate	2016 Aug 11	28	08 COC238077 FRA 2016	LR027528	
09	13¶	1.93/F	Ambulatory (Mirecourt)	HFMD	Mouth swab	2016 Aug 30	32	09 PMB250101 FRA 2016	LR027523	
10	14§	1.39/M	Ambulatory (Mirecourt)	HFMD	Throat swab	2016 Aug 30	29	10 PMB250102 FRA 2016	LR027524	
11	15§	0.11/M	Hospital Cochin (Paris)	Fever, hypotonia	Blood	2016 Sep 5	30	11 COC259064 FRA 2016	LR027525	
12	16¶	0.03/M	Hospital (Toulouse)	Acute meningitis, HFMD	Throat swab	2016 Sep 7	33	12 TOU307023 FRA 2016	LR027526	
13	17§	1.37/M	Ambulatory (Toulouse)	HFMD	Mouth swab	2016 Sep 14	29	13 PMB263109 FRA 2016	LR027534	
14	18§	0.18/F	Hospital Cochin (Paris)	Fever	Feces	2016 Sep 27	20	14 COC286037 FRA 2016	LR027533	
15	19§	1.77/F	Daycare (Volvic)	None reported	Feces	2016 Oct 4	29	15 VSV286030 FRA 2016	LR027530	
16	20§	1.39/M	Daycare (Volvic)	None reported	Feces	2016 Oct 4	31	16 VSV286032 FRA 2016	LR027536	
17	21§	1.69/M	Daycare (Volvic)	None reported	Feces	2016 Oct 4	31	17 VSV286034 FRA 2016	LR027537	
18	22§	2.38/F	Daycare (Volvic)	None reported	Feces	2016 Oct 4	29	18 VSV286035 FRA 2016	LR027535	
19	23§	2.05/M	Daycare (Volvic)	None reported	Feces	2016 Oct 4	31	19 VSV286036 FRA 2016	LR027538	
20	24	0.08/M	Hospital (Toulouse)	Sepsis-like disease	Throat swab	2016 Oct 5	33	20 TOU307031 FRA 2016		
21	25	0.04/M	Hospital (Versailles)	Diarrhea	Cerebrospinal fluid	2016 Oct 9	35	21 VER294019 FRA 2016		
22	26	14.7/M	Hospital (Toulouse)	Acute meningitis, cerebellitis	Throat swab	2016 Oct 10	36	22 TOU307034 FRA 2016		
22	27	14.7/M	Hospital (Toulouse)	Acute meningitis, cerebellitis	Feces	2016 Oct 10	30	22 TOU307035 FRA 2016		
23	28§	0.25/F	Hospital (Toulouse)	Fever	Throat swab	2016 Oct 11	27	23 TOU307036 FRA 2016	LR027532	
23	29	0.25/F	Hospital (Toulouse)	Fever	Feces	2016 Oct 12	29	23 TOU307037 FRA 2016		
24	30§	5/M	Hospital (Amiens)	Myelitis	Nasopharyngeal swab	2016 Oct 18	30	24 AMI302002 FRA 2016	LR027546	
24	31§	5/M	Hospital (Amiens)	Myelitis	Feces	2016 Oct 20	33	24 AMI302001 FRA 2016	LR027531	
25	32§	0.92/F	Ambulatory (Montesson)	Atypical HFMD, herpangina	Throat swab	2017 Jul 3	22	25 PMB501259 FRA 2017	LR027539	

*C_t, cycle threshold; EV-A71, enterovirus type A71; HFMD, hand, foot and mouth disease; NR, not reported.

†Patients 15–19 were children in the same day care facility, who had no evidence of clinical disease.

‡Virus genomes were not determined in 12 clinical specimens because gene amplifications were unsuccessful.

§Specimens for which the complete viral genomes (including the full 5' and 3' untranslated regions) were determined.

¶Specimens for which partial genomes were determined.

Table 2. Genomes of virus isolates determined from cell culture supernatants to investigate their genetic relationships with the EV-A71 C1v2015*

Sample no.	Sample designation	Collection year	Clinical specimen	Country (city)	Enterovirus type subgenogroup	GenBank accession nos., earlier partial sequence data	GenBank accession no., this study
1	CF097017 FRA 2003	2003	Stool	France (Clermont-Ferrand)	EV-A71 C1	HG934182.1	LR027544.1
2	STU562356 DEU 2003	2003	Unknown	Germany (Stuttgart)	EV-A71 C1	HG934192.1	LR027542.1
3	1480 NLD 2005	2005	Unknown	Netherlands (Eindhoven)	EV-A71 C1	AB524174.1	LR027541.1
4	CF210042 FRA 2006	2006	Pharynx	France (Clermont-Ferrand)	EV-A71 C1	HG934219.1	LR027547.1
5	GRE29 FRA 2007	2007	Pharynx	France (Grenoble)	EV-A71 C1	HG934229.1	LR027545.1
6	STU546711 DEU 2008	2008	Stool	Germany (Stuttgart)	EV-A71 C1	HG934246.1	LR027543.1
7	37183 TKM 2010	2010	Stool	Turkmenistan	CV-A7	KC879521.1, KC879366.1, KC879445.1	LR027540.1
8	41963 RUS 2011	2011	Stool	Russia	CV-A2	KC879551.1, KC879399.1, KC879478.1	LR027549.1
9	42115 RUS 2011	2011	Stool	Russia	CV-A2	KC879553.1, KC879401.1, KC879480.1	LR027551.1
10	41149 RUS 2011	2011	Stool	Russia	CV-A2	KC879544.1, KC879390.1, KC879469.1	LR027550.1
11	41143 RUS 2011	2011	Stool	Russia	CV-A5	KC879543.1, KC879389.1, KC879468.1	LR027548.1
12	40428 TKM 2011	2011	Stool	Turkmenistan	CV-A6	KC879540.1, KC879386.1, KC879465.1	LR027552.1

*EV-A71, enterovirus A71.

Appendix Table 3. Primers used in this study for gene amplification and amplicon sequencing*

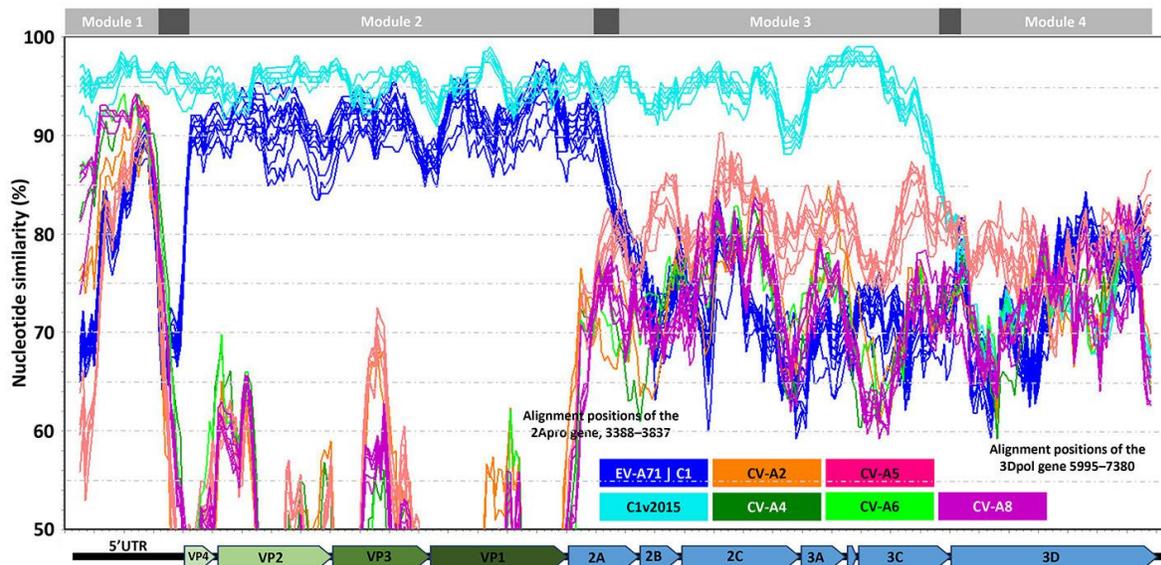
Genomic region†	Primer designation‡	Sequence (5'→3')	Location§	Use
5' UTR to 2C	A.F	TAAACAGCCTGTGGGTTG	2–20	Amplification
	A.R	CATGCAGTTCAAGAGCAARCACCG	4,409–4,432	Amplification
	A.F.1	GACGTCCGGCCCCTGAATGCGGCTAATCC	447–475	Sequencing
	A.R.1	GCGGAACCGACTACTTTGGGTGTCCGGAATTC	536–567	Sequencing
	A.F.2	TGGCTATGGTGAGTGGCC	1,049–1,066	Sequencing
	A.R.2	GCTAGTGACGAGAGTATG	2,610–2,627	Sequencing
	A.F.3	GTCAGATCCCCAGCACAGG	2,987–3,006	Sequencing
	A.R.3	GTCAGATCCCCAGCACAGG	2,987–3,006	Sequencing
	A.F.4	GGATACCTCGCCCGATGCGC	3,220–3,239	Sequencing
	A.R.4	GCYCAAGGHTGYGACACGATWGCTC	3,468–3,492	Sequencing
A.F.5	TCATTGATTGGCTCAAGGAG	4,159–4,178	Sequencing	
2C to 3' UTR	B.F	CATGCAGTTCAAGAGCAARCACCG	4,409–4,432	Amplification
	B.R	GTGGGGGTAATTTGTTATAACCAGAATAGC	7,378–7,408	Amplification
	B.F.1	GTGTGTGGCAAGGCCATTAG	4,929–4,949	Sequencing
	B.R.1	GTGTGTGGCAAGGCCATTAG	4,929–4,949	Sequencing
	B.R.2	GTGATCAACACAGAGCACATGCC	5,694–5,716	Sequencing
	B.F.2	CCRACCCGCACTAARCTTGARCCAG	5,994–6,019	Sequencing
	B.F.3	GGCCTTGACCTYCCYACTCYAC	6,381–6,403	Sequencing
	B.R.3	GGCCTTGACCTYCCYACTCYAC	6,381–6,403	Sequencing
	B.R.4	GGAGYAARYTACCRATYCTACTCCAGGATCGCTC	6,589–6,623	Sequencing
	B.F.4	CGGTGGACTAAGGACGCACGCAAC	7,143–7,166	Sequencing

*UTR, untranslated region.

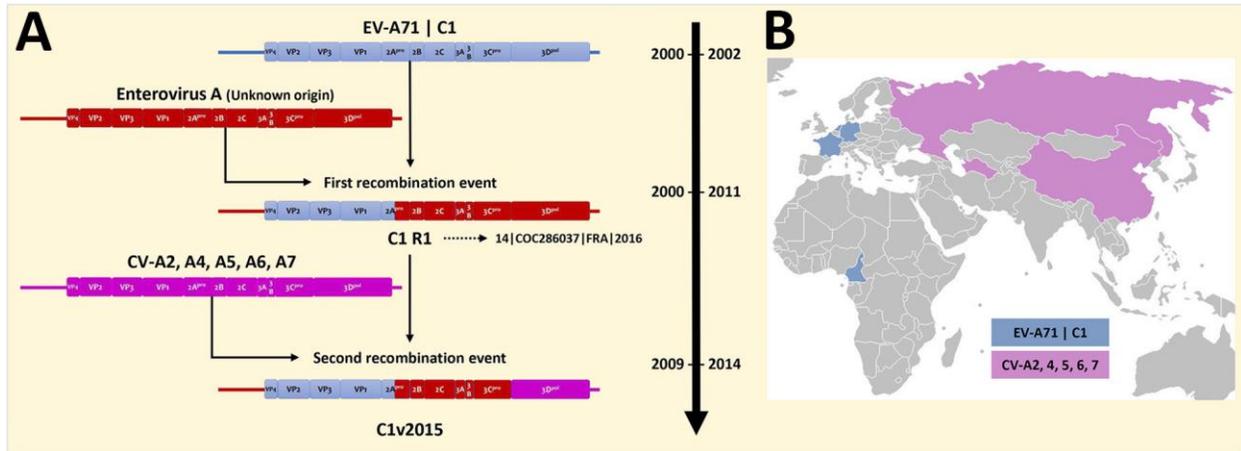
†A schematic of gene organization of the enterovirus genome is shown in Figure 2 (main text).

‡A.F and A.R: primers used for gene amplification of the 5' UTR-2C region. A.F.1–5, forward primers and A.R.1–4, reverse primers used for sequencing the 5' UTR-2C amplicon. B.F and B.R: primers used for amplification of the 2C-3' UTR region. B.F.1–4, forward primers and B.R.1–4, reverse primers used for sequencing the 2C-3' UTR amplicon.

§The location of primers indicates nucleotide positions in the genome of the enterovirus A71 BrCr prototype strain (GenBank accession no. U22521.1).



Appendix Figure 1. Nucleotide similarity plots determined for the EV-A71 C1v2015 genome 14|COC286037|FRA|2016 as query (patient 14, infant fever, stool specimen, collection year 2016). The nucleotide similarity was calculated by the sliding window method (window of 200 nucleotides moving every 20 nucleotides). The similarity plots determined for the other C1v2015 genomes were similar. The different enterovirus types and lineages selected for the analysis are color-coded. The mean genetic distance between the C1v2015 and C1 viruses, calculated with the P1 sequences, was <9% nucleotide differences. A schematic diagram of the enterovirus genome is shown at the bottom of the figure. Four genomic modules with different genetic origins were identified as indicated. The 99% confidence intervals (CIs) of the nucleotide boundaries assessed for the genomic modules (indicated in dark gray) were determined as described in Hassel et al. (1). The 3' boundary of module 1 and the 5' end of module 2 were located within a segment at the end of the 5' UTR but were not determined precisely. The 3' boundary of module 2 was located between alignment positions 3,532 and 3,722. The 5' boundary of module 4 was assessed at the end of 3Cpro gene (alignment positions 5,968–6,044). EV-A71, enterovirus A71; UTR, untranslated region.



Appendix Figure 2. Scenario of the possible origin and evolution of EV-A71 C1v2015. A) Sequential recombination events that drove emergence of C1v2015. B) Geographic distribution of current gene pools from which the C1v2015 genome was derived: capsid protein genes, countries colored in blue; 3Dpol gene, countries colored in purple. CV-A, coxsackievirus A; EV-A71, enterovirus A71; VP, viral protein.