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Sustained circulation of enterovirus D68 in Europe in 2023 and the continued evolution of enterovirus D68 B3-lineages associated with distinct amino acid substitutions in VP1 protein

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ABSTRACT

Background: Enterovirus D68 (EV-D68) causes respiratory disease ranging from mild to severe and in rare cases a paralytic syndrome, called acute flaccid myelitis (AFM). Since the global EV-D68 outbreak in 2014, the virus has mainly circulated in biennial epidemic cycles with peaks detected during even years. However, following the COVID-19 pandemic, the seasonal pattern of EV-D68 has been characterized by large yearly upsurges. Here, we describe the circulation of EV-D68 in Europe in 2023 and track its genetic evolution.

Study design: Data was compiled from members of the European Non-Polio Network (ENPEN). This included monthly data on the total number of EV samples tested, EV positive samples, EV-D68 positive samples and cases, and other EV positive samples detected in 2023. Information on sample types and surveillance system was recorded. Sequence data from the VP1 gene was used for phylogenetic and amino acid sequence analysis.

Results: EV was detected in 13,585 out of 203,622 diagnostic samples tested (6.7 %), of which 402 (3.0 %) were determined as EV-D68, representing 386 cases. EV-D68 infections peaked in October 2023 (136/386; 35.2 %). 267/386 (69.2 %) of EV-D68 cases were captured through clinical EV surveillance, almost all of which (202/204 of positive samples with sample type information) were detected in respiratory specimens. Phylogenetic analysis performed on 99 VP1 sequences revealed a distinct B3-derived lineage with a previously undescribed residue change, D554E, in Europe.

Conclusions: The study documents sustained circulation of EV-D68 in Europe in 2023, the evolution of B3-derived lineages, and appearance of previously undescribed amino acid substitutions in Europe. This stresses the need for continuous EV-D68 surveillance and harmonization of EV-D68 detection practices towards better data comparability across countries.

1. Background

Enterovirus D68 (EV-D68) is a non-enveloped, positive-sense single-stranded RNA virus which belongs to the species *Enterovirus deconjuncti* within the *Picornaviridae* family [1,2]. EV-D68 is one of the four known human *E. deconjuncti* types, with others being EV-D70, EV-D94, and EV-D111 [2]. The fifth genotype, EV-D120, has only been identified in the stool samples of non-human primates [3].

Analysis of the evolutionary history and emergence of new strains of EV-D68 is primarily based on phylogeny of the 1D gene that encodes one

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of the four capsid proteins, viral protein 1 (VP1) [4]. VP1 is the principal determinant of virus antigenicity, receptor binding and, as recently described, neuropathogenesis [5–7]. It differentiates EV-D68 into four primary genotypes: A, B, C, and D (also termed A2) [5,6]. Genotypes A, B and D are further divided into subgenotypes A1, A2, B1-B3, D1 (or A2/D1), and D2 (or A2/D2). Since 2017, B3 and A2/D2 are the only two genotypes that remain circulating in Europe, Asia, and the United States [8,9].

EV-D68 primarily causes acute respiratory disease in humans which can range from mild to severe. EV-D68 preferentially infects the epithelium in the upper respiratory tract and spreads through respiratory secretions [10]. Unlike other EVs, EV-D68 can be acid labile, thus it is rarely detected in stool samples [11]. Clinical manifestations are similar to a common cold. Children under the age of five are at the highest risk of developing severe respiratory disease, with a significant proportion needing intensive care. Severe cases have also been reported in adults, particularly in elderly, immunosuppressed individuals or those with underlying chronic disease [9,12]. In rare cases, EV-D68 infection can also lead to neurological complications, including acute flaccid myelitis (AFM), a polio-like condition [13]. The neuropathogenic features of the virus have been associated with specific amino acid residues. Notably, Leser and colleagues recently identified four neurovirulence-associated amino acid residue changes within the VP1 gene, namely I553L, D554N, A650T and K835E, between a paralysis-causing EV-D68 isolate (US/IL/14-18952) and a non-paralytic EV-D68 isolate (US/CA/14-4231) [7]. These findings allow the prediction of likely clinical outcomes of future EV-D68 epidemics and suggest potential prevention and treatment strategies for EV-D68 infections.

The past decade has witnessed the global re-emergence, accelerated spread and sustained circulation of EV-D68. Between its initial identification in California in 1962, up until 2008, the virus had only caused sporadic cases and minor outbreaks. In Europe, EV-D68 was first reported between 2008 and 2010 [14,15]. Since the global EV-D68 outbreak in 2014, the virus has mainly circulated in biennial epidemic cycles [16]. The link between EV-D68 circulation and incidence of AFM was first described at that time [17,18]. The seasonality of the virus occurs typically between August and October [9]. Despite the biennial pattern with circulation in even years, in 2019, 93 cases were reported across five European countries. Notably, five of these had severe neurologic manifestations [8]. No EV-D68 cases were reported in 2020, likely due to COVID-19 control measures and limited testing beyond SARS-CoV-2 [19–21]. Following the easing of these interventions in the fall of 2021, a major EV-D68 outbreak across Europe was detected, alongside the emergence of two novel B3-derived lineages [19,20]. Since then, the seasonal pattern of EV-D68 has continued with yearly upsurges, with cases reported in 2022 [20]. Here, we report the upsurges of EV-D68 cases in Europe in 2023 and track the ongoing evolution of B3-lineages.

2. Study design

2.1. Data collection and analysis

An invitation to participate in this study was sent by email to members of the ENPEN (https://escv.eu/european-non-polio-enterovirus-network-enpen/).

Data from 41 institutions in 19 European countries was compiled. This included monthly data on the total number of samples tested for EVs, the total number of EV positive samples, total number of EV-D68 positive samples and cases, and total number of other EV positive samples detected between January 1, and December 31, 2023. Along with epidemiological data, details on sample types tested and surveillance system were collected (Table S2). Three main surveillance systems were defined (A–D; Table S1) according to WHO surveillance systems for EVs [22,23]. For institutions using more than one surveillance system codes have been combined (AB, AC, and BC). Sequence data of EV-D68 cases for phylogenetic analysis included 300 bp VP1, complete VP1, and full-genome.

The data collected is summarized in Fig. 1.

2.2. Phylogenetic analysis

Phylogenetic analysis was carried out using MEGA7 [24]. The neighbour-joining tree for VP1 study sequences (n = 99, 14 institutions, 8 countries) was reconstructed using maximum composite likelihood, with pairwise deletion and 100 bootstrap iterations together with 3978 publicly available sequences extracted from GenBank (as available June 22nd, 2024). The analysis was conducted for complete VP1 study sequences covering nucleotides 2390 through 3322 and partial VP1 study sequences covering nucleotides 2519 through 2864 numbered relative to the VP1 genomic region of the Fermon strain (GenBank ID: AY426531). Evolutionary divergence was calculated with mean pairwise p-distances between sequence groups.

2.3. Amino acid sequence analysis

To identify specific amino acid substitutions contributing to the evolution of the B3–3 lineage, sequence analysis was performed on complete genome study sequences (n = 25; 6 institutions, 3 countries) from this study and 16 EV-D68 B3 genotype sequences extracted from GenBank. EV-D68 amino acid sequences were analysed relative to the coding sequence of the Fermon strain (GenBank ID: AY426531). A random subset of complete B3 genome sequences from the 2019, 2021, and 2022 upsurges were extracted from GenBank (n = 3, n = 6, n = 6, respectively; GenBank IDs: MH674155, MN935870, MN726800, OQ139570. OQ139571, OP267493, OP267494, OL829841, OL829844, OP389245, OP389246, OP321139– OP321142). The clinical isolate US/IL/14-18952 (GenBank ID: KM851230) from the 2014 EV-D68 epidemic, investigated in Leser et al. [7], was used to identify amino acid residue changes possibly associated with neuropathogenesis (i.e. I553L, D554N, A650T, and K835E). Amino acid composition scan was carried out using SSE [25]. All study sequences (n = 99, 14 institutions, 8 countries) and 1041 publicly available complete genome sequences extracted from GenBank on June 22nd, 2024, were assessed.

2.4. GenBank accession numbers

Sequences were deposited in GenBank under the following accession numbers:

OR571424, OR656579, PP548243–PP548246, PP947794, PP947795, PQ483175–PQ483183, PQ585546–PQ585569, PQ596446–PQ596503.

3. Results

3.1. Data collection

Data was available from 41 institutions from 19 European countries (Tables 1 and S2). Complete data was received from 23/41 institutions (13 countries). A total of 203,622 samples subjected to EV testing were reported from January 1 to December 31, 2023 (Table 2, Fig. 1). There were large differences between the number of samples among institutions, ranging from 267 (FI02) to 25,443 samples (UK05) (Table S2), yet all institutions showed similar seasonality for sample testing (data not shown). The monthly average of samples tested was relatively constant throughout the year for the whole dataset (average = 15,663 samples/month; standards deviation (SD) = 3608).

EV testing revealed a total of 13,585 EV positive samples (6.7 %; Table 1, Fig. 1) in 2023. Of the EV positive samples subjected to typing, 402 were EV-D68 positive (3.0 %; Table 1, Fig. 1) representing 386 cases. These were reported by 38/41 institutions (92.6 %) from 16 countries including Austria, Belgium, Germany, Denmark, Spain,

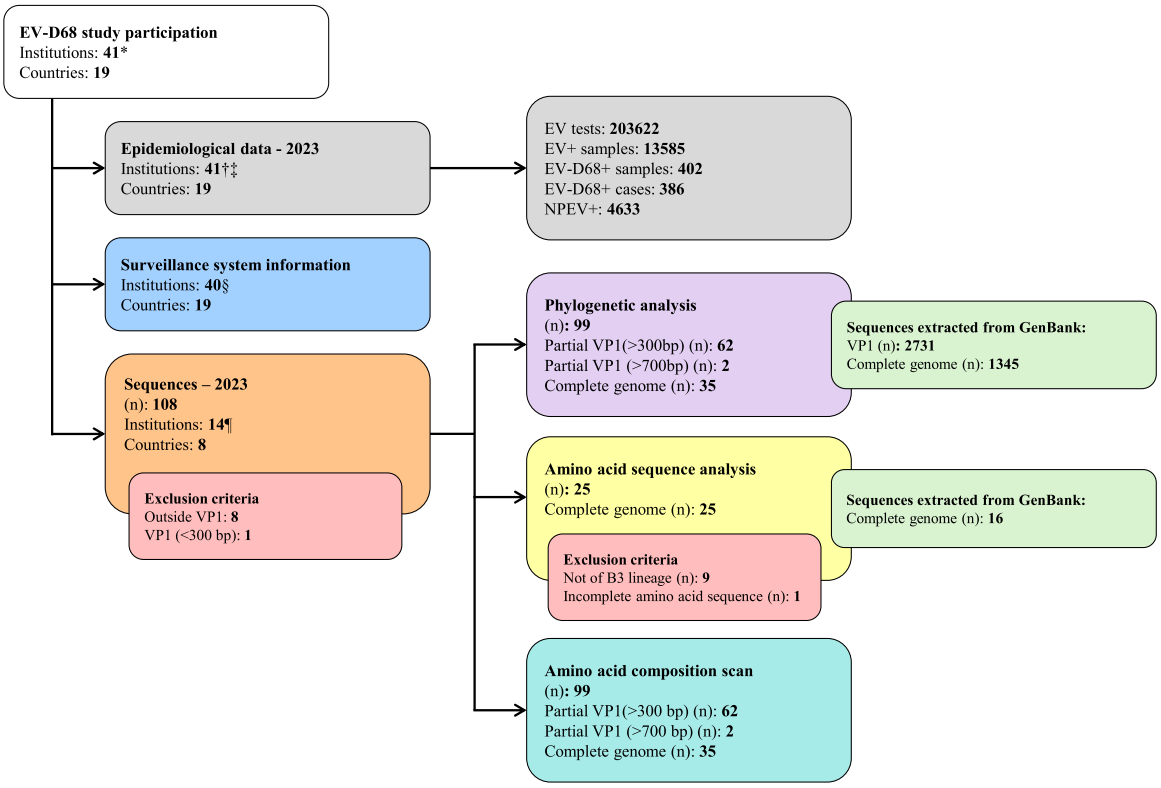


Fig. 1. Diagram of the information collected. Epidemiological data (grey boxes), surveillance system information (blue box), and sequence data (orange box) with associated metadata used for the analysis of the EV-D68 upsurge in Europe, in 2023. Sequence information is represented separately for phylogenetic reconstruction (purple box), the amino acid analysis (yellow box), and composition scan (teal box). Publicly accessible sequences are reported in green boxes while exclusion criteria in red boxes. * 9 institutions, 6 countries, included from ENPEN neonatal 2018–2023 study [30]. † Complete data for all variables, until December 2023 was received from 23 institutions; 13 countries; monthly testing data from 37 institutions; 19 countries; monthly EV+ data from 40 institutions; 19 countries; monthly EV-D68+ sample data from 38 institutions; 19 countries; monthly EV-D68+ case data from 37 institutions; 19 countries; monthly NPEV+ data from 36 institutions; 17 countries. ‡ Neonatal data included for 6 institutions; 5 countries. § Surveillance system information included from EV-D68 2021 study for 22 institutions; 15 countries. ¶ Data from NL02 used only for sequence analysis as background of labs that are not in the study.

Table 1
Epidemiological data of EV-D68 reported by 19 European countries between January 1, and December 31, 2023. The number of total samples tested, EV positive samples, EV-D68 samples and cases, and other EV-D68 samples. Data by Austria was reported quarterly in the first month of each quarter (January, April, July, October), and no data on the total number of tests was reported from Denmark. EV, enterovirus; NPEV, non-polio enterovirus; NA, not available.

			Tested (n)	EV+ (n)	EV+ (%)	Typing: EV-D68+ (n)		Typing: other NPEV+ (n)	
Country	Country code	Institutions (n)	Samples	Samples	Samples	Samples	Cases	Samples	Sequences (n)
Austria	AT	1	879	134	15.2 %	3	3	131	NA
Belgium	BE	2	17,533	1481	8.4 %	96	96	222	NA
Bulgaria	BG	1	349	13	3.7 %	0	0	11	NA
Czech Republic	CZ	1	324	192	59.3 %	0	0	62	NA
Germany	DE	4	13,396	642	4.8	11	11	417	NA
Denmark	DK	1	NA	1400	NA	15	14	360	7
Spain	ES	4	27,453	1020	3.7 %	88	87	698	18
Finland	FI	3	3920	135	3.4 %	3	3	5[a]	1
France	FR	1	23,012	558	2.4 %	17	16	335	13
Croatia	HR	1	12,511	1066	8.5 %	9	9	1057	NA
Iceland	IS	1	6824	116	1.7 %	5	4	111	NA
Italy	IT	2	5581	740	13.3 %	18	18	188	9
Luxembourg	LU	1	688	96	14.0 %	0	0	0	NA
Netherlands	NL	8	19,191	1075	5.6 %	66	56	434[a]	57
Norway	NO	2	9326	203	2.2 %	10	10	13	NA
Portugal	PT	1	1034	68	6.6 %	1	1	50	NA
Sweden	SE	1	15,138	2097	13.9 %	3	2	0	2
Slovenia	SI	3	10,121	332	3.3 %	13	13	68[a]	NA
United Kingdom[b]	UK	3	36,342	2217	6.1 %	44	43	471[a]	1
Total		41	203,622	13,585	6.7 %	402	386	4633	108

[a] Includes data from ENPEN neonatal study 2018–2023 [30].
[b] England, Northern Ireland, and Scotland.

Table 2
Epidemiological data of EV-D68 reported by month of detection between January 1, and December 31, 2023, in Europe. The number of total samples tested, EV positive samples, EV-D68 samples and cases, and other EV-D68 samples. EV, enterovirus; NPEV, non-polio enterovirus.

	Tested (n)	EV+ detection (n)	EV+ detection (%)	Typing: EV-D68+ (n)		Typing: other NPEV+ (n)
Month	Samples	Samples	Samples	Samples	Cases	Samples
January	18,388	583	3.2 %	14	14	205
February	16,462	658	4.0 %	3	3	262
March	17,794	785	4.4 %	7	7	247
April	14,553	585	4.0 %	0	0	198
May	14,018	891	6.4 %	0	0	309
June	13,948	1396	10.0 %	0	0	570
July	13,303	1228	9.2 %	1	1	600
August	13,390	1020	7.6 %	19	18	346
September	15,124	1450	9.6 %	90	88	348
October	17,765	2113	11.9 %	144	136	755
November	19,462	1566	8.0 %	94	90	485
December	21,953	1127	5.1 %	30	29	306
Unknown	7462	183	2.5 %	0	0	2
Average	15,663					
Standard deviation (SD)	3608					
Total	203,622	13,585	6.7 %	402	386	4633

Finland, France, Croatia, Iceland, Italy, the Netherlands, Norway, Portugal, Sweden, Slovenia, and the United Kingdom (England, Northern Ireland and Scotland) (Table 1, Fig. 2A, Table S2). No EV-D68 positive cases were detected in Bulgaria, Czech Republic, and Luxembourg; noting the numbers of samples subjected to EV testing in these countries were amongst the smallest (Table 1, Fig. 2A). The highest number of cases were reported by Belgium (n = 96) followed by Spain (n = 87), the Netherlands (n = 56), and the United Kingdom (n = 43) (Table 1, Fig. 2A).

Information on the surveillance systems in use was available for 40/41 institutions (97.6 %, 19 countries; Fig. 1, Table S2). The majority of institutions performed EV clinical surveillance (n = 30; Fig. 2C), three together with AFP surveillance, and three in combination with respiratory surveillance. AFP surveillance alone was carried out by one institution (BG01) while respiratory surveillance alone by eight institutions.

3.2. Epidemiology

In 2023, 366 EV-D68 cases were reported during the period of July to December (Fig. 2; Table 2). Seasonal incidence was bell-shaped, starting with sporadic cases in July (n = 1, Iceland) and August (n = 18). An exponential upsurge was seen in September, peaking in October (138/391; 35.2 %; Table 2, Fig. 2B). Case counts in November resembled September and the number of cases fell close to zero, with only sporadic appearances in December (n = 28). During this time, the number of samples tested for EV also increased slightly (Fig. 2B). Cases from January to March 2023 (n = 15, 3, 7, respectively) were a spillover from the previous EV-D68 season in 2022. The seasonal pattern was consistent for all institutes and countries (not shown). The majority of EV-D68 positives were reported through clinical EV surveillance (267/386, 69.2 %, 23 institutions; Fig. 2C). An additional 48 (12.4 %) cases may have also been identified under clinical EV surveillance, however since the labs in question also perform AFP and/or respiratory surveillance, the origin of cases could not be confirmed. 54 (14.0 %) EV-D68 case detections were made by respiratory surveillance, while 15 (3.9 %) either by AFP or respiratory surveillance, again due to the inability to discriminate their source. A higher detection count through a determinate surveillance system was not proportional to a higher number of institutions reporting through that surveillance system.

3.3. Sample types tested

Sample type information was available for 40/41 institutions (97.6 %; Table S2). Most institutions tested multiple sample types (31/41 institutions, 75.6 %, Table S2). Of these, 16 institutions (51.6 %) reported that they tested various samples without further specifications (Fig. 3). However, in this case, the proportion of each sample tested could not be determined. Only eight institutions exclusively analysed respiratory samples (Table S2).

Overall, the most common sample type tested was respiratory (23/41 institutions, 56.1 %, Fig. 3) followed by cerebrospinal fluid (CSF) and stool, respectively (16/41 and 13/41 institutions, 39.0 % and 31.7 %, respectively; Fig. 3). Only five institutions described blood samples, five institutions vesicle/skin samples, one institution a urine sample (ES05), and two autopsy samples (NO02B and FI02) (Fig. 3). The number of samples tested for each sample type was not specified.

Of the 402 EV-D68 positive samples, 204 had available sample type data. All except two of 204 EV-D68 positive samples were reported in respiratory specimen (202/204, 99.0 %; Fig. 3). The remaining two were from stool samples (Fig. 3).

3.4. Clinical characteristics of EV-D68 cases

Of 402 EV-D68 positive samples, clinical case descriptions were provided for 37 cases by 7 institutions (6 countries). For 34 (91.9 %) cases, the age was known: 19 children younger than 5 years, six children aged 5–12, one young adult (19-year-old), and eight adults (42–65 years) (Table S3).

Respiratory symptoms were noted in 27/37 (73.0 %) cases; respiratory infection (n = 17), pneumonia (n = 5), bronchopneumonia (n = 1), bronchitis (n = 1), bronchospasm (n = 2), wheeze (n = 2), asthma (n = 2), acute respiratory insufficiency (n = 1), and acute/severe respiratory infection (n = 2). Three cases presented fever and two cases manifested exanthema. Admission to the ICU and/or need of oxygen support was required in four cases. The sex was noted in 31/37 (83.8 %) of cases; 18 were male and 13 were female (Table S3). None of the cases reported neurological symptoms or AFM.

3.5. Phylogenetic analysis

Of 108 sequences, 99 were submitted for analysis (14 institutions, 8 countries; Fig. 1, Table S2). Nine sequences were excluded, eight being outside the VP1 region and one being shorter than 300 bp (Fig. 1). Phylogenetic analysis of the VP1 gene demonstrated that the majority of strains fell into the B3 subgenotype clade (69/99, 69.7 %) but forming a genetically distinct lineage within it, designated lineage 3 (B3–3). The divergence between previous lineages (lineage 1 and 2) and lineage 3 was over 7 % (SD = 0.009) based on the VP1 region. The remaining EV-D68 variants were of the A2/D2 genotype (30/99, 30.3 %). Both the B3 and A2/D2 strains were mainly detected during the peak season from

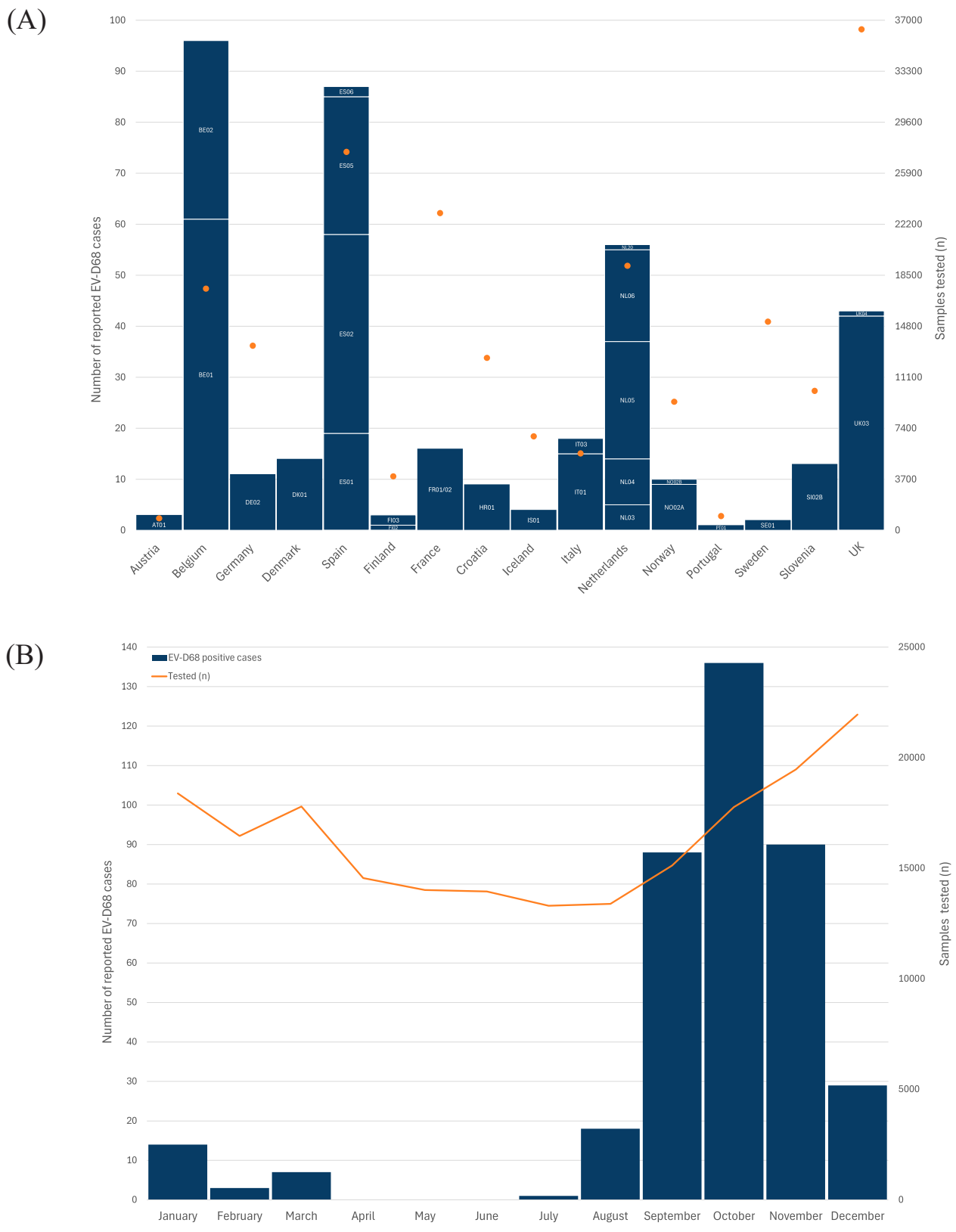
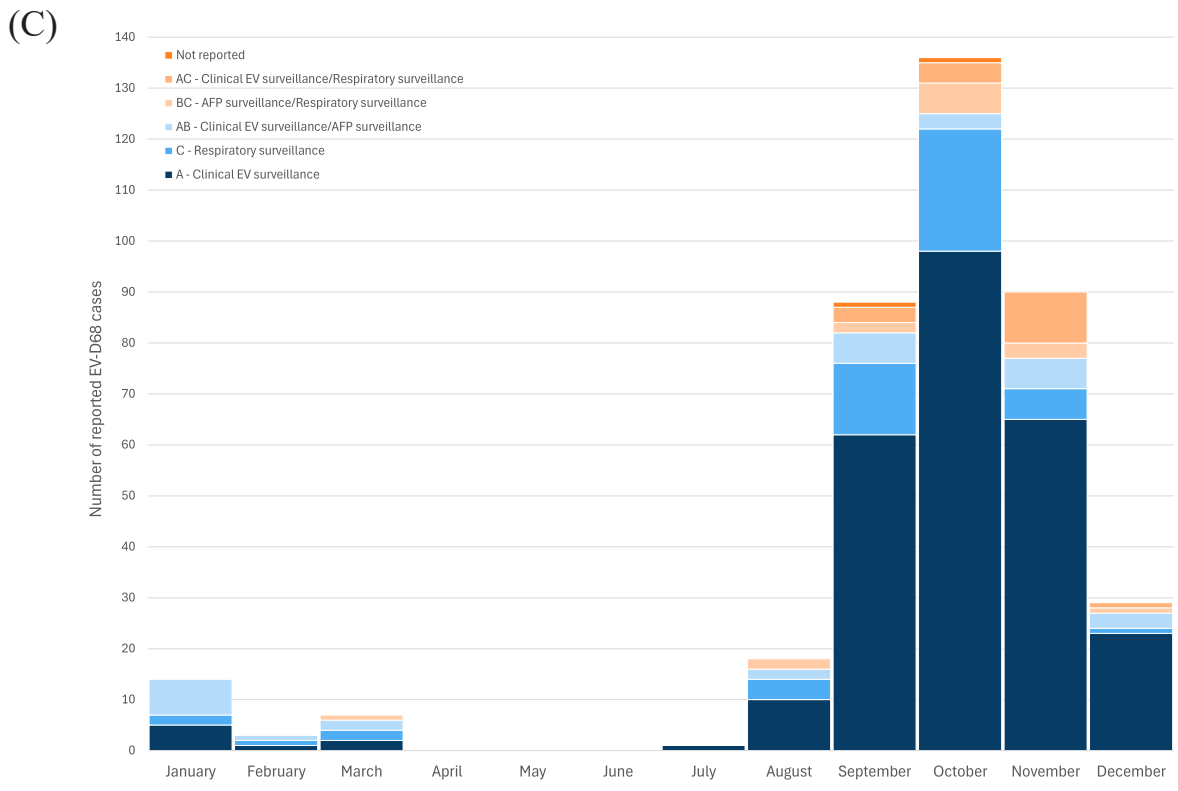


Fig. 2. Epidemiology of EV-D68 between January 1, and December 31, 2023, in Europe. (A) Number of cases positive for EV-D68 (blue bars) and EV samples tested (orange dots) reported by 38 institutions from 16 European countries. None detected in Bulgaria, Czech Republic, and Luxembourg. Denmark did not report on the total samples tested. UK = United Kingdom (B) Monthly reporting of the number of cases positive for EV-D68 (blue bars) and total number of samples tested (orange line). No cases detected in April, May, and June. (C) Number of cases positive for EV-D68 reported by surveillance system and month; A = Clinical enterovirus surveillance, B = Acute flaccid paralysis surveillance, C = Respiratory surveillance (includes ILI/ARI surveillance). (D) Temporal distribution of the number of EV-D68 sequences returned by genotype and month.



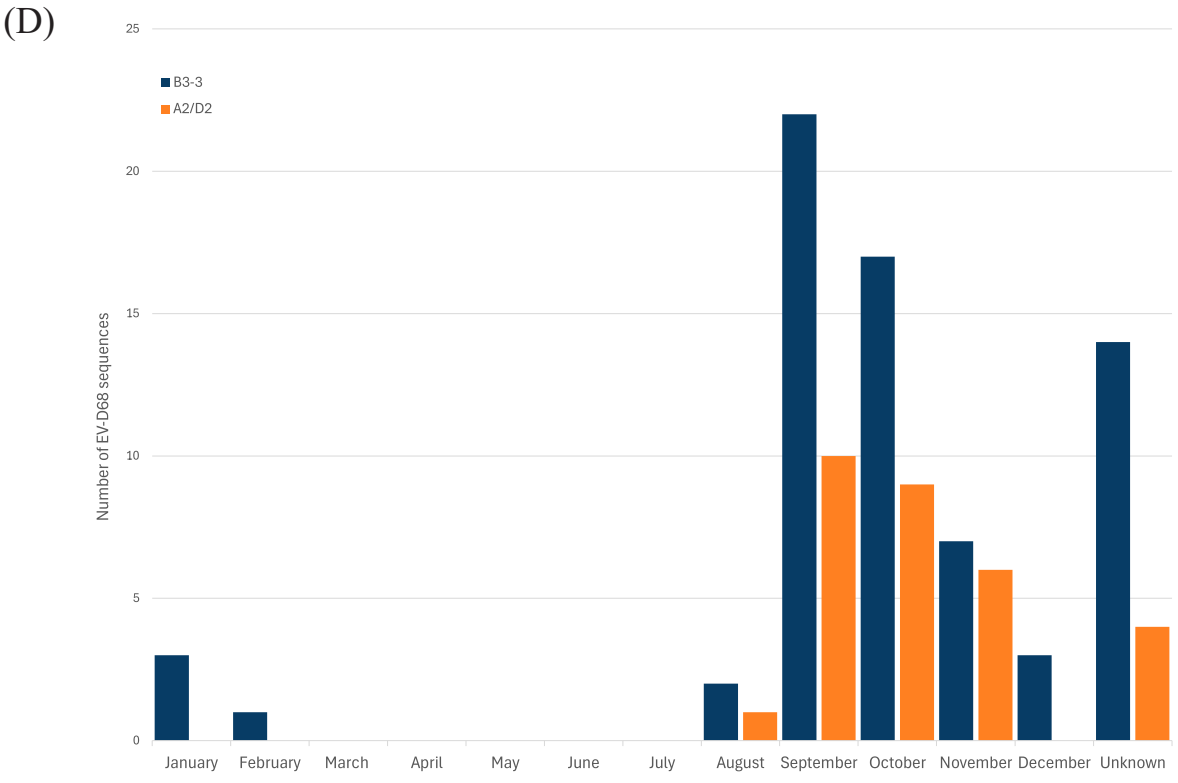


Fig. 2. (*continued*).

August to November (Fig. 2D).

The phylogenetic tree showed the continued evolution of a distinct B3–3 lineage (Fig. 4A). The analysis of the VP1 genomic region revealed that the European EV-D68 from 2023 formed a new cluster together with the US 2022 sequences within the B3 genotype. Other European EV-D68 sequences from 2023 clustered in A2/D2 group in two existing sub-lineages, one dominated by the presence of European 2021 sequences, the other by European and US sequences from 2018 to 2021 (Fig. 4A).

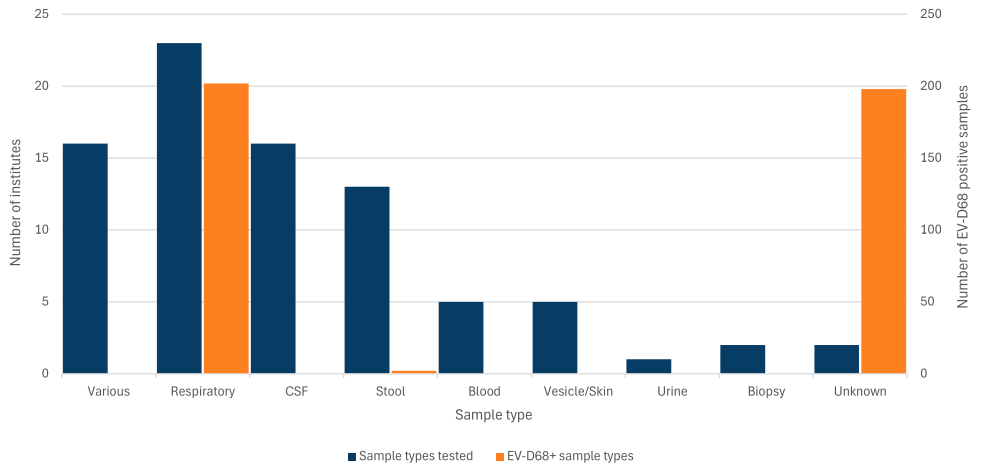


Fig. 3. Sample types tested in the period between January 1, and December 31, 2023, in Europe.
Number of institutions (blue bars) and number of EV-D68 positive cases (orange bars) returned by sample type tested. EV-D68 was only detected in respiratory and two stool samples.

3.6. Amino acid sequence analysis

An analysis of possible amino acid signature changes of B3–3 lineage was carried out for 25 complete genome study sequences of the B3 genotype, all of the B3–3 lineage (6 institutions; Fig. 1), and 16 sequences included from GenBank. These sequences showed 98 % similarity in their amino acid signatures. This was true also for the US 2022 sequences, used as reference, present within that cluster (Fig. 4B).

The examination of the EV-D68 coding sequence (VP1–4) revealed 25 characteristic substitutions, of which seven were specific to the B3–3 lineage, with respect to the sample of B3 sequences from 2019 to 2022 included in the analysis. Five of seven substitutions were found in more than 90 % of sequences: one in VP3 (V364I), VP1 (D554E), and 3A (I1445V) and two in 3D (I1931T, I1943M/V) (Fig. 4B). The two remaining substitutions were present in more than 45 % of sequences, one each in VP2 (I177V) and 3A (D1457E) (Fig. 4B). The five amino acid residue changes were present also in the US 2022 sequences, part of the emerging B3 lineage 3 cluster (B3–3). Lineage 3 sequences encoded a previously undescribed substitution, D554E, in VP1, not found in previous European B3 lineages (Fig. 4B and C).

Leser and colleagues identified four amino acid substitutions possibly related to neuropathogenesis of EV-D68 in mouse models i.e. I553L, D554N, A650T, and K835E (Fig. 4C) [7]. Three of these four substitutions were identified in the B3–3 sequences in this study (I553L, A650T, K835E; Fig. 4B and C). I553L was found only in one study sequence, A650 in all, while K835E in all except two (Fig. 4B). Amino acid at site 553 has maintained a non-neurovirulent signature, while sites 650 and 835 tend towards neurovirulent signatures (Fig. 4C). The D554N substitution has evolved into non-virulent D554E that was present in all B3–3 sequences of this study (Fig. 4C). Previous lineages present the non-neurovirulent amino acid, aspartic acid (D).

4. Discussion

This study documents the sustained circulation of EV-D68 in Europe in 2023 and the continued evolution of B3-derived lineages identified in previous years [19,20]. Nearly 400 EV-D68 cases were recorded by 38 institutions across 16 European countries predominantly during the fall of 2023.

In 2023, four countries, Spain, Belgium, the Netherlands, and the United Kingdom, reported over 70 % of EV-D68 cases. For all other countries documentation of EV-D68 circulation was substantially lower, ranging from one (Portugal) to 18 (Italy) cases. EV-D68 detections rates varied widely between and within countries. Large differences were also seen between the number of samples tested across institutions. Higher sample counts did not always mean more positive detections, suggesting variability in the effectiveness of diagnostic screening primarily related to the choice of sample type rather than differences in the intensity of transmission. The study shows that the primary specimen for detection of EV-D68 should be respiratory and the inclusion of testing data for stool and CSF created a heterogenous denominator. Data was biased also due to non-standardized testing and differing institute capacities.

Although clinical data was not collected as part of this study, descriptions of EV-D68 cases align with previous studies [8,19,20,26]. Severe respiratory symptoms included wheeze, acute/severe respiratory insufficiency, pneumonia, asthma, and bronchospasm, requiring hospitalizations and/or the need of oxygen support. Case data demonstrated the usual male predominance of EV-D68 infections, young age (<5 years of age), and presence of underlying medical conditions which have been seen to play a role in the severity of the disease outcome. None of the institutions described neurological disease or paralysis associated with EV-D68 infections, but this may have gone unreported (Helfferich J, unpublished).

In Europe, circulation of EV-D68 was monitored through testing of various sample types obtained via clinical EV or other surveillance systems. Over 75 % of institutions analysed multiple sample types. By and large, the most common sample type was respiratory (56.1 %) followed by CSF (39.0 %) and stool (31.7 %). Respiratory samples were analysed almost exclusively by clinical EV surveillance and ILI/ARI surveillance, while stool samples were related to clinical EV surveillance and AFP surveillance. Furthermore, almost 70 % of EV-D68 positive detections were reported through clinical EV surveillance, with potentially an additional 12 % from institutions combining also AFP and/or respiratory surveillance. 14 % of detections were exclusively through respiratory surveillance. The temporal trends of these two surveillance systems align to those seen for 2023, indicating these might be the most effective in catching EV-D68. Their focus is also on routine surveillance of mostly respiratory samples, and not solely on case-based syndromic surveillance as with AFP surveillance [27]. While participating institutions showed some commonalities in surveillance and detection strategies for EV-D68 infections, there is still a need for a systematic approach. The large proportion of unknown EV-D68 positive sample types and the aggregated reporting through multiple surveillance systems skews the accuracy of conclusions. The establishment of a standardized EV-D68 surveillance system that combines both routine and case-based surveillance would aid in better registration of cases [20,22,27].

Based on phylogenetic analysis, studied EV-D68 strains belonged to

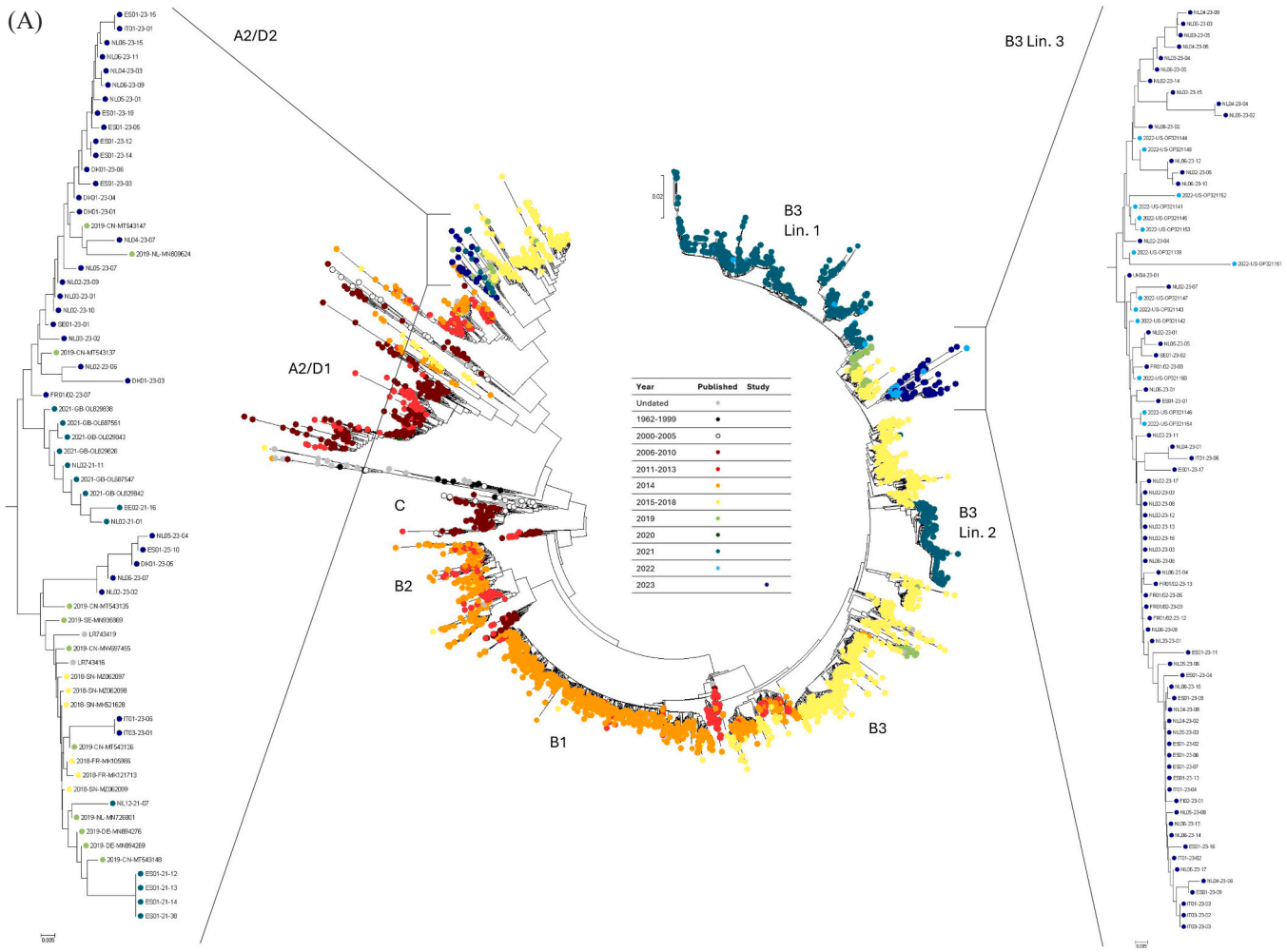
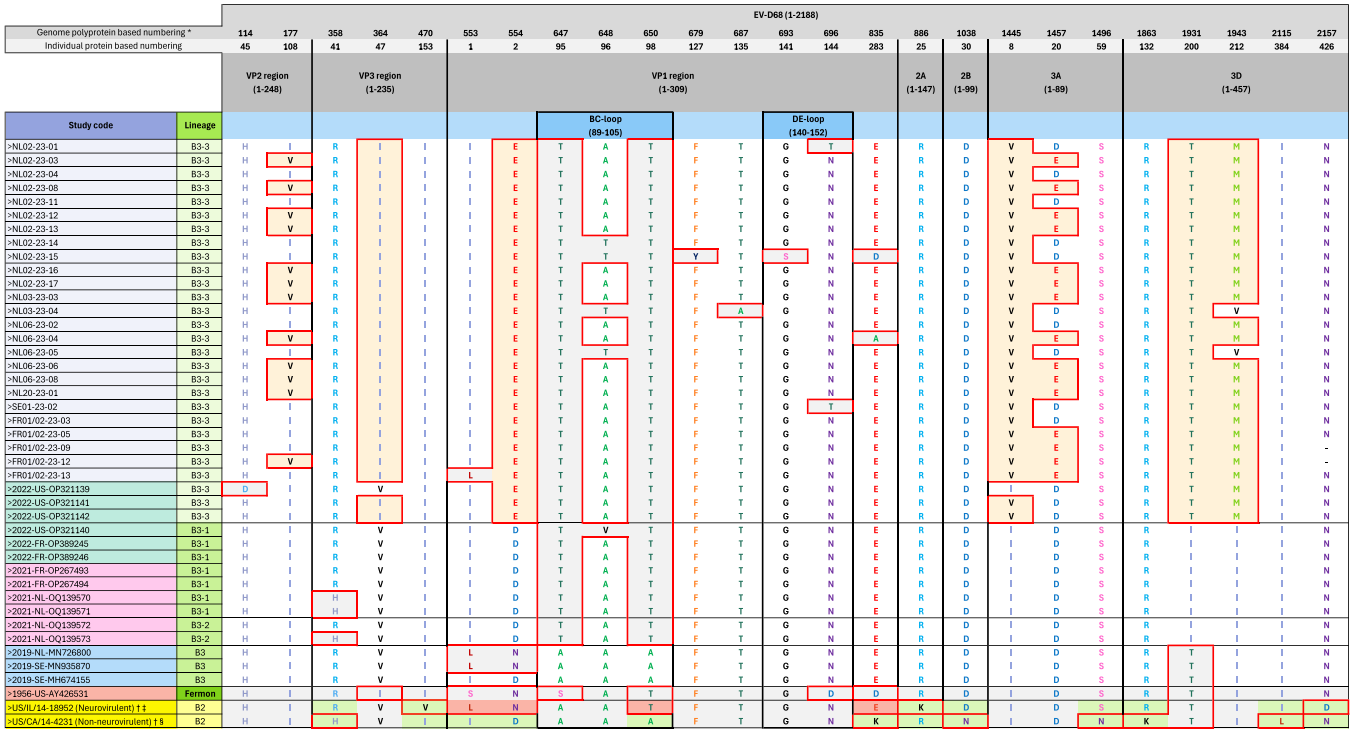


Fig. 4. Phylogenetic and amino acid analysis for sequences from January 1, and December 31, 2023, in Europe. (A) Neighbour-joining phylogenetic tree of the complete and partial VP1 region of EV-D68, reconstructed using maximum composite likelihood, with pairwise deletion and 100 bootstrap iterations, nucleotides 2390 through 3322, relative to the VP1 genomic region of the Fermon strain (GenBank ID: KU844178). 99 study sequences and 3978 publicly available sequences were included. Blow-ups of the A2/D2 cluster (left) and B3-derived lineage (lineage 3; right) are shown. Labels are indicated in the table. (B) Amino acid sequence analysis covering the coding region of EV-D68, including BC and DE immunogenic loops of the VP1 protein (black box), in 2023 (n = 25) in Europe, and in 2019–2022 (n = 15) in Europe and US, all relative to the 1956 Fermon strain (GenBank ID: AY426531). Numbering of amino acid residues is relative to the start of reading frame of the Fermon strain b (genome polyprotein- and individual protein-based numbering). All sequences are part of the B3 genotype, subdivided by lineage (B3–1 to B3–3; green column). Amino acid substitutions are indicated in red boxes. Mutations characteristic to the B3–3 lineage are marked by orange cells. Residue changes in the BC and DE immunogenic loops are outlined in black. No differences were seen in the VP4 region. The clinical isolates US/IL/14-18952 and US/CA/14-4231 (yellow rows) investigated in Leser et al. [7] were used to look into amino acid residue changes associated with neuropathogenesis. Mutations linked to neuropathogenesis are reported by red cells, those not associated by green cells. Incomplete sequences are marked with dashes (-). (C) Composition scan output of the four neurovirulence-associated amino acid residue changes (I553L, D554N, A650T, and K835E) identified by Leser and colleagues [7] (†) for 99 study sequences (EVD68-2023) and 1041 publicly available complete genome sequences (previous) returned by genotype. Numbering of amino acid residues is relative to the start of reading frame of the Fermon strain (genome polyprotein- and individual protein-based numbering). Mutations linked to neuropathogenesis in Leser et al. [7] are marked by red cells, those not associated by green cells. NV=neurovirulent. * Genome polyprotein-based numbering of the EV-D68 virus. † Leser et al. [7]. ‡ Paralysis-causing EV-D68 isolate. § Non-paralytic EV-D68 isolate.

two genotypes, B3 (69.7 %) and A2/D2 (30.3 %). Evolution of the A2/D2 genotype has continued as reported in 2021 in Europe [20]. Meanwhile, a distinct B3-derived lineage (lineage 3) was observed within the B3 genotype, previously undescribed in Europe. The sequences clustered together exclusively with US 2022 B3–3 sequences. Furthermore, most closely related ancestors to this distinct lineage fell within the US cluster circulating in 2018. Hodcroft and colleagues found that diversification of variants begins up to 18 months prior to the first diagnostic detection during an EV-D68 season, explaining the diverse pattern of the European 2023 B3–3 lineage [28]. It may have started to diversify as early as 2022 in the US but were not reported from Europe until now. Alternatively, the pattern could suggest a very low circulation of strains of this cluster, or a lack of sustained global and European-level surveillance of EV-D68 in 2022, with few sequences submitted to GenBank. The two previously described novel post-pandemic B3-derived lineages (lineage 1 and 2) were not detected in 2023 and may no longer be circulating, or circulating only at very low levels [8,20]. All this points to the continued evolution and spread of EV-D68, and necessitates comprehensive surveillance of EV-D68 in order to track permanence of novel lineages and emergence of other novel variants in the near future with potentially increased transmissibility or enhanced pathogenicity.

Seven characteristic substitutions distinguished the B3–3 lineage from the B3–1 and B3–2 lineages circulating in the previous years. The same residue changes were present in the US 2022 sequences indicating

(B)



Genome polyprotein based numbering *		EV-D68 (1-2188)																								
Individual protein based numbering		114	177	358	364	470	553	554	647	648	650	679	687	693	696	835	886	1038	1445	1457	1496	1863	1931	1943	2115	2157
		45	108	41	47	153	1	2	95	96	98	127	135	141	144	283	25	30	8	20	59	132	200	212	384	426
		VP2 region (1-248)		VP3 region (1-235)			VP1 region (1-309)										2A (1-147)	2B (1-99)	3A (1-89)			3D (1-457)				
Study code	Lineage								BC-loop (89-105)					DE-loop (140-152)												
>NL02-23-01	B3-3	H	I	R	I	I	I	E	T	A	T	F	T	G	T	E	R	D	V	D	S	R	T	M	I	N
>NL02-23-03	B3-3	H	V	R	I	I	I	E	T	A	T	F	T	G	N	E	R	D	V	E	S	R	T	M	I	N
>NL02-23-04	B3-3	H	I	R	I	I	I	E	T	A	T	F	T	G	N	E	R	D	V	D	S	R	T	M	I	N
>NL02-23-08	B3-3	H	V	R	I	I	I	E	T	A	T	F	T	G	N	E	R	D	V	E	S	R	T	M	I	N
>NL02-23-11	B3-3	H	I	R	I	I	I	E	T	A	T	F	T	G	N	E	R	D	V	D	S	R	T	M	I	N
>NL02-23-12	B3-3	H	V	R	I	I	I	E	T	A	T	F	T	G	N	E	R	D	V	E	S	R	T	M	I	N
>NL02-23-13	B3-3	H	V	R	I	I	I	E	T	A	T	F	T	G	N	E	R	D	V	E	S	R	T	M	I	N
>NL02-23-14	B3-3	H	I	R	I	I	I	E	T	T	T	F	T	G	N	E	R	D	V	D	S	R	T	M	I	N
>NL02-23-15	B3-3	H	I	R	I	I	I	E	T	T	T	Y	T	S	N	D	R	D	V	D	S	R	T	M	I	N
>NL02-23-16	B3-3	H	V	R	I	I	I	E	T	A	T	F	T	G	N	E	R	D	V	E	S	R	T	M	I	N
>NL02-23-17	B3-3	H	V	R	I	I	I	E	T	A	T	F	T	G	N	E	R	D	V	E	S	R	T	M	I	N
>NL03-23-03	B3-3	H	V	R	I	I	I	E	T	A	T	F	T	G	N	E	R	D	V	E	S	R	T	M	I	N
>NL03-23-04	B3-3	H	I	R	I	I	I	E	T	T	T	F	A	G	N	E	R	D	V	D	S	R	T	V	I	N
>NL06-23-02	B3-3	H	I	R	I	I	I	E	T	A	T	F	T	G	N	E	R	D	V	D	S	R	T	M	I	N
>NL06-23-04	B3-3	H	V	R	I	I	I	E	T	A	T	F	T	G	N	A	R	D	V	E	S	R	T	M	I	N
>NL06-23-05	B3-3	H	I	R	I	I	I	E	T	T	T	F	T	G	N	E	R	D	V	D	S	R	T	V	I	N
>NL06-23-06	B3-3	H	V	R	I	I	I	E	T	A	T	F	T	G	N	E	R	D	V	E	S	R	T	M	I	N
>NL06-23-08	B3-3	H	V	R	I	I	I	E	T	A	T	F	T	G	N	E	R	D	V	E	S	R	T	M	I	N
>NL20-23-01	B3-3	H	V	R	I	I	I	E	T	A	T	F	T	G	N	E	R	D	V	E	S	R	T	M	I	N
>SE01-23-02	B3-3	H	I	R	I	I	I	E	T	A	T	F	T	G	T	E	R	D	V	D	S	R	T	M	I	N
>FR01/02-23-03	B3-3	H	I	R	I	I	I	E	T	A	T	F	T	G	N	E	R	D	V	D	S	R	T	M	I	N
>FR01/02-23-05	B3-3	H	I	R	I	I	I	E	T	A	T	F	T	G	N	E	R	D	V	E	S	R	T	M	I	N
>FR01/02-23-09	B3-3	H	I	R	I	I	I	E	T	A	T	F	T	G	N	E	R	D	V	E	S	R	T	M	I	-
>FR01/02-23-12	B3-3	H	V	R	I	I	I	E	T	A	T	F	T	G	N	E	R	D	V	E	S	R	T	M	I	-
>FR01/02-23-13	B3-3	H	I	R	I	I	L	E	T	A	T	F	T	G	N	E	R	D	V	E	S	R	T	M	I	N
>2022-US-OP321139	B3-3	D	I	R	V	I	I	E	T	A	T	F	T	G	N	E	R	D	I	D	S	R	T	M	I	N
>2022-US-OP321141	B3-3	H	I	R	I	I	I	E	T	A	T	F	T	G	N	E	R	D	V	D	S	R	T	M	I	N
>2022-US-OP321142	B3-3	H	I	R	I	I	I	E	T	A	T	F	T	G	N	E	R	D	V	D	S	R	T	M	I	N
>2022-US-OP321140	B3-1	H	I	R	V	I	I	D	T	V	T	F	T	G	N	E	R	D	I	D	S	R	I	I	I	N
>2022-FR-OP389245	B3-1	H	I	R	V	I	I	D	T	A	T	F	T	G	N	E	R	D	I	D	S	R	I	I	I	N
>2022-FR-OP389246	B3-1	H	I	R	V	I	I	D	T	A	T	F	T	G	N	E	R	D	I	D	S	R	I	I	I	N
>2021-FR-OP267493	B3-1	H	I	R	V	I	I	D	T	A	T	F	T	G	N	E	R	D	I	D	S	R	I	I	I	N
>2021-FR-OP267494	B3-1	H	I	R	V	I	I	D	T	A	T	F	T	G	N	E	R	D	I	D	S	R	I	I	I	N
>2021-NL-OQ139570	B3-1	H	I	H	V	I	I	D	T	A	T	F	T	G	N	E	R	D	I	D	S	R	I	I	I	N
>2021-NL-OQ139571	B3-1	H	I	H	V	I	I	D	T	A	T	F	T	G	N	E	R	D	I	D	S	R	I	I	I	N
>2021-NL-OQ139572	B3-2	H	I	R	V	I	I	D	T	A	T	F	T	G	N	E	R	D	I	D	S	R	I	I	I	N
>2021-NL-OQ139573	B3-2	H	I	H	V	I	I	D	T	A	T	F	T	G	N	E	R	D	I	D	S	R	I	I	I	N
>2019-NL-MN726800	B3	H	I	R	V	I	L	N	A	A	A	F	T	G	N	E	R	D	I	D	S	R	T	I	I	N
>2019-SE-MN935870	B3	H	I	R	V	I	L	N	A	A	A	F	T	G	N	E	R	D	I	D	S	R	T	I	I	N
>2019-SE-MH674155	B3	H	I	R	V	I	I	D	A	A	A	F	T	G	N	E	R	D	I	D	S	R	T	I	I	N
>1956-US-AY426531	Fermon	H	I	R	I	I	S	N	S	A	T	F	T	G	D	D	R	D	I	D	S	R	T	I	I	N
>US/IL/14-18952 (Neurovirulent) † ‡	B2	H	I	R	V	V	L	N	A	A	T	F	T	G	N	E	K	D	I	D	S	R	T	I	I	D
>US/CA/14-4231 (Non-neurovirulent) † §	B2	H	I	H	V	I	I	D	A	A	A	F	T	G	N	K	R	N	I	D	N	K	T	I	L	N

(C)

Genome polyprotein based numbering *			553				554				650				835			
VP1 protein based numbering			1				2				98				283			
Year	Genotype	Total	NV	Frequency	Non-NV	Frequency	NV	Frequency	Non-NV	Frequency	NV	Frequency	Non-NV	Frequency	NV	Frequency	Non-NV	Frequency
1962-2022	Prototype	2	L	100 %			N	100 %			T	100 %					D	100 %
1962-2022	A1	55	L	95 %	S	4 %	N	4 %	D	96 %	T	98 %	I	2 %	E	98 %	K	2 %
1962-2022	A2/D1	4	L	100 %					E	100 %	T	50 %	I	50 %	E	100 %		
1962-2022	A2/D2	37	L	100 %					E	100 %	T	24 %	V	41 %	E	100 %		
2023	A2/D2	30	L	100 %					E	100 %			I	100 %	E	63 %	D	37 %
1962-2022	B1	312	L	98 %	I	1 %	N	1 %	D	99 %	T	2 %	A	98 %	E	99 %	K	<1%
1962-2022	B2	33	L	15 %	I	85 %			D	100 %	T	67 %	A	33 %	E	76 %	K	9 %
1962-2022	B3	437	L	29 %	I	71 %	N	4 %	D	91 %	T	45 %	A	55 %	E	99 %	K	<1%
1962-2022	B3-1	15	L	73 %	I	20 %			D	100 %			A	100 %	E	100 %		
1962-2022	B3-2	64	L	2 %	I	98 %	N	2 %	D	98 %	T	100 %			E	100 %		
1962-2022	B3-3	16			I	100 %			E	88 %	T	88 %	A	12 %	E	100 %		
2023	B3-3	69	L	7 %	I	93 %			E	100 %	T	94 %	A	3 %	E	93 %	D	4 %
1962-2022	C	66	L	97 %	S	3 %			D	100 %	T	100 %			E	94 %	K	6 %

Fig. 4. (*continued*).

high evolutionary closeness already seen in the phylogenetic analysis. Substitutions may have occurred within or outside the European region, and crossover with US 2022 sequences is seen only now. Surprisingly, one novel residue change was found, in VP1 (D554E), previously undescribed European lineages. The VP1 genomic region has been involved in defining antigenicity, receptor binding and its increased variability has been proposed to be the reason for co-circulation of the different phylogenetic lineages of EV-D68 [5–7]. The non-virulent D554E resides in the VP1 region, and it may be of immunological relevance. The conservative non-neurovirulent substitution of D554E in the circulating B3–3 lineage may explain why no AFM cases were reported in US 2022 or in this study.

Leser and colleagues further identified three amino acid signatures (I553L, A650T, K835E) potentially associated with neurovirulence in mice [7]. In this study, all three substitutions were found in cases of EV-D68 without being associated with neurovirulence in vivo. Site 553 seems to be evolving towards the non-neurovirulent amino acid residue, isoleucine (I). Moreover, a loose tendency towards neurovirulent changes was seen at two of the four sites, as site 650 seems to be going back to threonine (T), while site 835 has strongly evolved towards glutamic acid (E) in all EV-D68 genotypes. Overall, with the exception of K835E in the mouse neurovirulent strain, the amino acid changes are quite biochemically conservative. These substitutions could affect the secondary structure of VP1 and may perhaps be more connected with contact sites in receptor interactions [29]. Further research on different combinations of these substitutions is needed, i.e. are all four substitutions, a combination of some, or only one needed for paralysis. In addition, combining findings with demographic, clinical, and AFM data may provide crucial information on their role in disease severity and development of neurologic disease.

In conclusion, this study documents sustained circulation of EV-D68 in Europe in 2023, as well as the continued evolution of B3-derived lineages. The appearance of previously undescribed amino acid substitutions in Europe highlights the rapid adaptation and evolution of EV-D68, even during years of presumed low viral activity. Altogether, this stresses the need for continuous EV-D68 surveillance and phylogenetic monitoring beyond expected peak years. In addition, it demands awareness, adequate diagnostic procedures for virus identification and estimation of the clinical burden. This enables cultivation of strategies for the prevention and treatment of EV-D68 infection including neurologic disease, caused by currently circulating EV-D68 strains in Europe. In this light, ENPEN seeks to bring together European institutions to harmonize EV-D68 detection practices and move towards better data comparability across countries.

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CRediT authorship contribution statement

Heli Harvala: Writing – review & editing, Supervision, Methodology, Formal analysis, Conceptualization. **Kimberley S.M. Benschop:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Conceptualization. **Aurora Hirvonen:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Caroline Klint Johannesen:** Writing – review & editing, Conceptualization. **Peter Simmonds:** Writing – review & editing, Validation, Supervision, Methodology, Formal analysis, Data curation. **Thea K. Fischer:** Writing – review & editing, Supervision, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jcv.2025.105785.

Data availability

Data are available from the corresponding author on reasonable request.

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