The Journal of Infectious Diseases

MAJOR ARTICLE

Epidemiological and clinical insights into the enterovirus D68 upsurge in Europe 2021/22 and the emergence of novel B3-derived lineages, ENPEN multicentre study

Margarida Pires Simoes [1, 2], Emma B. Hodcroft [3-5], Peter Simmonds [6], Jan Albert [7, 8], Enagon K. Alidjinou [9], Katia Ambert-Balay [10], Cristina Andrés [11], Andrés Antón [11], Christelle Auvray [10], Jean-Luc Bailly [12], Fausto Baldanti [13, 14], Capser Bastings [15], Stuart Beard [16], Carla Berengua Pereira [17], Natasa Berginc [18], Mandy Bloemen [19], Soile Blomqvist [20], Froukje Bosma [21], Sindy Böttcher [22], Laura Bubba [23], Stafan Buderus [24], Maria Cabrerizo [26], Christina Calvo [27], Christina Celma [16], Ferruccio Ceriotti [28], Gemma Clark [29], Inës Costa [30], Marianne Coste-Burel [31], Karen Couderé [32], Jeroen Cremer [1], Margarita del Cuerpo Casas [17], Theo Daehne [33], Jessica de Beer [21], Maria de Ceano-Vivas [27], Cillian De Gascun [34], Alexis de Rougemont [10], Johnathan Dean [34], Jennifer L. Dembinski [35], Sabine Diedrich [22], Javier Diez-Domingo [36], Lena Dillner [37], Dagny H. Dorenberg [35], Alexandra Ducancelle [38], Susanne Dudman [39, 40], Robert Dyrdak [7, 8], Anna-Maria Eis-Huebinger [41], Iker Falces-Romero [42], Agnes Farkas [25], Susan Feeney [43], Maria D. Fernandez-Garcia [44], Jacky Flipse [45], Kristina T. Franck [46], Cristina Galli [47], Isabelle Garrigue [48], Felix Geeraedts [21], Irina Georgieva [49], Federica Giardina [13], Raquel Guiomar [30], Elenor Hauzenberger [37], Esther Heikens [50], Cécille Henquell [51], Didier Hober [9], Amrio Hönemann [52], Hannah Howson-Wells [29], Željka Hruškar [53], Niina Ikonen [20], Berthemarie Imbert [31], Arjan R. Jansz [15], Marion Jeannoël [54], Helena Jiřincová [55], Laurence Josset [54], Kathrin Keeren [56], Naomie Kramer-Lindhout

Corresponding author: Kimberley S.M. Benschop (kim.benschop@rivm.nl), Centre for infectious Disease Control (Cib) | Centre for Infectious Disease Research, Diagnostics and laboratory Surveillance (IDS), Dutch National Institute for Public Health and the Environment, PO Box 1 | 3720 BA Bilthoven | the Netherlands, Antonie van Leeuwenhoeklaan 9 | 3721 MA Bilthoven | the Netherlands

DOI: 10.1093/infdis/jiae154

[57], Sidsel Krokstad [58], Mouna Lazrek [9], Hélène Le Guillou-Guillemette [38], Caroline Lefeuvre [38], Andreas Lind [39], Maja M. Lunar [59], Melanie Maier [52], Stéphanie Marque-Juillet [60], C. Patrick McClure [29, 61], James McKenna [43], Adam Meijer [1], Ana Menasalvas Ruiz [62], Beatriz Mengual-Chuliá [36], Sofie Midgley [46], Audrey Mirand [12, 51], Richard Molenkamp [63], Milagrosa Montes [64], Antonio Moreno-Docón [65], Ursula Morley [34], Jean-Luc Murk [32], Ana Navascués-Ortega [66], Roel Nijhuis [67], Lubomira Nikolaeva-Glomb [49], Svein A. Nordbø [68], Sanela Numanovic [35], Massimo Oggioni [69], Eider Oñate Vergara [64], Jordi Pacaud [48], Marie L. Pacreau [60], Marcus Panning [33], Elena Pariani [47], Lili Pekova [70], Laura Pellegrinelli [47], Miroslav Petrovec [71], Corinna Pietsch [52], Léa Pilorge [72], Luis Piñeiro [64], Antonio Piralla [13], Mario Poljak [59], Birgit Prochazka [73], Nuria Rabella [17], Janette C. Rahamat-Langendoen [63], petra Rainetova [55], Marijke Reynders [74], Annelies Riezebos-Brilman [21], Lieuwe Roorda [75], Carita Savolainen-Kopra [20], Isabelle Schuffenecker [54], Leo C. Smeets [76], Ayse Stoyanova [49], Karl Stefic [77], Caroline Swanink [45], Irena Tabain [53], Jeroen Tjhie [15, 32], Luc Thouault [72], Camille Tumiotto [48], Sara Uceda Renteria [28], Tina Uršič [71], Sophie Vallet [72], Marc Van Ranst [19], Peter Van Wunnik [76], Jaco J. Verweij [32], Jorgina Vila [11], Bas Wintermans [57], Elke Wollants [19], Katja C. Wolthers [78], F. Xavier López-Labrado [36], Thea Kolsen Fischer [79, 80], Heli Harvala [81, 82], Kimberley S.M. Benschop [1].

[1] Centre for infectious Disease Control (Cib), Dutch National Public Health Insitute (RIVM), Bilthoven, the Netherlands; [2] European Program for Public Health Microbiology Training (EUPHEM), European Centre for Disease Prevention and Control, (ECDC), Stockholm, Sweden; [3] Geneva Center of Emerging Viral Diseases, HUG, and University of Geneva, Geneva, Switzerland; [4] Swiss Institute of Bioinformatics, Lausanne, Switzerland; [5] Institute of Social and Preventive Medicine, University of Bern, Bern, Switzerland; [6] Nuttfield Department for Medicine, University of Oxford, Oxford, United Kingdom; [7] Department of Clinical Microbiology, Karolinska University Hospital, Stockholm, Sweden; [8] Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm, Sweden; [9] University of Lille, CHU Lille, Laboratoire de Virologie EA3610, France; [10] National Reference Centre for gastroenteritis viruses. Laboratory of Virology-Serology, University Hospital of Dijon Bourgogne, Dijon, France; [11] Respiratory Virus Unit, Microbiology Department, Vall d'Hebron Institut de Recerca (VHIR), Vall d'Hebron Barcelona Hospital Campus, Barcelona, Spain; [12] Université Clermont Auvergne, CNRS, LMGE-EPIE team, Clermont-Ferrand, France; [13] Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy; [14] Department of Clinical Surgical Diagnostic and Pediatric Sciences, Università degli Studi di Pavia, Pavia, Italy; [15] PAMM, Veldhoven, the Netherlands; [16] Enteric Virus Unit, UK Health Security Agency, Colindale, United Kingdom; [17] Microbiology Department Hospital Universitari de la Santa Creu i Sant Pau, Universitat Autònoma de Barcelona, Barcelona, Spain; [18] National Laboratory of Health, Environment and Food, Laboratory for Public Health Virology, Ljubljana, Slovenia; [19] Clinical and Epidemiological Virology, Rega Institute KU Leuven, Leuven, Belgium; [20] National Institute

for Health and Welfare, Helsinki, Finland; [21] Laboratory for Medical Microbiology and Public Health, Hengelo, the Netherlands; [22] National Reference Laboratory for Poliomyelitis and Enteroviruses, Robert-Koch Institute, Berlin, Germany; [23] European Non-Polio Enterovirus Network; [24] GFO Kliniken Bonn Betriebsstätte St Marien, Bonn, Germany. Robert-Koch-Straße 1, D-53115 Bonn (Venusberg); [25] National Public Health Center, Budapest, Hungary; [26] National Centre for Microbiology, Instituto de Salud Carlos III, Enterovirus and Viral Gastroenteritis Unit/Polio National Lab, Madrid, Spain. CIBERESP; [27] Pediatric and Infectious Diseases Department. Hospital Universtiario La Paz, Fundación IdiPaz, CIBERINFEC ISCIII, Madrid, Spain; [28] Virology Unit, Division of Clinical Laboratory, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy; [29] Clinical Microbiology, Nottingham University Hospitals NHS Trust, Nottingham, NG7 2UH, United Kingdom; [30] National Reference Laboratory for Influenza and Other Respiratory Viruses, National Institute of Health Dr Ricardo Jorge (INSA), Lisbon, Portugal; [31] Virology Department, CHU Hôtel Dieu, University Hospital, Nantes, France; [32] ElisabethTweesteden Hospital, Tilburg, the Netherlands; [33] Institute of Virology, Medical Center - University of Freiburg, Faculty of Medicine, University of Freiburg, Freiburg, Germany; [34] National Virus Reference Laboratory, University College Dublin, Dublin, Ireland; [35] Department of Virology, Norwegian Institute of Public Health, Norway; [36] Center for Public Health Research (FISABIO-Public Health), Generalitat Valenciana, Valencia, Spain; [37] The Public Health Agency of Sweden, Solna, Sweden; [38] Laboratoire de Virologie, Département de Biologie des Agents Infectieux, CHU Angers, Angers, France; [39] Oslo University Hospital, Oslo, Norway; [40] University of Oslo, Oslo, Norway; [41] Institut für Virologie Universitätsklinikum Bonn, Bonn, Germany; [42] Microbiology Department, Hospital Unviersitario La Paz, CIBERINFEC ISCIII, Madrid, Spain; [43] Regional Virus Laboratory, Belfast Health and Social Care Trust (BHSCT), Royal Victoria Hospital, Belfast, United Kingdom; [44] National Centre for Microbiology, Instituto de Salud Carlos III, Ctra. Majadahonda-Pozuelo, Km 2, 28220 Majadahonda, Madrid, Spain; [45] Laboratory for Medical Microbiology and Immunology, Rijnstate, Velp, the Netherlands; [46] The Danish WHO National Reference Laboratory for Poliovirus, Statens Serum Institut, Copenhagen, Denmark; [47] Department of Biomedical Sciences for Health, University of Milan, Milano (MI), Italy; [48] Universitary Hospital of Bordeaux, France; [49] National Reference Laboratory for Enteroviruses, National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria; [50] St Jansdal Hospital, the Netherlands; [51] CHU Clermont-Ferrand, National Reference Centre for enteroviruses and parechoviruses – Associated laboratory, Clermont-Ferrand, France; [52] Institute of Medical Microbiology and Virology, University of Leipzig, Leipzig, Germany; [53] Department of Virology, Croatian Institute of Public Health, Zagreb, Croatia; [54] National Reference Center for Enteroviruses and Parechoviruses, Institut des Agents Infectieux, Hospices Civils de Lyon, Lyon, France; [55] National Reference Laboratory for Enteroviruses, National Institute of Public Health, Prague, Czech Republic; [56] Secretary of the commission for Polio Eradication in Germany, Robert-Koch Institute, Berlin, Germany; [57] Admiraal de Ruijter Hospital, Goes, the Netherlands; [58] Department of Medical Microbiology, St. Olavs Hospital, Trondheim University Hospital, Norway; [59] Institute of Microbiology and Immunology, Faculty of Medicine, University of

Ljubljana, Slovenia; [60] Service de Biologie, Centre Hospitalier de Versailles, 78157 Le Chesnay, France; [61] Wolfson Centre for Global Virus Research, School of Life Sciences, University of Nottingham, Nottingham, United Kigndom; [62] Pediatric Infectious Diseases Unit. Hospital Universitario Virgen de la Arrixaca, Carretera Murcia-Cartagena s/n 30120 El Palmar; Murcia, Spain; [63] Erasmus Medical Center, Rotterdam, the Netherlands; [64] Microbiology Department, Donostia University Hospital and Biodonostia Health Research Institute, San Sebastián, Spain; [65] Microbiology Department.Hospital Clínico Universitario Virgen de la Arrixaca. IMIB-Arrixaca, Murcia University, Murcia, Spain; [66] Microbiology Department, Hospital Universitario de Navarra, Spain; [67] Meander Medical Center, Amersfoort, the Netherlands; [68] Norwegian University of Science and Technology, Trondheim, Norway; [69] Microbiology and Virology Unit, Department of diagnostic services, ASST-Brianza, Vimercate, Italy; [70] Clinic of Infectious Diseases, University Hospital "Prof. Dr. Stoyan Kirkovich" AD - Stara Zagora, Bulgaria; [71] Institute of Microbiology and Immunology, Laboratory for the Diagnosis of Viral Infections, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia; [72] Département de Microbiologie (Pr Payan), Laboratoire de Virologie, CHRU de Brest, France; [73] NRCP Vienna at AGES - Austrian Agency for Health and Food Safety, Institute of Medical Microbiology and Hygiene, Vienna, Austria; [74] Laboratory Medicine, Molecular Microbiology, AZ St.Jan Brugge-Oostende AV, Bruges, Belgium; [75] Maasstad Hospital, Rotterdam, the Netherlands; [76] Reinier Haga Medical Diagnostic Center, Delft, the Netherlands; [77] INSERM U1259, Université de Tours, Tours, France - Laboratoire de Virologie, Centre Hospitalier Régional Universitaire de Tours, Tours, France; [78] Amsterdam University Medical Centers, location AMC, Amsterdam, the Netherlands; [79] Department of Clinical Research, Nordsjællands Hospital, Hilleroed, Denmark; [80] Department of Public Health, University of Copenhagen, Copenhagen, Denmark; [81] Nuffield Division of Clinical Laboratory Sciences, Radcliffe Department of Medicine, Oxford, United Kingdom; [82] NHS Blood and Transplant, Microbiology Services, Colindale, United Kingdom.

Enterovirus D68 (EV-D68) infections are associated with severe respiratory disease and acute flaccid myelitis (AFM). The European Non-Polio Enterovirus Network (ENPEN) aimed to investigate the epidemiological and genetic characteristics of EV-D68 and its clinical impact during the fall-winter season of 2021/22.

From 19 European countries, 58 institutes reported 10,481 (6.8%) EV-positive samples of which 1,004 (9.6%) were identified as EV-D68 (852 respiratory samples). Clinical data was reported for 969 cases. 78.9% of infections were reported in children (0-5 years); 37.9% of cases were hospitalised. Acute respiratory distress was commonly noted (93.1%) followed by fever (49.4%). Neurological problems were observed in 6.4% of cases with six reported with AFM. Phylodynamic/Nextstrain and phylogenetic analyses based on 694 sequences showed the emergence of two novel B3-derived lineages, with no regional clustering.

In conclusion, we describe a large-scale EV-D68 European upsurge with severe clinical impact and the emergence of B3-derived lineages.

Keywords: enterovirus D68 (EV-D68); respiratory infection; non-polio enterovirus (NPEV); European Non-Polio Enterovirus Network (ENPEN); acute flaccid paralysis (AFP); acute flaccid myelitis (AFM); surveillance; B3-derived lineages; coronavirus disease caused by SARS-CoV-2 (COVID-19).

BACKGROUND

Enterovirus D68 (EV-D68) primarily infects the human upper respiratory tract and is mainly associated with mild to moderately severe upper respiratory symptoms including sore throat, cough, congestion and fever. However, infections can also be associated with lower respiratory tract infections and severe neurological conditions such as meningitis, encephalitis or acute flaccid myelitis (AFM) [1-3]. Children up to 5 years of age are most commonly affected and are at greatest risk of developing severe disease [4]. Nevertheless, severe forms of EV-D68 have also been observed in adults, especially in elderly, immunosuppressed or those with other underlying clinical conditions [2, 5].

EV-D68 is a member of the species *Enterovirus D* in the genus *Enterovirus,* family *Picornaviridae* [6]. The genus *Enterovirus* comprises 9 species and more than 200 types. Molecular detection is the gold standard to diagnose EV-D68 infections, either by an EV-D68 specific RT-PCR or by an EV-generic assay targeting the conserved 5' untranslated region followed by genotyping of the genes encoding capsid proteins VP1 or VP4-VP2 [7].

Phylogenetic analysis of VP1 sequences enables the differentiation of EV-D68 strains into genotypes A through D. The B genotype is further divided into subgenotypes/clades B1, B2, and B3, while the A subgenotype/clade is divided into A1 and A2, where A2 has been further divided in D1 and D2 (also referred to as A2/D1 and A2/D2, respectively). The most common EV-D68 subgenotype/clade circulating worldwide is B3, followed by A2/D2 [8].

The first EV-D68 reported outbreaks of acute respiratory disease in Europe occurred between 2008 and 2010 in the Netherlands and Italy [9-11]. Prior to that, clinical EV-D68 cases were rarely reported [12]. Since 2008, the epidemiology of EV-D68 in Europe and North America has shown a biennial epidemic cycle [13], with infections occurring predominantly in early fall and winter [14-17]. From 2014 onwards, the biennial EV-D68 outbreaks have been accompanied by reports of AFM cases testing positive for EV-D68 in the USA where AFM is subject to enhanced surveillance (reviewed in [16, 18]). AFM cases associated with EV-D68 were first reported in Europe in 2014 [19-21]. In 2019, a disruption of the biennial cycle was noted when an upsurge of EV-D68 infections with 93 cases was reported in five European countries [22]. The study also identified five EV-D68 infected children with severe neurological disease and the circulation of B3-derived clusters, designated US18, EU18 and EU19.

During 2020, the first year of the SARS-CoV-2 pandemic, no EV-D68 cases were reported in Europe. However, during the fall of 2021, when countries eased non-pharmaceutical COVID-19 interventions, a substantial rise in EV infections was detected, in part due to increased EV-D68 circulation in some countries [4, 23]. The aim of this study was to examine the epidemiological, clinical and molecular characteristics of this EV-D68 upsurge in Europe.

METHODS

Data collection and analysis

An invitation to participate in the study was sent to members of the European Non-Poliovirus Enterovirus Network (ENPEN). ENPEN brings together specialists from different fields including clinical virology, neurological and paediatric infectious diseases, academic/molecular virology, epidemiology and public health [12, 24].

Institutes provided information on the source of the samples, sample types screened, and detection and typing methodology (Supplementary Table 1). Epidemiological data on the total number of samples tested and the total number of EV and EV-D68 positive samples were collected and analysed per month from January through December 2021. All participating institutes were coded using the two-digit country code, followed by a sequential number (e.g. XX99, Supplementary Table 1).

Demographic and clinical data was reported in aggregated format or pseudonymized manner and included cases from 2021 and 2022 (until February, Table 1 and Table 2). The data collected is summarized in Figure 1.

EV-D68 phylodynamic and phylogenetic analysis

Sequences were processed for phylodynamic analysis with the Nextstrain augur pipeline to show the time-related divergence [15, 22, 25]. Briefly, the pipeline combines EV-D68 sequences from this study and those publicly available EV-D68 sequences from NCBI GenBank (via ViPR) that were randomly sub-sampled down to 240 sequences per country per year to try to reduce disparity in representation between countries with different levels of sequencing. Sequences were then aligned using IQTree, and a time-resolved phylogeny was produced with Treetime, along which ancestral sequences were reconstructed also using Treetime (both programs as implemented in Nextstrain). EV-D68 subgenotypes/clades were assigned using mutational markers on the phylogeny. Sequence analysis included metadata on country, sample type, date of collection and age groups. Three analyses were performed based on the length of sequence available: (1) >300bp, which included the partial VP1 sequences >300bp, the near complete VP1 sequences >700bp and the complete genomes (n=692); (2) > 700bp, which included the near complete VP1 sequences >700bp and the complete genomes (n=210); (3) only complete genomes (n=82) (Figure 1). Near complete VP1 sequences and complete genome sequences were used to achieve a higher

phylogenetic resolution. In the VP1 analyses, we included all available VP1 study sequences longer than 300bp or 700bp and sequences >700bp extracted from GenBank on 21 Sept 2022 (n=3740) (subsampled as described earlier) (Figure 1). In the full-genome analysis we included all available study sequences longer than 6Kbp and all available sequences longer than 6 kb from GenBank on 3 Sept 2022 (n=976) (Figure 1). The code used to run the analysis is available at https://github.com/emmahodcroft/ev_d68_enpen2022.

In order to highlight the evolutionary divergence shown in Nextstrain, neighbour-joining trees (Jukes-Cantor corrected) using MEGA 7 software were constructed [26]. Mean pairwise (uncorrected *p* distances) distances between sequence groups were calculated using SSE software [27]. VP1 study sequences that were >80% complete between nucleotide positions 2501-2842 (numbering based on the Fermon prototype sequence, KU844179) were analysed together with 3300 publicly available sequences between these positions (>90% complete) extracted from GenBank on 31 October, 2022 (Figure 1). Complete genome sequences were analysed together with 1,025 publicly available complete genomes with known dates extracted from GenBank on 31 October, 2022 (Figure 1).

Genbank Accession Numbers

Sequences were deposited in GenBank under the following accession numbers: OM811651-OM811652, OM831155-OM831207, ON006421-ON006422, OP267493-OP267535, OQ120627-OQ127239, OQ139546-OQ139558, OQ139565-OQ139630, OQ148174-OQ148306, OQ148307-OQ586804, and OQ589870.

Ethical statement and privacy

Patients' privacy and confidentiality issues, according to General Data Protection Regulation, were managed in compliance with national/European legislation. Approval from an ethics committee and informed consent for virus screening was attained in accordance to participating institutes' regulations.

RESULTS

Detection frequencies of EV and EV-D68

A total of 58 institutions from 19 European countries participated in this study (Table 1 and supplementary table S1). Tested samples ranged from respiratory (852/969 (88%) majority) to faeces and cerebral spinal fluid, depending on institute/country and diagnostic/surveillance system. EV-D68 laboratory confirmation was mostly based on respiratory samples. Of the institutes that reported the sample type information (n=49), respiratory samples comprised 66% of the samples tested (supplementary table S1). Testing more than 150,000 samples revealed a total of 10,481 EV positive samples (6.8%) from 1st January through 31st December 2021. Of the EV positive

samples, 1,004 were confirmed as EV-D68 positive (9.6%). Large differences in the number of samples tested and proportions of EV and EV-D68 positive samples were observed among countries/reporting institutes (Table 1 and Supplementary table S1, respectively). However, data could not be compared due to different catchment population and testing strategies. Most institutes tested multiple sample types, and higher proportions of respiratory samples tested did not reflect a higher proportion in EV-D68 positive detections. Notably, the two institutes that only performed testing on cerebrospinal fluid (CSF) samples did not report any EV-D68 cases.

Seasonality of EV and EV-D68

The number of samples tested for EV remained similar throughout the first eight months of 2021 (average 10,000 tests/month) during the period when an increasing number and proportion of EV positive samples were observed (Figure 2). The first EV-D68 positive sample was detected in June 2021 and EV-D68 positive samples were sporadically detected from June through August 2021. From September 2021 onwards, the number of samples tested for EV increased accompanying a higher number of EV positive samples. During this time, the number of EV-D68 positive samples increased exponentially and reached a peak in October 2021 (405/1,004, 40%).

Clinical characteristics of EV-D68 cases

Clinical data was reported by 41 institutes (13 countries) for 969 EV-D68 cases (Figure 1, Table 1, Supplementary Table 1). Most EV-D68 cases were identified by testing a respiratory sample (n=852, sample type known for 870 cases; 98%) whereas faecal (n=16), vesicle (unknown origin; n=1) or plasma (n=1) samples were positive in the remaining cases. Most infections were reported in children between 2 and 5 years of age (41.1%) followed by children between 3 and 12 months of age (22.2%) (Table 2). In total, 79% (n=765) of EV-D68 cases were in the age group 0-5 years (median age of 2.9 years, range from new-borns to 93 years). More than half of the cases were male (54%). Detailed clinical information was available for 668 EV-D68 cases showing respiratory distress as the predominant symptom (93.1%).The second most common clinical sign was fever being reported in approximately half of these cases.

As shown in Table 2, co-infections were reported for 241/969 (24.9%) of EV-D68 cases of which almost half were also infected with human rhinovirus (RV). More than two co-infecting viruses were reported in 69 cases (28.6%).

369 of 969 (38.1%) of EV-D68 cases were hospitalized between 0.5 to 136 days (interquartile range, 0.5-3 days). A total of 249 individuals with EV-D68 infection (25.7%) had known underlying medical conditions, for example prematurity, congenital malformations, asthma and different cancer types (Table 2).

Neurological conditions were identified in 43 patients (6.4%), most of which were in the age group 0-5 years (n=34; 79%). The neurological problems ranged from headache, dizziness and agitation to seizures. One case was reported with encephalitis (8 years of age with comorbidities) and four

cases were diagnosed with meningitis (up to 5 months of age). AFM was reported in six children: five cases up to 5 years of age and one in an older child (6-15 years of age). Patients with neurological disorders typically showed respiratory distress (32/43; 74.4%), fever (26/43; 60.5%), enteric symptoms (12/43; 27.9%) or rash (7/43, 16.3%). 37.2% (16/43) had an underlying medical condition. 28 out of 43 patients had severe neurological disorders requiring intensive care unit (ICU) admission, 82.1% (23/28) being between 0 and 5 years of age and 39.3% (11/28) with known comorbidities.

Phylodynamics and divergence of EV-D68 strains

To investigate the circulation of EV-D68 strains in this study we included a maximum of 694 sequences of the received 744 sequences for analysis (Figure 1). A majority of the strains were reported by submitting institutes as B3, and eight strains as A2/D2. The B3 strains were detected throughout the study period. The A2/D2 strains were detected in October through December (data not shown). Figure 3 shows a screenshot of the Nextstrain build of the 300bp VP1 EV-D68 sequences over time, accessible at https://nextstrain.org/community/enterovirus-phylo/evd68-2022/vp1-300. All of the Nextstrain runs are available at https://github.com/enterovirus-phylo/evd68-2022. The phylogenetic tree showed a temporal ladder-like evolution and the emergence of two novel B3-derived lineages, designated lineage 1 and lineage 2 for this study. These patterns are visible in both of the VP1 analyses and the full-genome analysis.

The divergence between these lineages was estimated to be 4.2% based on the complete genome (5.2% based on the partial VP1). Lineage 1 descended from B3 strains reported in 2019 (previously designated US18 with the 2019/2020 upsurge) and was detected across Europe (Figure 4) predominantly in the UK, Netherlands, France and Spain from August 2021 throughout January 2022. For the four AFM cases with sequence data available, all fell into the B3-derived lineage 1.

Lineage 2 was distantly related to a B3 sample from the 2016 outbreak in Europe and predominantly detected in South-Western European countries, such as Spain and France (Figure 4) from June 2021 through January 2022, with similar kinetics to lineage 1 variants (data not shown). Notably, lineage 2 showed a deletion at VP1:S143 not widely seen elsewhere on the phylogeny.

DISCUSSION

In this study, we describe valuable information on the demographic and clinical features of nearly a thousand EV-D68 cases mostly reported in the fall and winter of 2021/22 by 13 of 19 European countries participating in the study, and the emergence of novel B3-derived lineages. For the first time, the collection of denominator data, i.e., the number of EV and EV-D68 samples tested across Europe, allowed for a depiction of the proportion of both EV and EV-D68 infections found within different institutes in 2021. However, it should be noted that catchment population and testing

strategies varied and data could not be compared directly between institutes nor among countries. Nevertheless, the study revealed the importance of sharing data across Europe, which aimed to improve diagnostic awareness based on the increased circulation of EV-D68. It also enforced our understanding of the epidemiology and evolution of EV-D68. The study also revealed gaps in data comparability and the need for better harmonized medical and diagnostic practices.

Based on the biennial circulation pattern of EV-D68 previously recorded in Europe, an EV-D68 upsurge was expected in summer/fall of 2020. However, this 2-year cycle had already been disrupted by the previous EV-D68 upsurge in Europe in 2019 [22] and was disturbed further by COVID-19 non-pharmaceutical interventions. For EV-D68 (and other viral pathogens), the disruption in their circulation is also hypothesised to have led to a much larger EV-immune naïve cohort compared to the ones found in previous incidence cycles [15, 23, 28]. As a result, the easing of COVID-19 non-pharmaceutical interventions may have spurred new upsurges far greater than in previous cycles in Europe and beyond [4, 29-31]. Similarly, increased detections of other pathogens has been noted due to resumption of community circulation [32-34]. An additional contributory factor to the greater number of samples tested for EV and EV-D68 detections may have been the increased number of respiratory samples that were collected for syndromic testing on respiratory viruses, including SARS-CoV-2.

EV-D68 infections were predominantly associated with respiratory symptoms (93.1%), and nearly a quarter of EV-D68 infections were in individuals with underlying medical conditions which/that may have played a role in the severity of EV-D68 infections [5, 16, 35, 36]. Similar proportions of underlying medical conditions among EV-D68 infections related to severity were also reported in previous studies albeit populations studied were different [16]. Most cases were detected by diagnostic testing of respiratory samples, highlighting the importance of including respiratory samples in EV surveillance as EV-D68 RNA is rarely detected in faecal and CSF samples, even in patients with neurological disease [7, 37]. Noteworthy, rhinovirus was the predominant co-infection, which was also seen in other studies [36, 38]. It should be considered which underlying medical conditions and co-infections to be included in the data collection to best promote standardization and to be implemented for further EV-D68 studies [7 ,12].

A total of 43 EV-D68 patients (6.4%) displayed neurological disorders, and half of them were diagnosed with seizures, encephalitis, meningitis or AFM, providing further evidence for a potentially neurovirulent property of this virus [39]. Although data is reported on only 6 AFM cases with confirmed EV-D68 infection, it was noted that for several other patients with similar paralytic clinical presentations laboratory testing for EV-D68 was not performed or failed due to the delayed onset of neurological symptoms (unpublished data; [40]). This could have resulted from inappropriate sampling for EV-D68 testing or clinical presentations that were not identified as AFM due to lack of clinical awareness. Furthermore, AFM initially starts with a respiratory prodromal phase and samples may not have been collected at an appropriate time before onset of AFM [41]. Inappropriate sampling and diagnosis can lead to underdiagnosing and underreporting [42, 43] and thus, clear guidelines how to diagnose AFM are required.

Of the reported clinical data, over 38% EV-D68 cases required hospitalisation and 17% of them at ICU level revealing a substantial utilisation of healthcare resources from EV-D68 infections in our study population, especially in young children. These results are consistent with previous studies [36]. The high proportions of hospitalisation can be the result of a sampling bias due to testing strategy. As such standardized surveillance that includes the general asymptomatic population is essential to estimate the true burden of disease. The EV-D68 hospitalisation rate is concordant with that associated with influenza virus infections (34%) [31], and ICU admission rates due to RSV infections resemble those of EV-D68 (both 17%) [44].

By comparing the 2021/22 sequence data to previous EV-D68 strains, we were able to detect a similar stepwise diversification of EV-D68 as observed with other viruses [45-47]. In this study, the majority of the sequences encompassed partial VP1, as most institutes use the assay developed by Nix and colleagues [48].

Despite the interruption in EV-D68 circulation during the COVID-19 pandemic, EV-D68 evolution continued, resulting in the emergence of two novel post-pandemic B3-derived lineages. Lineage 1 clearly originated from the pre-pandemic strains designated as B3-US18 by Midgley et al (2020). In that study, two other clusters/lineages were observed and designated as B3-EU18 and B3-EU19 [15, 22]. These were not observed in 2021/22, and may no longer be circulating, or circulating only at very low levels.

In contrast, another B3-derived lineage (lineage 2) was identified in 2021 and is less clearly linked to recent outbreaks, with a common ancestor in 2016. This highlights the need for more comprehensive surveillance of EV-D68 in order to better understand where such lineages may have circulated before becoming widespread. In order to track whether these novel lineages persist in the coming years or are replaced by other novel variants. vigilant monitoring and rapid molecular characterization shared among institutes is required.

This study has a number of limitations, particularly related to differences in surveillance systems without an uniform case definition and sampling strategy, and differences in screening and typing methods across institutes. In addition, not all institutes were able to provide detailed monthly testing data. Clinical records were incomplete in some cases due to different reasons such as General Data Protection Regulation (GDPR) or constraints of the reporting systems used by the institutes. The varied and incomplete reporting may have led to biased data, therefore/so we propose the standardisation of data collection with comprehensive reporting in order to better determine the disease burden of EV-D68 infections. Finally, EV-D68 samples are generally only collected from symptomatic and hospitalised individuals, which most likely do not fully reflect the demographics or overall geographic distribution. Despite these limitations, the epidemiological and clinical data show the considerable disease burden of this infection, especially in younger children, as well its re-emergence and continued evolution during and after the alleviation of non-pharmaceutical interventions in the wake of the SARS-CoV-2 pandemic. With globalisation and human connectedness, pathogenic agents are also constantly on the move and continue to evolve.

EV-D68 should be accounted in the differential diagnosis, especially when attending children with respiratory and/or neurological symptoms. It is our understanding that timely diagnosis could improve medical handling, in particular when neurological signs are present, Concurrently, standardizing sampling, clinical and viral diagnosis, and typing requirements all account towards better data which can then contribute towards the improved understanding of the clinical and public health impact of EV-D68 and other enteroviruses [7, 12].

CONCLUSION

This study substantiates and extends the previous description of an upsurge in EV-D68 infections in September 2021 [23] which raised awareness of EV-D68-associated respiratory and neurological infections in many countries and led to enhanced vigilance. The data shown in this study underlie the clinical and public health impact of EV-D68. The observation of rapid genetic diversification of EV-D68 into novel B3-derived lineages emphasises the value of continued phylogenetic monitoring of EV-D68 and calls for further genomic analyses to investigate potential strain-associated differences in neuropathogenicity suspected in previous outbreaks [1, 2]. Overall, we highlight the need for the implementation of a mandatory and harmonised pan-European EV surveillance system.

Conflict of interest

All authors declare that there is no conflict of interest.

Funding

The study was funded by: Austrian Ministry of Health (AT01), Czechia Ministry of Health (CZ01), and Project PI18CIII-00035, Foundation for Influenza Epidemiology for data collection and CIBEResp (Instituto de Salud Carlos III, Spain; ES05) for research researcher grants (FXL, JDD and BMC ES06); Santé Publique France (FR01); EU partial funding within the NextGeneration EU-MUR PNRR Extended Partnership initiative on Emerging Infectious Diseases (Project no. PE00000007, INF-ACT, IT01 and IT02); Ministry of Health, Welfare and Sport, The Netherlands (NL02) and National Health insurance (NL01, NL03-NL12); National Surveillance program (SI01), internal funds and by the Slovenian Research Agency [grant number P3-0083] (SI02); and, by The UK Clinical Virology Network (UK04). EBH is supported by the Swiss National Science Foundation through grant number 31CA30_196046.

Acknowledgements

The authors wish to thank the European Society for Clinical Virology for hosting the European Non-Polio Enterovirus Network (ENPEN). The authors would also like to thank everybody who contributed to the collection, testing and reporting of epidemiological, clinical and virological data presented in this study.

The project has also been supported by the European Programme for Public Health Microbiology Training (EUPHEM), ECDC and National Public Health Institutes of the authors' countries. We would like to acknowledge Aura Andreasen (EPIET/EUPHEM ECDC Fellowship Programme) for the careful revision of this manuscript.

We would specifically like to acknowledge all the clinical microbiology laboratories for sending samples, the following teams and staff from participating institutes: Jorn Hellemans (BE01), Sandra Bergs (DE02), Anna Creus-Costa and Maria Carmen Martín (Hospital Universitari Vall d'Hebron – ES01), Valencia Hospital Network for the Study of Influenza and Respiratory Viruses (VAHNSI, ES06); Adeline Duard and Nathalie Rodde for helpful technical assistance in virus molecular typing (FR01); Valeria Primache and Arlinda Seiti for laboratory testing and Sandro Binda (IT01); Guglielmo Ferrari for laboratory testing (IT02); Shukriya Barzinci and Tina Christoffersen for the laboratory analysis and sequencing, as well as all clinical microbiology departments for forwarding samples to SSI (DK01); Adri van der Zanden (NL12), Carlos Gustavo Cilla Eguiluz (ES02) and Christine Jonassen (NO01) for valuable collaboration. Also, to the General Practitioner Sentinel Network – Portugal and to the Team of the National Reference Laboratory for Influenza and Other Respiratory Viruses (Aryse Melo, Camila Henriques, Patrícia Conde; PT01). Rolf Ingemarsson, Guillermo Martinez Gonzalez, Anders Jonsson and Szilvia Szilágyi for laboratory testing (SE01). EBH was also supported by the Johanna Dürmüller-Bol award from the Department of BioMedical Research at the University of Bern

Authors' contributions

KB, HH and TKF initiated the study. KB and MS collected and analysed the data. EH, KB and PS analysed the sequence data. KB and MS wrote the first draft, prepared the tables and figures, as well as the final manuscript. EH, HH, PS and TKF helped with drafting the manuscript. All other authors were responsible for diagnostics, testing and data collection at partner sites. All other authors provided and checked data, critically reviewed and edited manuscript. All authors have accepted the final version of the manuscript.

References

1. Hixon AM, Frost J, Rudy MJ, Messacar K, Clarke P, Tyler KL. Understanding Enterovirus D68-Induced Neurologic Disease: A Basic Science Review. Viruses **2019**; 11.
2. Sooksawasdi Na Ayudhya S, Laksono BM, van Riel D. The pathogenesis and virulence of enterovirus-D68 infection. Virulence **2021**; 12:2060-72.
3. Elrick MJ, Pekosz A, Duggal P. Enterovirus D68 molecular and cellular biology and pathogenesis. J Biol Chem **2021**; 296:100317.
4. Zhang W, Luan R. Estimating the impact of non-pharmaceutical interventions against COVID-19 on mumps incidence in Sichuan, China. BMC Infect Dis **2021**; 21:886.
5. Larsson SB, Vracar D, Karlsson M, Ringlander J, Norder H. Epidemiology and clinical manifestations of different enterovirus and rhinovirus types show that EV-D68 may still have an impact on severity of respiratory infections. J Med Virol **2022**; 94:3829-39.

6. Zell R, Delwart E, Gorbalenya AE, et al. ICTV Virus Taxonomy Profile: Picornaviridae. J Gen Virol **2017**; 98:2421-2.
7. Harvala H, Broberg E, Benschop K, et al. Recommendations for enterovirus diagnostics and characterisation within and beyond Europe. J Clin Virol **2018**; 101:11-7.
8. Andres C, Vila J, Creus-Costa A, et al. Enterovirus D68 in Hospitalized Children, Barcelona, Spain, 2014-2021. Emerg Infect Dis **2022**; 28:1327-31.
9. Meijer A, van der Sanden S, Snijders BE, et al. Emergence and epidemic occurrence of enterovirus 68 respiratory infections in The Netherlands in 2010. Virology **2012**; 423:49-57.
10. Piralla A, Lilleri D, Sarasini A, et al. Human rhinovirus and human respiratory enterovirus (EV68 and EV104) infections in hospitalized patients in Italy, 2008-2009. Diagn Microbiol Infect Dis **2012**; 73:162-7.
11. Poelman R, Schuffenecker I, Van Leer-Buter C, et al. European surveillance for enterovirus D68 during the emerging North-American outbreak in 2014. J Clin Virol **2015**; 71:1-9.
12. Fischer TK, Simmonds P, Harvala H. The importance of enterovirus surveillance in a post-polio world. Lancet Infect Dis **2022**; 22:e35-e40.
13. Kramer R, Sabatier M, Wirth T, et al. Molecular diversity and biennial circulation of enterovirus D68: a systematic screening study in Lyon, France, 2010 to 2016. Euro Surveill **2018**; 23.
14. Cottrell S, Moore C, Perry M, Hilvers E, Williams C, Shankar AG. Prospective enterovirus D68 (EV-D68) surveillance from September 2015 to November 2018 indicates a current wave of activity in Wales. Euro Surveill **2018**; 23.
15. Hodcroft EB, Dyrdak R, Andres C, et al. Evolution, geographic spreading, and demographic distribution of Enterovirus D68. PLoS Pathog **2022**; 18:e1010515.
16. Holm-Hansen CC, Midgley SE, Fischer TK. Global emergence of enterovirus D68: a systematic review. Lancet Infect Dis **2016**; 16:e64-e75.
17. Xiang Z, Wang J. Enterovirus D68 and Human Respiratory Infections. Semin Respir Crit Care Med **2016**; 37:578-85.
18. Messacar K, Tyler KL. Enterovirus D68-Associated Acute Flaccid Myelitis: Rising to the Clinical and Research Challenges. JAMA **2019**; 321:831-2.
19. Helfferich J, de Lange MM, Benschop KS, et al. Epidemiology of acute flaccid myelitis in children in the Netherlands, 2014 to 2019. Euro Surveill **2022**; 27.
20. Lang M, Mirand A, Savy N, et al. Acute flaccid paralysis following enterovirus D68 associated pneumonia, France, 2014. Euro Surveill **2014**; 19.
21. Pfeiffer HC, Bragstad K, Skram MK, et al. Two cases of acute severe flaccid myelitis associated with enterovirus D68 infection in children, Norway, autumn 2014. Euro Surveill **2015**; 20:21062.
22. Midgley SE, Benschop K, Dyrdak R, et al. Co-circulation of multiple enterovirus D68 subclades, including a novel B3 cluster, across Europe in a season of expected low prevalence, 2019/20. Euro Surveill **2020**; 25.
23. Benschop KS, Albert J, Anton A, et al. Re-emergence of enterovirus D68 in Europe after easing the COVID-19 lockdown, September 2021. Euro Surveill **2021**; 26.
24. Benschop KSM, Broberg EK, Hodcroft E, et al. Molecular Epidemiology and Evolutionary Trajectory of Emerging Echovirus 30, Europe. Emerg Infect Dis **2021**; 27:1616-26.
25. Huddleston J, Hadfield J, Sibley TR, et al. Augur: a bioinformatics toolkit for phylogenetic analyses of human pathogens. J Open Source Softw **2021**; 6.

26. Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol **2016**; 33:1870-4.
27. Simmonds P. SSE: a nucleotide and amino acid sequence analysis platform. BMC Res Notes **2012**; 5:50.
28. Tedcastle A, Wilton T, Pegg E, et al. Detection of Enterovirus D68 in Wastewater Samples from the UK between July and November 2021. Viruses **2022**; 14.
29. Park SW, Pons-Salort M, Messacar K, et al. Epidemiological dynamics of enterovirus D68 in the United States and implications for acute flaccid myelitis. Sci Transl Med **2021**; 13.
30. van Summeren J, Meijer A, Aspelund G, et al. Low levels of respiratory syncytial virus activity in Europe during the 2020/21 season: what can we expect in the coming summer and autumn/winter? Euro Surveill **2021**; 26.
31. Wang LP, Han JY, Zhou SX, et al. The changing pattern of enteric pathogen infections in China during the COVID-19 pandemic: a nation-wide observational study. Lancet Reg Health West Pac **2021**; 16:100268.
32. Liu P, Xu M, Lu L, et al. The changing pattern of common respiratory and enteric viruses among outpatient children in Shanghai, China: Two years of the COVID-19 pandemic. J Med Virol **2022**; 94:4696-703.
33. Olsen SJ, Winn AK, Budd AP, et al. Changes in influenza and other respiratory virus activity during the COVID-19 pandemic-United States, 2020-2021. Am J Transplant **2021**; 21:3481-6.
34. Honemann M, Thiem S, Bergs S, et al. In-Depth Analysis of the Re-Emergence of Respiratory Syncytial Virus at a Tertiary Care Hospital in Germany in the Summer of 2021 after the Alleviation of Non-Pharmaceutical Interventions Due to the SARS-CoV-2 Pandemic. Viruses **2023**; 15.
35. Cassidy H, Poelman R, Knoester M, Van Leer-Buter CC, Niesters HGM. Enterovirus D68 - The New Polio? Front Microbiol **2018**; 9:2677.
36. Penela-Sanchez D, Gonzalez-de-Audicana J, Armero G, et al. Lower Respiratory Tract Infection and Genus Enterovirus in Children Requiring Intensive Care: Clinical Manifestations and Impact of Viral Co-Infections. Viruses **2021**; 13.
37. Freeman MC, Wells AI, Ciomperlik-Patton J, et al. Respiratory and intestinal epithelial cells exhibit differential susceptibility and innate immune responses to contemporary EV-D68 isolates. Elife **2021**; 10.
38. Giardina FAM, Piralla A, Ferrari G, Zavaglio F, Cassaniti I, Baldanti F. Molecular Epidemiology of Rhinovirus/Enterovirus and Their Role on Cause Severe and Prolonged Infection in Hospitalized Patients. Microorganisms **2022**; 10.
39. Sinclair W, Omar M. Enterovirus. StatPearls. Treasure Island (FL): StatPearls Publishing Copyright © 2022, StatPearls Publishing LLC., **2022**.
40. Christy A, Messacar K. Acute Flaccid Myelitis Associated With Enterovirus D68: A Review. J Child Neurol **2019**; 34:511-6.
41. Knoester M, Helfferich J, Poelman R, et al. Twenty-nine Cases of Enterovirus-D68-associated Acute Flaccid Myelitis in Europe 2016: A Case Series and Epidemiologic Overview. Pediatr Infect Dis J **2019**; 38:16-21.
42. Helfferich J, Knoester M, Van Leer-Buter CC, et al. Acute flaccid myelitis and enterovirus D68: lessons from the past and present. Eur J Pediatr **2019**; 178:1305-15.

43. Murphy OC, Messacar K, Benson L, et al. Acute flaccid myelitis: cause, diagnosis, and management. Lancet **2021**; 397:334-46.
44. Rha B, Curns AT, Lively JY, et al. Respiratory Syncytial Virus-Associated Hospitalizations Among Young Children: 2015-2016. Pediatrics **2020**; 146.
45. Chen Z, Schiffman M, Herrero R, et al. Classification and evolution of human papillomavirus genome variants: Alpha-5 (HPV26, 51, 69, 82), Alpha-6 (HPV30, 53, 56, 66), Alpha-11 (HPV34, 73), Alpha-13 (HPV54) and Alpha-3 (HPV61). Virology **2018**; 516:86-101.
46. Graf T, Bello G, Venas TMM, et al. Identification of a novel SARS-CoV-2 P.1 sub-lineage in Brazil provides new insights about the mechanisms of emergence of variants of concern. Virus Evol **2021**; 7:veab091.
47. Huang C, Yu J, Hause BM, et al. Emergence of new phylogenetic lineage of Influenza D virus with broad antigenicity in California, United States. Emerg Microbes Infect **2021**; 10:739-42.
48. Nix WA, Oberste MS, Pallansch MA. Sensitive, seminested PCR amplification of VP1 sequences for direct identification of all enterovirus serotypes from original clinical specimens. J Clin Microbiol **2006**; 44:2698-704.

Table 1. Enterovirus testing and EV-D68 detection in participating European countries between January 2021 and February 2022

			Sampling and Testing Data					Clinical records		
			Enterovirus (EV) 2021			Enterovirus D68 (EV-D68) 2021		Enterovirus D68 (EV-D68) Total, 2021-2022		
Country	Country code	Institutes (n)	Samples tested (n)	Positive samples (n)	EV positive detection (%)	Positive samples (n)	EV-D68 % of EV	Clinical cases (n)	AFM cases/ acute myelitis (n)	Sequences (n)
Austria	AT	1	381	2	0.5%	1	50.0%	1	0	0
Belgium	BE	2	11,275	1,038	9.2%	100	9.6%	98	0	18
Bulgaria	BG	1	399	13	3.3%	0	*NA*	0	0	0
Croatia	HR	1	106	14	13.2%	0	NA	0	0	0
Czechia	CZ	1	465	120	25.8%	0	*NA*	0	0	0
Denmark	DK	1	NR	431	NA**	17	3.9%	12[#]	0	11
Finland	FI	1	176*	NA*	NA*	0	NA*	0	0	0
France	FR	10	28,237	1,921	6.8%	156	8.2%	153[#]φ	1	132
Germany	DE	4[^]	6,064	149	2.5%	5	3.4%	5	1	5
Hungary	HU	1	360	6	1.7%	0	*NA*	0	0	0
Ireland	IE	1	10,075	1,964	19.5%	14	0.7%	16[#]	0	16
Italy	IT	3	5,296	415	7.8%	20	4.8%	24[#]	0	8

Netherlands	NL	11	18,258	1,004	5.5%	106	10.6%	105	2	97
Norway	NO	3	8,289	205	2.5%	32	11.7%	15[#]	0	1
Portugal	PT	1	1,040	5	0.5%	0	*NA*	3[#µ]	0	2
Slovenia	SI	3	5,629	163	2.9%	0	*NA*	0	0	0
Spain	ES	8[^]	39,738	1,194	3.0%	248	20.5%	253[#]	1	249
Sweden	SE	2	6,122	84	1.4%	11	13.1%	11	1	1
United Kingdom	UK	3	11,533	1,753	15.2%	294	16.8%	273[#]	0	204
	Total	**58**	**153,443**	**10,481**	**6.8%**	**1,004**	**9.5%**	**969**	**6**	**744**

^ One contributor reported for other institutes
* Finland only reported on tested samples for EV-D68
** Denmark did not report on the total samples tested
[#] Countries reporting EV-D68 cases in 2022
φ French institute reporting EV-D68 cases only in 2022
µ Portuguese institute only reportingEV-D68 cases in 2022
NA – Not applicable
NR – Not reported

Table 2. Demographic and clinical characteristics of EV-D68 cases reported by 14 European countries between January 2021 through February 2022 (n=968)

		EV-D68 cases (n)	Proportion of cases
Demographic	**Age group**		
	0–2 months	79	8.2%
	3-23 months	288	29.7%
	3–12 months	215	22.2%
	13–23 months	73	7.5%
	2–5 years	398	41.1%
	6–15 years	101	10.4%
	16–25 years	14	1.4%
	26–45 years	34	3.5%
	46-65 years	24	2.5%
	> 65 years	17	1.8%
	Unknown	14	1.4%
	Total	*969*	
	Sex		
	Male	524	54.08%
	Female	361	37.25%
	Unknown	84	8.67%
Clinic	**Symptoms (data reported for)**		
	Any symptom reported	668	68.9%
	Respiratory	622	93.1%

	Fever	330	49.4%
	Enteric	99	14.8%
	Neurological [a]	43	6.4%
	Rash	28	4.2%
	Co-infections		
	Any co-infection reported	241	24.9%
	Rhinovirus	115	47.7%
	Adenovirus	45	18.7%
	RSV	33	13.7%
	CoV (OC43, 229E and Sars-CoV-2)	12	5.0%
	Clinical history and hospital information		
	Pre-existing condition [b]	249 **	25.7%
	Hospitalised	369	38.1%
	Hospital stay (IQR days)	2.8	
	Intensive Care Unit admission	61	16.5%

RSV: Respiratory Syncytial virus; CoV: Coronavirus.

[a] Reported neurological symptoms included: headache, dizziness and agitation, seizures, encephalitis, meningitis, acute myelitis and AFM/AFP

[b] Reported pre-existing conditions were asthma, congenital malformations, epilepsy, prematurity and cancer.

** 235 patients with comorbidities displaying respiratory signs

FIGURES AND TABLES - LEGENDS

Figure 1. Diagram of the information collected: epidemiological data, clinical records and sequence data with associated clinical metadata used for the analysis of the EV-D68 upsurge in Europe, 2021-2022. Sequence information is also represented separately for phylogenetic reconstruction (yellow) and divergence analysis (blue). Publicly accessible sequences used for epidemiologic analysis depicted in green boxes alongside exclusion criteria (orange boxes). * Monthly testing data was received from 55 institutes, 18 countries; and monthly EV positive data received from 57 institutes, 19 countries. # Two institutes only reported EV-D68 cases for 2022 (one also representing the country cases)

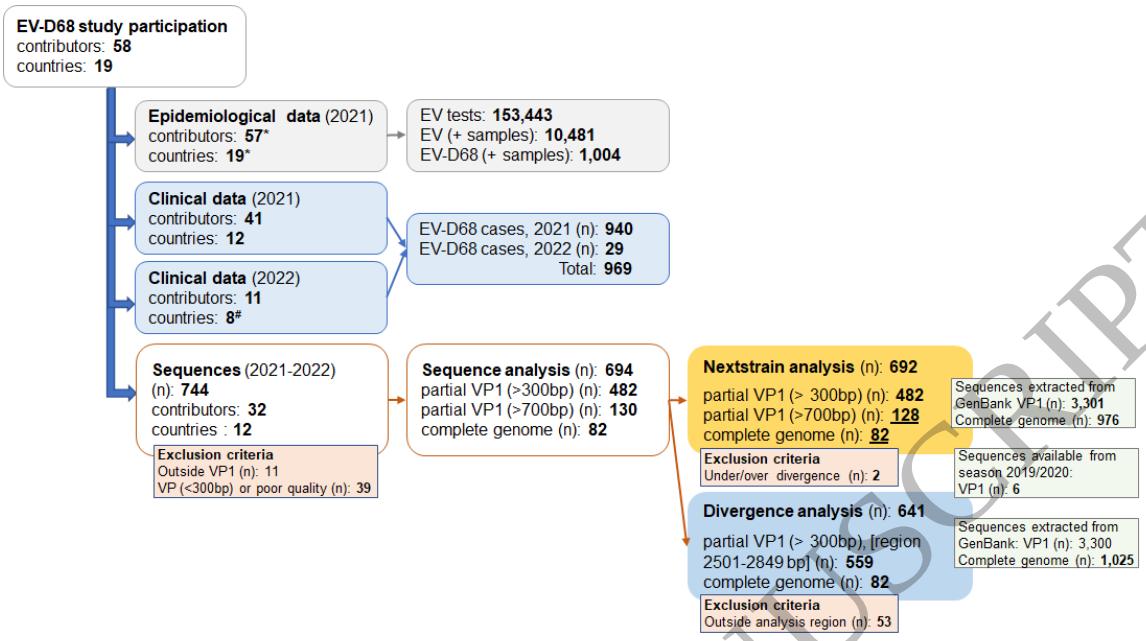

Figure 2. Proportion of samples found positive for EV (yellow bars, % of EV positive samples/number of samples tested) and EV-D68 (grey bars, % of EV-D68 positive samples/number of EV positive samples), and monthly totals of EV tests (blue line, n) reveal an increasing trend from September 2021 onwards. Highest number of EV and EV-D68 detections was observed in October 2021.

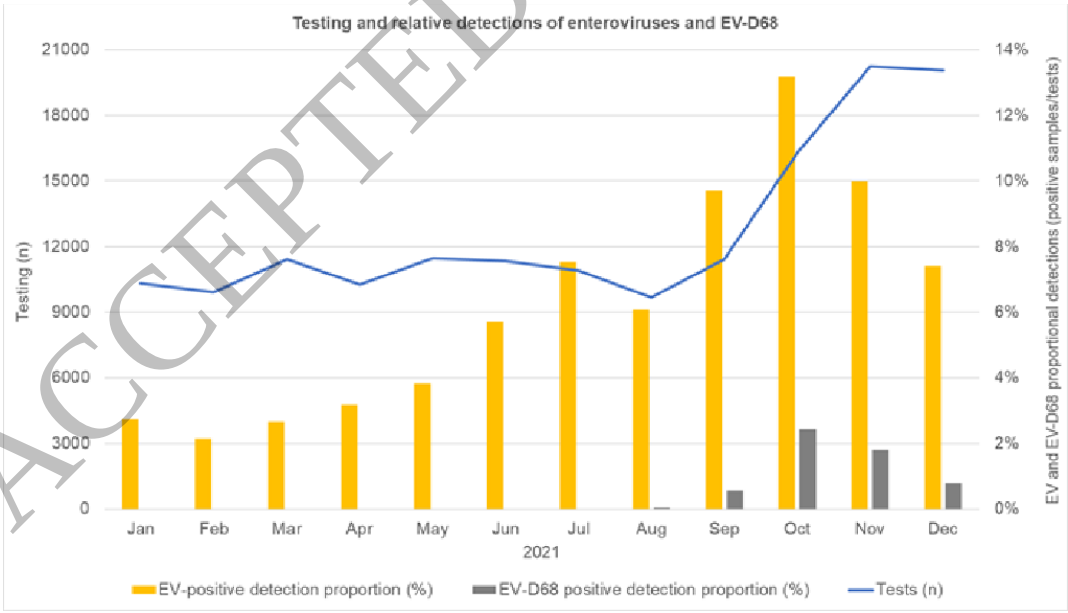

Figure 3 **(A-C)** Phylodynamic analysis of EV-D68 with Nextstrain using 692 partial study VP1 sequences > 300bp and 3,307 publicly available VP1 sequences. Insets B and C show zoomed views of areas of the tree containing the two novel lineages. **(D)** Neighbour-joining phylogenetic tree of complete genome from the study samples (n=82) and 1,025 sequences with data annotations from GenBank. **(E)** Neighbour-joining phylogenetic tree of VP1 region sequences (positions 2501-2842, numbered using the Fermon prototype sequence, KU844179) from the study samples (n=559) and 3,306 sequences with data annotations from GenBank.

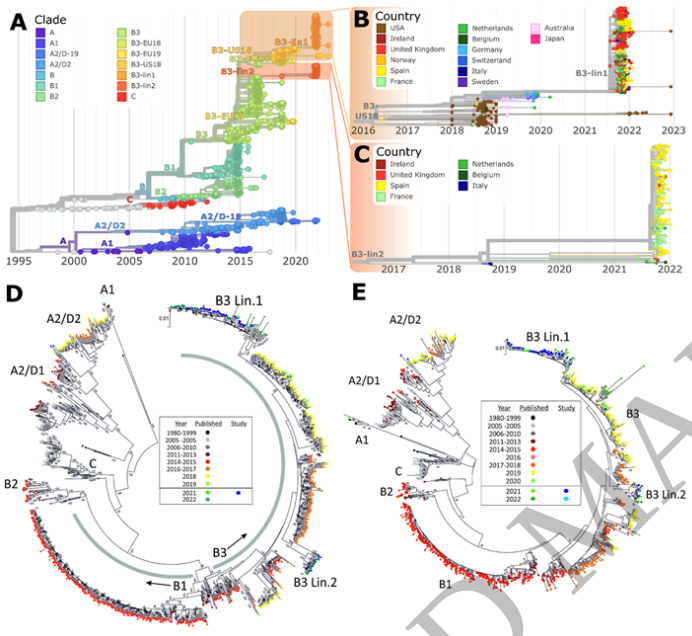

Figure 4 Maps showing the geographic distribution of the two novel EV-D68 B3 derived lineages, lineage 1 (A) and lineage 2 (B).