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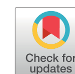
A decade of enterovirus genetic diversity in Belgium



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ABSTRACT

Background: Enteroviruses are responsible for a wide range of clinical symptoms.Enterovirus D68 was already known to cause mild to severe respiratory infections, but in the last few years, it has also been associated with neurological symptoms and acute flaccid paralysis.
Objectives: In this epidemiological surveillance in Belgium, 1521 enteroviruspositive samples were genotyped.
Study design: Enterovirus-positive patient samples were collected from the University Hospitals Leuven and other hospitals and medical practices in Belgium from 2007 to 2018. Molecular typing was done by RT-PCR using different primers sets. EV-A and EV-B were typed by sequencing part of VP1. For EVC and EV-D, the VP4/VP2 region was used together with the non-coding region.
Results: In this epidemiological survey with samples collected over 12 years, 35 different EV types were detected in 1521 patient samples. Enterovirus species B was by far the most dominant species in our samples (93%). Echovirus 30 was most frequently found (24%), followed by echovirus 6 (8%) and echovirus 9 (7%). In 2018, there was an outbreak for the first time of enterovirus D68 with severe respiratory infections but no acute flaccid paralysis. Phylogenetic analyses showed that the collected outbreak strains coexist in different clades.
Conclusions: For more than a decade, the circulating enterovirus strains were investigated in Belgium. During this time span, echovirus 30 was the most frequently detected and peaked every 3 years. Enterovirus D68 began an upsurge in 2018, but thus far without being clinically associated with acute flaccid paralysis.

1. Background

Each year, human enteroviruses (EV) infect millions of people worldwide. They can be responsible for a common cold, meningitis, encephalitis, paralysis, and even death. Enteroviruses B are the most common cause of aseptic meningitis worldwide, which mainly affects young children and is an important cause of hospitalization [1]. Enterovirus D68 (EV-D68) and enterovirus A71 (EV-A71) most commonly causes respiratory illness and hand, foot, and mouth disease (HFMD), respectively. Sometimes they are linked to acute flaccid paralysis (AFP) but also other non-polio enteroviruses (NPEV) can be responsible for paralysis [2].

Molecular techniques such as reverse transcriptase PCR (RT-PCR) and subsequent nucleotide sequencing have gained significant importance above the conventional techniques of virus culture and seroneutralization testing for the diagnosis and characterization of enterovirus infections [3]. Molecular sequence data and comparative genomics (sequence homology search in GenBank and phylogenetic analysis) are the methodologies preferentially used to identify the enterovirus type [4,5].

The second half of the 5′noncoding region (5′NCR) is extremely conserved among all enteroviruses and therefore used extensively in diagnostic RT-PCR assays. Because of the genetic conservation in the 5′NCR, sequencing of this region does not allow accurate identification of the enterovirus type. The standard method for genotyping of enteroviruses is to sequence the amino terminal part of the VP1 capsid protein [6,7]. However, the lower sequence conservation in this region complicates the amplification of all enteroviruses using one primer set

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[8]. The VP1 gene encodes important serotype-specific neutralization epitopes and therefore its sequence has shown to correlate very well with the classical serotype classification [9]. Sequence homology criteria have been defined: a VP1 nucleotide sequence identity of more than 75% to a certain reference strain in GenBank indicates that the sample is homologous to that specific serotype, provided that the second-highest identity score is less than 70% [5]. Sequencing part of the VP1 is the gold standard for EV typing [9,3]. The protocol of Nix et al. [10] is adopted by the WHO as recommendation for EV typing and is widely used in national reference laboratories (WHO EV surveillance guidelines 2015). This protocol is rather time consuming and therefore a non-nested one-step RT-PCR with different primer sets was used for typing directly from clinical specimens.

Since 2014, EV-D68 gained epidemiological and clinical relevance because of a large outbreak of severe respiratory infections associated with nervous system diseases in the USA [11]. Since then, EV-D68 is reported all over Europe [12–14]. Outbreaks can include genetically different strains, belonging to different clades of the phylogenetic tree. EV-D68 is divided into four clades (A, B, C and D), the B clade is further divided into three subclades (B1, B2 and B3) [15]. Some papers divides the A cluster into two subclades A1 and A2 [16]. Other papers describe clade D which is divided into two subclades (D1 end D2) [17,15]. Since the epidemiology of EV-D68 in Belgium is unknown, we performed phylogenetic analysis of the Belgian EV-D68-positive samples to investigate the clustering of the samples in the different clades.

2. Objectives

Our laboratory started collecting EV-positive samples for epidemiological surveillance purposes in 1999 [8]. To further investigate the epidemiology of enteroviruses, EV-positive samples collected in Belgium over more than 10 years were molecularly typed. Different types of patient samples with diverse clinical manifestations were used for typing purposes. Since 2010, our laboratory serves as the Belgian National Reference Center for enteroviruses, including polioviruses and parechoviruses. Therefore, enterovirus-typing results are officially reported to the Belgian Institute of Health (Sciensano) every year. Since 2017, our laboratory joined the European non-polio enterovirus network (ENPEN) which is a large collaborative network providing researchers in Europe a platform for EV surveillance and data sharing [3].

3. Study design

Our surveillance study consist out of 1521 samples, 80% originated from patients admitted to the University Hospital Leuven, and 20% came from other regional hospitals and laboratories.

3.1. Sample collection and preparation

Samples were collected from patients hospitalized at the University Hospital, Leuven, from 2007 until 2018. In addition, samples from external laboratories were collected since 2010. Different types of samples such as cerebrospinal fluid (CSF), feces, skin lesions, and samples from the lower and upper respiratory tract were used for the diagnosis of EV infections.

Viral RNA was extracted from CSF and respiratory samples with an easyMAG instrument (bioMérieux, Marcy l'Etoile, France). The viral RNA extraction kit (QIAGEN Benelux) was used for all other sample types.

3.2. Diagnostic qPCR and cell cultures in the University Hospital Leuven

3.2.1. Diagnostic qPCR

In a decade of EV detection, different primer and probe sets were used in a qPCR (supplementary data). CSF samples were tested with an EV qRT-PCR targeting the 5′ NCR. Before 2013, primers E4KB-F/E1R were used with probe EVTM2. From 2013 primer set EVWV1/EVWV2 and probe EVWVSC from Verstrepen et al. [18] were used in order to detect EV-D68. From 2016 onwards, the forward primer EVWV1 was slightly adapted to primer EVfw1. From 2016, respiratory samples were tested with a lab-developed respiratory panel detecting 29 different respiratory pathogens (including EV-D68) in 12 multiplex reactions (not published). In this panel, the RHEV-1/2/3 primer set was used together with probe RHEV-VT for the detection of all enteroviruses and rhinoviruses.

3.2.2. Cell culture

Respiratory samples (before the introduction of the respiratory panel in 2016) and fecal samples were filtered and inoculated on three different cell-lines: PLC/PRF-5, RD and Hela. Cell cultures showing typical cytopathogenic effect (CPE) for enterovirus were diagnosed as positive. All positive cell cultures were molecularly typed. Supernatant of the enterovirus-positive cell culture was 1/100 diluted with PBS and directly used for one-step RT-PCR, no extraction step was needed for this type of samples. This method reduces workload and avoids contamination during extraction. All cell-cultured samples yielded a positive NCR PCR.

3.3. RT-PCR of the NCR, VP1 and VP4/VP2

A 231bp gene fragment in the second half of the 5′ NCR was amplified through the QIAGEN® One-step RT-PCR (QIAGEN Benelux) using primers E4KB-F and E1R and PCR conditions as described previously [8]. To detect rhinoviruses and EV-D68 (formally rhinovirus 89), the NCR primers from Kiang et al. [19] were additionally used since 2014.

Only the inner degenerate primer set ENTNES-F/R of a previously developed nested PCR [8] was used for the amplification of the amino terminal part of VP1 (350bp). The ENTNES primers detect only group B enteroviruses. If this primer set failed to amplify the RNA, different primer pairs were used based on the results of the 5′NCR (Fig. 1). For sequencing the VP4/VP2 region the nested PCR protocol was used of Wisdom et al [20].

3.4. Sequence and sequence analysis

PCR amplicons (5 μl) were purified with 1 μl ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) and Sanger sequenced with the respective PCR primers using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Life Technologies) according to manufactures protocol. Sequencing products were analyzed in an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). From 2017, purified PCR products were send to the Macrogen sequencing facility.

Chromatogram sequencing files were inspected with Chromas (Technelysium Pty Ltd, Tewantin Qld, Australia) and compared to all corresponding EV sequences available in GenBank using BLAST (Basic Local Alignment Search Tool). In order to identify the EV type, a VP1 nucleotide sequence similarity of more than 75% to a certain reference strain in GenBank must be achieved. The partial VP1 sequence was also submitted to the Enterovirus Genotyping Tool: https://www.rivm.nl/mpf/typingtool/enterovirus/ [21].

3.5. Sequence strategy

The VP1 is most applicable for typing; ENTNES F/R was therefore the first primer set used for genotyping positive samples. At the same time, a NCR enterovirus (E4KB-F/E1R) and rhinovirus (RV-5′UTR F/R) PCR was performed (Fig. 1). When the VP1 PCR was negative, different primers sets were used depending on the sequence outcome of the NCR. This conserved region among enteroviruses has been extensively used for diagnostic purposes but cannot be used for molecular typing. However, the NCR result can be an indication of EV species (A, B, C or

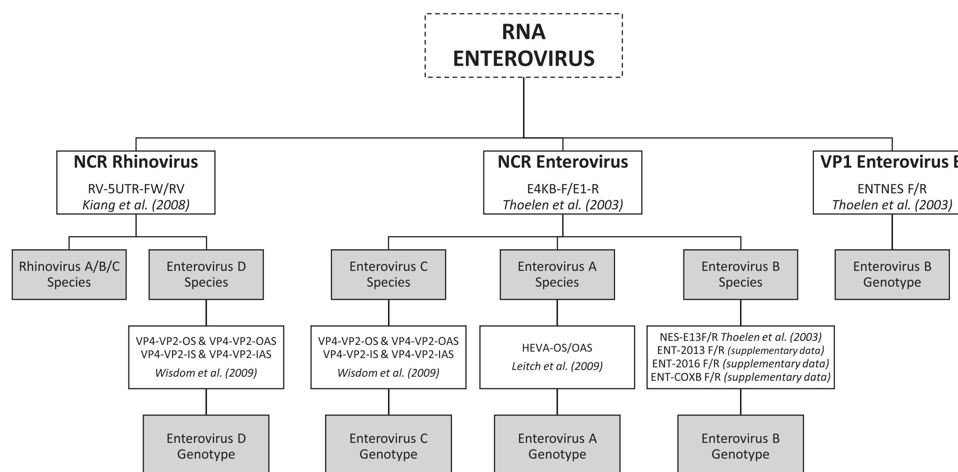

Fig. 1. Flowchart of the strategy for genotyping Enteroviruses.

D) after BLAST analyses or the Enterovirus Genotyping Tool. If the species was indeed an EV-B type, other primers sets were used that were slightly adapted from the ENTNES primers over the years (supplementary data). If an Enterovirus A was the result of the NCR sequence, the primer set of Leich et al. [22] (HEVA-OS and HEVA-OAS) was used to identify the genotype in the VP1. EV-D68 was detected with the sequence of the NCR RV primers [19] and confirmed with the sequencing of the VP4/VP2 PCR product. All EV-C were detected with the NCR EV primers [8] and also confirmed with the Wisdom primers [20]. A flow chart of this sequence strategy is included in Fig. 1. All primers are available in the supplementary data. In some cases, a phylogenetic tree was constructed using 5′NCR sequences of unknown samples together with samples that were already typed with the VP1 region. A dataset of more than 1000 partial 5′NCR sequences was created (data not shown).

All the graphical representation of the data was performed in R using the ggplot2 package [23,24].

3.6. Phylogenetic analyses of EV-D68

The VP4/VP2 region was used for the phylogenetic analyses of EV-D68. Alignments were made with our samples and other strains from GenBank including several EV-D68 strains belonging to different clades and subclades [14,25,26,17].

A phylogenetic tree was constructed with the maximum-likelihood method after model testing in Mega10 [27]. The Kimura 2 parameter model with gamma distribution was the best fitting model for our data set.

4. Results

From the beginning of 2007 until the end of 2018, 1521 EV positive Belgian samples were molecularly typed, presenting 35 different enteroviruses as showed in Table 1. Our sample set exist out of 64% CSF and 36% other sample types (respiratory samples, fecal samples or skin lesions) and were associated with several clinical manifestations. In total, over a decade, 89% (1521/1702) of all EV-positive samples that our lab received were molecularly typed with our method, with a decrease of 19% (28/144) non-genotyped samples in 2007 to 2% (4/166) non-genotyped samples in 2018. EV-B is the causal agent of the majority of aseptic meningitis cases. Our sample set consisted mostly of CSF from patients with meningitis. The single RT-PCR with the inner primer set (ENTNES F/R) [8] gave an adequate positive result in approximately 70% of our samples, because most of the Belgium strains are EV-B species. The other samples were genotyped with different primer sets (Fig. 1). EV-B was the predominant species in our 12 year-survey; in 1419/1521 samples (93.3%). EV-A was detected in 63 cases (4.1%), EV-D was detected in 36 cases (2.4%) and EV-C was only detected in three cases (0.2%).

The heat map (supplementary data) represents the percentage of samples positive for the different EV genotypes per year from 2007 to 2018. The five most abundant enteroviruses in our data set are echovirus 30 (E-30), echovirus 6 (E-6), echovirus 9 (E-9), echovirus 11 (E-11) and coxsackievirus B5 (CV-B5). The abundance percentage of these four enteroviruses was projected over a 12-year window in a line plot (Fig. 2). Every three years we see a peak abundance for E-30 (2007, 2010, 2013, and 2016). For E-6, a 2-, 4- and 2-year interval was seen with peaks in 2009, 2011, 2015 and 2017. E-9 peaked in 2011 and 2014, mostly together with the peak of E-6 and in between the E-30 peaks. The heat map in Fig. 3 represents the percentage of positive samples in different age categories. All EV genotypes can infect all ages. E-30 was more present in children and adults than in infants. In 12 years, EV-A71 was detected in 23 samples (1.5%) and was mostly detected in children under the age of four. EV-D68 was detected in 36 samples (2.4%) and infects almost all ages but mostly the age group between 40–80 years. Another heat map was created to show the distribution of different genotypes on different sample types in Fig. 4. E-30 was mostly detected in CSF but also in feces samples and in the upper respiratory tract. EV-D68 was detected in the lower and upper respiratory tract. CV-A6 was mostly detected in skin lesions.

There was a sampling bias for EV-D68 because we received 20 positive respiratory samples from AZ Sint.Jan Brugge for sequencing. In 2018, this hospital in Bruges detected more than 80 positive EV-D68 samples in daily practice by using TAC (Taqman Array Card) technology for broad respiratory screening of all received respiratory samples (83/7986 samples). In 2015 a specific real-time PCR for EV-D68 [12] was integrated on this micro-array card additional to several broader PCR's for rhino-, entero-and parechovirus screening. Before the outbreak of 2018, EV-D68 was only detected sporadically in Belgium. Not all samples from Bruges were sequenced in the VP4/VP2 region; only 20 samples are included in our data set. In Fig. 5, the phylogenetic analyses show that the respiratory samples from 2016 cluster in subclade B3. Only one sample of 2014 clusters in clade A1, this strain originated from a baby with neurological problems. Two strains from 2017 cluster in A2 (D2) and 2018 samples cluster in subclades B3 and A2 (D1), regardless of the location of detection, Leuven (L) or Bruges (B). All patients in 2018 had mild to severe respiratory symptoms but no neurological symptoms or AFP was reported.

5. Discussion

Typing enteroviruses is important for studying the relationship

Table 1
Overview of all enterovirus types that were found in 12 years. The numbers indicate the number of samples that were typed per year.

SPECIES	Enterovirus	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	Total
EVA	Enterovirus A71	0	0	0	8	0	0	3	0	4	2	0	6	23
EVA	Coxsackie A6	1	0	0	0	0	0	1	0	1	3	5	7	18
EVA	Coxsackie A10	0	0	0	1	0	0	0	2	0	1	0	1	5
EVA	Coxsackie A16	0	0	0	0	0	0	2	0	0	0	2	1	5
EVA	Coxsackie A2	0	0	0	0	0	0	0	1	1	0	2	1	5
EVA	Coxsackie A4	0	0	0	0	0	0	0	0	2	1	0	0	3
EVA	Coxsackie A8	0	0	0	0	0	0	1	0	0	0	0	1	2
EVA	Coxsackie A5	0	0	0	0	0	0	0	0	0	0	1	0	1
EVA	Coxsackie A7	0	0	0	0	0	0	0	0	0	0	1	0	1
EVB	Echovirus 30	89	4	14	52	1	4	40	2	10	123	4	22	365
EVB	Echovirus 6	1	4	8	2	10	2	8	21	21	5	27	11	120
EVB	Echovirus 9	2	3	2	1	8	5	2	42	4	11	8	19	107
EVB	Echovirus 5	0	0	0	0	0	37	1	0	11	13	36	0	98
EVB	Coxsackie B5	1	7	5	5	3	8	7	17	3	11	21	1	89
EVB	Echovirus 11	4	7	5	8	2	20	0	2	15	1	0	20	84
EVB	Coxsackie B4	1	0	10	8	7	5	1	15	2	13	5	9	76
EVB	Coxsackie B1	0	38	0	3	8	1	1	2	5	1	0	2	61
EVB	Coxsackie B3	0	7	0	10	2	1	13	4	2	10	5	3	57
EVB	Echovirus 18	0	1	4	0	1	9	2	7	1	3	23	1	52
EVB	Echovirus 7	0	7	3	0	9	0	0	11	11	2	6	0	49
EVB	Coxsackie A9	3	0	0	16	2	0	14	0	3	0	2	8	48
EVB	Coxsackie B2	4	2	5	10	1	5	5	3	1	2	3	4	45
EVB	Echovirus 16	0	0	0	0	0	0	21	8	0	0	0	0	29
EVB	Echovirus 25	2	0	1	9	3	1	1	4	0	1	0	7	29
EVB	Echovirus 21	0	0	0	0	4	15	1	0	0	1	6	1	28
EVB	Echovirus 20	1	0	0	0	4	9	0	2	1	0	0	7	24
EVB	Echovirus 14	2	5	0	0	0	1	0	1	1	6	0	1	17
EVB	Echovirus 13	5	0	0	0	0	0	1	0	4	0	0	2	12
EVB	Echovirus 17	0	1	0	8	3	0	0	0	0	0	0	0	12
EVB	Echovirus 3	0	2	0	0	5	0	0	1	0	3	1	0	12
EVB	Echovirus 4	0	0	0	0	0	0	0	0	0	1	2	1	4
EVB	Echovirus 2	0	0	0	0	0	0	0	1	0	0	0	0	1
EVC	Coxsackie A21	0	0	0	0	0	0	0	0	0	0	1	1	2
EVC	Enterovirus C104	0	0	0	0	0	0	0	0	0	0	0	1	1
EVD	Enterovirus D68	0	0	0	0	0	0	0	2	0	8	2	24	36
Total typed		116	88	57	141	73	123	125	148	103	222	163	162	1521
Not typed		28	23	17	25	12	9	21	25	6	6	5	4	181
Total samples		144	111	74	166	85	132	146	173	109	228	168	166	1702

between EV genotype and clinical syndrome, for finding new types or variants, and for epidemiological surveillance. By directly using the nucleotide sequence of the VP1-amplicons in BLAST and/or the Enterovirus Genotyping Tool, most of the EV-B samples were molecularly typed with only one PCR. When the VP1 RT-PCR was negative, the highly conserved 5′NCR was used as a prediction of the enterovirus type [28]. A major drawback of using 5′NCR-based RT-PCR assays is their inability to identify the enterovirus type, furthermore, recombination of EV is a frequent event and appears in the same species between structural and non-structural region mostly in species A–C [29]. However, the predictive value of the 5′NCR can be exploited in subsequent VP1 molecular typing strategies [28].

Our approach is very sensitive and a rapid method to identify the EV type in different types of patient samples. All EV positive samples are typed and reported to the physician in less than one week. The most frequently isolated EV type in our surveillance study of more than a decade is E-30. This EV type is a known cause of massive outbreaks of aseptic meningitis in temperate climates. The genotype distribution we observed largely corresponds with observations in other European countries and in the United States [30–32]. The predominant enterovirus type varies from year to year. Every 3 years E-30 is the most prevalent EV in our dataset alternated with E-6 and E-9 peaks. E-30 epidemics occurs usually as repeated cycles of emerging and dominating virus lineages that causes outbreaks every 3–5 years. They can cover large geographical areas and can disappear to re-emerge later [33,34].

EV-C104 was found once in 1521 samples. This relatively rare EV was first described in 2009 in Switzerland [35] since then, it was found only sporadically. In Europe, there were some cases reported with EV-C104 in Italy [36], the Netherlands [37] and Denmark [38].

Outbreaks of EV-D68 are detected since 2014 all over Europe. In 2016, there was an outbreak in the Netherlands [39], in Stockholm [14] and in Italy [25]. In our data set, there was only an upsurge in 2018, all with severe respiratory symptoms but no neurological problems or paralysis. Phylogenetic analyses showed that all Belgium samples are located in three different clades A1, B3 and A2 (D1). Only the one strain in clade A1 gave neurological symptoms but no AFP. The samples from the outbreak in 2018 coexisted in two subclades, B3 and A2 (D1) and only mild to severe respiratory infections were seen. To improve the monitoring of the clinical and molecular epidemiology of EV-D68 a global consensus of the nomenclature of clades and subclades should be pursued.

During a decade of EV surveillance, our EV genotyping strategy allowed 89% of all EV-positive samples to be molecularly typed. This strategy improved over time and genotyped 98% of the EV strains in 2018. Between 2007 and 2018, EV-B was the most detected species and E-30 the most identified genotype with epidemic peaks every three years. In total, 35 different EV genotypes were encountered. Typing enteroviruses remains an interesting challenge and next generation sequencing will continue to improve genotyping in the future.

Author statement

All authors provided critical feedback and helped shape this manuscript.

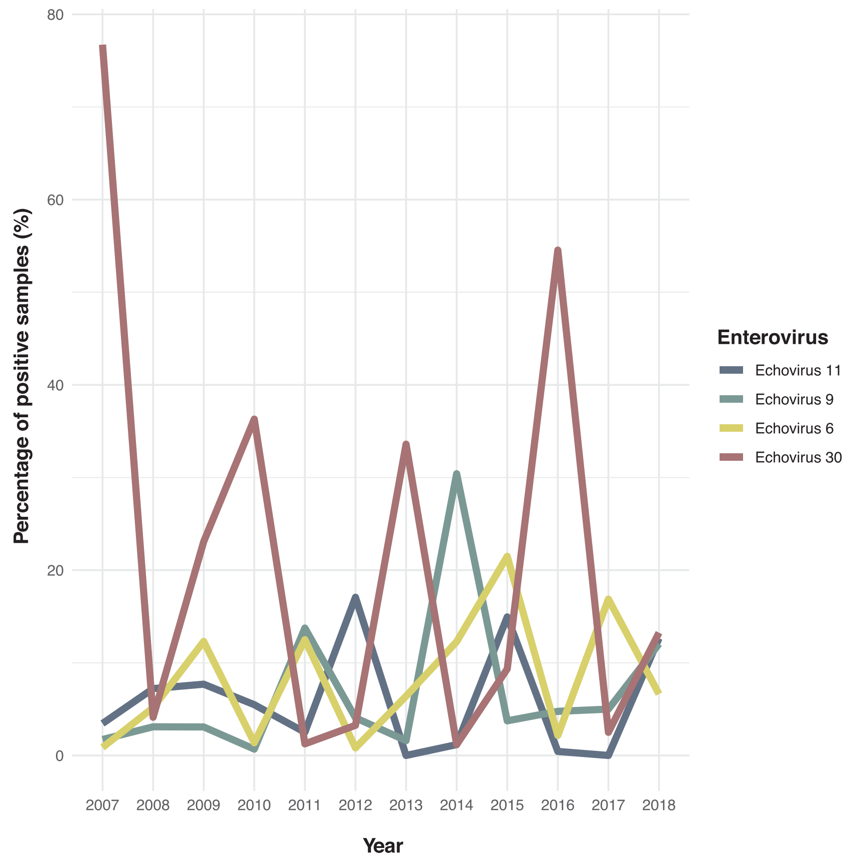

Fig. 2. Line plot showing the percentage of samples that are positive for the four most abundant enteroviruses (E-30, E-6, E-9 and E-11) detected from 2007 to 2018.

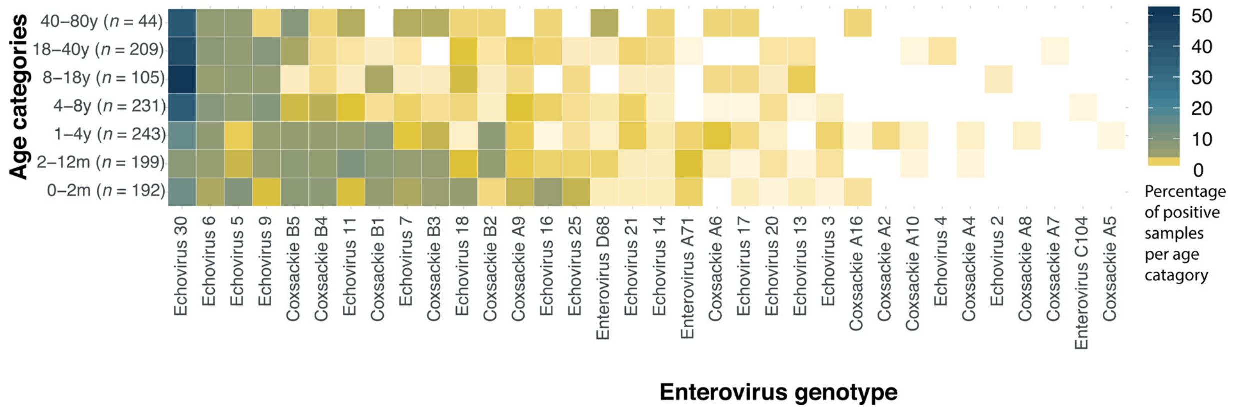

Fig. 3. Heat map of EV genotypes in relation with the age distribution. The color bar indicates the percentage of samples per age category (y-axis) that were positive for every enterovirus (x-axis).

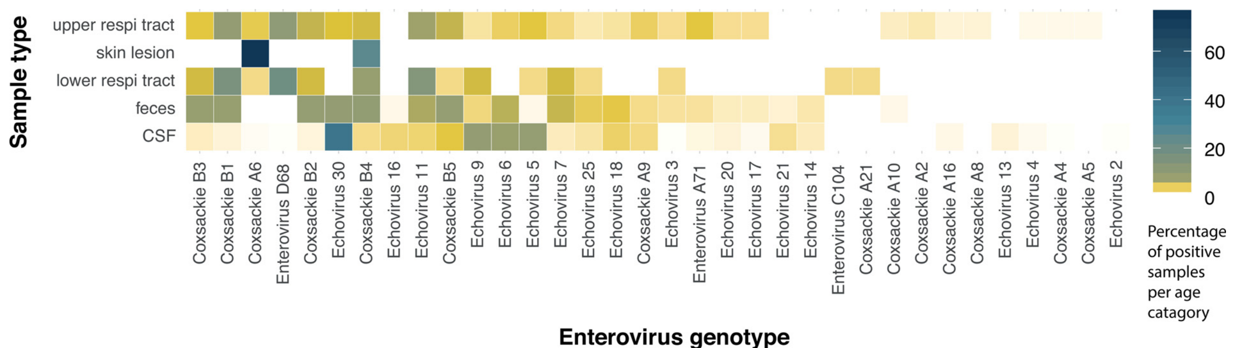

Fig. 4. Heat map of EV genotypes in relation with the sample type distribution. The color bar indicates the percentage sample type (y-axis) that were positive for every enterovirus (x-axis).

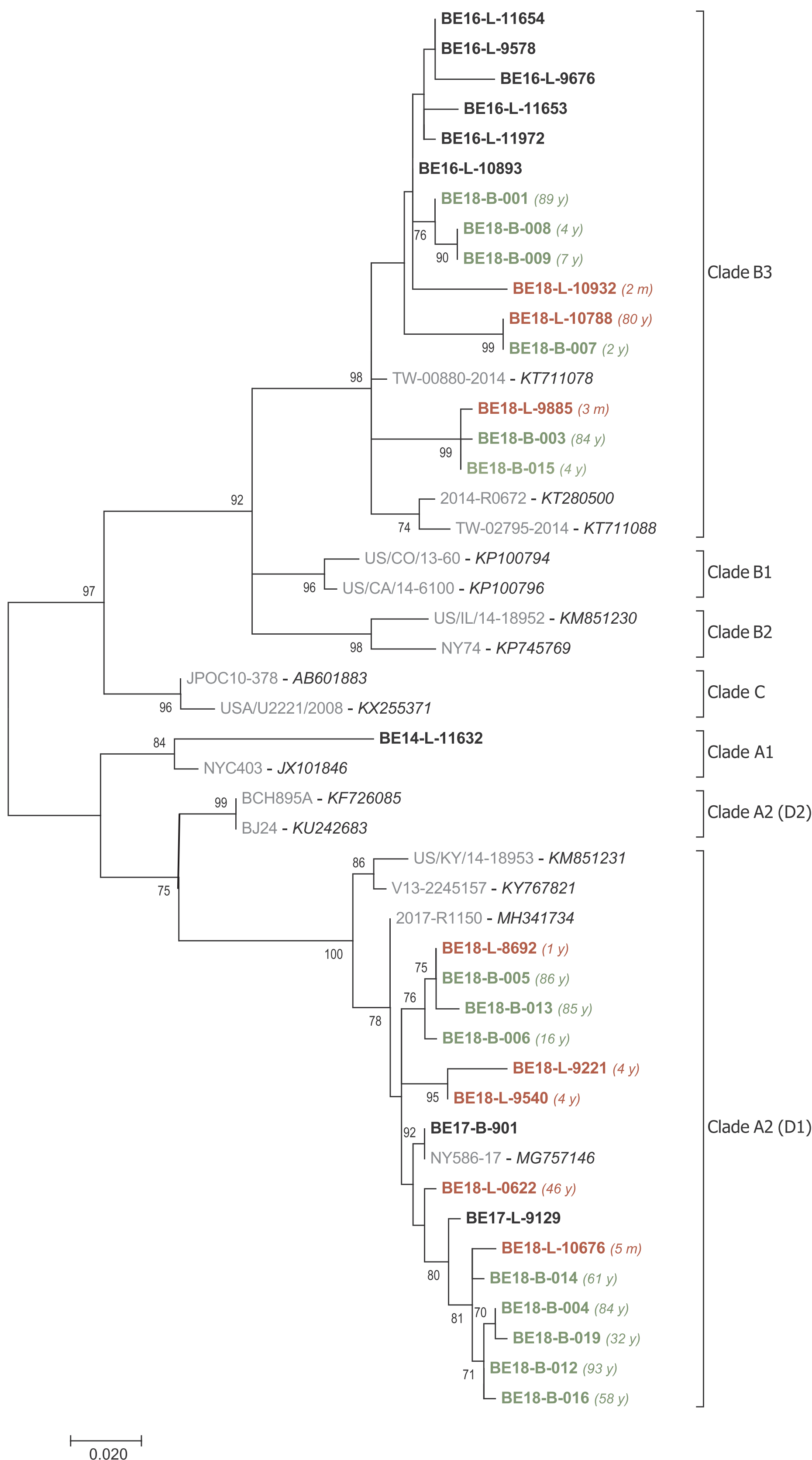

Fig. 5. Phylogenetic analysis using the VP4/VP2 gene of 47 EV-D68 strains (557 nucleotides). The trees were constructed after model testing using the Maximum Likelihood method and Kimura 2-parameter model with Gamma distribution 5. Bootstrap values calculated from 1000 trees. Evolutionary analyses were conducted in MEGA X. Different clades were detected and Belgium strains are indicated in bold as BE and the year of origin, place of detection L = Leuven, B = Bruges with the age of the patient. The color green indicates samples from Bruges and red are the samples from Leuven in the upsurge of 2018. We submitted our EV-D68 sequences to GenBank under accession numbers MN310412-MN310440.

Transparency document

The Transparency document associated with this article can be found in the online version.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jcv.2019.104205.

References

[1] H.A. Rotbart, H.A. Rotbart, Meningitis and Encephalitis, ASM Press, washington DC, 1995.
[2] S. Suresh, S. Forgie, J. Robinson, Non-polio Enterovirus detection with acute flaccid paralysis: a systematic review, J. Med. Virol. 90 (2018) 3–7, https://doi.org/10.1002/jmv.24933.
[3] H. Harvala, E. Broberg, K. Benschop, N. Berginc, S. Ladhani, P. Susi, C. Christiansen, J. McKenna, D. Allen, P. Makiello, G. McAllister, M. Carmen, K. Zakikhany, R. Dyrdak, X. Nielsen, T. Madsen, J. Paul, C. Moore, K. von Eije, A. Piralla, M. Carlier, L. Vanoverschelde, R. Poelman, A. Anton, F.X. Lopez-Labrador, L. Pellegrinelli, K. Keeren, M. Maier, H. Cassidy, S. Derdas, C. Savolainen-Kopra, S. Diedrich, S. Nordbo, J. Buesa, J.L. Bailly, F. Baldanti, A. MacAdam, A. Mirand, S. Dudman, I. Schuffenecker, S. Kadambari, J. Neyts, M.J. Griffiths, J. Richter, C. Margaretto, S. Govind, U. Morley, O. Adams, S. Krokstad, J. Dean, M. Pons-Salort, B. Prochazka, M. Cabrerizo, M. Majumdar, G. Nebbia, M. Wiewel, S. Cottrell, P. Coyle, J. Martin, C. Moore, S. Midgley, P. Horby, K. Wolthers, P. Simmonds, H. Niesters, T.K. Fischer, Recommendations for enterovirus diagnostics and characterisation within and beyond Europe, J. Clin. Virol. 101 (2018) 11–17, https://doi.org/10.1016/j.jcv.2018.01.008.
[4] V. Caro, S. Guillot, F. Delpeyroux, R. Crainic, Molecular strategy for “serotyping” of human enteroviruses, J. Gen. Virol. 82 (2001) 79–91, https://doi.org/10.1099/0022-1317-82-1-79.
[5] M.S. Oberste, K. Maher, M.R. Flemister, G. Marchetti, D.R. Kilpatrick, M.A. Pallansch, Comparison of classic and molecular approaches for the identification of untypeable enteroviruses, J. Clin. Microbiol. 38 (2000) 1170–1174 http://www.ncbi.nlm.nih.gov/pubmed/10699015.
[6] H. Norder, L. Bjerregaard, L.O. Magnius, Homotypic echoviruses share aminoterminal VP1 sequence homology applicable for typing, J. Med. Virol. 63 (2001) 35–44 http://www.ncbi.nlm.nih.gov/pubmed/11130885.
[7] M.S. Oberste, K. Maher, D.R. Kilpatrick, M.A. Pallansch, Molecular evolution of the human enteroviruses: correlation of serotype with VP1 sequence and application to picornavirus classification, J. Virol. 73 (1999) 1941–1948 http://www.ncbi.nlm.nih.gov/pubmed/9971773.
[8] I. Thoelen, P. Lemey, I. Van Der Donck, K. Beuselinck, A.M. Lindberg, M. Van Ranst, Molecular typing and epidemiology of enteroviruses identified from an outbreak of aseptic meningitis in Belgium during the summer of 2000, J. Med. Virol. 70 (2003) 420–429, https://doi.org/10.1002/jmv.10412.
[9] M.S. Oberste, W.A. Nix, K. Maher, M.A. Pallansch, Improved molecular identification of enteroviruses by RT-PCR and amplicon sequencing, J. Clin. Virol. 26 (2003) 375–377 http://www.ncbi.nlm.nih.gov/pubmed/12637088.
[10] W.A. Nix, M.S. Oberste, M.A. Pallansch, Sensitive, seminested PCR amplification of VP1 sequences for direct identification of all enterovirus serotypes from original clinical specimens, J. Clin. Microbiol. 44 (2006) 2698–2704, https://doi.org/10.1128/JCM.00542-06.
[11] K. Messacar, M.J. Abzug, S.R. Dominguez, Outbreak of enterovirus D68 in North America, J. Med. Virol. 88 (2016) (2014) 739–745, https://doi.org/10.1002/jmv.24410.
[12] R. Poelman, E.H. Scholvinck, R. Borger, H.G. Niesters, C. van Leer-Buter, The emergence of enterovirus D68 in a Dutch University Medical Center and the necessity for routinely screening for respiratory viruses, J. Clin. Virol. 62 (2015) 1–5, https://doi.org/10.1016/j.jcv.2014.11.011.
[13] L. Gimferrer, M. Campins, M.G. Codina, J. Esperalba, M.D. Martin, F. Fuentes, T. Pumarola, A. Anton, First Enterovirus D68 (EV-D68) cases detected in hospitalised patients in a tertiary care university hospital in Spain, October 2014, Enferm. Infecc. Microbiol. Clin. (2015), https://doi.org/10.1016/j.eimc.2015.01.008.
[14] R. Dyrdak, M. Grabbe, B. Hammas, J. Ekwall, K.E. Hansson, J. Luthander, P. Naucler, H. Reinius, M. Rotzen-Ostlund, J. Albert, Outbreak of enterovirus D68 of the new B3 lineage in Stockholm, Sweden, August to September 2016, Euro Surveill. 21 (2016), https://doi.org/10.2807/1560-7917.ES.2016.21.46.30403.
[15] Y.N. Gong, S.L. Yang, S.R. Shih, Y.C. Huang, P.Y. Chang, C.G. Huang, K.C. Kao, H.C. Hu, Y.C. Liu, K.C. Tsao, Molecular evolution and the global reemergence of enterovirus D68 by genome-wide analysis, Medicine 95 (2016) e4416, https://doi.org/10.1097/MD.0000000000004416.
[16] R. Dyrdak, M. Mastafa, E. Hodcroft, R. Neher, J. Albert, Comprehensive analysis of intra- and interpatient evolution of enterovirus D68 by whole-genome deep sequencing, BioRxiv (2018), https://doi.org/10.1101/420836.
[17] C.C.Y. Yip, J.Y.C. Lo, S. Sridhar, D.C. Lung, S. Luk, K.H. Chan, J.F.W. Chan, V.C.C. Cheng, P.C.Y. Woo, K.Y. Yuen, S.K.P. Lau, First report of a fatal case associated with EV-D68 infection in Hong Kong and emergence of an interclade recombinant in China revealed by genome analysis, Int. J. Mol. Sci. (2017), https://doi.org/10.3390/ijms18051065.
[18] W.A. Verstrepen, P. Bruynseels, A.H. Mertens, Evaluation of a rapid real-time RT-PCR assay for detection of enterovirus RNA in cerebrospinal fluid specimens, J. Clin. Virol. 25 (Suppl 1) (2002) S39–S43 http://www.ncbi.nlm.nih.gov/pubmed/12091080.
[19] D. Kiang, I. Kalra, S. Yagi, J.K. Louie, H. Boushey, J. Boothby, D.P. Schnurr, Assay for 5′ noncoding region analysis of all human rhinovirus prototype strains, J. Clin. Microbiol. 46 (2008) 3736–3745, https://doi.org/10.1128/JCM.00674-08.
[20] A. Wisdom, E.C. Leitch, E. Gaunt, H. Harvala, P. Simmonds, Screening respiratory samples for detection of human rhinoviruses (HRVs) and enteroviruses: comprehensive VP4-VP2 typing reveals high incidence and genetic diversity of HRV species C, J. Clin. Microbiol. 47 (2009) 3958–3967, https://doi.org/10.1128/JCM.00993-09.
[21] A. Kroneman, H. Vennema, K. Deforche, H. v d Avoort, S. Penaranda, M.S. Oberste, J. Vinje, M. Koopmans, An automated genotyping tool for enteroviruses and noroviruses, J. Clin. Virol. 51 (2011) 121–125, https://doi.org/10.1016/j.jcv.2011.03.006.
[22] E.C. Leitch, H. Harvala, I. Robertson, I. Ubillos, K. Templeton, P. Simmonds, Direct identification of human enterovirus serotypes in cerebrospinal fluid by amplification and sequencing of the VP1 region, J. Clin. Virol. 44 (2009) 119–124, https://doi.org/10.1016/j.jcv.2008.11.015.
[23] R Core Team, R, A Language and Environment for Statistical Computing, (2018) https://www.r-project.org/.
[24] L. Wilkinson, ggplot2: Elegant Graphics for Data Analysis by WICKHAM, H., Biometrics, (2011), https://doi.org/10.1111/j.1541-0420.2011.01616.x.
[25] A. Piralla, N. Principi, L. Ruggiero, A. Girello, F. Giardina, E. De Sando, S. Caimmi, S. Bianchini, G.L. Marseglia, G. Lunghi, F. Baldanti, S. Esposito, Enterovirus-D68 (EV-D68) in pediatric patients with respiratory infection: the circulation of a new B3 clade in Italy, J. Clin. Virol. 99–100 (2018) 91–96, https://doi.org/10.1016/j.jcv.2018.01.005.
[26] R. Dyrdak, M. Rotzen-Ostlund, A. Samuelson, M. Eriksson, J. Albert, Coexistence of two clades of enterovirus D68 in pediatric Swedish patients in the summer and fall of 2014, Infect. Dis. 47 (2015) 738–742, https://doi.org/10.3109/23744235.2015.1047402.
[27] S. Kumar, G. Stecher, M. Li, C. Knyaz, K. Tamura, M.E.G.A. X, Molecular evolutionary genetics analysis across computing platforms, Mol. Biol. Evol. (2018), https://doi.org/10.1093/molbev/msy096.
[28] I. Thoelen, E. Moës, P. Lemey, S. Mostmans, E. Wollants, A.M. Lindberg, A.-M. Vandamme, M. Van Ranst, Analysis of the serotype and genotype correlation of VP1 and the 5′ noncoding region in an epidemiological survey of the human enterovirus B species, J. Clin. Microbiol. 42 (2004), https://doi.org/10.1128/JCM.42.3.963-971.2004.
[29] E.C. McWilliam Leitch, M. Cabrerizo, J. Cardosa, H. Harvala, O.E. Ivanova, S. Koike, A.C. Kroes, A. Lukashev, D. Perera, M. Roivainen, P. Susi, G. Trallero, D.J. Evans, P. Simmonds, The association of recombination events in the founding and emergence of subgenogroup evolutionary lineages of human enterovirus 71, J. Virol. 86 (2012) 2676–2685, https://doi.org/10.1128/JVI.06065-11.
[30] M.S. Oberste, K. Maher, M.L. Kennett, J.J. Campbell, M.S. Carpenter, D. Schnurr, M.A. Pallansch, Molecular epidemiology and genetic diversity of echovirus type 30 (E30): genotypes correlate with temporal dynamics of E30 isolation, J. Clin. Microbiol. 37 (1999) 3928–3933 http://www.ncbi.nlm.nih.gov/pubmed/10565909.
[31] G. Trallero, I. Casas, A. Tenorio, J.E. Echevarria, A. Castellanos, A. Lozano, P.P. Brena, Enteroviruses in Spain: virological and epidemiological studies over 10 years (1988-97), Epidemiol. Infect. 124 (2000) 497–506 http://www.ncbi.nlm.nih.gov/pubmed/10982074.
[32] C. Centers for Disease, Prevention, Outbreaks of aseptic meningitis associated with echoviruses 9 and 30 and preliminary surveillance reports on enterovirus activity–United States, 2003, MMWR Morb. Mortal. Wkly. Rep. 52 (2003) 761–764 http://www.ncbi.nlm.nih.gov/pubmed/12917581.
[33] E.C. McWilliam Leitch, J. Bendig, M. Cabrerizo, J. Cardosa, T. Hyypia, O.E. Ivanova, A. Kelly, A.C. Kroes, A. Lukashev, A. MacAdam, P. McMinn, M. Roivainen, G. Trallero, D.J. Evans, P. Simmonds, Transmission networks and population turnover of echovirus 30, J. Virol. 83 (2009) 2109–2118, https://doi.org/10.1128/JVI.02109-08.
[34] E.K. Broberg, B. Simone, J. Jansa, C. The Eu/Eea member state, upsurge in echovirus 30 detections in five EU/EEA countries, April to September, 2018, Euro Surveill. 23 (2018), https://doi.org/10.2807/1560-7917.ES.2018.23.44.1800537.
[35] C. Tapparel, T. Junier, D. Gerlach, S. Van Belle, L. Turin, S. Cordey, K. Mühlemann, N. Regamey, J.D. Aubert, P.M. Soccal, P. Eigenmann, E. Zdobnov, L. Kaiser, New respiratory enterovirus and recombinant rhinoviruses among circulating picornaviruses, Emerg. Infect. Dis. (2009), https://doi.org/10.3201/eid1505.081286.
[36] A. Piralla, F. Rovida, F. Baldanti, G. Gerna, Enterovirus genotype EV-104 in humans, Italy, 2008-2009, Emerg. Infect. Dis. (2010), https://doi.org/10.3201/eid1606.091533.
[37] C.C. Van Leer-Buter, R. Poelman, R. Borger, H.G.M. Niesters, Newly identified enterovirus C genotypes, identified in the Netherlands through routine sequencing of

all enteroviruses detected in clinical materials from 2008 to 2015, J. Clin. Microbiol. (2016), https://doi.org/10.1128/jcm.00207-16.
[38] C. Barnadas, S.E. Midgley, M.N. Skov, L. Jensen, M.W. Poulsen, T.K. Fischer, An enhanced Enterovirus surveillance system allows identification and characterization of rare and emerging respiratory enteroviruses in Denmark, 2015–16, J. Clin. Virol. (2017), https://doi.org/10.1016/j.jcv.2017.05.017.
[39] M. Knoester, E.H. Scholvinck, R. Poelman, S. Smit, C.L. Vermont, H.G. Niesters, C.C. Van Leer-Buter, Upsurge of enterovirus D68, the Netherlands, 2016, Emerg. Infect. Dis. 23 (2017) 140–143, https://doi.org/10.3201/eid2301.161313.