030

Genetic Diversity of Enterovirus in Belgium

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1. BACKGROUND

Human enteroviruses (EV) infect millions of people worldwide each year. Some infections are asymptomatic but enteroviruses have been associated with a wide spectrum of common and uncommon illnesses, such as common cold, acute hemorrhagic conjunctivitis, myocarditis and poliomyelitis. Enteroviruses B are the most common cause of aseptic meningitis worldwide. This mainly affects young children and in most cases requires hospitalization. Enteroviral meningitis often appears in the form of outbreaks and peaks during the summer and early fall. Enterovirus D68 (EVD68) is associated with respiratory illness and neurological complications and enterovirus A 71 (EVA71) is a common causative agent of hand, foot, and mouth disease (HFMD). We investigated the different EV genotypes that were circulating in Belgium in 2016.

2. METHODS

2.1. Sample collection

Samples were collected from different hospitals in Belgium but mostly patients hospitalized at UZ Leuven during 2016. Different kinds of samples such as cerebrospinal fluid (CSF), feces, pharyngeal swabs and aspirates were used for the diagnosis of EV infection.
CSF and respiratory samples were diagnosed using a 5'-NCR qRT-PCR. Fecal samples and other respiratory samples were inoculated on different cells (PLC/PRF5, RD and Hela). Cell cultures showing typical cytopathogenic effect typical for EV were diagnosed as positive at the university hospital.

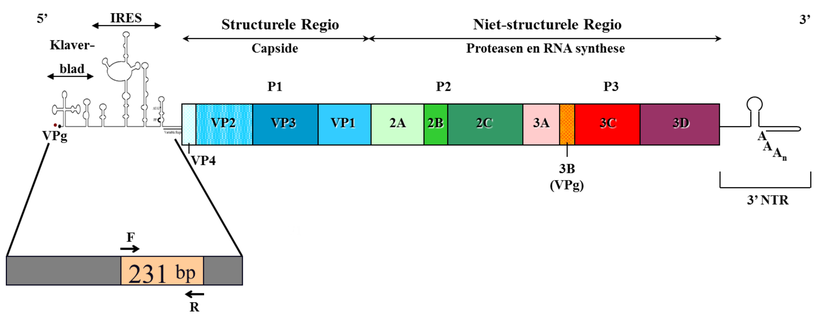
2.2. Sample preparation

Viral RNA was extracted from CSF and respiratory samples at the university hospital Leuven with an easyMAG instrument (bioMérieux, Marcy l'Etoile, France).
Supernatant of enterovirus-positive cell cultures was diluted 1/100 with PBS and directly used for one-step RT-PCR, no extraction step is needed.

2.3. Diagnostic 5'-NCR RT-PCR

A 231 bp gene fragment in the second half of the 5' noncoding region (Fig. 1) was amplified through Qiagen® OneStep RT-PCR (Qiagen Benelux) using 5 µl viral RNA template and a 5'-NCR-specific primer set: E4KB-F and E1R (Thoelen et al., 2003). The PCR was carried out in a 25 µl reaction volume containing 5 µl 5X Qiagen OneStep RT-PCR buffer, 1 µl dNTP mix containing 200 µM of each dNTP, 1 µl Qiagen OneStep RT-PCR enzyme mix, 60 pmol of each primer and RNase-free water. The amplification profile involved a reverse transcription step at 50°C for 30 min, followed by PCR activation at 95°C for 15 min, 40 cycles of amplification (94°C, 30 sec; 57°C, 30 sec; 72°C, 1 min) and a final extension of 10 min at 72°C in a Biometra T3000 thermocycler (Biometra, westburg NV, Netherlands). The PCR products were run on a polyacrylamide gel, stained with ethidium bromide and visualized under UV light.

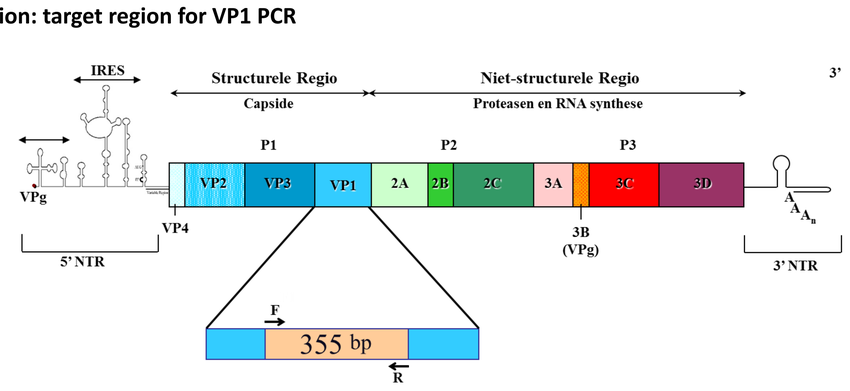
Figure 1: EV genome organization: target region for 5'-NCR PCR



2.4. Typing VP1 RT-PCR

The inner degenerate primer pair ENTNES-F/R of a formerly developed nested PCR (Thoelen et al., 2003) was used for the amplification of the amino-terminal part of VP1 (Fig. 2).
A VP1 OneStep RT-PCR (Qiagen Benelux) was carried out using 5 µl viral RNA template in a 25 µl reaction volume containing 5 µl 5X Qiagen OneStep RT-PCR buffer, 1 µl dNTP mix containing 200µM of each dNTP, 1 µl Qiagen OneStep RT-PCR enzyme mix, 60 pmol of each primer and RNase-free water. The 355 bp PCR amplicons were subjected to polyacrylamide gel electrophoresis and visualized. The ENTNES primers detect only group B enteroviruses. If this primer set failed to amplify the RNA, additional primer sets were used (Table 1).

Figure 2: EV genome organization: target region for VP1 PCR



2.5. Sequence analysis

PCR amplicons (5µl) were purified with 1 µl ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) and sequenced with their respective PCR primers using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Life Technologies) according to the manufacturer's protocol. Sequencing products were analyzed on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems).
Chromatogram sequencing files were inspected with chromas 2.6.2 (Technelysium Pty Ltd, Tewantin Qld, Australia) and compared to all corresponding EV sequences available in GenBank using BLAST (Basic Local Alignment Search Tool). To positively identify the EV type, a VP1 nucleotide sequence identity of more than 75% to a reference strain in GenBank had to be achieved. The partial VP1 sequence was also used in the online enteroviral typing tool (Kroneman et al., 2011).

Table 1: Oligonucleotide primers for NCR and VP1 RT-PCR

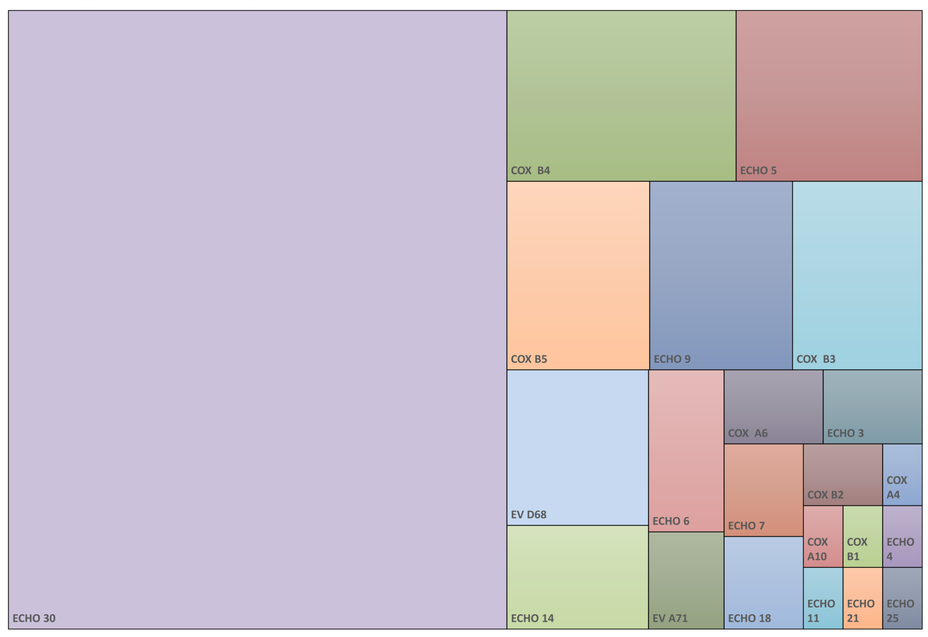
Primer	Enterovirus specificity	Sequence	Reference
E4KB-F	All	AAG GTG YGA AGA GYC TAT TGA GCT A	Thoelen et al., 2003
E1-R	All	CAC CGG ATG GCC AAT CCA	
ENTNES-F	Group B	GAY ACW ATG CAR ACV MGR CAY GT	Thoelen et al., 2003
ENTNES-R	Group B	GRG CAT TVC CYT CTG TCC A	
HEVA-OS	Group A	CCN TGG ATH AGY AAC CAN CAY T	Leitch et al., 2009
HEVA-AOS	Group A	GGR TAN CCR TCR TAR AAC CAY TG	
RV-5UTR-FW	EVD68	CAA GCA ACT TCT GTT TCC C	Kiang et al., 2008
RV-5UTR-RV	EVD68	CAC GGA CAC CCA AAG TAG T	

3. RESULTS

In 2016, positive enterovirus samples were molecularly typed based on the nucleotide sequence of a VP1 amplicon, obtained using the ENTNES primers, by using BLAST and/or the enterovirus typing tool. If the VP1 RT-PCR was negative, the highly conserved sequence of the 5'-NCR was used to predict the enterovirus type. Enterovirus species (A, B, C, D, ..) can be determined using this NCR sequence. A phylogenetic tree was also constructed using partial 5'-NCR sequences of samples that were typed based on the VP1 region. This information can be used to decide which additional primer sets to use in the VP1 RT-PCR. The primers that were mostly used are presented in table 1, all other used primers will be published (manuscript in preparation).
In total, 247 positive enterovirus samples were analyzed. 231 samples could be assigned to different genotypes. For 16 samples, only the species could be assigned based on the 5'-NCR sequence (7 EVA and 9 EVB), probably because the viral load was too low.

Table 2: Distribution of enterovirus genotypes in Belgium 2016

Genotype	N	%
Echovirus 30	126	54,5
Coxsackievirus B4	16	6,9
Echovirus 5	13	5,6
Echovirus 9	11	4,8
Coxsackievirus B5	11	4,8
Coxsackievirus B3	10	4,3
Enterovirus D68	9	3,9
Echovirus 14	6	2,6
Echovirus 6	5	2,2
Enterovirus A71	3	1,3
Echovirus 7	3	1,3
Echovirus 3	3	1,3
Echovirus 18	3	1,3
Coxsackievirus A6	3	1,3
Coxsackievirus B2	2	0,9
Echovirus 4	1	0,4
Echovirus 25	1	0,4
Echovirus 21	1	0,4
Echovirus 11	1	0,4
Coxsackievirus A4	1	0,4
Coxsackievirus B1	1	0,4
Coxsackievirus A10	1	0,4
TOTAL	231	100,0



Twenty-two different genotypes were identified in Belgium in 2016, with echovirus 30 being the most prevalent one (54,5%), followed by coxsackievirus B4 (6,9%), echovirus 5 (5,6%), echovirus 9 and coxsackievirus B5 (4,8%).
EVD68 was detected in 9 respiratory samples (3,9%). These were sporadic cases in July, August and September, mostly from UZ Leuven hospitalized transplant patients.
EVA71 was detected in only 3 cases (1,3%), 2 in respiratory samples in March and June and 1 in a fecal sample in March.

4. CONCLUSIONS

Typing enteroviruses is important for studying the relationship between EV genotypes and clinical syndromes, for finding new types or variants, and for epidemiological surveillance.
Enterovirus D68 was already known to be the cause of mild to severe respiratory infections, but in the last few years it has also been associated with acute flaccid myelitis, a polio-like disorder. Enterovirus A71 is associated with HFMD outbreaks with fatal neurological complications.
As the Belgian national reference center for enteroviruses, it is our responsibility to, each year, report all detected enteroviruses to the scientific institute for public health (WIV-ISP).

The assays used were developed in a previous study aiming near-universal enteroviral diagnosis and adequate molecular typing of the most common species B EV directly from clinical specimens. For detection of EVD68 and EVA71 other primer sets were used with the same RT-PCR conditions. Only 5,2 % tested positive for these genotypes and no neurological problems were detected in these patients.

The predominant EV type was echovirus 30. This is the most frequently isolated EV and 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.

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