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National Surveillance of Enterovirus D68 Upsurge, France, 2024

Appendix

Reverse transcription and amplification protocols of the complete genome sequencing of EV-D68 in the National Reference Laboratory 1 (NRL1, Clermont-Ferrand, France).

In NRL1, complete genome sequencing was done after amplification of full-length genome. For reverse transcription, 5.5 µL of extracted RNA was incubated at 65°C for 5 minutes with 0.5 µL of the reverse primer polyT3NC_EVD68_R (5′-TTTTTTTTTTTTTTTTTTTTTTTTTGGYCCCAAGTGRCCAA AATTTACCTC-3′, 2 µM) and 0.5 µL of dNTPs (10 mM), then held at 4°C. Subsequently, 0.5 µL of SuperScript IV reverse transcription (200 U/µL; Thermo Fisher Scientific), 0.5 µL of RNaseOUT (40 U/µL; Thermo Fisher Scientific), 0.5 µL of DTT (0.1 M), and 2 µL of 5× reaction buffer were added to the reaction mix, yielding a final volume of 10 µL. Reverse transcription was performed at 55°C for 30 minutes, followed by enzyme inactivation at 80°C for 10 minutes. For near full-length genome amplification, 2 µL of cDNA was mixed with 6 µL of nuclease-free water, 10 µL of 2× SuperFi II PCR Master Mix (Thermo Fisher Scientific), and 1 µL each of the forward primer IFT7 EVD68_S (5′-GACAGCTTATCATCGTAATACGACTCACTATAGGGTTAAAACAGCCTTGGGGTTG-3′) and reverse primer R3D-EVD68 (5′-CGTCTAAGACTAGARTATGCAGGTAGTG-3′). The thermal cycling protocol was as follows: initial denaturation at 98°C for 1 minute; 41 cycles of denaturation at 98°C for 5 seconds, annealing at 60°C for 10 seconds, and extension at 72°C for 4 minutes and 30 seconds; followed by a final extension at 72°C for 5 minutes. To amplify the 3′ terminal region of the genome (~150 nt), cDNA synthesis was done as described above, using the reverse primer M13-dT18_R (5′-CAGGAAACAGCTATGACCGTTTTTTTTTTTTTTTTTT-3′). PCR amplification was then performed using SuperFi I DNA polymerase (Thermo Fisher Scientific), the forward primer

EVD68_7029_S (5′-ATTAGTAATGACACCAGC-3′), and the reverse primer M13-dT18_R, under the following cycling conditions: 98°C for 1 minute; 41 cycles of 98°C for 5 seconds, 55°C for 10 seconds, and 72°C for 20 seconds; followed by a final extension at 72°C for 5 minutes.

Sequencing libraries were prepared using the Illumina DNA Prep kit according to the manufacturer's instructions. Sequencing was carried out on the Illumina NextSeq 550 platform using a MidOutput flow cell (2×75 bp paired-end reads). Raw sequencing reads were demultiplexed using **bcl2fastq** (v2.20.0.422), and quality was assessed using **FastQC** (v0.11.9). Adaptor trimming and quality filtering were performed with **Trimmomatic**, and de novo assembly was conducted using **SPAdes** (v3.15.3).

Seven amplicons were sequenced with Oxford Nanopore technology as previously described (Mirand et al., 2025; doi: 10.1080/22221751.2025.2525266).

Appendix Table. EV-D68 subgenotype proportions among children and adults, France, 2024

EV-D68 infections with known subgenotype	Subgenotype B3, no. (%)	Subgenotype A2, no. (%)	Total
Children	227 (61.8)	140 (38.2)	367
Adults	37 (10.5)	317 (89.5)	354
Total	269 (36.7)	452 (63.3)	721