

# Adeno-Associated Virus 2 and Human Adenovirus F41 in Wastewater during Outbreak of Severe Acute Hepatitis in Children, Ireland

## Appendix

### Additional Materials and Methods

#### Calculation of Limit of Detection and Limit of Quantification for qPCR

The amplification efficiency of each assay was calculated with the slope of the standard curve using the equation  $E = 10^{(-1/\text{slope})}$  (1). All reaction efficiencies were between 90 and 110%. The limit of quantification (LOQ) was specified as the lowest concentration of DNA quantified within 0.5 standard deviations of the  $\log_{10}$  concentration, and the limit of detection (LOD) was specified as the lowest concentration of DNA detected in 95% or more of replicates (2,3).

#### Nanopore Sequencing of HAdV Hexon Subgenomic Fragments from Wastewater

200 fmol of amplicons were end-repaired by the NEBNext Ultra II End Repair/dA-Tailing Module (New England Biolabs, <https://www.neb.com>) with 1.75  $\mu\text{l}$  of buffer and 0.75  $\mu\text{l}$  enzyme made up to 15  $\mu\text{l}$  with nuclease-free water and incubated at 20°C for 15 min and 65°C for 15 min. Native barcodes were ligated to the amplicons employing 5  $\mu\text{l}$  of NEB Blunt/TA Ligase Master Mix with 0.9  $\mu\text{l}$  of end-prepped DNA, 1.25  $\mu\text{l}$  of individual barcode for each sample made up to 10  $\mu\text{l}$  and incubated at 20°C for 20 mins and at 65°C for 10 mins. The barcoded library was pooled and mixed with 0.4 volumes of AMPure XP magnetic beads (Beckman Coulter, <https://www.beckmancoulter.com>) and incubated on a HulaMixer for 30 minutes. The pellet was spun down and separated on a magnetic rack for 5 mins, then washed twice in Short Fragment Buffer (SFB; Oxford Nanopore Technologies, <https://nanoporetech.com>), then washed with 100  $\mu\text{L}$  80% [v/v] ethanol, and finally eluted in 30  $\mu\text{l}$  of EB (Oxford Nanopore Technologies) and quantified on a Qubit fluorometer. Adaptor

ligation was performed with the NEBNext Quick Ligation Module (New England Biolabs) by incubating the entire barcoded library with 5 µl of Adaptor Mix II (AMII), 10 µl of NEBNext Quick Ligation Reaction Buffer and 5 µl of Quick T4 DNA Ligase (New England Biolabs) in a 50 µl reaction volume and incubated at room temperature for 10 mins. Twenty µl of AMPure XP beads were added and incubated on a HulaMixer for 30 minutes, then washed twice in 125 µl of SFB and finally eluted in 15 µL of EB and quantified. Fifteen ng of the prepared library was loaded onto a FLO-MIN106D flow cell (Oxford Nanopore Technologies). Data acquisition was executed for 36 hours and subsequently called bases and demultiplexed with Guppy Basecalling Software version 6.3.7 (Oxford Nanopore Technologies), and adapters were trimmed with porechop version 0.3.2pre (<https://github.com/rrwick/Porechop>).

## References

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**Appendix Table 1.** Oligonucleotide primers and probes and thermocycling parameters for qPCR and dPCR assays\*

| Target  | Primer and probe sequences, 5'-3' | PCR type | Primer, probe concentration      | LOD/LOQ slope, efficiency             | Thermocycling conditions   | Reference |
|---|-----------------------------------|----------|----------------------------------|---------------------------------------|--|-----------|
| HAdV-F  |                                   | qPCR     |                                  | 7.6 GC/ $\mu$ L<br>-3.3142<br>100.3%  | 95°C, 120 s, 40 cycles at 95°C, 15 s; 60°C, 30 s                   | (4)       |
|   | Forward<br>Reverse<br>Primer      |          | 300 nmol<br>300 nmol<br>100 nmol |                                       |  |           |
| CACTTAATGCTGACACGGGC<br>ACTGGATAGAGCTAGCGGGC<br>FAM-TGCACCTCTTGG ACTA<br>GT-IBFQ              |                                   |          |                                  |                                       |  |           |
| AAV2 VP1  |                                   | qPCR     |                                  |                                       | 50°C, 120 s;<br>95°C, 20 s; 40 cycles at 95°C, 3 s; 60°C 30 s      | RIVM†     |
|   | Forward<br>Reverse<br>Primer      |          | 400 nmol<br>400 nmol<br>100 nmol |                                       |  |           |
| TACCTCAAGTACAACCAC GC<br>CCTCTTTTTSGCCTG GAAGA<br>FAM-ATACGTCTTTTGGGGGC<br>AACCTCG – BHQ-1    |                                   |          |                                  |                                       |  |           |
| AAV2 VP1  |                                   | dPCR     |                                  |                                       | 95°C, 120 s; 40 cycles at 95°C, 5 s; 60°C, 30s                     | RIVM†     |
|   | Forward<br>Reverse<br>Primer      |          | 600 nmol<br>600 nmol<br>200 nmol |                                       |  |           |
| TACCTCAAGTACAACCAC GC<br>CCTCTTTTTSGCCTG GAAGA<br>FAM-ATACGTCTTTTGGGGGC<br>AACCTCG – BHQ-1    |                                   |          |                                  |                                       |  |           |
| AAV2 NSP  |                                   | qPCR     |                                  |                                       | 50°C, 120 s;<br>95°C, 20 s; 40 cycles at 95°C, 3 s; 60°C, 30 s     | RIVM†     |
|   | Forward<br>Reverse<br>Primer      |          | 400 nmol<br>400 nmol<br>100 nmol |                                       |  |           |
| AAGGTCACCAAGCAGGAAGT<br>CGTTTGGGCTCACTTATATCTG<br>FAM-ACCCCGCATTACGTTTGG<br>TGGACC - IBFQ     |                                   |          |                                  |                                       |  |           |
| AAV2 NSP  |                                   | dPCR     |                                  |                                       | 95°C, 120 s; 40 cycles at 95°C, 5 s; 60°C, 30 s                    | RIVM†     |
|   | Forward<br>Reverse<br>Primer      |          | 600 nmol<br>600 nmol<br>200 nmol |                                       |  |           |
| AAGGTCACCAAGCAGGAAGT<br>CGTTTGGGCTCACTTATATCTG<br>FAM-ACCCCGCATTACGTTTGG<br>TGGACC - IBFQ     |                                   |          |                                  |                                       |  |           |
| SARS-CoV-2 N1   |                                   | qPCR     |                                  | 5 GC/ $\mu$ L;<br>-3.2177<br>99%      | RT: 50°C, 600 s;<br>95°C, 30 s; 45 cycles at 95°C, 5 s; 60°C, 30 s | (5)       |
|   | Forward<br>Reverse<br>Primer      |          | 500 nmol<br>500 nmol<br>125 nmol |                                       |  |           |
| GACCCAAAATCAGCGAAAT<br>TCTGGTTACTGCCAGTTGAATCT<br>G<br>FAM- CTACGTCAAAAAGGGTGGGA<br>GC- BHQ-1 |                                   |          |                                  |                                       |  |           |
| crAss_2   |                                   | qPCR     |                                  | 1.56 GC/ $\mu$ L;<br>-3.3068<br>99.4% | 40 cycles at 95°C, 15 s; 60°C, 60 s                                | (6)       |
|   | Forward<br>Reverse<br>Primer      |          | 950 nmol<br>950 nmol<br>250 nmol |                                       |  |           |
| AGGAGAAAGTGAACGTGGAAC<br>A<br>AACGAGCACCAACTTTAAGCTTT<br>A<br>FAM-AGGATTTGGAGAAGGAA-<br>MGB   |                                   |          |                                  |                                       |  |           |

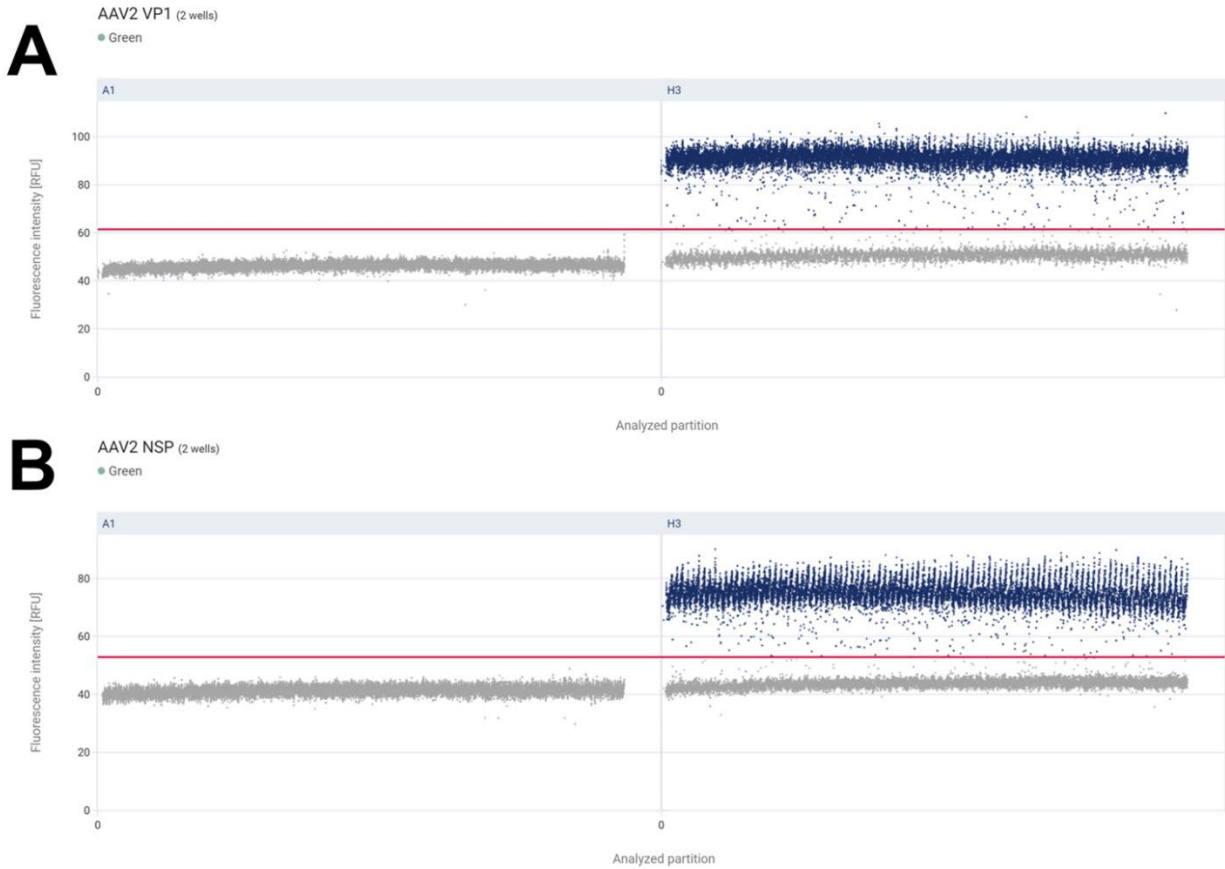
\*AAV2, adeno-associated virus type 2; BHQ-1, blackhole quencher-1; GC, genome copies; HAdVF, human adenovirus type F; IBFQ, Iowa black FQ quenchers; N1, nucleocapsid protein 1; NSP, nonstructural protein; RIVM, Rijksinstituut voor Volksgezondheid en Milieu Hygiene, the Netherlands  
†AAV2-VP1 and AAV2-NSP oligonucleotide primer and probe sequences were kindly provided by Dr. Harry Vennema at RIVM, through the European Centre for Disease Control and Prevention EpiPulse portal.

**Appendix Table 2.** Human adenovirus hexon amino acid sequences used to identify HAdV types in the wastewater samples

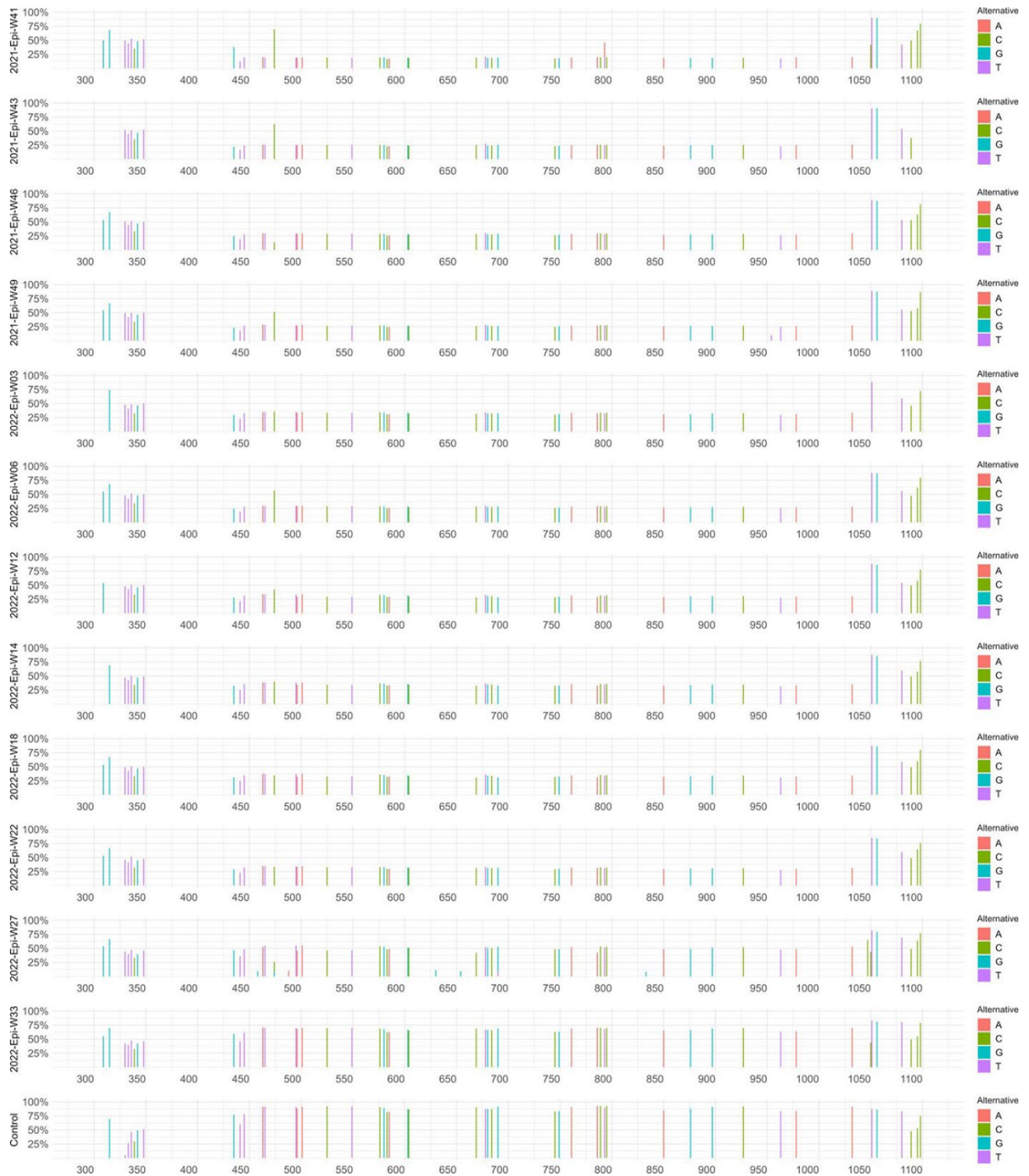
| No. | Accession No. | HAdV species | Type |
|-----|---------------|--------------|------|
| 1   | BAG48789      | A            | 12   |
| 2   | NP_040924     | A            | 12   |
| 3   | BAG48808      | A            | 31   |
| 4   | CAO78638      | A            | 31   |
| 5   | AKL80488      | B            | 3    |
| 6   | BAG48780      | B            | 3    |
| 7   | AGT77341      | B            | 7    |
| 8   | BAG48784      | B            | 7    |
| 9   | AAN62515      | B            | 11   |
| 10  | AAP49209      | B            | 11   |
| 11  | BAG48788      | B            | 11   |
| 12  | QZA82869      | B            | 11   |
| 13  | ACO81799      | B            | 14   |
| 14  | AFK92966      | B            | 14   |
| 15  | BAG48791      | B            | 14   |
| 16  | BAG48793      | B            | 16   |
| 17  | BAG48798      | B            | 21   |
| 18  | BAB20014      | B            | 34   |
| 19  | BAG48811      | B            | 34   |
| 20  | AAP92351      | B            | 35   |
| 21  | AP_000585     | B            | 35   |
| 22  | BAB20015      | B            | 35   |
| 23  | BAG48812      | B            | 35   |
| 24  | BAG48827      | B            | 50   |
| 25  | AGT55553      | B            | 55   |
| 26  | AET87230      | B            | 68   |
| 27  | BAG48778      | C            | 1    |
| 28  | QDO16041      | C            | 1    |
| 29  | BAG48779      | C            | 2    |
| 30  | AP_000211     | C            | 5    |
| 31  | BAG48782      | C            | 5    |
| 32  | BAG48783      | C            | 6    |
| 33  | BAU36782      | C            | 6    |
| 34  | AHI45188      | C            | 57   |
| 35  | ANW61309      | D            | 8    |
| 36  | BAG48785      | D            | 8    |
| 37  | BAE66671      | D            | 9    |
| 38  | BAG48786      | D            | 9    |
| 39  | AFK92137      | D            | 10   |
| 40  | BAG48787      | D            | 10   |
| 41  | BAM66756      | D            | 10   |
| 42  | AFK92177      | D            | 13   |
| 43  | BAG48790      | D            | 13   |
| 44  | BAG48792      | D            | 15   |
| 45  | ADY18429      | D            | 17   |
| 46  | BAG48794      | D            | 17   |
| 47  | BAG48795      | D            | 18   |
| 48  | BAG48796      | D            | 19   |
| 49  | BAG66282      | D            | 19   |
| 50  | AFK92257      | D            | 20   |
| 51  | BAG48797      | D            | 20   |
| 52  | ACL13145      | D            | 22   |
| 53  | ACR78217      | D            | 22   |
| 54  | BAG48799      | D            | 22   |
| 55  | AFK92297      | D            | 23   |
| 56  | BAG48800      | D            | 23   |
| 57  | AFK92336      | D            | 24   |
| 58  | BAG48801      | D            | 24   |
| 59  | AFK92376      | D            | 25   |
| 60  | BAG48802      | D            | 25   |
| 61  | ABO61316      | D            | 26   |
| 62  | BAG48803      | D            | 26   |
| 63  | AFK92416      | D            | 27   |
| 64  | BAG48804      | D            | 27   |
| 65  | BAG48805      | D            | 28   |
| 66  | AFK92456      | D            | 29   |
| 67  | BAG48806      | D            | 29   |
| 68  | AEY79573      | D            | 30   |

| No. | Accession No. | HAdV species | Type |
|-----|---------------|--------------|------|
| 69  | BAG48807      | D            | 30   |
| 70  | AFK92496      | D            | 32   |
| 71  | BAG48809      | D            | 32   |
| 72  | AFK92576      | D            | 33   |
| 73  | BAG48810      | D            | 33   |
| 74  | ACY04472      | D            | 36   |
| 75  | BAG48813      | D            | 36   |
| 76  | ABK59070      | D            | 37   |
| 77  | BAG48814      | D            | 37   |
| 78  | BAH19144      | D            | 37   |
| 79  | ABO47729      | D            | 38   |
| 80  | AFK92616      | D            | 38   |
| 81  | BAG48815      | D            | 38   |
| 82  | AFK92656      | D            | 39   |
| 83  | BAG48816      | D            | 39   |
| 84  | AFK92696      | D            | 42   |
| 85  | BAG48819      | D            | 42   |
| 86  | AFK92736      | D            | 43   |
| 87  | BAG48820      | D            | 43   |
| 88  | AFK92776      | D            | 44   |
| 89  | BAG48821      | D            | 44   |
| 90  | AFK92816      | D            | 45   |
| 91  | BAG48822      | D            | 45   |
| 92  | BAG48823      | D            | 46   |
| 93  | AFK92536      | D            | 47   |
| 94  | BAG48824      | D            | 47   |
| 95  | BAG48825      | D            | 48   |
| 96  | BAG48826      | D            | 49   |
| 97  | AFK92856      | D            | 51   |
| 98  | BAG48828      | D            | 51   |
| 99  | BBW89580      | D            | 53   |
| 100 | YP_003038612  | D            | 54   |
| 101 | AGM61353      | D            | 56   |
| 102 | ADW95419      | D            | 58   |
| 103 | AEI91289      | D            | 59   |
| 104 | AEL78878      | D            | 60   |
| 105 | AFA46691      | D            | 64   |
| 106 | AFK92217      | D            | 69   |
| 107 | AGT76762      | D            | 71   |
| 108 | BAO53845      | D            | 81   |
| 109 | AZI15535      | E            | 4    |
| 110 | BAG48781      | E            | 4    |
| 111 | BBH49391      | E            | 4    |
| 112 | AAC13967      | F            | 40   |
| 113 | BAG48817      | F            | 40   |
| 114 | CAA36077      | F            | 40   |
| 115 | ACH90432      | F            | 41   |
| 116 | BAG48818      | F            | 41   |

\*HAdV, human adenovirus.



**Appendix Figure 1.** Adenovirus-associated virus 2 genes detected in wastewater, Ireland. A) AAV2 VP1; B) AAV2 NSP. Genes were detected by dPCR assays using nuclease free water as negative control (left panels; A1) and AAV2–positive plasma clinical sample as the positive control (right panels; H3). AAV2, adeno-associated virus type 2; N1, nucleocapsid protein 1; NSP, nonstructural protein.



**Appendix Figure 2.** Human adenovirus type F41 (HAdV-F41) single nucleotide variant frequencies per wastewater sample, Ireland. The 13 panels correspond to the samples from each epidemiology week, as indicated on the left of each panel. The vertical axes indicate the frequency of the SNV in each sample and the horizontal axes are aligned across panels displaying the position relative to the hexon nucleotide position in the HAdV-F41 (GenBank accession no. ON442316). The bars representing the SNVs are color-coded according to the alternative nucleotide found in the reads.