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RESEARCH ARTICLE

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Postpandemic rebound of adeno-associated virus type 2 (AAV2) infections temporally associated with an outbreak of unexplained severe acute hepatitis in children in the United Kingdom

Shannah Gates[1] | Julien Andreani[2,3] | Rebecca Dewar[4] | Donald B. Smith[3] | Kate Templeton[4] | Harry T. Child[5] | Judy Breuer[6] | Tanya Golubchik[7,8] | Irene Bassano[9] | Matthew J. Wade[9] | Aaron R. Jeffries[5] | Peter Simmonds[3] | Heli Harvala[1,10]

[1]Microbiology Services, National Health Service (NHS) Blood and Transplant, London, UK

[2]Centre Hospitalier Universitaire (CHU) Grenoble-Alpes, Grenoble, France

[3]Nuffield Department of Medicine, Peter Medawar Building for Pathogen Research, University of Oxford, Oxford, UK

[4]Specialist Virology Centre, Royal Infirmary Edinburgh, NHS Lothian, Edinburgh, UK

[5]Biosciences, Faculty of Health and Life Sciences, University of Exeter, Exeter, UK

[6]Division of Infection and Immunity, University College London, London, UK

[7]Faculty of Medicine and Health, Sydney Infectious Diseases Institute, University of Sydney, Sydney, Australia

[8]Nuffield Department of Medicine, Big Data Institute, Oxford, UK

[9]Analytics & Data Science Directorate, UK Health Security Agency, Nobel House, London, UK

[10]Radcliffe Department of Medicine, Nuffield Division of Clinical Laboratory Sciences, University of Oxford, Oxford, UK

Correspondence
Shannah Gates, Microbiology Services, National Health Service (NHS) Blood and Transplant, London, UK.
Email: Shannah.Gates@nhsbt.nhs.uk

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Abstract

Over 1000 cases of unexplained severe acute hepatitis in children have been reported to date worldwide. An association with adeno-associated virus type 2 (AAV2) infection, a human parvovirus, prompted us to investigate the epidemiology of AAV in the United Kingdom. Three hundred pediatric respiratory samples collected before (April 03, 2009–April 03, 2013) and during (April 03, 2022) the COVID-19 pandemic were obtained. Wastewater samples were collected from 50 locations in London (August 2021–March 2022). Samples were tested for AAV using real-time polymerase chain reaction followed by sequencing. Selected adenovirus (AdV)-positive samples were also sequenced. The detection frequency of AAV2 was a sevenfold higher in 2022 samples compared with 2009–2013 samples (10% vs. 1.4%) and highest in AdV-positive samples compared with negatives (10/37, 27% vs. 5/94, 5.3%, respectively). AAV2-positive samples displayed high genetic diversity. AAV2 sequences were either very low or absent in wastewater collected in 2021 but

increased in January 2022 and peaked in March 2022. AAV2 was detected in children in association with AdV of species C, with a highest frequency in 2022. Our findings are consistent with the expansion of the population of children unexposed to AAV2, leading to greater spread of the virus once distancing restrictions were lifted.

KEYWORDS

AAV2, adeno-associated virus, adenovirus, epidemiology, parvovirus, rebound, unexplained hepatitis

1 | INTRODUCTION

Adeno-associated virus (AAV) is a member of *Parvoviridae*, a family of small DNA viruses infecting humans and a range of other mammalian species.[1] In contrast to autonomous parvoviruses such as parvovirus B19V, AAV and other members of the genus *Dependoparvovirus* typically only replicate in the presence of helper factors, often provided by co-infections with helper viruses, most commonly herpesviruses (e.g., human herpesvirus 6) or adenoviruses (AdVs), or through the effects of cellular stress from physical or chemical carcinogens.[2] In the absence of helper functions, AAV persists as a latent infection as an episome or less frequently through integration into the host's genome.[3,4] The replication, tropism, and normally minimal pathogenicity of AAV are all well characterized since its extensive use as a vector for gene therapy.

AAVs are genetically diverse, with at least 13 serologically distinct types.[5,6] Human infection with AAV is common, with up to 80% of individuals having neutralizing antibodies to one or more serotype, the majority being directed against AAV2, although with considerable variation in prevalence depending on age, sex, and geographical region.[7]

Infections are generally believed to be apathogenic, evoking only a minor inflammatory response, although they have been associated with mild hepatitis when used in gene therapy.[8–10] However, AAV2 has recently been implicated in the etiology of the 2022 outbreak of unexplained severe acute hepatitis in children (UHC).[11–14] Infections with human AdV, specifically AdV type F-41, were initially suspected as it was detected in the majority of cases and at rates statistically significantly higher than control groups,[15] although generally with low virus loads. However, two studies in the United Kingdom and one in the United States have found a much more specific association with AAV2 infection.[11–14] In the United Kingdom studies, high levels of AAV2 DNA were detected in all nine cases requiring liver transplant, and also in 15 out of 16 children with unexplained hepatitis who recovered without needing a transplant. Both UK studies documented very low AAV2 infection frequencies in non-UHC control cohorts, including AdV-F41-positive children without hepatitis. AAV2 DNA was present at much lower levels in these control groups than in UHC cases. In both of these studies, the majority of UHC cases also tested positive for AdV (predominantly AdV-F41) or for HHV-6. These studies suggested that AAV2 was the primary cause of UHC, albeit requiring co-infection with a helper virus such as AdVF-41 and/or HHV-6 for replication. In addition, Ho et al.[11] found an association between disease and the class II human leukocyte antigen (HLA) DRB1*04:01 allele, present in 89% of cases, compared with a background frequency of 15.6% in the general Scottish population. A US study observed similar results, AAV2 was detected in 13 out of the 14 UHC cases studied (93%) whilst it was only detected in 4 of 113 controls (3.5%).[14]

Using wastewater samples and clinical samples obtained from children under the age of 5 years, we investigated more broadly whether there had been a systematic change in AAV2 incidence in early 2022 compared with previous years. We chose to study samples from children below the age of 5 years as most cases of UHC were reported in this group, and that is the age when AAV infections are most likely acquired.[16,17]

2 | MATERIALS AND METHODS

2.1 | Pediatric respiratory samples

Anonymized pediatric respiratory samples were obtained from the Edinburgh Specialist Virology Centre sample archive. Approval was obtained from the Lothian Regional Ethics Committee (approval number 08/S11/02/2) to retain the collected information for epidemiological purposes but to make the information anonymous to protect patient confidentiality. The stored data included age band, partial postcode, any recorded symptoms or clinical information, referral source, month of sample collection, and the results of other virological tests for each sample. For the samples from 2022, the majority were nose and throat swabs (117) with the remainder consisting of nasopharyngeal secretions (14), nose swabs (10), throat swabs (6), and bronchoalveolar lavage (BAL) (2). For the control cohort, the samples predominantly comprised nasopharyngeal aspirates or swabs (130/141) and were routinely screened for the following respiratory viruses by previously described polymerase chain reaction (PCR) assays[18,19]: AdV, influenza A and B viruses, parainfluenza virus type 1 (PIV-1), PIV-2, PIV-3, and respiratory syncytial virus (RSV).

Nucleic acid extraction was carried out from 200 µL of patient sample using the ThermoFisher MagMAX Viral/Pathogen nucleic acid isolation kits with MagMAX magnetic beads using the automated ThermoFisher Kingfisher Apex magnetic particle processor. Extracted nucleic acids were eluted into 30 µL of DNase/RNase-free water.

2.2 | Wastewater sample collection processing

Wastewater sample collection, processing, and nucleic acid extraction were carried out by the Environmental Monitoring for Health Protection (EMHP) program in England.[20] Wastewater samples of 1 L were collected from 42 locations across the sewer network in London, United Kingdom between October 2, 2021 and March 29, 2022, as well as eight hotel wastewater sites close to Heathrow Airport, London, United Kingdom between December 8 and 16, 2021. Wastewater was transported and stored at 4–6°C and processed within 24 h to avoid excessive RNA degradation. Viral enrichment and nucleic acid extraction methods have been described previously in full.[21] Nucleic acid samples, collected weekly, were pooled into 27 separate lots containing between 7 and 16 sites in each (Supporting Information: Table S1). Potential PCR inhibitors were reduced by a 1.8X NGS Magbind Total-Pure bead (VWR) cleanup, followed by LunaScript (NEB) reverse transcription.[22] Library preparation was then performed using the Illumina RNA prep with Enrichment kit and Illumina Respiratory Virus Oligo Panel for target enrichment (Illumina). The resulting library was sequenced on an Illumina Novaseq. 6000 using a 2 × 150 bp read format, obtaining a mean sequencing depth of 106 million (Standard deviation = 3.4 million) reads. Reads were trimmed using *fastp* v0.23.1[23]; parameters -l 75 -q 20), before being taxonomically assigned using *kraken2* v2.1.2[24] to a virus and bacteria database built on June 4, 2022.

2.3 | Real-time PCR screening for AAV

AAV screening of samples was conducted using real-time PCR. Primers and probe were used at a final concentration of 0.4 and 0.2 µM respectively. Primers were designed within a region of the AAV VP1 gene conserved between serotypes. Forward- 5′-CCGAYGGAGTGGGTAATKCCTC-3′ (5′ based at position 2855 in the AF043303 reference sequence); reverse- 5′-TTGTTGTTGATGAGTCKYTGCCA-3′ (position 3110); probe- 5′-HEX-TTTGACTTYAACAGATTCCACTGCCACTT-TAMRA-3′ (position 3046) (Sigma-Aldrich/Merck). The total reaction volume was 20 µL including 2.5 µL of template. Real-time PCRs were carried out using an Applied Bioscience StepOnePlus Thermocycler, performing the following protocol: initial DNA polymerase activation for 15 min at 95°C, followed by 45 cycles of denaturation at 95°C for 10 s and 60°C for 40 s for annealing and elongation.

2.4 | AAV typing by nested PCR and Sanger sequencing

The AAV REP region of AAV-positive samples was amplified by nested PCR and sequenced. The outer primer sequences were: 5′-GAGCTYCAGTGGGCGTGGAC-3′ (5′- position 817 relative to AF043303 reference sequence), 5′-GGGAAAGTTYTCRTTGGTC-CAGTTBAC-3′ (position 1415). The inner primer sequences were: 5′-CSGARAAGCARTGGATYCAGG-3′ (position 1033), 5′-ATGGCY-TCCGCGATGTTGGTC-3′ (position 1360). For the first round of PCR 10 µL of template was used and for the second round 2 µL of first-round PCR product was used as the template. For both PCRs the total reaction volume was 50 µL. Reaction mix was made using Promega's 5X Colorless GoTaq® DNA polymerase reaction buffer and contained a final concentration of forward and reverse primers 0.2 µM, $MgCl_2$ 1.5 mM, dNTPs 0.2 mM, 1.25 U of GoTaq G2 DNA polymerase (Promega). The first round of PCR was carried out using the following parameters: 95°C for 120 s; followed by 45 cycles of 95°C for 30 s, 58°C for 30 s, and 73°C for 90 s; then a final extension at 73°C for 5 min. The same parameters were used for the second round of PCR except that the elongation time was 60 s instead of 90. PCR products were run on 2% agarose gels prestained with GelRed® nucleic acid gel stain to identify positive samples with a predicted size of 327 bp. PCR products were sent for Sanger Sequencing by Source BioScience.

2.5 | AdV typing by nested PCR and Sanger sequencing

Samples that were positive for AAV and AdV underwent AdV nested PCR for nucleotide sequencing, type identification, and genomic characterization. Nested PCR was carried out targeting the AdV hexon gene region. The outer primer sequences were: 5′-ATGTAYTAYAAYAGYACTGGHAAYATGGG-3′ (5′ based at position 1033 in the FJ349096 reference sequence), 5′-CCCARVAGCATGGANCGGTARCGC-3′ (position 1685). The inner primer sequences were: 5′- GTGGTBGACTTGCARGACAGAAAYAC-3′ (position 1096), 5′-CGCARVCCMGCRTTGCGGTGGTG-3′ (position 1664). For the first round of PCR 2 µL of template was used and for the second round 2 µL of first-round PCR product was used as the template. For both PCRs the total reaction volume was 50 µL. Reaction mix was made using Promega's 5X Colorless GoTaq® DNA polymerase reaction buffer and contained a final concentration of forward and reverse primers 0.2 µM, $MgCl_2$ 1.5 mM, dNTPs 0.2 mM, 1.25 U of GoTaq G2 DNA polymerase (Promega). The first round of PCR was carried out using the following parameters: 95°C for 120 s; followed by 45 cycles of 95°C for 30 s, 51°C for 30 s, and 73°C for 90 s; then a final extension at 73°C for 5 min. The same parameters were used for the second round of PCR except that the anneal temperature was 65°C instead of 51°C. PCR products were run on 2% agarose gels prestained with GelRed® nucleic acid gel stain to identify positive samples with a predicted size of 568 bp. PCR products were sent for Sanger Sequencing by Source BioScience.

2.6 | Phylogenetic analysis

Nucleotide sequences in the AdV hexon gene (positions 1122-1641, numbered based on FJ349096) were aligned with representative sequences from all human and nonhuman primate AdVs classified into the species *Human mastadenovirus A–G* as listed in the International committee on taxonomy of viruses virus metadata resource (VMR) (https://ictv.global/vmr). AAV sequences between positions 1054–1339 (numbered in the AF043303 AAV2 reference sequence) were aligned with all available AAV2 sequences available on GenBank (downloaded August 5, 2022) together with representative sequences of other AAV types listed in the VMR.

Phylogenetic trees were constructed by neighbor-joining of maximum composite likelihood distances from 100 bootstrap resamples using the MEGA (version 7.0) software package with pairwise deletion for missing data.[25]

3 | RESULTS

3.1 | AAV detection frequency in respiratory samples

One hundred and forty-nine respiratory samples were collected in March and April 2022 in Edinburgh and South-East Scotland, of which 24.8% (38) had been previously tested as positive for AdV and 12.1% (18) positive for SARS-CoV-2. The majority of samples were from children under the age of 5 years (Table 1). AAV detection frequencies in these samples were compared with those in 141 samples collected between March and April in 2009, 2010, and 2013, primarily from children under the age of 5 years. Of these control samples, 54.6% were positive for AdV DNA on routine diagnostic screening.

Of the 2022 samples, 10.1% (15/149) were positive for AAV2 (Figure 1). Of the 15 AAV-positives, 10 were positive for AdV, 4 were AdV-negative, and 1 undetermined. For the 2009–2013 cohort 1.4% (two) of the samples were positive for AAV2 ($n = 141$); both of the AAV2-positive samples were also AdV positive. Samples from 2022 were significantly more likely to be positive for AAV2 if they were also positive for AdV (Fisher's exact test, $p = 0.0004$). The detection frequency of AAV in 2022 was significantly higher than in 2009–2013, most notably in those co-infected with AdV (Fisher's exact test, $p = 0.0002$).

Analysis of samples by age indicated that the majority of AAV2-positive cases were in children aged 2 years (8/14; 57.1%; Figure 1). Both AAV2 positives from the 2009–2013 cohort were in the age category of 1–3 years.

3.2 | AAV and AdV types and genetic diversity

All samples that were AAV-positive by quantitative polymerase chain reaction (qPCR) were confirmed by nested PCR targeting the REP region and typed by Sanger sequencing; all were AAV2 (Figure 2A). AAV2 variants from the 2022 timepoint were genetically diverse and interspersed with variants detected in previous years or from other geographical regions. AAV2 strains obtained in the current study infrequently matched variants from previously reported UHC cases, which were equally genetically diverse despite their UK origin and similarly narrow 3 months sampling window (May 3, 2022).

AdV types in individuals co-infected with AAV2 were assigned by phylogenetic analysis of hexon gene sequences (Figure 2B). Of the samples positive for both AAV2 and AdV, which the AdV hexon gene could be amplified and sequenced, six were AdV-C1, and four were AdV-C2. For a further four AdV-positive but AAV2-negative samples, three were serotype AdV-C1 and one was AdV-C5.

3.3 | AAV2 in wastewater samples

As a population-scale metric of AAV and enteric AdV circulation, metagenomic sequencing using a hybrid-capture enrichment approach targeting human respiratory viruses was conducted on pooled wastewater sampled in London, United Kingdom between October 2021 and March 2022. AAV2 reads were either very low or absent for samples taken between October and December 2021. The number of AAV2 reads then increased, with peaks in January 2022 and March 2022 (Figure 3A). To corroborate the quantitation provided by metagenomic read totals, viral loads in these samples were measured by AAV qPCR. Quantitation by the two methods was concordant both in the timing and magnitude of the peaks in detection. Comparable numbers of AdV-F41 reads were obtained in the same wastewater samples, although peaks did not precisely coincide with those of AAV2.

The timing of the major peak of AAV2 detection in mid-March 2022 coincided with the very rapid increase in SARS-CoV-2 incidence based on Office for National Surveillance data based on randomized testing of respiratory samples in the United Kingdom. These increases followed shortly after the relaxation of distancing restrictions on February 24, 2022 (Figure 3B) and immediately preceded the outbreak of jaundice and unexplained hepatitis in

TABLE 1 Comparison of the age distributions of cohort and control samples.

Age	2009–2013/number of subjects (%)	2022/number of subjects (%)
0–6 months	30 (21.3)	21 (14.1)
7–12 months	36 (25.5)	25 (16.8)
1–3 years	45 (31.9)	49 (32.9)
3–5 years	30 (21.3)	18 (12.1)
5–10 years	0 (0)	31 (20.8)
10–15 years	0 (0)	5 (3.4)

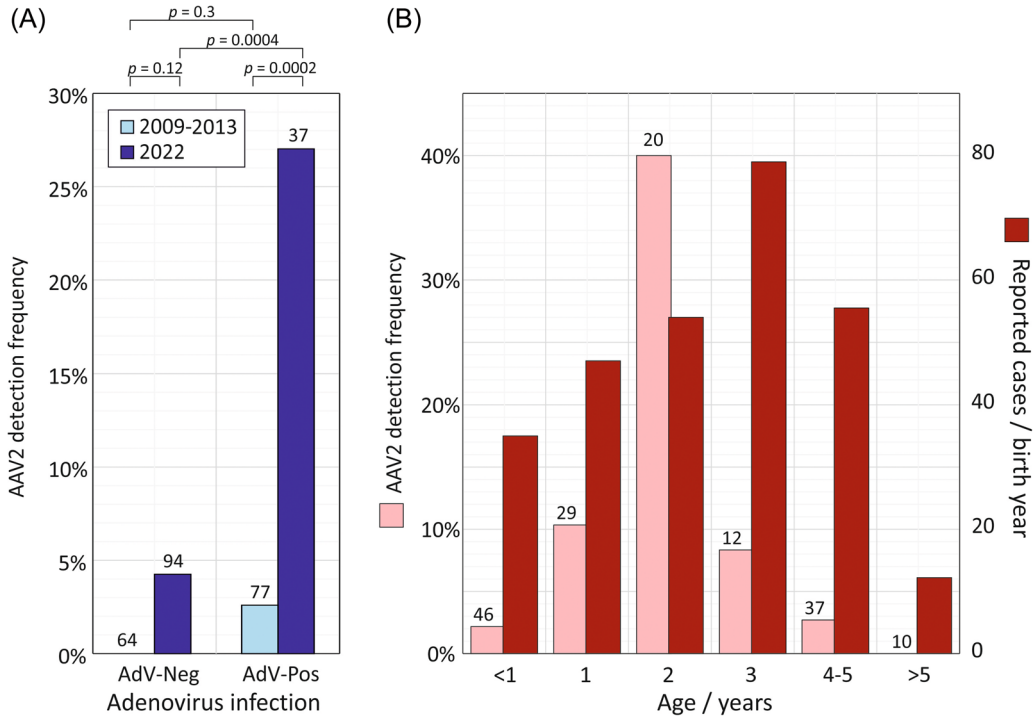

FIGURE 1 Detection frequencies of adeno-associated virus (AAV) in association with adenovirus (AdV) coinfection, age, and unexplained hepatitis. (A) Frequencies of samples positive for AAV in individuals with and without AdV co-detection in pre- (2009–2013, $n = 141$) and postpandemic (2022, $n = 144$) periods. AAV detection frequencies were compared using Fisher's exact probability test for pairwise category comparison (p values shown above graph). (B) Frequencies of respiratory samples positive for AAV DNA in March 2022 (y-axis) in different age ranges. Total number of cases of unexplained hepatitis reported by WHO[26] were split into the same age ranges for comparison. Total number of samples tested are shown on top.

children, that peaked in incidence in the United Kingdom in April and May of 2022 (Figure 3B).

4 | DISCUSSION

Nonpharmaceutical interventions including mask mandates and social distancing intended to limit the transmission of SARS-CoV-2 have been associated with decreased incidence or cessation of community circulation of a range of communicable diseases. As a result, the proportion of the population susceptible to these diseases has increased, particularly children in the age range 1–3. This cohort may have escaped exposure to normal childhood infections during COVID lockdown and are now beyond the period of maternal antibody protection. Postlockdown resurgence of infections has already been described for several transmissible pathogens.[28] In New Zealand, RSV cases postpandemic were more than five times the 2015–2019 peak average.[29] Similarly, rhinovirus, enterovirus, and RSV infections in Germany were greatly increased post-pandemic compared with prepandemic levels.[30] In China, there were 118.7% and 75.8% rises in RSV and human parainfluenza virus cases, respectively, in hospitalized children during the 19 months postlockdown in comparison with the prepandemic period.[31] The recent surge in cases of group A streptococcal infections seen in children in the United Kingdom in late 2022 may also be a postpandemic rebound.[32] Lack of pre-existing immunity through reduced exposure of communicable diseases during the COVID pandemic will be more evident in younger children as they will have spent a greater proportion of their lives under social restrictions. Supporting this, the age range with the highest incidence of AAV2 detection was indeed in the 1–3-year-old age group (Figure 1), a distribution that matches that of children with jaundice and unexplained hepatitis, who were primarily <5 years of age (Figure 1B).[11,13,14]

As proposed by Morfopoulou et al.[13], the surge in cases of children with unexplained hepatitis may represent a post-restriction rebound event. The current study demonstrates an increase in AAV2 circulation immediately before the unexplained hepatitis outbreak. Furthermore, wastewater sampling allowed us to study circulation of AAV2 in the general population in 2021 and 2022 and revealed a peak in AAV2 in late March 2022. These events coincided closely with the end of lockdown restrictions and a rapid increase in the number of SARS-CoV-2 infections recorded by the Office for National Surveillance in the United Kingdom, suggesting that this rebound was likely due to the lifting of those restrictions.

The analysis of wastewater samples collected in London indicated an increase in AAV2 prevalence in March 2022, consistent with findings from a study in Ireland where a sharp and substantial increase in AAV2 detection in wastewater was reported during early

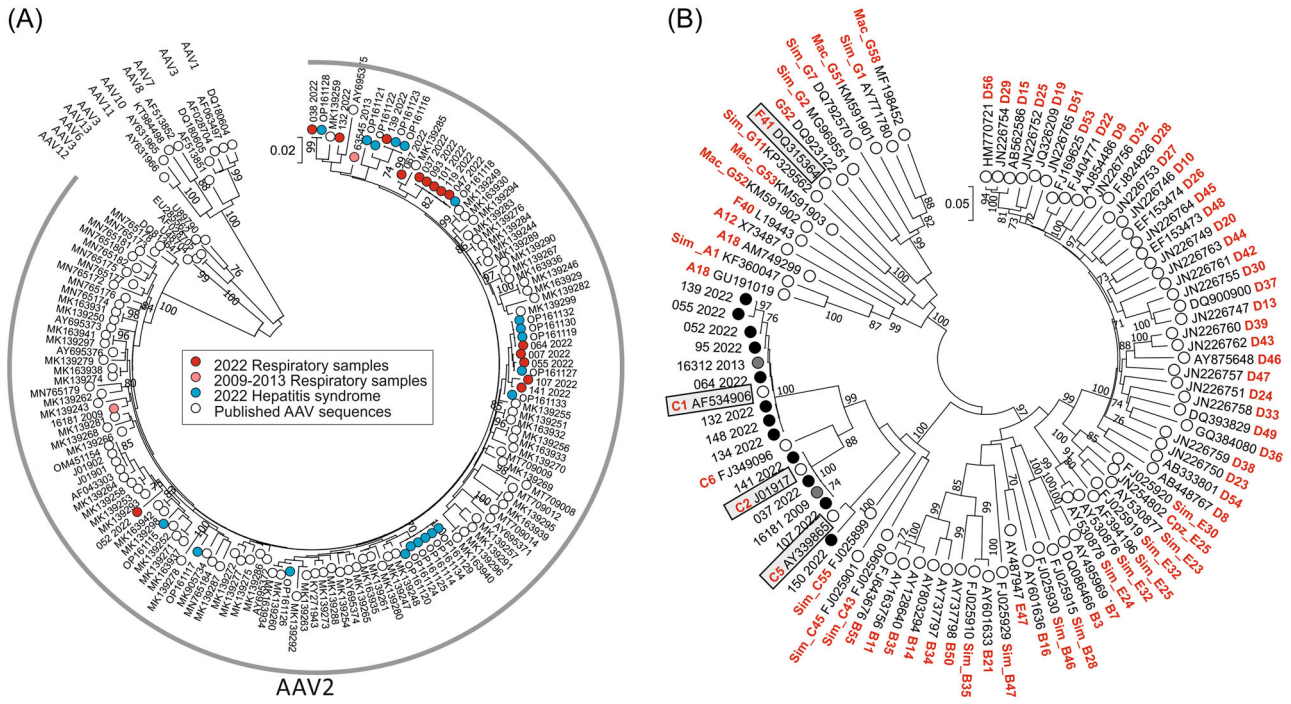

FIGURE 2 Phylogenetic analysis of adenovirus (AdV) and adeno-associated virus (AAV) sequences from the study samples. Phylogenetic analysis of (A) Adeno-associated virus 2 (AAV2) sequences from the *Rep* gene of study samples (red) along with study samples from unexplained hepatitis in children cases[13] available published AAV2 sequences and representative sequences of other AAV types, and (B) hexon gene sequences (Adenovirus) amplified from the study samples (black circle: co-infection with AAV2; gray circle: no co-infection with AAV2) and representative sequences of each classified type in species *Human mastadnovirus A–G. Genotype names shown after the Genbank submission number; Sim referring to Simian viruses, Mac to Macaques and Cpz to Chimpanzees for nonhuman genotypes*. Trees were constructed as described in Section 2.

2022 and peaking in April 2022.[33] Little or no AAV2 was detected in wastewater in the period back to July 2020. As AAV infections generally do not associate with disease and are rarely clinically tested, wastewater surveillance could be used as a potentially valuable tool with which to monitor the circulation of AAV2 in the community.

Phylogenetic analysis of REP gene sequences of AAV2 sampled over the period of the unexplained hepatitis outbreak showed considerable within-type genetic diversity and shared no common ancestor distinct from other AAV2 strains. Similar diversity amongst AAV2 sequences was also found in children with hepatitis syndrome.[13] These observations provide no evidence for the existence or emergence of a uniquely transmissible or pathogenic strain of AAV2 underlying hepatitis syndrome. For the reasons discussed above, we propose the increase in cases of previously circulating variants of AAV2 arose through rapid spread of normally-common viruses through a very large immune-naïve population.

The requirement of helper viruses for AAV replication entails co-infection with AdV, herpesviruses, or cellular stress. The frequent co-detection of AdV and AAV in unexplained hepatitis cases[11,13,14] may arise through the requirement of AAV for a helper virus rather than AdV being the direct causative agent as proposed by UKHSA and others.[15] This explanation is supported by the low viral loads of AdV, even in severely affected tissue such as the liver in hepatitis cases and in which high viral loads of AAV2 are reported.[11,13,14] In our study, the frequency of AAV detection was similarly higher in AdV co-infection, although in contrast to previous studies, AAV infections in the respiratory tract were associated with coinfections with species A and C AdVs (Figure 2) rather than AdV-F41.

The association of AAV2 infection with UHC stimulated us to understand the normal patterns of circulation of AAV2, a subject which has been little studied due to the general belief that AAV2 is apathogenic. Our study demonstrated a temporal association between a peak in AAV2 cases and the peak in unexplained hepatitis cases. This data supports its recently proposed etiological link[11,13,14] and the potential of AAV2 to cause severe disease in certain circumstances. These include host genetic background and greater postpandemic host susceptibility.[11] The frequent descriptions of dose-dependent hepatitis in recipients of AAV-vectored vaccines is further consistent with its aetological role in UHC[34,35] The influence of host factors on AAV2 infection outcomes is supported by the observation that AAV2 strains characterized in this study and in UHC cases[13] were genetically heterogeneous and interspersed phylogenetically with AAV2 strains previously characterized from asymptomatic individuals in past decades. This suggests that the unexplained hepatitis outbreak is not due to the emergence of a new, more infectious strain of AAV2. Our data supports further the idea that AAV2 infections coincided with AdV species A or C infections, likely followed by AdV-F41 infection. These data show our limited

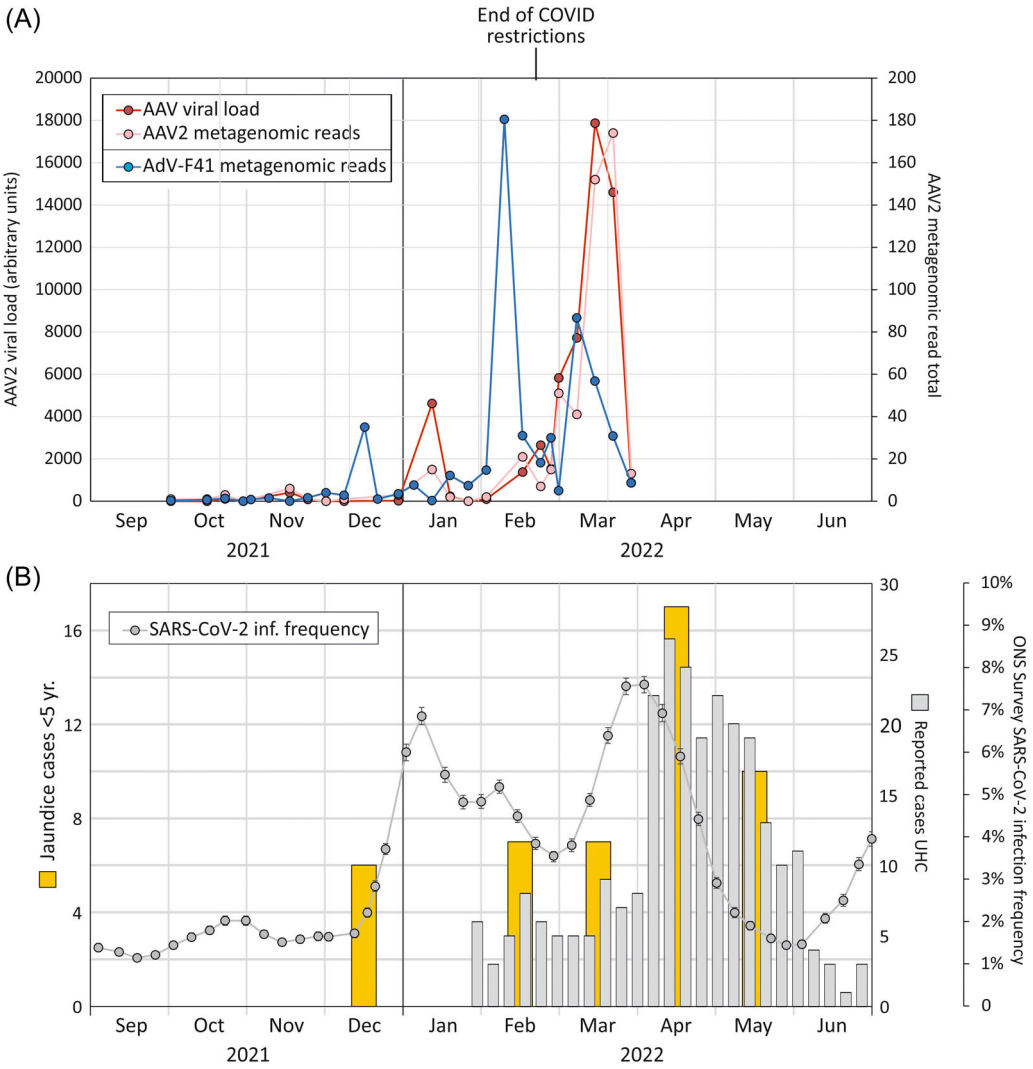

FIGURE 3 Temporal association of adeno-associated virus (AAV) and adenovirus (AdV) detection in wastewater with the postpandemic resurgence of SARS-CoV-2 and the outbreak of unexplained hepatitis in children (UHC). (A). Detection of AAV and AdV DNA sequences in wastewater by enumeration of AAV2 reads by metagenomic sequencing and by quantitative polymerase chain reaction for AAV. (B) SARS-CoV-2 incidence of infection (frequency of respiratory sample positivity in the UK population reported by Office for National Statistics[27] and reported numbers of UHC diagnoses based on the current UK Health Security Agency (UKHSA) case definition, and numbers of emergency cases of jaundice in under 5-year-olds (monthly totals of >5 shown).[15]

understanding of the complexity of simultaneous viral infections, where the outcomes may well be coupled with the primed immunity either by previous infection or host genetics. More detailed investigation of infection outcomes in previously unexposed individuals with host genetic susceptibilities (such as HLA) would be of considerable value in understanding more about the pathogenesis of dependoviruses and their interplay with host immune responses, in addition to other viral infections.

AUTHOR CONTRIBUTIONS

Study conception and design: Peter Simmonds, Heli Harvala, Shannah Gates, and Julien Andreani. *Data collection*: Shannah Gates, Julien Andrani, Harry T. Child, Donald B. Smith, Kate Templeton, Rebecca Dewar, Irene Bassano, Matthew J. Wade, and Aaron R. Jeffries. *Analysis and interpretation of results*: Peter Simmonds, Heli Harvala, Shannah Gates, Julien Andrani, Harry T. Child, Donald B. Smith, Kate Templeton, Rebecca Dewar, Irene Bassano, Matthew J. Wade, Aaron R. Jeffries, Judy Breuer, and Tanya Golubchik. *Draft manuscript preparation*: Peter Simmonds, Heli Harvala, and Shannah Gates. All authors reviewed the results, provided feedback, and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

Lothian Regional Ethics Committee (approval number 08/S11/02/2).

ORCID

Peter Simmonds http://orcid.org/0000-0002-7964-4700

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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