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Adeno-associated virus type 2 in US children with acute severe hepatitis

Venice Servellita*[1], Alicia Sotomayor Gonzalez*[1], Daryl M. Lamson*[2,] Abiodun Foresythe[1], Hee Jae Huh[1,3], Adam L. Bazinet[4], Nicholas H. Bergman[4], Robert L. Bull[5], Karla Y. Garcia[4], Jennifer S. Goodrich[4], Sean P. Lovett[4], Kisha Parker[4], Diana Radune[4], April Hatada[6], Chao-Yang Pan[6], Kyle Rizzo[6], J Bradford Bertumen[6,11], Christina Morales[6], Paul E. Oluniyi[1], Jenny Nguyen[1], Jessica Tan[1], Doug Stryke[1], Rayah Jaber[7], Matthew T. Leslie[8], Zin Lyons[9], Hayden D. Hedman[10,11], Umesh Parashar[11], Maureen Sullivan[12], Kelly Wroblewski[12], M. Steven Oberste[11], Jacqueline E. Tate[11], Julia M. Baker[11], David Sugerman[11], Caelin Potts[11], Xiaoyan Lu[11], Preeti Chhabra[11], Pediatric Hepatitis of Unknown Etiology Working Group, L. Amanda Ingram[13], Henry Shiau[14], William Britt[14], Luz Helena Gutierrez Sanchez[14], Caroline Ciric[15], Christina A. Rostad[15], Jan Vinjé[11], Hannah L. Kirking[11], Debra A. Wadford[6], R. Taylor Raborn[4], Kirsten St. George[2, 16], Charles Y. Chiu[1,17,18]

*contributed equally

1. Department of Laboratory Medicine, University of California, San Francisco, San Francisco, CA, USA.
2. Wadsworth Center, New York State Department of Health, David Axelrod Institute, Albany, NY, USA.
3. Department of Laboratory Medicine and Genetics, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, South Korea
4. National Biodefense Analysis and Countermeasures Center (NBACC), Frederick, MD, USA
5. Federal Bureau of Investigation Laboratory Division / Scientific Response and Analysis Unit, Quantico, VA, USA
6. California Department of Public Health, Richmond, CA, USA

7. Florida Department of Health, Tallahassee, USA
8. Illinois Department of Public Health, Chicago, IL, USA
9. North Carolina Department of Health and Human Services, Raleigh, NC, USA
10. South Dakota Department of Health, Pierre, SD, USA
11. Centers for Disease Control and Prevention, Atlanta, CA, USA
12. Association for Public Health Laboratories, Silver Spring, MD, USA
13. Alabama Department of Public Health, Montgomery, AL, USA
14. University of Alabama at Birmingham, Birmingham, AL, USA
15. Department of Pediatrics, Emory University School of Medicine and Children's Healthcare of Atlanta, Atlanta, GA, USA.
16. Department of Biomedical Science, University at Albany, SUNY, Albany, NY, USA
17. Department of Medicine, Division of Infectious Diseases, University of California, San Francisco, San Francisco, CA, USA.
18. Chan-Zuckerberg Biohub, San Francisco, CA, USA.

Correspondence to:
Charles Chiu
charles.chiu@ucsf.edu

ABSTRACT

As of August 2022, clusters of acute severe hepatitis of unknown etiology in children have been reported from 35 countries, including the United States[1,2]. Previous studies have found human adenoviruses (HAdVs) in the blood from cases in Europe and the United States[3-7], although it is unclear whether this virus is causative. Here we used PCR testing, viral enrichment based sequencing, and agnostic metagenomic sequencing to analyze samples from 16 HAdV-positive cases from October 1, 2021 to May 22, 2022, in parallel with 113 controls. In blood from 14 cases, adeno-associated virus 2 (AAV2) sequences were detected in 93% (13 of 14), compared to 4 (3.5%) of 113 controls (P<0.001) and to 0 of 30 patients with hepatitis of defined etiology (P<0.001). In controls, HAdV-41 was detected in blood from 9 (39.1%) of the 23 patients with acute gastroenteritis (without hepatitis), including 8 of 9 patients with positive stool HAdV testing, but co-infection with AAV2 was observed in only 3 (13.0%) of these 23 patients versus 93% of cases (P<0.001). Co-infections by Epstein-Barr virus (EBV), human herpesvirus 6 (HHV-6), and/or enterovirus A71 (EV-A71) were also detected in 12 (85.7%) of 14 cases, with higher herpesvirus detection in cases versus controls (P<0.001). Our findings suggest that the severity of the disease is related to co-infections involving AAV2 and one or more helper viruses.

Viral hepatitis is an inflammation of the liver most caused by one of the major hepatitis viruses (A-E). Since October 2021, clusters of acute non-A-E severe hepatitis of unknown etiology in children have been reported in 35 countries, including the United States (US)[1,2]. As of August 17, 2022, 358 persons under investigation (PUIs) have been reported in the United States, of whom 22 (6%) required a liver transplant and 13 (4%) died[8].

HAdVs are non-enveloped, double-stranded DNA viruses that cause a variety of infections in both adults and children, including respiratory tract infection, conjunctivitis, and gastroenteritis (mainly from HAdV-40 and HAdV-41)[9]. Although a potentially life-threatening disease in immunocompromised patients[7,9], hepatitis from adenovirus infection has been thought to be rare in immunocompetent children without underlying comorbidities[10]. HAdVs, particularly HAdV-41, have been found in blood from clusters of acute severe hepatitis cases from Scotland, the United Kingdom (UK), and the US[3-7], but whether the virus is causative remains unclear. Adeno-associated viruses (AAVs) are small, single-stranded DNA parvoviruses that are considered non-pathogenic in humans and for this reason have been widely used as vectors for gene therapy[11]. Importantly, AAVs require a helper virus, such as a herpesvirus or adenovirus, for productive infection of liver tissue[12]. AAVs have been previously reported in children with acute severe hepatitis in a study of 9 patients from the UK[18], often in association with adenovirus or human herpesvirus 6 (HHV-6) infection[13].

In this study, 27 samples (21 whole blood, 2 plasma, 1 liver tissue, 1 nasopharyngeal swab, and 2 stool sample(s)) from 16 children with acute severe hepatitis of unknown etiology were analyzed **(Figure 1)**. All children met the clinical case definition established by the CDC, including lack of a confirmed etiology, liver enzyme levels (aspartate aminotransferase (AST) or alanine aminotransferase (ALT)) >500 U/L, age<10 years, and onset on or after October 1, 2021[2,14]. Cases were enrolled from 6 states (Alabama, California, Florida, Illinois, North Carolina, and South Dakota) from October 1, 2021 to May 22, 2022. All 16 cases had positive testing for HAdV from blood, and thus HAdV infection was over-represented compared to the overall affected population, of which HAdV is detected in 45-90%[3-7]. The median age of affected children was 3 years; 56% were female and 44% male (**Table 1**). Mean elevations in ALT and AST were 2,293 +/- 1,486 U/L (normal range: 4-36 U/L) and 2,652 +/- 1,851 U/L (normal range: 8-33 U/L), respectively **(Supplementary Table 1)**. A liver biopsy was performed in 8 patients; viral PCR testing of the liver biopsies for HAdVs,

herpesviruses, enteroviruses, and SARS-CoV-2 was incomplete, but yielded positive results for HAdV in 3 patients with no other viruses detected **(Supplementary Table 1 and Figure 2B)**. Two children underwent liver transplantation, but none died from complications of liver failure.

Controls (n=113) consisted of 78 whole blood, 1 serum, and 34 plasma samples. Many controls were enrolled from California (n=54) and Georgia (n=24) to be geographically similar to the cases, with the remaining controls enrolled from Ohio (n=12), Texas (n=14), and Washington (n=9). Sixty-nine of 113 controls (61.0%) were collected over the same time frame as the cases (i.e., collected between October 1, 2021 – May 22, 2022). The 113 controls consisted of 42 patients (37%) without hepatitis, 30 (26.5%) patients with acute hepatitis (ALT > 100 U/L) of defined etiology, 23 (21%) patients with acute gastroenteritis (12 with positive HAdV stool testing), and 18 (16%) blood donors. Differences in age and gender between cases and controls were non-significant overall, although cases were significantly younger and older, respectively, than controls with hepatitis of defined etiology and those with acute gastroenteritis **(Table 1).** Among reported symptoms, only jaundice was significantly more associated with cases than controls **(Table 1)**. Mean elevations in ALT and AST in the 30 hepatitis controls were 291 +/- 288 U/L and 455 +/- 833 U/L, respectively, and significantly lower than in cases ($P < 0.0001$) **(Table 1 and Supplementary Table 1)**.

Virus detection in hepatitis cases

Metagenomic sequencing for agnostic detection of all viruses[15], tiling multiplex PCR amplicon sequencing for AAV2 and HAdV-41[16], metagenomic sequencing with probe capture viral enrichment[17], and virus-specific polymerase chain reaction (PCR) testing for HAdV with genotyping by hexon gene sequencing were performed to identify AAV2 and HAdV viruses in clinical samples from cases **(Figure 2A).** We detected AAV2 from available whole blood or plasma samples in 13 (93%) of 14 cases and confirmed detection of HAdV in all 14 cases **(Figure 2B).** Ten (71%) of the 14

cases were typed as HAdV-41, one (7%) as HAdV-40, and one (7%) as HAdV-2; one (7%), sample NC_14, was untypeable. AAV2 and HAdV-41 enrichment were performed using either tiled multiplex PCR amplicon sequencing targeting the genomes of HAdV-41 and AAV (n=9 samples, with a mean 6.6±1.5 (SD) million raw reads generated per sample) or probe capture viral enrichment targeting 3,153 species (n=4, with a mean 128±34 (SD) million raw reads generated per sample). Mean normalized viral counts expressed as reads per kilobase per million mapped reads (RPKM) for AAV2 ($\bar{x}$=4,063) were approximately 12.7 times greater than for HAdV-41 ($\bar{x}$=320) **(Supplementary Table 1).** AAV2 was detected in both liver tissue and whole blood from one case and in 1 of 2 plasma samples, but neither AAV2 nor HAdV-41 were detected in nasopharyngeal swab and stool samples. No reads from AAVs other than AAV2 were detected, despite using methods that would enrich for other AAV subtypes. Among the 4 cases analyzed using metagenomic sequencing with probe capture viral enrichment (13_FL, 14_NC, 15_IL, and 16_SD), EBV was also detected in the blood sample from 13_FL.

Metagenomic sequencing was less sensitive than viral enrichment and targeted sequencing for detection of AAV2 (8 of 14 cases, 57%) and HAdV-41 (0 of 13 cases, 0%) **(Figure 2B and Supplementary Table 1)**. However, reads from additional viruses were identified including EBV (n=2), CMV (n=1), HAdV-2 (adenovirus type C) (n=1), and enterovirus A71 (EV-A71) (n=1) from whole blood, HAdV-1 (adenovirus type C) (n=1) from nasopharyngeal swab, and picobirnavirus (n=1) from stool. EV-A71 was detected as a co-infection in an HAdV-41/AAV2 case, while HAdV-2 was detected in the single AAV2-negative case.

Among the 113 controls, AAV2 was detected in 4 (3.5%), including 2 (16.7%) of 12 children with acute HAdV-positive gastroenteritis, 1 (9.1%) of 11 hospitalized children with HAdV-negative gastroenteritis, and 1 donor control who was also positive for HAdV **(Figure 2B and Supplementary Table 1).** The AAV2-positive child with HAdV-negative gastroenteritis was HAdV-negative from stool

but both HADV41-positive and CMV-positive from blood and had been discharged from the hospital with liver failure (although additional clinical details were not available). Of note, neither AAV2 nor HAdV41 was detected among the 30 pediatric controls with acute hepatitis of defined etiology and 42 hospitalized children without hepatitis.

We next performed virus-specific PCR testing for EBV, CMV, HHV-6, and SARS coronavirus 2 (SARS-CoV-2) for all available cases and controls **(Figure 2B and Supplementary Table 1).** EBV and HHV-6 were detected from blood in 11 (79%) of 14 and 7 (50%) of 14 cases, respectively, versus 1 (0.88%) of 113 controls for each virus. No tested cases were positive for CMV compared to 2 (1.8%) of 113 controls. SARS-CoV-2 was not detected in blood from cases or controls **(Figure 2B)**, nor from liver biopsy tissue **(Supplementary Table 1).**

Virus associations with hepatitis cases

We used Fisher's Exact Test on cases (n=14) and controls to investigate associations between detected viruses in blood and cases of acute severe hepatitis. Controls were stratified into 4 groups for comparison against cases (**Figure 2C, Supplementary Table 2**). Three viruses, AAV2, EBV and HHV-6, were significantly associated with cases when compared to each control group, except for HHV-6 detection between cases and donors ($p=0.010$, Bonferroni-corrected significance level of $p<0.002$). As all 14 cases were known to be HAdV-positive *a priori*, statistical analysis was not performed for HAdV-41.

Phylogenetic and mutation analyses

We performed multiple sequence alignment and phylogenetic analysis of 13 recovered AAV2 genomes from 12 cases with >25% breadth of coverage **(Figure 3 and Extended Data Figure 1)**. Multiple sequence alignment was performed in parallel with all complete 119 AAV2 reference

genomes deposited in GenBank as of August 18, 2022. A whole-genome nucleotide phylogenetic tree revealed that the 13 genomes were located within a distinct subgroup of a large human-infecting AAV2 clade **(Figure 3A).** Other previously sequenced AAV2 genomes from France and United States from patients without hepatitis were also found within this subgroup. Amino acid phylogenetic trees of the VP1 and AAP proteins showed slightly different topologies **(Supplemental Figure 1)**, but the 13 genomes were still positioned within a distinct subgroup.

To explore the underlying basis behind the observed phylogenetic clustering, we performed a multiple sequence alignment of the 13 genomes from cases. We then searched for shared coding mutations relative to the AAV2 reference genome that were found in ≥50% of genomes with coverage at that mutational site. This analysis yielded 35 mutations that were unevenly distributed across the viral genome, with 15 of 35 (42.9%) in the assembly activating protein (AAP), 14 of 35 (40%) in the VP1 protein, and 6 of 35 (17.1%) in the Rep78 protein **(Figure 3B and Supplementary Table 3)**. Clusters of mutations were found to be located within hypervariable regions of the capsid VP1 and AAP proteins[18]. Of note, 25 (71.4%) of the 35 mutations were shared with those identified in an independent study of severe pediatric hepatitis from the UK[13].

Discussion

Here we report virus findings from PCR, metagenomic, and targeted sequencing of samples from 16 pediatric cases of acute severe hepatitis of unknown etiology that were first identified in the US in October 2021. After combining these three diagnostic modalities, AAV2 was detected in 93% (13 of 14) of cases for whom whole blood or plasma samples were available (P<0.001). Conversely, AAV2 was detected in only 4 (3.5%) of 113 controls and HAdV-41 in 9 (8%) of 113. Notably, AAV2 was not detected in whole blood from 30 pediatric controls with hepatitis of defined etiology. HAdV sequences were detected in all 14 cases, of which 11 (79%) were genotyped as HAdV-41. The 100%

detection rate of HAdV is expected given that all 14 cases were known to be HAdV positive by clinical testing *a priori.* Other co-infecting viruses, including EBV and HHV-6, were detected by PCR in many cases but rarely in controls (P<0.001); EV-A71 was also detected by metagenomic sequencing in one case but not in any control. Taken together, these findings show a significant association between co-infection by AAV2 and one or more hepatotropic viral pathogens and the clinical manifestations of severe acute hepatitis, although a direct causal link has yet to be confirmed.

Initial epidemiological investigation and molecular testing have found adenovirus in 45-90% of severe acute hepatitis cases[3-7]. In the UK, 116 of 179 (64.8%) reported cases as of May 2022 have tested positive for HAdV[1]. This is in comparison to 8 of 9 (88.9%) positive cases in the United States corresponding to a cluster of cases who presented at a children's hospital in Alabama between October 2021 and March 2022[5], for which there is a substantial overlap with cases from our study. An expanded national investigation in the United States detected HAdV in slightly less than half (44.6%) of cases[3]. Consistent with prior reports[5,13], HAdV genotyping of our cases by sequencing confirmed that most of the detected serotypes were HAdV-41 (11 of 14, 79%).

Our results suggest that co-infection with AAV2 may cause more severe liver disease than infection by an adenovirus and/or herpesvirus alone. This condition may be analogous to the fulminant liver failure that can occur when hepatitis D virus (HDV, "delta") infection is superimposed on a chronic hepatitis B virus (HBV) infection[19]. Importantly, AAV2 was only seen in 2 (16.7%) of 12 pediatric controls with HAdV-associated acute gastroenteritis in the absence of liver inflammation, suggesting that co-infection with AAV2 may predispose patients to more severe disease. Here we also found that in cases of AAV2 / HAdV co-infection, viral loads for AAV2 were higher than for HAdV, with an approximately 12.7X increase in mean normalized read counts by targeted sequencing. This observation may be partially explained by previously published data showing that AAVs can suppress the replication of other hepatotropic viruses, including adenoviruses and herpesviruses[12]. Similarly,

patients co-infected with HDV usually exhibit low HBV viral loads due to suppression of HBV replication by HDV-induced interferons[20].

Among the AAVs, AAV2 is the most well-characterized AAV and has been shown to replicate to high titers in the liver and spleen[21]. Seroprevalence data from infants, children, and adults demonstrate that 30-80% of the general population is seropositive and that natural infection with AAV can occur at all ages[22]. However, there is a peak of AAV2 infection between the ages of 1 to 5 years old[23], consistent with the observed distribution of ages of cases in the current study.

Phylogenetic analysis reveals that the recovered AAV2 genomes from all cases map to a distinct subclade. The positioning is driven by groups of mutations located within hypervariable regions of the capsid VP1 and AAP proteins[18]. Notably, two of these mutations in the VP1 protein, R585S and R588T, are arginine-to-serine and arginine-to-threonine mutations that likely impact receptor binding, as these residues are necessary for the interaction of AAV2 with its heparan sulfate proteoglycan receptor[24]. These are shared not only among the US genomes in the current study but also overlap substantially with the mutations found in an independent study of acute severe hepatitis cases in children from the UK[13]. Interestingly, several of these shared capsid mutations (V151A, Q457M, S492A, E499D, F533Y, R585S, and R588T) are also found in a sublineage of AAV2 (AAVv66) that exhibits increased replication, virion stability, central nervous system transduction, and evasion of neutralizing antibodies relative to wild-type AAV2[25]. However, as other contemporary AAV2 genomes are not readily available, it is unclear whether the relatedness in AAV2 genomes across geographically dispersed regions and to AAVv66 merely represents detection of a predominant global circulating strain.

In the current study, hepatotropic viruses other than HAdV, including EBV, HHV-6, and EV-A71, were detected, albeit in a smaller proportion of cases. Infection by EBV or HHV-6 alone has been implicated in cases of liver failure requiring transplantation[26,27]. However, although we found

significant differences in the relative proportions of EBV and HHV-6 in cases compared to controls, we do not believe it likely that these viruses are the primary cause of acute severe hepatitis in these children. First, the viral loads for these herpesviruses were very low, with median PCR cycle threshold (C_t) values of 38.1 and 38 for EBV and HHV-6, respectively, and thus the borderline positive PCR results may represent detection of integrated proviral DNA rather than *bona fide* low-level herpesvirus viremia. Second, herpesvirus reactivation can be seen in various cytokine-associated inflammatory conditions such as COVID-19[28], and whether such reactivation contributes to disease pathogenesis is unclear. Third, herpesviruses were not detected in available liver biopsy tissue from cases, and the only viruses detected were HAdV (2 of 6, 33%) and AAV2 (1 of 1, 100%). Nevertheless, it is striking that among the 16 cases, dual, triple, and even quadruple infections with AAV2, adenovirus, EBV, and/or HHV-6 were detected in whole blood from at least 12 cases (75%). We postulate that the COVID-19 pandemic and more than 2 years of school and childcare closures and decreased social interactions with other children may have generated a vulnerable population of young children who failed to develop broad immunity to common viral pathogens due to lack of exposure. Interestingly, among the 9 cases with available information, 5 of 9 (55.6%) children had never attended school or a childcare center, whereas the remaining 4 were likely hindered from interacting with other children by social isolation measures enacted at the onset of the pandemic. Decreased immunity from lack of exposure to common viral pathogens may have predisposed cases to infection by multiple viruses, thus increasing the likelihood of more severe disease manifestations such as hepatitis.

Limitations of the study include (1) lack of availability and/or incomplete testing of liver biopsies, particularly for the presence or absence of AAV2, making it difficult to ascertain the pathogenetic mechanisms underlying the viral infection, (2) low titers associated with detected viral

pathogens, and (3) the retrospective study design and the inclusion of only cases that had previously tested positive for HAdV.

In summary, here we identify a distinct strain of AAV2 and co-infection with at least one helper virus in blood from US pediatric cases of acute severe hepatitis of unknown etiology. These results are consistent with findings from independent and contemporary studies of acute severe hepatitis in children from Scotland and the UK[13,29]. AAV2 infection may contribute to the pathogenesis and/or severity of the hepatitis, or alternatively, may be a non-pathogenic marker of liver inflammation. Further studies including serologic surveillance, viral culture, and animal models are needed to investigate the potential role AAV2 infection may play in this disease.

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TABLE AND FIGURE LEGENDS

Table 1. Demographic and clinical characteristics of cases and controls. Uncorrected *P*-values were calculated using two-tailed Fisher's Exact Test for categorical variables and two-tailed unpaired T-test for continuous variables. A Bonferroni-corrected significance level of $P < 0.001$ was considered significant (n=50 comparisons). Significant comparisons are denoted with an asterisk.

Characteristic		Cases (n=16)		Controls, all (n=113)			Controls, acute			Controls, acute		
		#	%	#	%	p-value	#	%	p-value	#	%	p-value
Sex	Female	9	56.3%	56	49.6%	0.79	15	50.0%	0.76	10	43.5%	0.52
	Male	7	43.8%	57	50.4%		15	50.0%		13	56.5%	
Age (years) mean ± SD		3.8 ± 2.0		7.4 ± 6.2		0.0239	10.7 ± 5.8		<0.0001*	1.6 ± 1.6		<0.0001*
Immunocom-promised	Yes	0	0.0%	15	13.3%	0.21	6	20.0%	0.08	0	0.0%	1
	No	16	100.0%	98	86.7%		24	80.0%		23	100.0%	
ICU stay	Yes	2	12.5%	30	26.5%	0.50	15	50.0%	0.08	1	3.3%	0.27
	No	10	62.5%	71	62.8%		15	50.0%		22	73.3%	
	unknown	4	25.0%	12	10.6%		0			0	0.0%	
ALT (U/L) mean ± SD		2,293 ± 1,486		N/A			291 ± 288		<0.0001*	N//A		
AST (U/L) mean ± SD		2,652 ± 1,851		N/A			455 ± 833		<0.0001*	N/A		
fever	Yes	6	37.5%	40	35.4%	0.78	16	53.3%	0.10	13	43.3%	0.51
	No	9	56.3%	46	40.7%		7	23.3%		10	33.3%	
	unknown	1	6.3%	27	23.9%		7	23.3%		0	0.0%	
diarrhea	Yes	8	50.0%	36	31.9%	0.58	10	33.3%	0.74	20	87.0%	0.03
	No	7	43.8%	45	39.8%		12	40.0%		3	13.0%	
	unknown	1	6.3%	32	28.3%		8	26.7%		0	0.0%	
nausea / vomiting	Yes	11	68.8%	38	33.6%	0.05	14	46.7%	0.50	17	73.9%	1
	No	0	0.0%	16	14.2%		2	6.7%		0	0.0%	
	unknown	5	31.3%	60	53.1%		14	46.7%		6	26.1%	
malaise / fatigue	Yes	10	62.5%	15	13.3%	0.016	9	30.0%	0.49	0	0.0%	N/A
	No	5	31.3%	35	31.0%		8	26.7%		0	0.0%	
	unknown	1	6.3%	63	55.8%		13	43.3%		23	100.0%	
abdominal pain	Yes	6	37.5%	22	19.5%	0.24	7	23.3%	0.71	2	8.7%	0.18
	No	10	62.5%	17	15.0%		7	23.3%		0	0.0%	
	unknown	0	0.0%	74	65.5%		16	53.3%		21	91.3%	
anorexia	Yes	10	62.5%	4	3.5%	0.08	3	10.0%	0.12	0	0.0%	N/A
	No	6	37.5%	10	8.8%		8	26.7%		0	0.0%	
	unknown	0		99	87.6%		19	63.3%		23	100.0%	
jaundice	Yes	13	81.3%	0	0.0%	<0.0001	0	0.0%	<0.0001*	0	0.0%	N/A
	No	2	12.5%	25	22.1%		10	33.3%		0	0.0%	
	unknown	1	6.3%	88	77.9%		20	66.7%		23	100.0%	
cough	Yes	5	31.3%	13	11.5%	0.29	10	33.3%	0.74	0	0.0%	N/A
	No	8	50.0%	45	39.8%		12	40.0%		0	0.0%	
	unknown	3	18.8%	55	48.7%		8	26.7%		23	100.0%	

Figure 1. **Epidemiology of cases and controls.** Geographic distribution of the 16 acute severe hepatitis cases and 113 controls in the study, showing the hospital or public health laboratory sites providing samples and associated clinical data from cases and/or controls.

Figure 2. **Sequencing and molecular-based testing for viruses performed on cases and controls. (A)** Analyses performed on the different sample groups. The specific assays performed for each group are shown in green while assays that are not done are shaded in black**. (B)** Graphical chart showing results of sequencing or PCR based assays for virus detection. Each circle represents a sequenced sample with at least 3 non-overlapping reads[30] aligning to a viral reference sequence. Circles are color-coded based on sample type and scaled according to normalized viral read counts. Viral PCR positive and negative results are denoted by plus and minus symbols, respectively. For the HAdV-F PCR, the HAdV subtype identified by hexon sequencing is shown to the left of the viral PCR result. **(C)** Associations between viruses detected in blood and cases of acute severe hepatitis of unknown etiology in children. Red and blue shading indicate positive and negative detection, respectively. Uncorrected *P*-values were calculated using two-tailed Fisher's Exact Test. A Bonferroni-corrected significance level of $P < 0.002$ was considered significant (n=24 comparisons). Exact *P* values are provided in **Supplementary Table 2.** Abbreviations: AAV, adeno-associated virus; AAV2, adeno-associated virus type 2; liver bx, liver biopsy; Ca, cases; Co, controls; CMV, cytomegalovirus; EBV, Epstein-Barr virus; enterovirus A71, EV-A71; HAdV-1, human adenovirus type 1; HAdV-40, human adenovirus type 40; HAdV-41, human adenovirus type 41; HAdV-F, human adenovirus type F; HHV-6, human herpesvirus 6; PBV, picobirnavirus; PCR, polymerase chain reaction; AL, Alabama; CA, California; FL, Florida; GA, Georgia; IL, Illinois; NC, North Carolina; OH, Ohio; SD, South Dakota; TX, Texas; WA, Washington; NS, non-significant; ***, p<0.001; ****, p<0.0001.

Figure 3. Phylogenetic and mutation analysis of AAV2 genomes. (A) Phylogenetic tree of 119 AAV2 whole genomes available from GenBank as of August 18, 2022. The 12 recovered genomes from this study with >25% coverage are denoted in red. The location of the AAV2 reference genome (NC_001401.2) is marked with a red asterisk. The phylogenetic tree was constructed by multiple sequence alignment of the AAV genomes or amino acid sequences using the MAFFT algorithm[31], followed by maximum likelihood based tree construction using IQ-TREE[32]. (B) Multiple sequence alignment of 13 AAV2 genomes recovered from cases. Nucleotide mismatches are represented by black-colored vertical lines, while areas of missing coverage are represented by gray rectangles. Amino acid variants with respect to the reference genome (NC_001401.2) are denoted in blue and red arrows; red arrows indicate shared mutations that were reported in another study from the UK and Scotland[13]. Mutational sites that were identified in 100% of cases with sufficient coverage in both studies are highlighted in boldfaced red text.

METHODS

Ethics Statement

Remnant clinical samples from cases with acute severe hepatitis were collected and analyzed under "no subject contact" protocols with waiver of informed consent approved by the institutional review boards (IRBs) of University of Alabama, Birmingham, California Department of Public Health, New York State Department of Health, and CDC. Whole blood samples from pediatric controls (age < 18) from Children's Healthcare of Atlanta were prospectively collected and analyzed under a protocol approved by the Emory IRB (STUDY00000723); parents or guardians of these children provided oral consent for study enrollment and collection and analysis of their samples. Remnant whole blood

samples from pediatric controls (age < 18) at University of California, San Francisco (UCSF) were collected, biobanked, and analyzed under a “no subject contact” protocol with waiver of informed consent approved by the UCSF IRB (protocol no. 11-05519). A subset of the control samples was provided by the CDC from children enrolled in the National Vaccine Surveillance Network (NVSN) study. Approval for the NVSN study was obtained from the institutional review board at each participating site and from the Centers for Disease Control and Prevention (protocol no. 6164). Parents or guardians of eligible children provided written informed consent for participant enrollment. Blood specimens were also collected as leftover samples from clinical procedures.

Subject Recruitment and Sample Collection

This was a retrospective observational case-control study using all available samples from cases and controls. A severe acute hepatitis case enrolled in this study was a person under investigation (PUI) by local, state, or federal public health agencies, defined as a person <10 years of age with elevated (>500 U/L) aspartate aminotransferase (AST) or alanine aminotransferase (ALT), an unknown etiology for the hepatitis, and onset on or after October 1, 2021[8]. All cases (n=16) were hospitalized with acute elevation in liver enzymes, asparate aminotransferase (AST) or alanine aminotransferase (ALT), and one or more of the following symptoms on presentation: nausea, vomiting, jaundice, generalized weakness, and abdominal pain. Cass were selected for inclusion into the current study if blood samples had previously tested positive for adenovirus by clinical testing, resulting in a selection bias that made case-control comparisons related to adenovirus detection not meaningful. Blood samples from cases meeting PUI criteria but testing negative for adenovirus were not available.

Controls from University of California, San Francisco (UCSF) in California (n=54) included children hospitalized with hepatitis of defined etiology (>100 U/L) or another inflammatory or non-

inflammatory condition. These controls were selected based on availability of convenience samples from a study of biomarkers of acute inflammatory disease, including severe COVID-19, in hospitalized patients. Controls were selected to be geographically similar (located within the same state) to cases from California. Remnant whole blood samples from controls from UCSF (n=54) were retrospectively biobanked and aliquoted with addition of 2X DNA/RNA Shield (Zymo Research) in a 1:1 ratio by volume and stored at -80°C until use.

Controls from Children's Healthcare of Atlanta (n=24) in Georgia included children hospitalized with hepatitis of defined etiology (>100 U/L) or another inflammatory or non-inflammatory condition (n=18) or blood donors (n=6). These controls were selected from available biobanked samples from consented subjects enrolled in a prospective study of COVID-19 and multi-system inflammatory syndrome in children (MIS-C). Controls were selected to be geographically similar (located within a neighboring state with similar demographic characteristics) to the cases from Alabama and Florida[33]. Collected samples were stored at -80°C until use.

Serum or plasma samples from children enrolled in the New Vaccine Surveillance Network (NVSN) were also included as controls. Three groups of children were selected: children admitted for acute gastroenteritis who tested positive for adenovirus in the stool (n=12), children admitted for acute gastroenteritis who tested negative for adenovirus in the stool (n=11), and blood donors (n=12). These children were enrolled from three sites (Seattle, Houston, and Cincinnati) from June 2021 to May 2022 and enrollment criteria have previously been described[34]. Collected samples were stored at -80°C until use.

Nucleic Acid Extraction

Whole blood and plasma samples collected from cases from Alabama were extracted at the Wadsworth Center laboratory using the NucliSENSÒ easyMAG "specific B" protocol (bioMerieux)

according to the manufacturer's instructions. Samples from cases from Florida, Illinois, North Carolina, and South Dakota were extracted using the Zymo Direct-zol™ DNA/RNA Miniprep Kit (Zymo Research) following the manufacturer's instructions. Briefly, 200 µL of sample was extracted and total nucleic acid was eluted in 60 µL and stored at -80°C until use.

Samples from cases form California were extracted at the California Department of Public Health. Whole blood samples (200 µL) were extracted using the Qiagen Blood Mini Kit (Qiagen) according to the manufacturer's instructions. Total nucleic acid was eluted in 100 µL and stored at -80°C until use. Respiratory samples, serum, plasma, and clarified stool suspensions were extracted using the NucliSENS easyMAG instrument (bioMerieux). Briefly, 300 µL of nasopharyngeal swab, serum, or plasma sample or 140 µL of clarified stool suspension was lysed with 1 mL of lysis buffer and incubated for 10 minutes at room temperature, followed by addition of 100 µL magnetic silica and transfer to the instrument to begin the automated extraction. Total nucleic acid was eluted in 110 µL for nasopharyngeal swab, serum, or plasma or 60 µL for stool and stored at -80°C prior to use.

For control samples from California or Georgia extracted at UCSF, total nucleic acid was extracted from the original sample using two different protocols for whole blood and plasma. Whole blood samples (400 µL) that had been pretreated with DNA/RNA Shield (Zymo Research) were extracted using the Quick-RNA Whole Blood Kit (Zymo Research) according to the manufacturer's instructions. Total nucleic acid was eluted in 15 µL and stored at -80°C until use. Plasma samples (200 µL) were extracted using the Mag-Bind Viral DNA/RNA 96 Kit (Omega Bio-Tek) on a KingFisher Flex instrument (Thermo-Fisher) according to the manufacturer's instructions. Total nucleic acid was eluted in 100 µL and stored at -80°C until use.

Viral PCR testing

Samples from California cases were screened for adenovirus using a pan-adenovirus PCR targeting all human adenoviruses[35] and/or a group F adenovirus (HAdV-40 / HAdV-41) real-time PCR[36]. A cycle threshold cutoff of ≤40 was used to call a positive result by PCR. PCR and Sanger sequencing of the HAdV hexon gene targeting hypervariable regions 1-6[37] were performed on all adenovirus positive samples with purified PCR products sequenced in-house or sent to an outside laboratory (Sequetech, Mountain View, CA) for sequencing. Sanger sequences were assembled and edited in Sequencher 5.2.4 (Gene Codes) and analyzed using the nucleotide BLAST aligner[38]. Samples from cases outside of California and all control samples were screened for adenovirus using a pan-adenovirus PCR assay[35] and sequenced using a nested PCR assay targeting HAdV hexon hypervariable regions 1-6, followed by Sanger sequencing[39]. PCR testing for detection of CMV, EBV, and HHV-6 was performed as previously described[40].

Viral Enrichment Based Targeted Sequencing

For cases from California and Alabama and all controls, viral enrichment followed by targeted sequencing of viral genomes was performed using custom spiked primers designed to target HAdV-41 or adeno-associated virus (AAV1-AAV8) genomes. Spiked primers were designed using the metagenomic sequencing with spiked primer enrichment (MSSPE) algorithm[16] as follows. For HAdV-41, 22 representative HAdV-41 genomes were aligned using MAFFT, while for AAV, 11 genomes representing AAV1-AAV8 were aligned using MAFFT. Next, automated primer design was performed by running the MSSPE algorithm with the following parameters: kmer size=25, segment window=500, e-value=0.1, dS=2, dG=-9000. The algorithm generated 219 HAdV and 150 AAV spiked primers **(Supplementary Table 4)**. PCR amplification from extracted DNA was performed as previously described[41], with the following modifications: the annealing temperature for PCR was 60°C for HAdV and 53°C for AAV. The HAdV primers were amplified in two different reactions using two different

sets of primers **(Supplementary Table 4, Set1 and Set2)**, while the AAV2 primers were amplified in a single reaction. Next-generation sequencing (NGS) libraries were prepared using the NEBNext Ultra II DNA Kit (New England Biolabs) and Revelo™ DNA-Seq Mech Kit on the MagicPrep™ NGS system (Tecan Genomics). Final libraries were quantified using the Qubit Flex instrument (Invitrogen) with the dsDNA HS Assay Kit (Invitrogen). Libraries were pooled and sequenced on an Illumina NextSeq using 300 base pair (bp) single-end sequencing. Negative template controls were included in every run to monitor for contamination. No contamination from HAdV-41 or AAV2 reads was detected in the negative template control libraries.

For cases from Florida, Illinois, North Carolina, and South Dakota, probe capture target enrichment of viral genomes was performed on metagenomic libraries using the Twist Comprehensive Viral Research Panel (Twist Biosciences), which covers reference genomes of 3153 viruses and 15,488 different strains[42]. Individual DNA or cDNA/RNA libraries were hybridized to CVRP probes according to the manufacturer's instructions. Libraries were then barcoded and sequenced on an Illumina MiSeq (250 bp paired-end sequencing) or an Illumina NextSeq (150 bp paired-end sequencing) (Illumina). For plasma sample 14_NC, nucleic acid carrier, either bacteriophage lambda DNA (New England Biolabs) or HeLa total RNA (Thermo-Fisher), was added during the library preparation step according to the manufacturer's instructions.

Viral Metagenomic Sequencing and Analysis

Metagenomic DNA and RNA libraries were prepared using the Revelo™ DNA-Seq Mech Kit on the MagicPrep™ NGS system (Tecan Genomics), NEBNext Ultra II DNA Library Prep Kit (New England Biolabs) and NEBNext Ultra II RNA Library Prep Kit (New England Biolabs), according to the manufacturer's instructions. Libraries were pooled and sequenced on a NextSeq 550 Sequencing

System using 150 bp single-end sequencing. Potential contamination was monitored in each run by processing negative water and controls in parallel with samples.

Sequencing data from all cases and controls were analyzed for viral nucleic acids using SURPI+ (v1.0.7-build.4)[43], an automated bioinformatics pipeline for pathogen detection and discovery from metagenomic data that has been modified to incorporate enhanced filtering and classification algorithms[30]. A threshold of ³3 non-overlapping reads was used for calling a positive virus detection[30].

Phylogenetic Analysis

Multiple sequence alignments were performed using MAFFT algorithm (v7.388)[31] as implemented in Geneious (version 10.0.9)[44]. Nucleotide and amino acid phylogenetic trees were inferred using a maximum likelihood method with ultrafast bootstrap approximation as implemented in IQ-TREE (version 1.6.1)[32] using 1000 bootstrap replicates. Trees were visualized using FigTree (version 1.4.4).

Viral Genome Assembly and Analysis

Binary base call (BCL) files generated by Illumina sequencers were simultaneously demultiplexed and converted to FASTQ files using bcl2fastq (version 2.20.0.422). Custom scripts were used to assemble AdV and AAV2 genomes as follows. Briefly, raw FASTQ reads were filtered using BBDuk (version 38.87)[45] for removal of adapters, primer sequences, and low-quality reads, and then HAdV-41 or AAV reads were identified by Bowtie2[46] alignment (parameters: -D 20 -R 3 -L11 -N 1) to a reference database consisting of 1,395 HAdV-41 or 3,600 AAV partial sequences / genomes, respectively. These aligned reads were then mapped to the HAdV-41 reference genome (accession DQ315364.2) or consecutively to AAV genomes 1-8. For all AAV genomes, the assembly with the highest breadth of coverage corresponded to the AAV2 reference genome (accession number

NC_001401.2). Consensus assemblies were generated using iVar[47] (parameters: -t 0.5 -m 1). AAV consensus genomes were further analyzed for shared mutations in the nucleotide and translated nucleotide (amino acid) sequences relative to the AAV2 reference by performing a multiple sequence alignment using the MAFFT algorithm[31], followed by visualization of the alignment using Geneious software (version 10.0.9)[44].

Statistical Analysis

Statistical analyses were performed using the Python scipy package (version 1.5.2)[48] and rstatix package (version 0.7.0) in R (version 4.0.3)[49]. Uncorrected *P*-values were calculated using two-tailed Fisher's Exact Test for categorical variables and two-tailed unpaired T-test for continuous variables, with the significance level determined after a Bonferroni correction for multiple comparisons.

Data Visualization

Plots were generated using matplotlib (version 3.3.2), seaborn (version 0.11.0) and plotly (version 5.6.0) packages in Python software (version 3.7.12), Jupyter notebook (version 6.1.4), RStudio (version 1.4) and Adobe Illustrator (version 26.4.1) software.

Data Availability

Raw metagenomic sequencing data with human reads removed have been deposited in the NCBI Sequence Read Archive (Bioproject accession number PRJNA918667, under umbrella BioProject accession number PRJNA171119). Consensus genome files, alignment files, phylogenetic tree files and supplementary tables have been uploaded to a Zenodo data repository (doi: 10.5281/zenodo.7089581).

Code Availability

Custom scripts for Ad41 and AAV genome assembly are available in a Zenodo data repository (doi: 10.5281/zenodo.7089581).

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673 **Pediatric Hepatitis of Unknown Etiology Working Group**

674 Olivia Almendares[11], Neha Balachandran[11], Jordan E. Cates[11], Brandi Freeman[11], Anita
675 Kambhampati[11], Rachel Marine[11], Everardo Vega[11], Mary E. Wikswo[11], Erin R. McKeever[11], David
676 Kelly[14], Julie A.[19], Leila C. Sahni[19], Janet A. Englund[20], Eileen J. Klein[20], Mary A. Staat[21], Maeve
677 Marsh (IL Department of Public Health)[8], Erin Ricketts9, Joshua L. Clayton[10], Natalia Cano[7], Miriam
678 Oseguera[1], and Robert Blattner[1]. Consortium representative: Olivia Alemendares[11], gtt1@cdc.gov.
679 1. Department of Laboratory Medicine, University of California, San Francisco, San Francisco,
680 CA, USA.
681 7. Florida Department of Health, Tallahassee, USA
682 8. Illinois Department of Public Health, Chicago, IL, USA
683 9. North Carolina Department of Health and Human Services, Raleigh, NC, USA
684 10. South Dakota Department of Health, Pierre, SD, USA
685 11. Centers for Disease Control and Prevention, Atlanta, CA, USA
686 14. University of Alabama at Birmingham, Birmingham, AL, USA
687 19. Baylor College of Medicine, Houston, TX
688 20. Seattle Children's Research Institute, Seattle, WA
689 21. Children's Hospital Medical Center, Cincinnati, OH
690

691 **AUTHOR CONTRIBUTIONS**

692 C.Y.C., J.V., T.R., and K.S.G. conceived and designed the study. C.Y.C., V.S., and A.S.G.
693 coordinated the study. V.S., A.S.G., A.F., H.J.H., P.E.O., and J.T. performed metagenomic
694 sequencing and targeted viral enrichment experiments. V.S. and D.S. ran the SURPI computational
695 pipeline for detection of viruses from metagenomic data. V.S. and C.Y.C. analyzed metagenomic and
696 targeted viral enrichment data, assembled AAV2 genomes, and performed phylogenetic and mutation

analyses. A.L.B., N.H.B., R.L.B., K.Y.G., J.S.G., S.P.L., K.P., D.R., and R.T.R. performed viral probe capture enrichment experiments and assembled AAV2 genomes. R.J., M.T.L., Z.L., and H.H. investigated acute severe hepatitis cases and collected samples and clinical metadata. U.P., M.S., K.W., M.S.O., J.E.T., J.M.B., D.S., C.P., X.L., P.C., J.V., H.L.K., and the CDC Pediatric Hepatitis of Unknown Etiology Working Group investigated acute severe hepatitis cases for the CDC and collected samples and clinical metadata. L.A.I., H.S., W.B., and L.H.G.S. investigated acute severe hepatitis cases in Alabama and collected samples and clinical metadata. C.C. and C.A.R. provided samples and clinical metadata from pediatric controls from Georgia. V.S., A.S.G., J.N., and C.Y.C. provided samples and clinical metadata from pediatric controls from California. A.H., C.-Y.P., K.R., J.B.B., C.M., and D.A.W. investigated acute severe hepatitis cases and performed viral PCR testing and HAdV genotyping at the California Department of Public Health. D.M.L. and K.S.G. performed viral PCR testing and HAdV genotyping at the Wadsworth Center / New York State Department of Public Health. C.Y.C. and V.S. wrote the initial draft of the manuscript and prepared the figures. C.Y.C., V.S., A.S.G., U.P., J.E.T., J.M.B., C.P., X.L., J.V., H.L.K., and R.T.R. edited the manuscript. All authors read the manuscript and agreed to its contents.

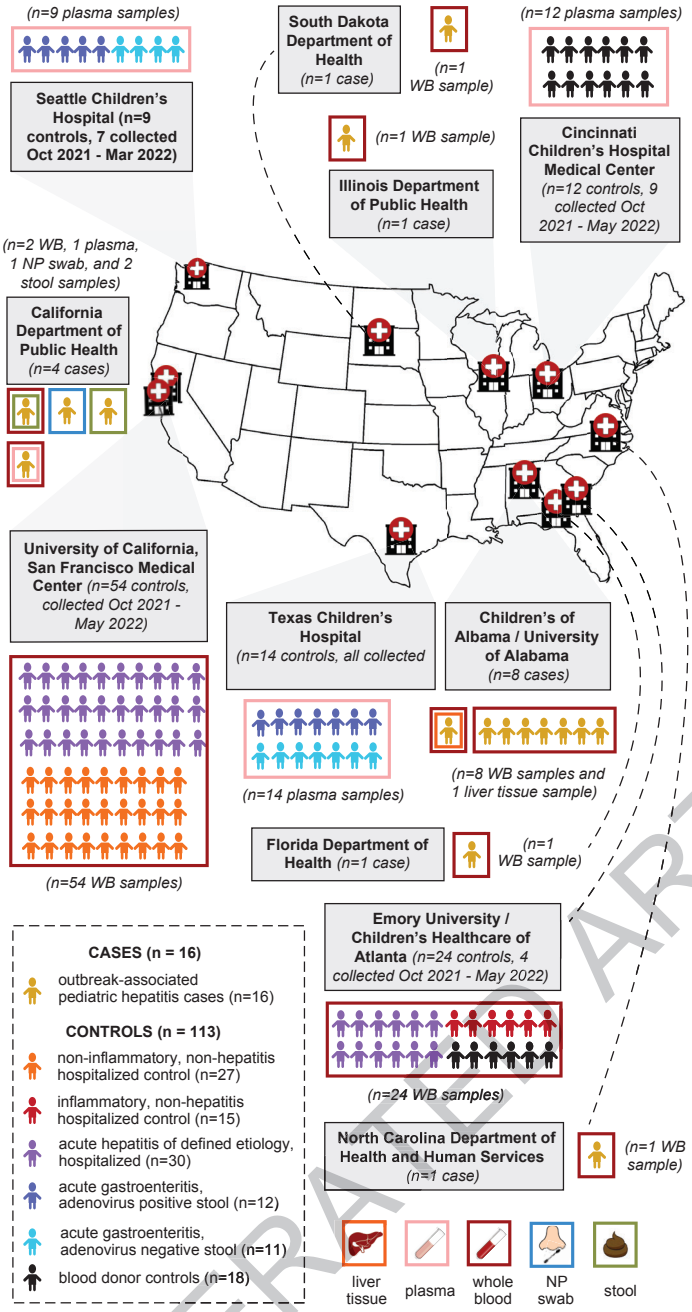
COMPETING INTERESTS

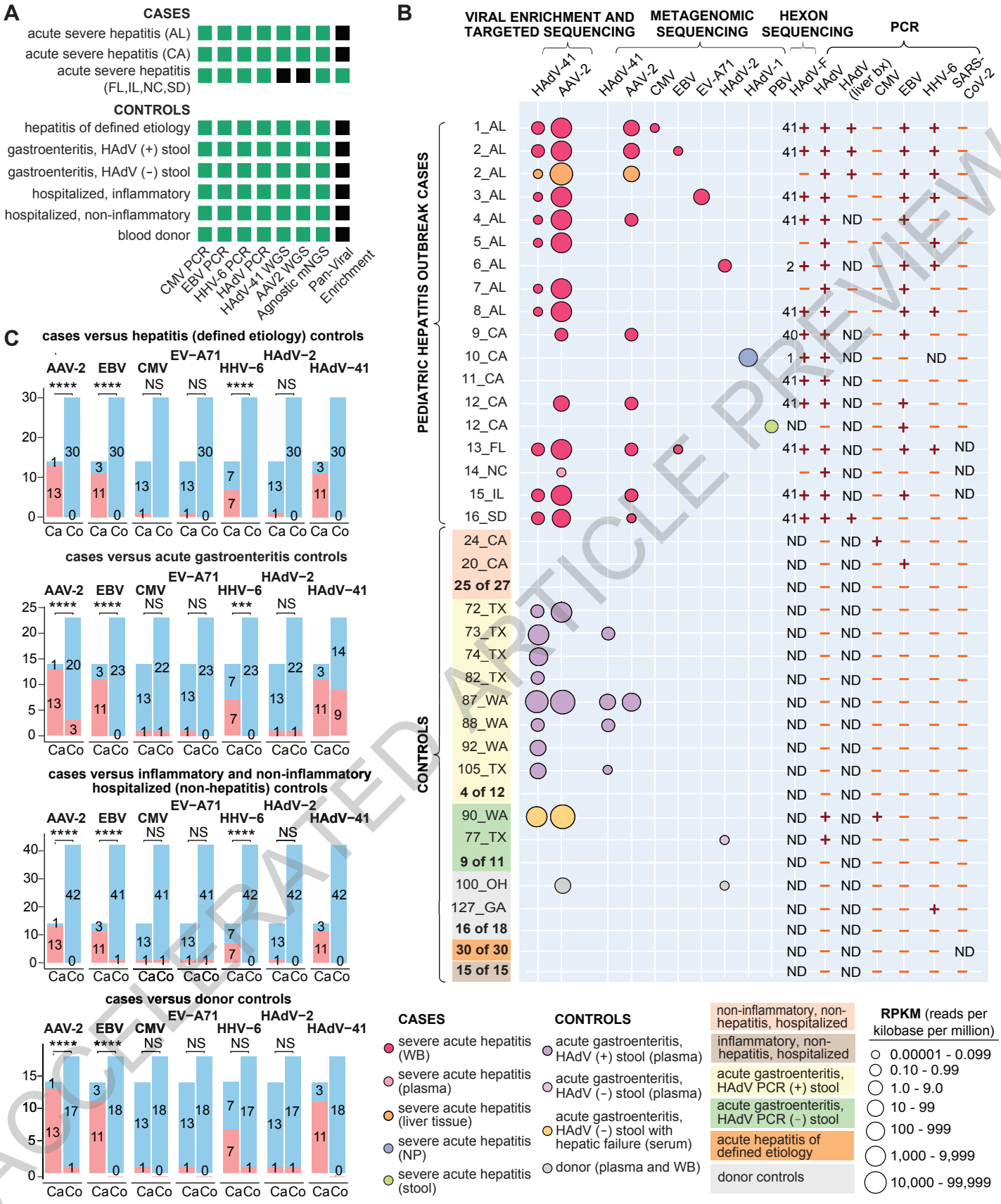
C.Y.C. is a founder of Delve Bio and on the scientific advisory board for Delve Bio, Mammoth Biosciences, BiomeSense, and Poppy Health. He is also a co-inventor on U.S. patent 11380421, “Pathogen Detection using Next Generation Sequencing”, under which algorithms for taxonomic classification, filtering, and pathogen detection are used by SURPI+ software. C.Y.C. receives reseach support unrelated to this manuscript from Abbott Laboratories, Inc. C.A.R.’s institution has received funds to conduct clinical research unrelated to this manuscript from BioFire Inc, GSK, Janssen, MedImmune, Merck, Moderna, Novavax, PaxVax, Pfizer, Regeneron, Sanofi-Pasteur. She

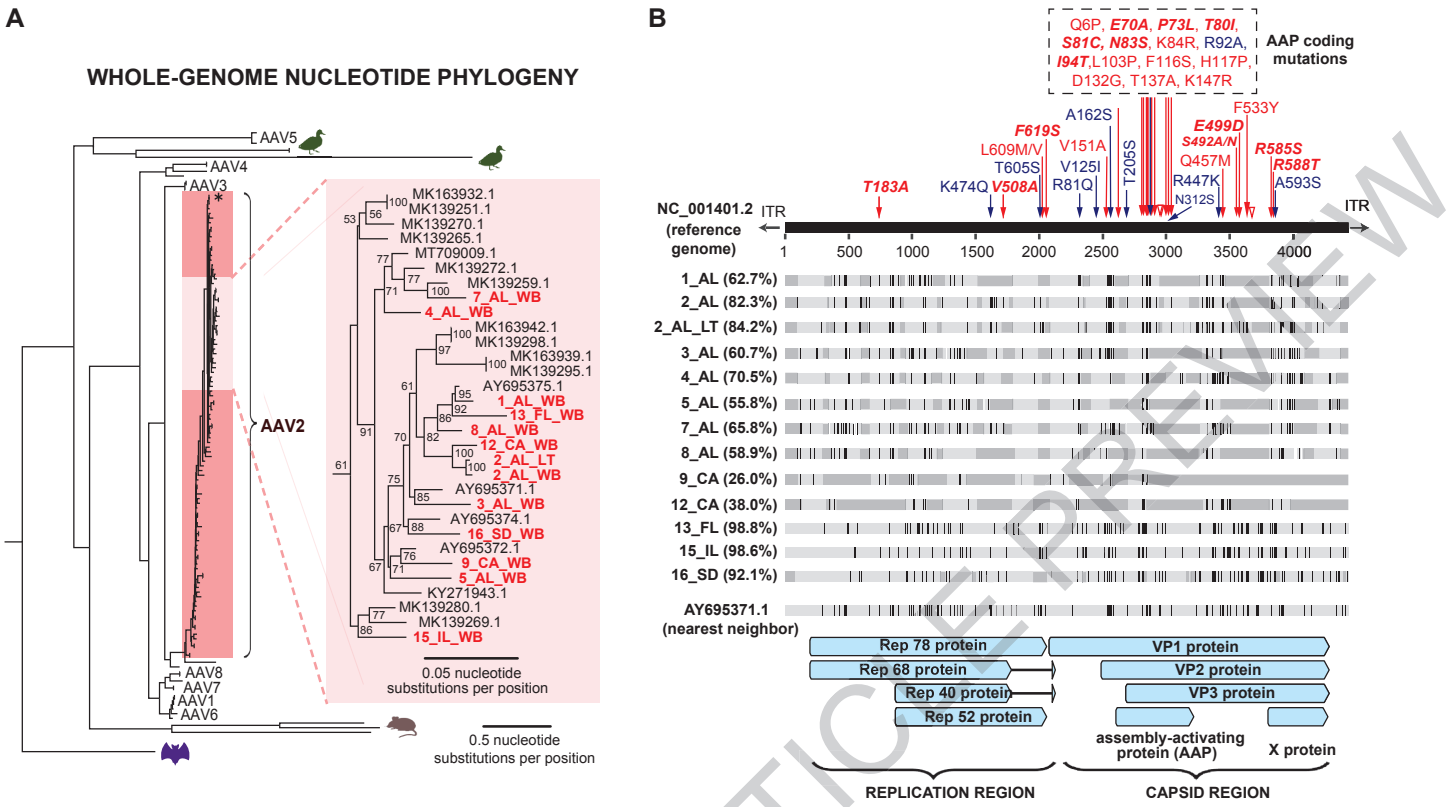
721 is co-inventor of patented RSV vaccine technology, which has been licensed to Meissa Vaccines, Inc.
722 K.S.G receives research support unrelated to this manuscript from Thermo-Fisher and has a royalty
723 generating collaborative agreement with Zeptometrix. The other authors declare no competing
724 interests.
725
726

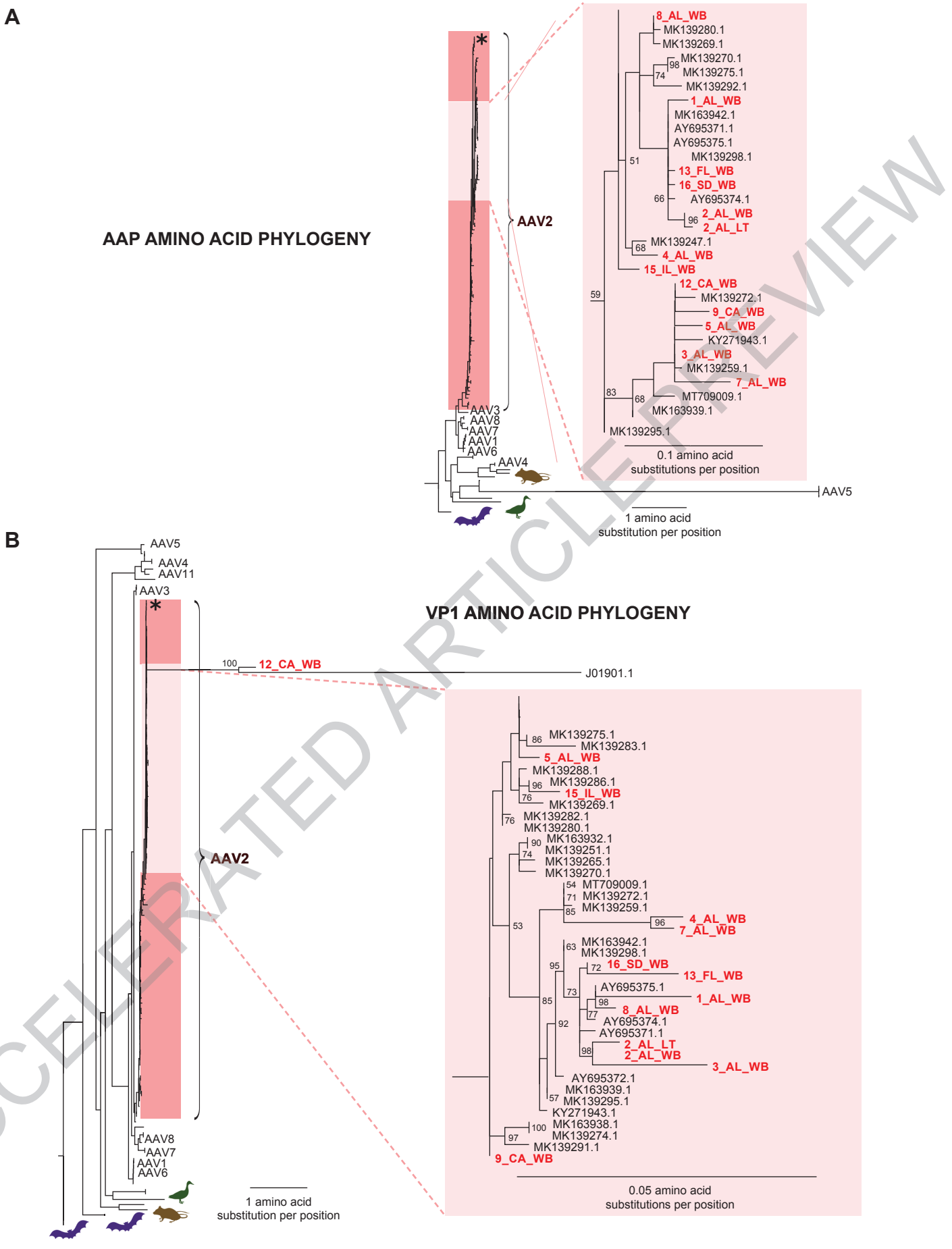
EXTENDED DATA FIGURES

Extended Data Figure 1. Amino acid phylogenetic analysis of AAV2 genomes from cases, related to Figure 3. (A) Phylogenetic tree corresponding to the Assembly Activating Protein (AAP) protein. **(B)** Phylogenetic tree corresponding to the VP1 protein. Phylogenetic trees was constructed by multiple sequence alignment of the AAV genomes or amino acid sequences using the MAFFT algorithm[31], followed by maximum likelihood based tree construction using IQ-TREE[32].









Extended Data Fig. 1

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Corresponding author(s): Charles Chiu

Last updated by author(s): 1/5/2023

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For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed	
☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒	A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒	The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☒	☐	A description of all covariates tested
☒	☐	A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒	A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒	For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

Viral PCR and hexon sequencing data were collected using manufacturer-provided software associated with the instrument. Metagenomic and whole-genome sequencing data were collected on Illumina sequencers (MiSeq or NextSeq Sequencing System) using manufacturer-provided software.

Data analysis

A cycle threshold cutoff of ≤40 was used to call a positive result by PCR. Sanger sequences were assembled and edited in Sequencher 5.2.4 (Gene Codes) and analyzed using the nucleotide BLAST aligner.

Binary base call (BCL) files generated by Illumina sequencers were simultaneously demultiplexed and converted to FASTQ files using bcl2fastq (version 2.20.0.422). Metagenomic sequencing data from all cases and controls were analyzed for viral nucleic acids using SURPI+ (v1.0.7-build.4), an automated bioinformatics pipeline for pathogen detection and discovery from metagenomic data that has been modified to incorporate enhanced filtering and classification algorithms. A threshold of ≥ 3 non-overlapping reads was used for calling a positive virus detection.

A custom script was used to assemble AdV and AAV2 genomes as follows. Briefly, raw FASTQ reads were filtered using BBDuk (version 38.87) for removal of adapters, primer sequences, and low-quality reads, and then HAdV-41 or AAV reads were identified by Bowtie2 alignment (parameters: -D 20 -R 3 -L11 -N 1) to a reference database consisting of 1,395 HAdV-41 or 3,600 AAV partial sequences / genomes, respectively. These aligned reads were then mapped to the HAdV-41 reference genome (accession DQ315364.2) or consecutively to AAV genomes 1-8. For all AAV genomes, the assembly with the highest breadth of coverage corresponded to the AAV2 reference genome (accession number NC_001401.2). Consensus assemblies were generated using iVar (parameters: -t 0.5 -m 1). AAV consensus genomes were further analyzed for shared mutations in the nucleotide and translated nucleotide (amino acid) sequences relative to the AAV2 reference by performing a multiple sequence alignment using the MAFFT algorithm (v7.388), followed by visualization of the alignment using Geneious

software (version 10.0.9).

Multiple sequence alignments were performed using MAFFT algorithm (v7.388) as implemented in Geneious (version 10.0.9). Nucleotide and amino acid phylogenetic trees were inferred using a maximum likelihood method with ultrafast bootstrap approximation as implemented in IQ-TREE (version 1.6.1). Trees were visualized using FigTree (version 1.4.4).

Statistical analyses were performed using the Python scipy package (version 1.5.2) and rstatix package (version 0.7.0) in R (version 4.0.3). Fisher's Exact Test was used to assess the association between variables. All statistical tests were conducted as two-sided at the 0.05 significance level.

Plots were generated using matplotlib (version 3.3.2), seaborn (version 0.11.0) and plotly (version 5.6.0) packages in Python software (version 3.7.12), Jupyter notebook (version 6.1.4), RStudio (version 1.4) and Adobe Illustrator (version 26.4.1) software.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

Raw metagenomic sequencing data with human reads removed have been deposited in the NCBI Sequence Read Archive (Bioproject accession number PRJNA918667, under umbrella BioProject accession number PRJNA171119). Consensus genome files, alignment files, phylogenetic tree files and supplementary tables have been uploaded to a Zenodo data repository (doi: 10.5281/zenodo.7089581).

Human research participants

Policy information about studies involving human research participants and Sex and Gender in Research.

Reporting on sex and gender	Sex and gender were not considered in study design. Analysis was performed on aggregated data..
Population characteristics	In this study, 27 samples (21 whole blood, 2 plasma, 1 liver tissue, 1 nasopharyngeal swab, and 2 stool sample(s)) from 16 children with acute severe hepatitis of unknown etiology were analyzed. All children met the clinical case definition established by the CDC, including lack of a confirmed etiology, liver enzyme levels (aspartate aminotransferase (AST) or alanine aminotransferase (ALT)) >500 UL, age<10 years, and onset on or after October 1, 2021. Cases were enrolled from 6 states (Alabama, California, Florida, Illinois, North Carolina, and South Dakota) from October 1, 2021 to May 22, 2022. All 16 cases had positive testing for HAdV from blood, and thus HAdV infection was over-represented compared to the overall affected population, of which HAdV is detected in 45-90%. The median age of affected children was 3 years; 56% were female and 44% male. Controls (n=113) consisted of 78 whole blood, 1 serum, and 34 plasma samples. Many controls were enrolled from California (n=54) and Georgia (n=24) to be geographically similar to the cases, with the remaining controls enrolled from Ohio (n=12), Texas (n=14), and Washington (n=9). Sixty-nine of 113 controls (61.0%) were collected over the same time frame as the cases (i.e., collected between October 1, 2021 – May 22, 2022). Differences in age and gender between cases and controls were non-significant. The 113 controls consisted of 42 patients (37%) without hepatitis, 30 (26.5%) patients with acute hepatitis (ALT > 100 U/L) of defined etiology, 23 (21%) patients with acute gastroenteritis (12 with positive HAdV stool testing), and 18 (16%) blood donors.
Recruitment	This was a retrospective observational case-control study using all available samples from cases and controls. A severe acute hepatitis case enrolled in this study was a person under investigation (PUI) by local, state, or federal public health agencies, defined as a person <10 years of age with elevated (>500 U/L) aspartate aminotransferase (AST) or alanine aminotransferase (ALT), an unknown etiology for the hepatitis, and onset on or after October 1, 20218. All cases (n=16) were hospitalized with acute elevation in liver enzymes, asparate aminotransferase (AST) or alanine aminotransferase (ALT), and one or more of the following symptoms on presentation: nausea, vomiting, jaundice, generalized weakness, and abdominal pain. Cass were selected for inclusion into the current study if blood samples had previously tested positive for adenovirus by clinical testing, resulting in a selection bias that made case-control comparisons related to adenovirus detection not meaningful. Blood samples from cases meeting PUI criteria but testing negative for adenovirus were not available. Controls from University of California, San Francisco (UCSF) in California (n=54) included children hospitalized with hepatitis of defined etiology or another inflammatory or non-inflammatory condition. These controls were selected to be geographically similar (located within the same state) to cases from California. Remnant whole blood samples from controls from UCSF (n=54) were retrospectively biobanked and aliquoted with addition of 2X DNA/RNA Shield (Zymo Research) in a 1:1 ratio by volume and stored at -80°C until use. Controls from Children's Healthcare of Atlanta (n=24) in Georgia included children hospitalized with hepatitis of defined etiology or another inflammatory or non-inflammatory condition (n=18) or blood donors (n=6). These controls were selected to be geographically similar (located within a neighboring state with similar demographic characteristics) to the cases from

Alabama and Florida. Collected samples were stored at -80°C until use.

Serum or plasma samples from children enrolled in the New Vaccine Surveillance Network (NVSN) were also included as controls. Three groups of children were selected: children admitted for acute gastroenteritis who tested positive for adenovirus in the stool (n=12), children admitted for acute gastroenteritis who tested negative for adenovirus in the stool (n=11), and blood donors (n=12). These children were enrolled from three sites (Seattle, Houston, and Cincinnati) from June 2021 to May 2022. Collected samples were stored at -80°C until use.

Ethics oversight

Remnant clinical samples from cases with acute severe hepatitis were collected and analyzed under "no subject contact" protocols with waiver of informed consent approved by the institutional review boards (IRBs) of University of Alabama, Birmingham, California Department of Public Health, New York State Department of Health, and CDC. Whole blood samples from pediatric controls (age < 18) from Children's Healthcare of Atlanta were prospectively collected and analyzed under a protocol approved by the Emory IRB (STUDY00000723); parents or guardians of these children provided oral consent for study enrollment and collection and analysis of their samples. Remnant whole blood samples from pediatric controls (age < 18) at University of California, San Francisco (UCSF) were collected, biobanked, and analyzed under a "no subject contact" protocol with waiver of informed consent approved by the UCSF IRB (protocol no. 11-05519). A subset of the control samples was provided by the CDC from children enrolled in the National Vaccine Surveillance Network (NVSN) study. Approval for the NVSN study was obtained from the institutional review board at each participating site and from the Centers for Disease Control and Prevention (protocol no. 6164). Parents or guardians of eligible children provided written informed consent for participant enrollment. Blood specimens were also collected as leftover samples from clinical procedures.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

No sample size was calculated. All available samples were processed. 27 samples (21 whole blood, 2 plasma, 1 liver tissue, 1 nasopharyngeal swab, and 2 stool sample(s)) from 16 pediatric patients with acute severe hepatitis of unknown etiology were analyzed. Affected children included in this analysis were admitted to tertiary care hospitals in 6 states (Alabama, California, Florida, Illinois, North Carolina, and South Dakota) from October 1, 2021 to May 22, 2022.
Controls (n=113) consisted of 78 whole blood, 1 serum, and 34 plasma samples. The majority of controls were enrolled from California (n=54) and Georgia (n=24) to be geographically similar to the cases, with the remaining controls enrolled from Ohio (n=12), Texas (n=14), and Washington (n=9). The controls consisted of a mix of remnant convenience samples (California) and samples from patients prospectively enrolled in a clinical (Georgia) or population-level surveillance (Ohio, Texas, and Washington) study.

Data exclusions

No data were excluded from the analysis.

Replication

Samples were processed only once but analyses were done on independent sample cohorts from 6 states (Alabama, California, Florida, Illinois, North Carolina, and South Dakota) and independent control cohorts from California, Georgia, Ohio, Texas and Washington state.

Randomization

Randomization is not applicable as this is a retrospective observational case-control study that used all available samples from cases (pediatric acute severe hepatitis) and controls.

Blinding

Researchers were blinded during processing as all samples were assigned a random unique ID. Cases and controls were analyzed in parallel. Samples were unblinded during analyses to interpret and visualize results and to determine associations between detection of virus and severe acute hepatitis.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☒	☐ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☒	☐ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging