Supplementary Appendix

Supplement to: Gutierrez Sanchez LH, Shiau H, Baker JM, et al. A case series of children with acute hepatitis and human adenovirus infection. N Engl J Med. DOI: 10.1056/NEJMoa2206294

This appendix has been provided by the authors to give readers additional information about the work.

Supplementary Appendix

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Centers for Disease Control (CDC) Investigation Policy

CDC policy: eg, 45 CFR part 46, 21 CFR part 56; 42 USC §241(d); 5 USC §552a; and 44 USC §3501 et seq.

University of Alabama (UAB) Diagnostic Virology Lab Human Adenovirus real-time PCR Assays for Whole Blood and Fresh Frozen Tissue

The HAdV qPCR testing done on whole blood and PCR testing on fresh frozen liver tissue was done at the UAB Diagnostic Virology Lab. UAB Diagnostic Virology Lab had previously validated a real-time PCR assay to quantify Human Adenovirus (HAdV) DNA in human blood specimens. For this assay, a conserved region of the hexon gene was amplified using HAdV-specific primers that detect all adenovirus types. The PCR reaction was composed of ELITech MGB Alert® Adenovirus primer mix, EliTech MGB Alert® FAM Probe, and MGB Alert® Platinum Master Mix. Nucleic acids from clinical specimens were extracted with the Roche MagNA Pure 96 automated extraction system. This DNA template was amplified through 45 successive cycles of PCR using the following protocol on an Applied Biosystems™ 7500 Real-Time PCR instrument (Thermo Fisher Scientific).

Table S1. DNA Amplification Protocol

Cycles	Step	Time	Temp. (°C)
1	PCR Enzyme Activation	4 min.	95
45	Denaturation	10 sec.	95
	Annealing	30 sec.	56
	Extension	15 sec	73

A laboratory-designed plasmid encoding target DNA sequence for the virus, was manufactured by IDT (Integrated DNA Technologies), was used to generate a standard curve to determine the absolute quantity of viral DNA in each qPCR assay. Results are reported as genome copy per ml of blood extracted.

The validation of this assay included comparison of 20 positive blood specimens that were shown to be positive for HAdV by an established qualitative method, and 20-deidentified samples from the laboratory of Dr. Keith Tardiff at ARUP Laboratories, Salt Lake City, Utah, shown to be HAdV positive by their validated qPCR assay, as well as 20 negative specimens known to be negative for HAdV by qualitative assay.

The assay described above to detect HAdV in blood is the same assay used to detect HAdV in the liver biopsy tissue. Liver tissue was placed in Viral Transport Media and processed using standard tissue grinder. This product was then subjected to total nucleic acid extraction using the Roche MagnaPure 96 instrument in the same manner as the blood samples.

US Centers for Disease Control and Prevention Testing on Formalin-Fixed, Paraffin Embedded (FFPE) Liver Biopsy Tissue

At the CDC Infectious Disease Pathology Branch, immunohistochemistry for HAdV was done on formalin-fixed, paraffin embedded (FFPE) liver biopsy tissues by using a mouse adenovirus monoclonal antibody with broad reactivity with adenovirus species (CDC Biological Products) at 1:2000 dilution and a Mach 4 Universal AP Polymer Kit (Biocare Medical) with Permanent Red Chromogen (Cell Marque/Millipore Sigma).[1] A conventional adenovirus specific PCR assay targeting the hexon gene, followed by Sanger sequencing of amplicons, was also performed on DNA extracted from FFPE liver tissue biopsies. This assay used previously published primers,[2] but the PCR was modified, validated and CLIA approved in the laboratory of CDC. Enterovirus and acute respiratory syndrome coronavirus 2 (SARS-CoV-2) RT-PCR assays were also performed on RNA extracted from FFPE liver tissue biopsies as previously described.[3,4]

Wadsworth Center Human Adenovirus Typing and Phylogenetics

Available residual blood specimens were sent to the Wadsworth Center, New York State Department of Health (Albany, NY) for HAdV typing. To determine HAdV type, a portion of the hexon gene that covers six of the seven hypervariable regions (HVRs) was amplified and bidirectional sequencing performed.[5] Sequences generated in this study were deposited in GenBank.

Nucleotide sequences were aligned using MAFFT v7.450, implemented in Geneious Prime. Phylogenetic trees were constructed by the maximum likelihood method using a Kimura 2-parameter model of nucleotide substitution with 5 rate categories of gamma distribution (K2+G) as implemented in MEGA7: Molecular Evolutionary Genetics Analysis version 7.0.

Severe Hepatitis Additional Diagnostic Evaluation

Autoimmune hepatitis serology included Antinuclear Antibodies (ANA), two children tested positive (2/9, 22%) with low titers (1:40 and 1:80); Smooth Muscle Antibody (SMA), two children tested positive (2/9, 22%) with low titers (1:80); all children were negative for Liver-Kidney Microsomal (LKM) antibody (0/9, 0%), Soluble Liver Antigen (SLA) antibody (0/3, 0%), Mitochondria antibody (0/3, 0%) and Actin antibody (0/3,0%). Wilson disease screening was performed by obtaining ceruloplasmin, which was above 20 mg/dL in all tested patients (6/6, 100%) with a median value of 37.5 mg/dL. Alpha-1-Antitrypsyn phenotype was PiMM in all tested patients (5/5,100%). All tested patients had undetectable acetaminophen level (7/7, 100%).

Metabolic work up included serum amino acids, which were normal or non-specific in all tested patients (5/5,100%); Urine organic acids were normal or non-specific in all tested patients (5/5, 100%) and acyl carnitine profile was normal in all tested patients (4/4, 100%). The three patients who presented with or developed acute liver failure underwent all metabolic testing.

Testing for underlying immunodeficiency using a primary immunodeficiency panel (PID, Invitae, San Francisco, CA) was performed in both patients (n=2) who progressed to acute liver failure. One child had five heterozygous variants of uncertain significance: CEBPE (c.130G>A, p.Ala44Thr), DNASE1L3 (c.274C>T, p.Arg92Trp), GUCY2C (C.1124T>C, P.Val375Ala), LIG1 (c.1331+3A>G, intronic), WIPF1 (c.601C>T, p. Arg201Trp). In the second child this panel demonstrated a heterozygous pathogenic variant in the RFXANK gene (c.454_455del, p.Ile152Profs*28). All other patients (n=6) did not undergo an immunodeficiency work-up, as they all recovered spontaneously and clinicians did not feel that further testing was necessary.

Methodology and Results of the Systematic Retrospective Review of Severe Hepatitis Cases from October 1, 2020 to September 30, 2021.

In order to obtain a baseline frequency of pediatric (<18 years) severe hepatitis at Children's of Alabama Hospital, a retrospective review of medical records was performed from October 1, 2020 to September 30, 2021, utilizing ICD-10 codes for hepatitis (K75.9), transaminitis (R74.01), elevated liver enzymes (R74.8), liver failure (K72.9) and acute liver failure (K72.00). Pertinent clinical information was extracted from the medical records.

From October 1, 2020 to September 30, 2021, eight children (age<18 years) meeting criteria for severe hepatitis were admitted to Children's of Alabama Hospital. Final diagnoses included acetaminophen overdose (n=3), autoimmune hepatitis (n=1), SARS-CoV-2 (n=1) and unknown (n=3). Seven children were tested for HAdV by whole blood qPCR, all were negative (0/7, 100%). Five children (5/8, 63%) met diagnosis for acute liver failure. None (0/8, 0%) required liver transplantation. Five (5/8, 63%) were females and three (3/8, 37%) were males. Four children (4/8, 50%) were white, three children (3/8, 37%) were black and one child (1/8, 13%) was multiracial. One (1/8, 13%) was Hispanic, and seven (7/8, 87%) were non-Hispanic.

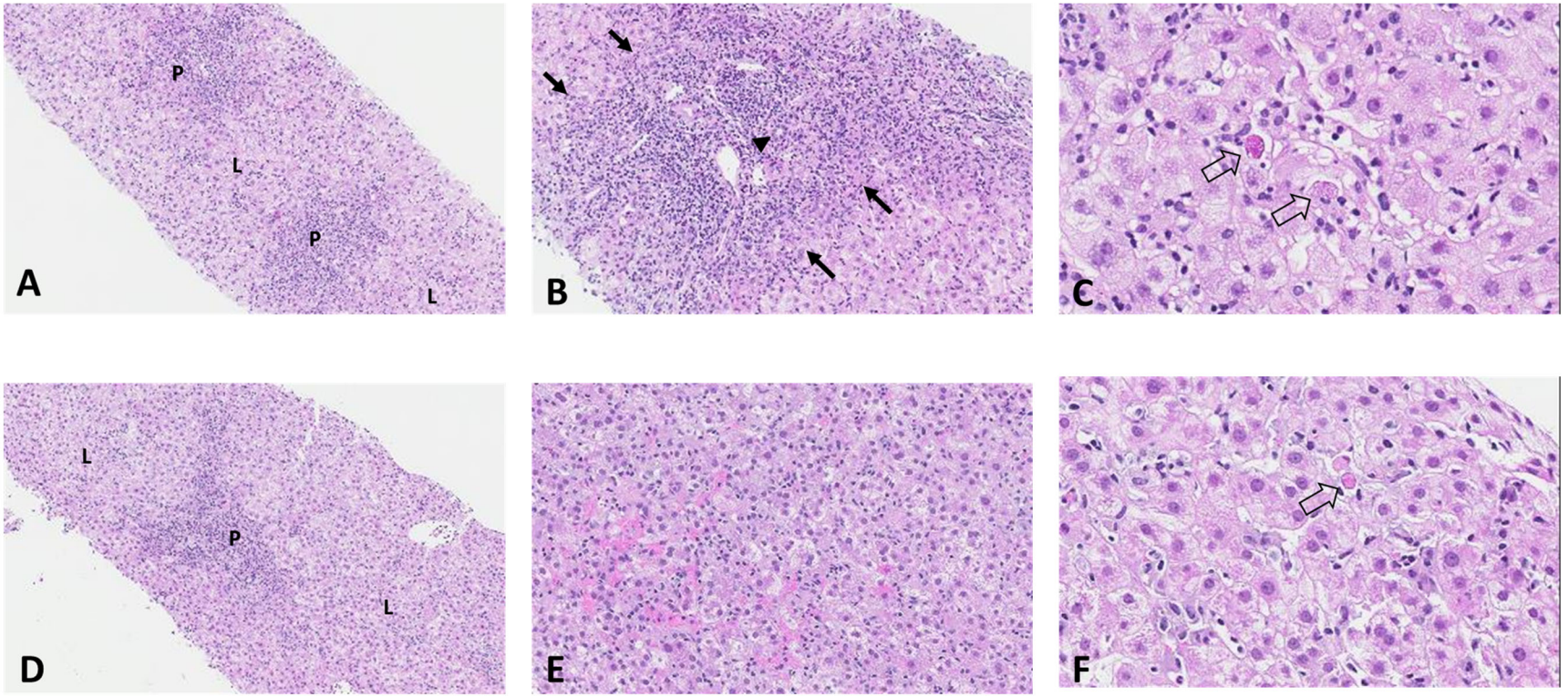

Figure S1. Liver biopsies of six children with severe hepatitis and human adenovirus viremia. Histopathology in liver biopsies from patients with hepatitis requiring subsequent liver transplant **(A-C)** or recovering without transplant **(D-F)**, hematoxylin and eosin (H&E) stain.

(A): Moderate to marked, portal (P) and lobular (L) inflammation. **(B)**: Portal tract with bile duct (arrowhead) and mixed inflammation comprising lymphocytes, histiocytes, and neutrophils. Interface activity (arrows) is present at the junction of the portal tract and lobule. **(C)**: Lobule shows inflammation and diffuse hepatocellular swelling with rare single cell apoptosis (open arrow). **(D)**: Moderate portal (P) and lobular (L) inflammation. **(E)**: Lobular infiltrates comprising lymphocytes, histiocytes, and neutrophils. **(F)**: Lobule shows inflammation and diffuse hepatocellular swelling with rare single cell apoptosis (open arrow).

References

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