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BRIEF REPORT

Clinical Correlation of Adenoviral Load in the Respiratory Tract Measured by Digital PCR in Immunocompromised Children

Diego R. Hijano,[1,*] Jose A. Ferrolino,[1,*] Jennifer Hidinger,[1] Jessica N. Brazelton,[2] Zhengming Gu,[2] Sri Suganda,[2] Heather L. Glasgow,[2] Kim J. Allison,[1] Megan Peterson,[1] Ronald H. Dallas,[1] Gabriela Maron,[1] and Randall T. Hayden[2]

[1]Department of Infectious Diseases, St. Jude Children Research Hospital, Memphis, Tennessee, USA, and [2]Department of Pathology, St. Jude Children Research Hospital, Memphis, Tennessee, USA

Immunocompromised patients can have life-threatening adenoviral infection. Viral load in blood and stool is commonly used to guide antiviral therapy. We developed and evaluated a digital polymerase chain reaction assay to quantify human adenovirus in the respiratory tract and showed that higher peak load correlates with disseminated infection, mechanical ventilation, and death.

Keywords. adenovirus; children; digital PCR; immunocompromised; viral load.

Human adenovirus (HAdv) infections are a common and important cause of illness in both immunocompetent and immunocompromised young children [1, 2]. Following initial infection, HAdV can persist in a latent state in various tissues, such as tonsillar, adenoidal, and intestinal T lymphocytes, as well as in epithelial cells. Reactivation can occur in the presence of immunosuppression, particularly in patients undergoing hematopoietic cell transplant (HCT) [3].

The overall incidence of HAdV infection among HCT patients ranges from 12% to 42%; HAdV most frequently presents in the first 100 days following HCT, and the reported mortality rate can exceed 50% [1, 2]. Risk factors for severe HAdV infection are T-cell depletion, grafts from unrelated donors or cord blood, presence of acute graft-vs-host disease, severe lymphopenia, and age [4]. High viral load in blood has been associated with worse clinical outcomes, and high viral load in stools has been associated with blood dissemination [5–7]. HAdV positivity in nasopharyngeal aspirates before HCT has been shown to predict DNAemia after HCT [8]. However, these data are limited, and there are no available data on the role of viral load in the respiratory tract to predict disease severity and death.

Polymerase chain reaction (PCR) is the standard methodology for diagnosis of adenovirus and can be designed to detect all HAdV types within a wide range of diagnostic material [9]. Digital PCR (dPCR) offers advantages over real-time PCR for quantitation, such as reduced susceptibility to assay inhibitors and target polymorphisms, which may increase the precision and accuracy of some assays. dPCR also eliminates the need to generate calibration curves for analyte quantitation. These characteristics are particularly amenable to use in sample types prone to inhibitory effects and for targets with high sequence heterogeneity, such as HAdV, which has numerous types [10], while offering potential diagnostic, prognostic, and predictive utility [11].

While current therapeutic options are limited and substantially toxic, timely initiation of antiviral therapy may be important to prevent disease progression, and initiation of treatment upon detection of HAdV DNAemia may be too late in some cases [4]. However, no controlled study has been performed despite the wide use of cidofovir in this population [12]. We designed, validated, and evaluated the clinical utility of a quantitative digital PCR assay to assess clinical disease in immunocompromised children with adenoviral respiratory tract infection.

METHODS

Study Design and Population

This was a retrospective cohort study performed at St. Jude Children's Research Hospital that included patients age 18 years or younger with hematologic malignancy and those who underwent HCT and had at least 2 respiratory samples positive for HAdV within a 4-week span. HAdV was diagnosed by PCR testing of respiratory tract secretions (nasopharyngeal wash, broncho-alveolar lavage, or tracheal aspirate) using the BioFire FilmArray Respiratory Panel 2 (bioMérieux, Marcy-l'Étoile, France). Digital PCR (dPCR) was performed on remnant respiratory samples left over after clinical diagnostic testing and stored in the microbiology laboratory for quality assurance purposes (additional information available in the Supplementary Methods).

Patient Consent

The study was approved by the St Jude Institutional Review Board, with a waiver of informed consent.

Received 19 December 2022; editorial decision 10 January 2023; accepted 19 January 2023; published online 23 January 2023

*Equal contribution

Correspondence: Randall T. Hayden, MD, Department of Pathology, Mail Stop 250, St Jude Children's Research Hospital, 262 Danny Thomas Place, Memphis, TN 38105 (randall.hayden@stjude.org); or Diego R. Hijano, MD, MSc, Department of Infectious Diseases, Mail Stop 230, St Jude Children's Research Hospital, 262 Danny Thomas Place, Memphis, TN 38105 (diego.hijano@stjude.org).

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https://doi.org/10.1093/ofid/ofad030

Digital PCR

Nucleic acids were purified from each 200-μL sample using the Generic Protocol of the bioMérieux NUCLISENS easyMAG (bioMérieux, Marcy-l'Étoile, France) nucleic acid extractor and stored at −20°C. An internal control, Taqman Exogenous Internal Positive Control (Applied Biosystems, Waltham, MA, USA), was added to each sample before extraction. Digital PCR was conducted using the QX200 digital PCR system (Bio-Rad Laboratories, Hercules, CA, USA), BioRad ddPCR Supermix for Probes, and altona RealStar ASR Adenovirus primers and probes (altona Diagnostics, Hamburg, Germany). Results of copies per reaction using QuantaSoft software (Bio-Rad) were converted to $\log_{10}$ copies per mL of the original patient sample pre-extraction (additional information available in the Supplementary Methods).

Statistical Analysis

Continuous variables were summarized using medians with ranges, and categorical factors were reported with percentages. To assess the association between viral load in the respiratory tract and detection of HAdV in blood/stool, patients with a prior positive blood or stool PCR result were excluded. Receiver operating characteristics (ROC) curves were generated using the highest HAdV viral load, and viral thresholds that best predicted outcomes were determined using the maximum Youden index method [13]. Stepwise logistic regression analysis was done to predict mechanical ventilation and HAdV-attributable death, and unadjusted and adjusted odds ratios (ORs) and 95% confidence intervals were calculated. $P < .05$ was considered statistically significant for all analyses. Data were analyzed using SAS (version 9.4; SAS Institute, Cary, NC, USA) and R (R Core Team 2020) [14] (additional information available in the Supplementary Methods).

RESULTS

Demographic and Clinical Characteristics

A total of 64 patients with 219 samples were included. Thirty-two of these (50%) received antiviral treatment. The median number of samples per patient in the treated group (range) was 3 (2–13) compared with those untreated (2 [1–10]). The median age of treated patients (range) was 8.5 (0–23) years, in comparison with 6 (0–17) years for the untreated. No significant differences were observed in gender. The most common diagnoses were hematologic malignancies in both groups. Twenty-eight (87.5%) treated patients were HCT recipients, compared with 19 (59.4%) untreated patients (Table 1).

A total of 15 patients did not have any respiratory symptoms at the time of positive PCR testing. Presence or absence of symptoms was not associated with increased viral load. Likewise, fever, nasal congestion, and cough were not associated with higher adenoviral load in respiratory specimens (Supplementary Figure 1).

Among treated patients, 17 (73.9%) had evidence of lower respiratory tract infection (LRTI), with 12 requiring mechanical ventilation. Among untreated patients, 16 (61.5%) had LRTI, with only 4 requiring mechanical ventilation. Although there was no significant difference in viral load based on location of respiratory infection, higher adenoviral loads were significantly associated with the need for mechanical ventilation (Figure 1*A*).

Coinfections with other respiratory viruses were present in 96 (43.84%) samples: 76 (79.17%) of these samples had 1 coinfecting virus, 15 (15.62%) had 2 coinfecting viruses, and 5 (5.21%) samples had 3 or more coinfecting viruses. The most common coinfecting virus was human rhinovirus/enterovirus (n = 63, 65.62%), followed by parainfluenza virus type 3 (n = 28, 29.17%) and respiratory syncytial virus (n = 12, 12.50%). Coinfection was not associated with need for mechanical ventilation, HAdV-attributable mortality, or all-cause mortality. Adenoviral load was higher when associated with viral coinfection, irrespective of the number of viruses present in the sample (Supplementary Figure 2).

HAdV load did not correlate with absolute lymphocyte count (ALC; Spearman's $\rho = -.034$; $P = .68$) (Supplementary Figure 3). There was also no difference in presenting clinical symptoms, specifically upper respiratory tract infection vs LRTI, between lymphopenic and nonlymphopenic patients. In addition, lymphopenia was not associated with the need for mechanical ventilation or HAdV-attributable death.

Twenty-eight patients (43.8%) had HAdV detected only in the respiratory tract. The remainder had HAdV in at least 1 additional site (stool and/or blood): 14.1% stool, 6.2% blood, and 9.4% both stool and blood. Seventeen patients (26.6%) had HAdV in stool and/or blood before detecting it in respiratory secretions. Among the 14 patients with DNAemia, 38.7% (n = 12) were in the treated cohort and 6.2% (n = 2) were in the untreated cohort. Four patients (12.9%) in the treated population, but none in the untreated group, had DNAemia before detection of HAdV in the respiratory tract. Patients with HAdV detected in stool or blood had a higher viral load in the respiratory tract (median [IQR], 6.93 [5.16–9.84]; $P = .043$; for stool HAdV; median [IQR], 8.98 [5.19–9.36]; $P = .078$; for DNAemia) (Figure 1*B* and *C*). A total of 8 patients died (7 [21.9%] from the treated group and 1 [3.1%] from the untreated group). Six of these deaths were attributable to adenoviral infection (5 from the treated group and 1 from the untreated group). The median time for adenoviral-attributable death from first positive sample (range) was 79 (34–103) days for those who received an antiviral (Table 1). ROC curve analysis determined that a viral RNA load >7 $\log_{10}$ copies/mL predicted need for mechanical ventilation, with a sensitivity of 78% and a specificity of 72% (AUC, 0.733) (Supplementary Figure 4).

Table 1. Comparison of Patient Characteristics Between Treatment Groups

	Treated	Untreated	*P*
Total subjects	32	32	
Age at diagnosis, median [range], y	8.76 [0.28–23.32]	6.56 [0.44–17.76]	.428
Sex, No. (%)			
Female	19 (59.38)	11 (34.38)	.08
Male	13 (40.62)	21 (65.62)	
Race, No. (%)			
White	24 (75.00)	14 (43.75)	.040
African American	3 (9.38)	7 (21.88)	
Other	5 (15.62)	11 (34.38)	
Diagnosis, No. (%)			
Leukemia	20 (62.50)	26 (81.25)	.567
ALL	9 (45.00)	15 (57.69)	
AML	9 (45.00)	8 (30.77)	
Mixed Phenotype	2 (10.00)	3 (11.54)	
Solid tumor	2 (6.25)	1 (3.12)	
Anemia	2 (6.25)	1 (3.12)	
Lymphoma	2 (6.25)	1 (3.12)	
MDS	1 (3.12)	2 (6.25)	
Lysosomal storage disease	2 (6.25)	0 (0.00)	
SCID	2 (6.25)	0 (0.00)	
CNS tumor	0 (0.00)	1 (3.12)	
Histiocytosis	1 (3.12)	0 (0.00)	
Transplant, No. (%)			
Nontransplant	4 (12.50)	13 (40.62)	.022
Transplant	28 (87.50)	19 (59.38)	
Clinical symptoms, No. (%)			
No	9 (28.12)	6 (18.75)	.555
Yes	23 (71.88)	26 (81.25)	
LRTI	17 (73.91)	16 (61.54)	.537
URTI	6 (26.09)	10 (38.46)	
GvHD at HAdV diagnosis, No. (%)			
No	20 (71.43)	12 (63.16)	.781
Yes	8 (28.57)	7 (36.84)	
O2 requirement, No. (%)			
None	12 (37.50)	27 (84.38)	<.001
Mechanical ventilation	12 (37.50)	4 (12.50)	
Supportive oxygen	8 (25.00)	1 (3.12)	
HAdV stool PCR, No. (%)			
Negative	6 (18.75)	23 (71.88)	<.001
Positive at baseline	14 (43.75)	6 (18.75)	
Positive	12 (37.50)	3 (9.38)	
HAdV blood PCR, No. (%)			
Negative	19 (61.29)	30 (93.75)	.004
Positive at baseline	4 (12.90)	0 (0.00)	
Positive	8 (25.81)	2 (6.25)	
ICU within 7 d, No. (%)			
No	19 (59.38)	29 (90.62)	.008
Yes	13 (40.62)	3 (9.38)	
ICU status, No. (%)			
No	19 (59.38)	29 (90.62)	.008
ICU at baseline	4 (12.50)	0 (0.00)	
ICU admission	9 (28.12)	3 (9.38)	
No. of samples, median [range]	3 [2–13]	2 [1–10]	.012
Earliest HAdV viral load, mean (SD)	4.70 (2.86)	4.74 (2.92)	.964
Highest HAdV viral load, mean (SD)	6.91 (2.47)	5.63 (2.99)	.081
Mean HAdV viral load, mean (SD)	4.89 (2.46)	4.38 (2.28)	.412

Table 1. Continued

	Treated	Untreated	*P*
All-cause death, No. (%)			
No	25 (78.12)	31 (96.88)	.053
Yes	7 (21.88)	1 (3.12)	
Time from first sample to death, median [range], d	79 [26–161]	32	.275
HAdV-attributable death, No. (%)			
No	27 (84.38)	31 (96.88)	.196
Yes	5 (15.62)	1 (3.12)	
Not attributable	2 (28.57)	0 (0.00)	.500
Probable	1 (14.29)	1 (100.00)	
Definite	4 (57.14)	0 (0.00)	
Time from first sample to death, median [range], d	79 [34–103]	32	.143

Abbreviations: ALL, acute lymphocytic leukemia; AML, acute myeloid leukemia; CNS, central nervous system; GvHD, graft-vs-host disease; HAdV, human adenovirus; ICU, intensive care unit; LRTI, lower respiratory tract infection; MDS, myelodysplastic syndrome; PCR, polymerase chain reaction; SCID, severe combined immunodeficiency; URTI, upper respiratory tract infection.

Higher respiratory adenoviral load was significantly associated with increased risk for mechanical ventilation (OR, 1.41; 95% CI, 1.09–1.91), all-cause mortality, and adenoviral-attributable mortality (OR, 1.98; 95% CI, 1.16–4.71) independent of age, presence of adenovirus in blood, and receipt of HCT (Figure 1*D*; Supplementary Table 1).

Performance Characteristics of dPCR

Analytic specificity for adenovirus was 100%. Positive predictive value was 1, and negative predictive value was 0.43 (Supplementary Table 2). Plotted linearity for adenovirus had an R^2 value that ranged from 0.94 to 0.99, depending on the viral serotype (Supplementary Figure 5). Precision analysis was measured by within-run and between run coefficient of variation (CV). Within-run variation was 1.20% CV based on results in $\log_{10}$ cp/mL data for the mid control (Supplementary Table 3). Between-run variation ranged from 2.19% CV to 2.57% CV for the high external control and from 4.48% CV to 4.85% CV for the low external control (Supplementary Table 4). The limit of detection, limit of quantitation, and cutoff for positivity were 2.30 $\log_{10}$ copies/mL. The sensitivity and specificity of the assay were 83.5% and 100%, respectively (Supplementary Table 2).

DISCUSSION

We describe the use of quantitative respiratory adenoviral testing and its correlation with clinical course and outcomes in pediatric immunocompromised patients. The role of viral load and its correlation to disease severity in respiratory viral infection remain controversial [15, 16]. Patients with a higher adenoviral load had an increased risk for requiring mechanical ventilation. Consideration of viral load in the upper respiratory tract, in addition to known risk factors, could help further discriminate patients at highest risk for progression to LRTI. The association of HAdV load with poor clinical outcomes is not limited to the respiratory tract. Higher HAdV load in stool strongly predicts impending adenoviremia, and high viral loads in blood are associated with worse clinical outcomes [2, 17].

Disseminated adenoviral disease has unfavorable outcomes, particularly in immunocompromised children undergoing HCT. Given the lack of approved therapies and the toxicity associated with the drugs currently used for treatment, universal prophylaxis for adenoviral infection in high-risk populations is not feasible. Hence, diagnostic tests that correlate with poor clinical outcome or predict impending adenoviremia may play a critical role in management of these patients. Serial testing of adenoviral load in stool has been recommended as an early indication for preemptive treatment [6]. Likewise, higher viral load in respiratory samples could be used to trigger the use of antivirals.

This study had several limitations. First, it was limited by the need for retrospective data abstraction. Second, the use of a convenience sample was limited by availability of remnant samples collected at different time points in the course of infection. Third, many samples had viral coinfection, which can limit clear attribution of symptoms to HAdV. Fourth, we did not include patients undergoing solid organ transplant. Finally, both sensitivity and specificity to predict mechanical ventilation were under 80%, and more studies with larger sample sizes will be needed to further evaluate this.

Virologic surrogates are lacking for clinical trials of antiadenoviral drugs in high-risk populations. The high variability and lack of standardization in viral load assays are a primary limitation in further advancing our knowledge on the role of viral load in predicting outcomes and guiding clinical care. The methodology described here can help increase the reproducibility of these data, both within and across different laboratories, facilitating the development of common interpretive thresholds.

This is the first study reporting the use of dPCR to determine adenoviral loads in the respiratory tract of pediatric immunocompromised patients. dPCR provides advantages such as absolute target quantitation without the need for calibration curves, improved precision, and reduced likelihood of

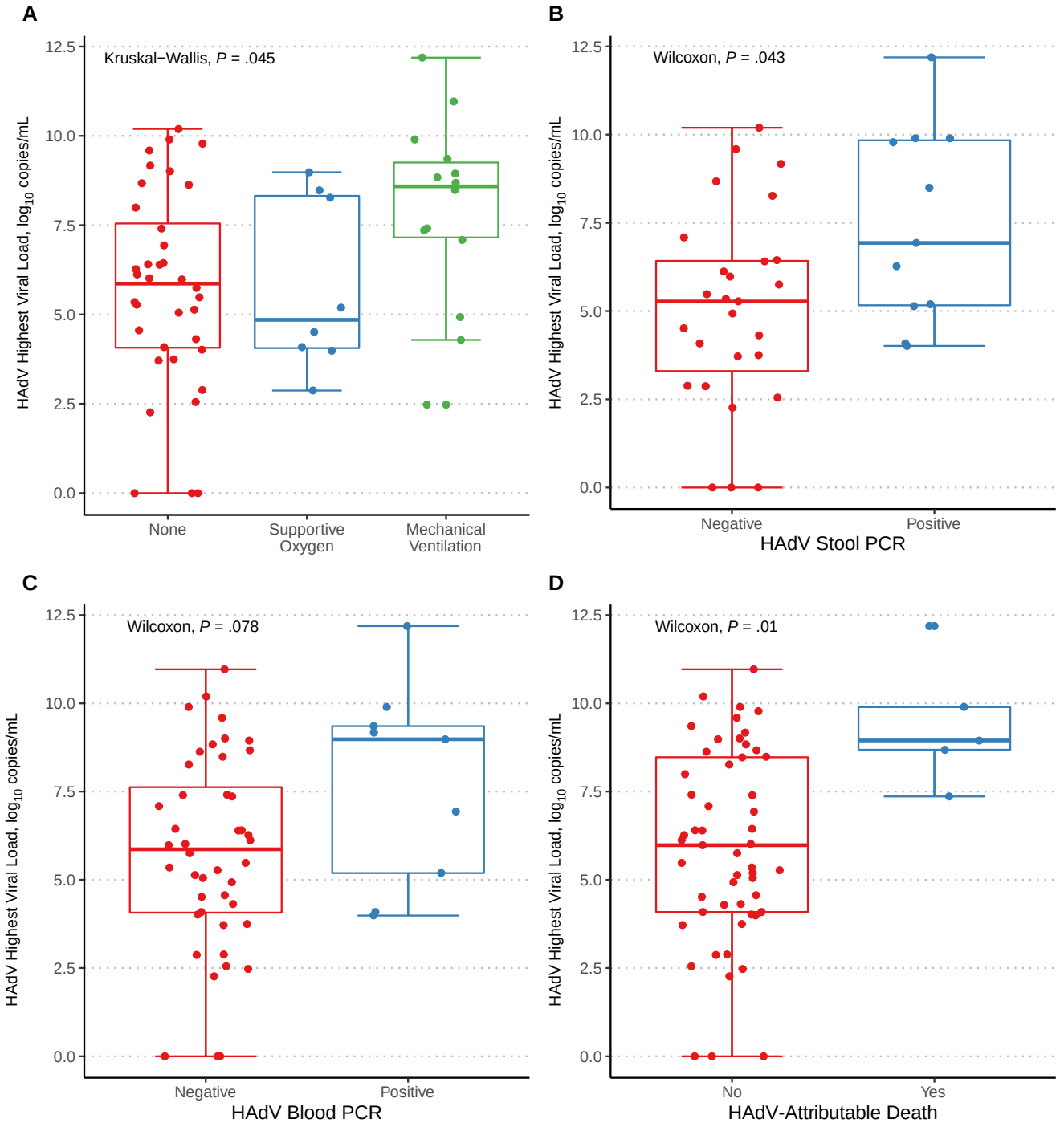

Figure 1. HAdV load in the respiratory tract is associated with disseminated infection, mechanical ventilation, and death. *A*, HAdV load and need for respiratory support. *B*, HAdV load and detection of HAdV in stool. *C*, HAdV load and detection of HAdV in blood. *D*, HAdV load and attributable death due to adenoviral infection. Abbreviations: HAdV, human adenovirus; PCR, polymerase chain reaction.

quantitative bias based on amplification efficiency or inhibition, providing potential advantages to real-time PCR. Adoption of dPCR may be challenging in some settings with a requirement for high throughput, those with limited staffing, and those with an inability to run high-complexity testing. Hands-on time is generally greater using dPCR compared with many of the automated and high-throughput platforms currently in use. The initial use case for quantitative viral testing of respiratory samples would likely be as a reflexive assay, run only on initially positive samples. The reagents and

equipment required for such testing are widely available and do not present significant technical hurdles for implementation [11, 18, 19].

Our findings suggest that higher viral load in respiratory secretions may be associated with poor clinical outcome, progression to DNAemia, need for mechanical ventilation, and death. Prediction of DNAemia, LRTI requiring intensive care unit, mechanical ventilation, and death through quantitative adenoviral PCR in respiratory samples could potentially guide clinical care and improve clinical outcomes. Future studies will focus on prospectively validating the use of quantitative HAdV testing by dPCR in the clinical setting.

Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Acknowledgments

We acknowledge the following members from St. Jude Children's Research Hospital for their commitment and support of this work: Donna Williams and Alicia Rodriguez.

Financial support. This work was supported in part by the American Lebanese Syrian Associated Charities (ALSAC) and The Children's Infection Defense Center (CIDC). No additional external funding was received for the study.

Potential conflicts of interest. R.H. has served as a consultant for Abbott Laboratories, Roche Diagnostics, T2 Diagnostics, and MiraVista Labs. G.M. receives research funding from SymBio Pharmaceuticals Inc. D.R.H., J.A.F., J.H., J.N.B., Z.G., S.S., H.L.G., K.J.A., M.P., R.H.D. have no conflicts of interest relevant to this article to disclose. All other authors report no potential conflicts.

Author contributions. Drs. Hijano, Maron, and Hayden conceptualized and designed the study. Drs. Hijano, Hidinger, and Hayden drafted the initial manuscript and reviewed and revised the manuscript. Drs. Hijano, Glasgow, Maron, and Hayden coordinated and supervised data collection and critically reviewed the manuscript for important intellectual content. Dr. Gu, Dr. Brazelton, and Ms. Suganda processed and analyzed the respiratory samples. Dr. Dallas, Dr. Ferrolino, Ms. Peterson, and Ms. Allison designed the data collection instruments, collected the data, and reviewed and revised the manuscript. Dr. Ferrolino carried out all the analyses. All authors approved the final manuscript as submitted and agreed to be accountable for all aspects of the work.

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