Virology

Clinical disease and viral load in children infected with respiratory syncytial virus or human metapneumovirus[☆]

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Abstract

The relationship between respiratory syncytial virus (RSV) and human metapneumovirus (hMPV) quantity in respiratory secretions and severity of illness in children remains unclear. We assessed the effect of hMPV and RSV viral load as determined by reverse transcriptase polymerase chain reaction on disease characteristics. Data were abstracted from medical records of 418 children with RSV and 81 children with hMPV; associations were evaluated in multivariate analyses, both continuously and comparing lower versus higher viral loads. Increasing viral load in hMPV-infected children was associated with increases in presence of fever, bronchodilator use, obtaining chest radiograph, and length of hospital stay. Increasing viral load in RSV-infected children was associated with decreases in inpatient admissions, use of antibiotics, and respiratory rate. Our study has described a significant relationship between viral load and markers of disease severity for both RSV and hMPV in a large population of children evaluated for respiratory disease.
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1. Introduction

Human metapneumovirus (hMPV) and respiratory syncytial virus (RSV) are important causes of respiratory disease, bronchiolitis, and hospitalization in young children worldwide. Respiratory syncytial virus has become increasingly recognized globally as an important viral pathogen in young children through the widespread use of RSV-specific rapid diagnostic tests, such as antigen enzyme immunoassay detection and direct fluorescent antibody (FA) detection (Piedra et al., 2002). However, rapid diagnostic tests are not widely used for the detection of newly recognized viruses such as hMPV (van Burik, 2006), coronavirus (Fouchier et al., 2004; van der Hoek et al., 2004; Woo et al., 2005), and bocavirus (Allander et al., 2005). This has led to the increasing use of sensitive viral detection methods such as reverse transcriptase polymerase chain reaction (RT-PCR) for the diagnosis of viral respiratory pathogens.

Real-time PCR detection methods are characterized by high levels of reproducibility, specificity, and sensitivity (Kehl et al., 2001; Watzinger et al., 2004) and do not require immediate processing or inoculation into tissue culture (Kuypers et al., 2004). Good correlation of viral load as determined by plaque assays on fresh aliquots and RT-PCR has also been demonstrated (Perkins et al., 2005), an important finding in light of the technical difficulties of plaque assays and the potential interference of neutralization or inactivation of virus by antibodies or antiviral agents. Molecular quantitative assays for pathogenic respiratory viruses have been advocated for the detection and monitoring of clinical disease, particularly in immunosuppressed pediatric patients (Watzinger et al., 2004), and RT-PCR lends itself to the quantification of viral load from a variety of clinical specimens, including nasal wash, endotracheal aspirate, and bronchoalveolar lavage.

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We assessed the effect of hMPV and RSV viral load, as determined by RT-PCR, on diverse clinical correlates of disease and epidemiologic attributes through a large retrospective cross-sectional study of children with respiratory disease from a single institution over 2 respiratory virus seasons to evaluate the relationship between viral load and severity of disease.

2. Materials and methods

2.1. Study design and population

All residual samples from consecutive nasal washes collected for diagnostic purposes from children with respiratory symptoms at Children's Hospital and Regional Medical Center (CHRMC), Seattle, WA, from November 2002 through September 2004 were included in this study. Children's Hospital and Regional Medical Center provides both primary and tertiary care for children from birth to 21 years old throughout the Pacific Northwest of the United States. Only one sample per child was selected for this analysis. When multiple samples were available for a single admission, the sample with the highest viral load was used for analysis. Children with insufficient clinical data for analysis were excluded.

2.2. Laboratory data collection

Samples were stored at −70 °C until retrospectively evaluated for hMPV and for RSV using quantitative real-time reverse-transcription PCR as previously described (Kuypers et al., 2004, 2005). These assays detect both subtypes of RSV and all 4 known subtypes of hMPV. For the quantitative assays, the threshold cycles of clinical samples were compared with standard curves generated by amplification of known numbers of RSV or hMPV RNA transcripts. Negative-sense RNA transcripts, which were synthesized from PCR amplicons, were purified and quantified by absorbance at 260 nm.

Reverse transcriptase PCR results were expressed as RSV or hMPV copies per milliliter of original sample. To ensure that negative results were not due to poor RNA extraction or inhibition of the PCR assay, we added 50 000 copies/mL (1000 copies/RT-PCR reaction) of EXO external control, a 130-base RNA transcript derived from jellyfish DNA (Kuypers et al., 2004), to the lysis buffer during RNA extraction. All samples with negative respiratory virus results required detection of EXO to be considered valid.

2.3. Clinical data collection

Clinical data were abstracted by medical record review using a uniform data collection form. The following variables were collected for each child: age at sample collection, gender, duration of hospitalization, admission unit, and admission and discharge International Classification of Diseases, 9th Revision (ICD-9) codes. Clinical disease correlates collected for the period of 24 h before to 24 h after the nasal sample included maximum temperature, maximum respiratory rate, use of bronchodilators, use of chest radiography examination, supplemental oxygen requirement, mechanical ventilation requirement, and use of antibiotics. Results of direct FA detection from each nasal sample determined in real time were also obtained. This research study was approved by our institutional review board.

2.4. Statistical analyses

Quantitative variables were described using mean or median, with SD or range. Categorical variables were described using frequency and percentage. All viral load numbers are presented as copies per milliliter. ICD-9 discharge and admission codes were grouped using the Complex Chronic Condition categories previously described by Feudtner et al. (2000) with the addition of a group for asthma (519.1, 493.0–493.9). Comparisons between types of infection (hMPV versus RSV) were assessed using linear regression for continuous data and logistic regression for binary data, controlling for age and for chronic underlying conditions. The burden of underlying disease in our study population was described for each group and compared between the hMPV and RSV groups using multivariate logistic regression, controlling for age.

The relationship between variables of interest and log 10-transformed quantitative viral load was assessed with linear and logistic regression as appropriate, with log viral load included as a continuous linear variable. In a secondary analysis intended to evaluate a possible threshold viral load necessary for presence of disease, differences in clinical correlates were compared between 2 viral load groups, lower than 1×10^5 copies and greater than or equal to 1×10^5 copies. Relationships were first assessed univariately and then in multivariate models controlling for age group and presence of chronic underlying conditions. Adjusted respiratory rate estimates were calculated as such, controlling for use of bronchodilators in addition. Analyses were performed using STATA version 9 (College Station, TX).

3. Results

Altogether, 1264 residual nasal washes collected between December 2002 and September 2004 (Fig. 1) from unique patients with clinical data were available for analysis. The majority of samples (88%) were collected within 1 day of admission. This study population had a median age of 14.3 months at the time of sampling; the oldest subject was 20 years and the youngest was 1 day (Table 1). Slightly more than half of the population was male (57%). The majority of patients were admitted to the inpatient medical ward (73%), but 17% were seen in the emergency department only, 10% were admitted to the intensive care unit, and 0.5% were seen as outpatients in specialty clinics.
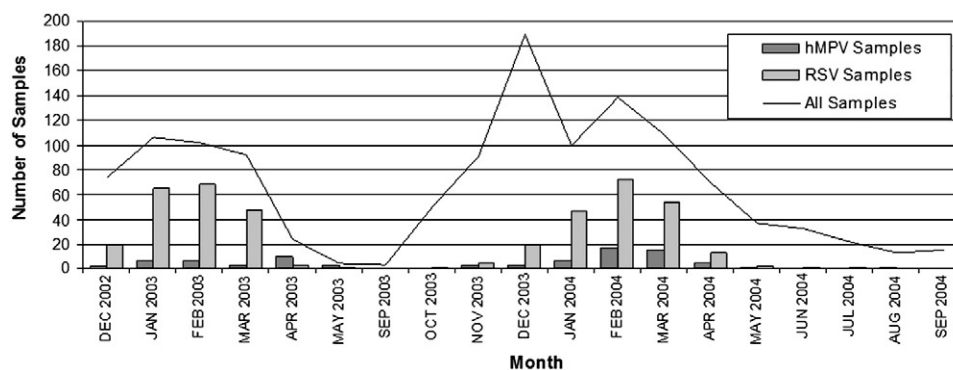
Fig. 1. Frequency of sample collection and virus detection by month.

Four hundred forty-five (35%) of the 1264 total children had a specimen with RSV detected by RT-PCR; 418 (94%) of those RSV samples had no viruses other than RSV detected by direct FA testing and did not have hMPV present by RT-PCR. Among the 418 samples used for this analysis, the mean RSV viral load was 5.3×10^8 copies/mL, with a median viral load of 4.5×10^7 (range, 850 to 2.1×10^{10} copies/mL) (Fig. 2). Ninety-eight percent of the 393 children with RSV viral loads $\geq 10^5$ had RSV detected by both FA as well as RT-PCR, whereas only 15 (60%) of the 25 children with RSV viral loads $< 10^5$ copies/mL had RSV detected by FA testing ($P < 0.001$, χ^2 test).

Only 110 (9%) of the 1264 children had a specimen with hMPV detected by RT-PCR. Of these samples with hMPV detected, 81 (74%) had no other viruses detected by FA and did not have RSV present as detected by FA or RT-PCR. Among these 81 samples, the mean hMPV viral load was 1.3×10^9 copies/mL, with a median viral load of 3.0×10^8 (range, 1.6×10^4 to 4.0×10^{10} copies/mL) (Fig. 2).

Children with only hMPV ($n = 81$) or only RSV ($n = 418$) were similar in gender distribution (57% and 55% male, respectively), although children with RSV were significantly younger ($P < 0.001$) (median, 8.2 months; range, 0.3 months to 18 years) compared with children with hMPV (median, 14.9 months; range, 0.7 months to 20 years) (Table 1). No significant differences were observed between these 2 groups for admission unit (outpatient clinic, emergency department, inpatient, or intensive care unit) or proportion of children with a specimen collected within 1 day of admission. In a univariate linear regression model evaluating the relationship between age and log viral load, every 6-month increase in age was significantly associated with a 0.1 log decrease in RSV viral load ($P = 0.027$). By contrast, no association between age and hMPV viral load was observed.

Of the 418 RSV-only infections, 370 (89%) episodes had admission ICD-9 diagnoses available; 83% of admission ICD-9 codes were for acute respiratory illnesses. An additional 9% of admissions were for asthma or asthma exacerbations, and 2% were for fever. Likewise, the 68 (84%) of 81 ICD-9 admission diagnoses available for children with hMPV-only infections showed that 74% of

admission codes were for acute respiratory illnesses, with 10% for asthma or asthma exacerbations and 9% for fever.

We characterized the burden of underlying chronic disease, including asthma, for all 1264 patients, as well as separately within the hMPV and RSV groups (Table 1). The presence of any underlying condition did not significantly differ between children with an RSV-only and those with an hMPV-only specimen ($P = 0.609$), after controlling for age and gender; however, condition-specific differences were identified. Eight (2%) children with only RSV detected had underlying chronic respiratory conditions compared with 8 (10%) children infected only with hMPV ($P = 0.002$). Neither hMPV nor RSV viral load differed between previously healthy children and the overall group of children with chronic conditions. However, children with malignancies and an RSV-only infection ($n = 5$) had a lower log viral load compared with other children with an RSV-only infection (median, 5.4 versus 7.7) that approached statistical significance ($P = 0.055$). Children with an

Table 1
Characteristics of study population

	Overall ($n = 1264$)	hMPV ($n = 81$)	RSV ($n = 418$)
Male, n (%)	716 (57)	46 (56.8)	231 (55)
Median age	14.3	14.9	8.2*
(range, interquartile range) (months)	(0–246, 4.4–35.6)	(0.7–246, 7.4–33.5)	(0.3–225, 2.6–20.5)
Admission unit, n (%)			
Clinic	6 (0.5)	0 (0)	0 (0)
Emergency room	214 (17)	13 (16.1)	48 (11)
Inpatient	921 (73)	65 (80.3)	343 (82)
ICU	123 (10)	3 (3.7)	27 (6)
Sample collected within 1 day of admission, n (%)	1120 (88)	77 (95)	401 (96)
Underlying chronic conditions, n (%)			
Asthma	235 (19)	18 (22)	75 (18)
Other	320 (25)	20 (25)	57 (14)

* $P < 0.001$ for age difference between hMPV and RSV groups by Mann–Whitney U test.
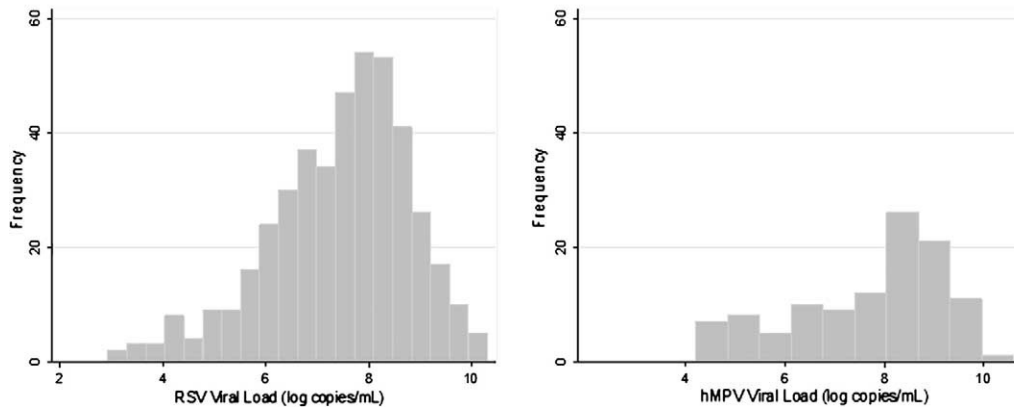
Fig. 2. Distribution of RSV and hMPV viral load. Only one specimen per patient was included in the analysis. For the 14% of individuals that had more than one specimen collected, we used the specimen with the highest viral load for this analysis.

underlying neurologic condition with an RSV-only infection ($n = 12$) had a significantly higher RSV log viral load than those without a neurologic condition (median, 8.3 versus 7.6; $P = 0.021$).

Several markers of disease severity increased significantly with each log increase of hMPV viral load, controlling for age and presence of underlying chronic disease (Table 2). The presence of fever at or above 38.0 °C, increased bronchodilator use, increased frequency of hospital stays greater than 2 days, and increased frequency of obtaining chest radiographs were related to hMPV viral load (odds ratio [OR] = 1.9, $P = 0.002$; OR = 1.6, $P = 0.019$; OR = 1.5, $P = 0.025$; and OR = 1.4, $P = 0.05$, respectively). When these estimates were reevaluated in the subgroup of children 12 months and younger, the magnitude of each odds ratio, except bronchodilator use, increased. In the RSV-only group, controlling for age and presence of underlying chronic conditions, the use of antibiotics, and the frequency of inpatient admission significantly decreased with each log increase in viral load (OR = 0.8, $P = 0.001$; OR = 0.8, $P = 0.047$) (Table 2). An increase in RSV viral load of 1 log was associated with a decrease in respiratory rate of 1.3 breaths per minute, controlling for bronchodilator use, age, and presence of underlying chronic conditions ($P = 0.008$).

In a secondary analysis, we evaluated differences in the clinical markers of disease severity discussed above for hMPV and RSV between children with a viral load less than 1.0×10^5 ($n = 6$ for hMPV, $n = 25$ for RSV) compared with children with a viral load of 1.0×10^5 or higher ($n = 75$ for hMPV, $n = 393$ for RSV). For children infected only with hMPV, increases in illness severity in the higher viral load group were observed for every variable evaluated, except antibiotic use and inpatient admission. However, these differences were not statistically significant when controlling for age and the presence of underlying chronic disease. This pattern was not observed among children infected only with RSV. Importantly, clinically significant illness was observed in children with fewer than 1×10^5 copies/mL with both hMPV and RSV infections. For example, 83% of the low-

copy hMPV cases were admitted to the inpatient ward and 33% required supplemental oxygen compared with 80% ($P = 0.993$) and 49% ($P = 0.423$), respectively, in the high-copy group. Fifty-two percent of the low-copy RSV cases had a fever within 24 h of the sample collection, 68% required bronchodilators, and 44% required supplemental

Table 2
Associations with viral load[a], controlling for age, and chronic disease

	hMPV ($n = 81$)	RSV ($n = 418$)
	OR, P (95% CI)	OR, P (95% CI)
Fever ≥38 °C	1.9, $P = 0.002$ (1.3–2.9)	0.95, $P = 0.494$ (0.8–1.1)
Bronchodilator use	1.6, $P = 0.019$ (1.1–2.3)	1.1, $P = 0.540$ (0.9–1.2)
Antibiotic use	0.8, $P = 0.265$ (0.6–1.1)	0.8, $P = 0.001$ (0.7–0.9)
Stay >2 days	1.5, $P = 0.025$ (1.1–2.2)	0.9, $P = 0.067$ (0.8–1.0)
Inpatient admission	1.2, $P = 0.355$ (0.8–1.7)	0.8, $P = 0.047$ (0.7–0.997)
ICU admission	0.8, $P = 0.525$ (0.4–1.6)	0.8, $P = 0.219$ (0.6–1.1)
Ventilator requirement[b]		
In underlying disease group	0.5, $P = 0.151$ (0.2–1.3)	1.6–$P = 0.119$ (0.9–3.0)
In previously healthy group	1.5, $P = 0.287$ (0.7–3.3)	0.7, $P = 0.150$ (0.5–1.1)
Oxygen requirement	1.0, $P = 0.941$ (0.7–1.30)	0.9, $P = 0.099$ (0.8–1.0)
Chest X-ray ordered	1.4, $P = 0.05$ (0.99–1.9)	0.9, $P = 0.373$ (0.8–1.1)
	β[c], P (95% CI)	β[c], P (95% CI)
RR-max[d]	0.22, $P = 0.84$ (−1.9 to 2.3)	−1.3, $P = 0.008$ (−2.3 to −0.4)

CI = confidence interval; ICU = intensive care unit; RR-max = maximum respiratory rate.

[a] Viral load was assessed as a predictor using a log10-transformed, linear variable.

[b] The presence of an underlying chronic condition was found to significantly modify the relationship between viral load and ventilator requirement. For this reason, the estimate is presented separately for the previously healthy and the underlying disease groups.

[c] β indicates the linear regression coefficient. For example, a 1 log increase in hMPV viral load corresponds to an increase in respiratory rate of 0.2 breaths per minute.

[d] Controlling for use of bronchodilators as well.

oxygen compared with 39% ($P = 0.205$), 75% ($P = 0.281$), and 50% ($P = 0.416$), respectively, in the high-copy cases.

4. Discussion

We used quantitative RT-PCR methods to quantify viral loads in 499 children with RSV or hMPV from clinical specimens obtained on or close to hospital admission. Respiratory syncytial virus and hMPV viral load and clinical disease severity were not consistently correlated among all the markers of severity we examined, and the relationship between viral load and markers of disease severity differed between RSV- and hMPV-infected children. Although increasing viral load was associated with increased presence of fever, bronchodilator use, use of chest radiography, and lengthened hospital stays for hMPV-infected children, similar associations were not seen for RSV-infected children. Conversely, decreased admission to the inpatient unit, use of antibiotics, and respiratory rate were significantly associated with increasing RSV but not hMPV viral load. Some clinical characteristics of respiratory disease were observed among children with low viral loads ($<1 \times 10^5$ copies/mL) and no other pathogens detected, making it difficult to establish a "minimum" viral load associated with clinical disease. In general, we found that the presence of virus even at low copy numbers may be associated with clinical symptoms of respiratory disease.

The clinical significance of RSV viral load has been studied in various populations of children. Respiratory syncytial virus quantity in respiratory secretions has been shown to be greater in infants younger than 1 month, based on quantitative culture techniques pioneered by Hall et al. (1976). A similar association of higher RSV viral load with younger age as detected by molecular detection methods has been previously reported (Kuypers et al., 2004).

However, the relationship between the quantity of virus in respiratory secretions and the severity of illness attributed to respiratory viruses remains unclear. Critically ill adult bone marrow transplant recipients have been shown to have severe disease with low viral loads, as assessed by plaque assays (Englund et al., 1996). Bennett et al. (2007) have found that RSV viral load by plaque assay was significantly correlated with cytokine levels of interleukin (IL)-8 and IL-10. Increased levels of IL-10 were, in turn, significantly inversely associated with the duration of supplemental oxygen requirement. In a study of 77 infants younger than 2 years conducted by Wright et al. (2002), illness severity (measured by respiratory illness scoring and duration of supplemental oxygen and ventilatory support) was correlated with younger age but not with RSV quantity determined by plaque assays on fresh nasal wash specimens. In another study of 141 untreated infants younger than 2 years, a higher RSV load in the nasal wash at admission, measured by fresh culture, was independently associated with longer hospitalization, respiratory failure, and need for intensive care, controlling for age

among other subject characteristics (DeVincenzo et al., 2005). These differences in findings may be due to characteristics other than age that may differ between the 2 populations studied. In the study by Wright et al., more than half of the infants ($n = 44$) had bronchopulmonary dysplasia or congenital heart disease, 2 conditions which were among the exclusion criteria for the study by DeVincenzo et al. For this reason, the populations used in the 2 studies are not directly comparable. Further differences in definitions of severity and the statistical methods used may also have contributed to the noncomparability of these 2 studies' results. Although we included children with underlying chronic conditions, similar to the Wright et al. study, we were able to control for this factor in a multivariate analysis, leading to some results that demonstrate that viral load may correlate inversely with some markers of RSV disease severity including inpatient admissions, use of antibiotics, and respiratory rate.

Limited data are available on the impact of viral load of hMPV. One study collected multiple samples from 8 Italian children and concluded that hMPV viral load correlated with the general severity of illness (Sarasini et al., 2006). Another study has reported hMPV viral load results in 37 children with varying ages and testing windows (Gerna et al., 2007).

In this study, we were able to use hospital discharge and admission records to characterize the underlying chronic disease conditions in our study population and to control for that factor in multivariate analyses, in contrast to other studies that chiefly evaluated previously healthy children. This approach allowed us to establish a larger and more diverse study population and enabled us to examine the effect of chronic disease on the relationship between viral load and disease severity. We found that the presence of an underlying chronic disease condition modified the relationship between viral load and the requirement for mechanical ventilation (Table 2), indicating that underlying health conditions should be considered carefully in the future when evaluating this relationship. We also described a significantly higher RSV viral load among children with neurologic conditions compared with children without. This is an interesting finding in light of recent data by Wilkesmann et al. (2007), demonstrating that children with clinically relevant neuromuscular impairment are at increased risk for severe RSV disease.

A younger median age was found in children infected with RSV compared with hMPV, but clinical factors related to acute respiratory disease such as duration of hospitalization, presence of fever, requirement of supplemental oxygen, assisted ventilation, and use of antibiotics were similar overall in children infected with RSV and those infected with hMPV. These findings are in agreement with those documented by other investigators (Klein et al., 2006; van den Hoogen et al., 2003; Wolf et al., 2006), as is our finding that RSV viral load was significantly higher in younger children (Kuypers et al., 2004).

A decrease in antibiotic use was documented in children with high viral loads of RSV. Although this group was likely

to be younger and thus potentially more likely to be considered for antibiotic therapy on the basis of age alone, these children were also more likely to have a positive rapid test by direct FA because of this high viral load. We attribute the decreased antibiotic use to timely notification of a laboratory diagnosis to the prescribing physician. No similar association between antibiotic use and hMPV viral load was present, presumably because no rapid test was then available at our hospital for hMPV. We speculate that the rapid identification of hMPV, as well as other viral respiratory pathogens currently not detectable using rapid tests, may ultimately assist in the use of appropriate antibiotic therapy in the hospital setting.

The measurement of viral load for infections such as human immunodeficiency virus, cytomegalovirus, and hepatitis B and C has now evolved into state-of-the art patient care and is now widely used as a standard of care for monitoring response to antiviral therapy. Respiratory viral load assessment as a marker of disease severity or response to antiviral therapy has also been used for the assessment of response to therapy with influenza antiviral agents (Hayden et al., 1999; Monto, 2005) and RSV disease in immunocompromised adults (Boeckh et al., 2007). The increased sensitivity of RT-PCR RSV tests in comparison to FA testing has lead to speculation that the presence of virus as detected by PCR may not be truly indicative of viral causality (Gerna et al., 2008). This is in contrast to studies using plaque reduction assays and PCR that demonstrated clinically significant disease at all detectable levels of virus (Buckingham et al., 2000; Wright et al., 2002). Our findings support this latter observation. Future evaluations of dual infections using multiple sequential samples with viral load assessments may help to further define the relationship between detectable virus and clinical disease.

Our study is limited by its retrospective cross-sectional design and the use of clinically ordered samples. Data on symptom onset before clinical presentations was not reliably available from the medical record. We likely excluded children with mild or asymptomatic disease for which the clinical provider did not think a viral test was necessary, and we did not test the samples for other known viruses that are increasingly identified in respiratory samples such as rhinoviruses, coronaviruses, or bocavirus.

In conclusion, our study has assessed the relationship between viral load and disease severity for both RSV and hMPV. Although clinical disease was identified even at low copy numbers, we found a significant association between viral load and several markers of disease severity. We have also highlighted the importance of accounting for the prevalence of underlying disease conditions when undertaking a study of this nature. Our data support the potential clinical importance of quantitative results from RT-PCR testing, and we hope that additional studies will help to further guide the interpretation and application of this information in a clinical setting.

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