Frequent and Prolonged Shedding of Bocavirus in Young Children Attending Daycare

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(See the editorial commentary by Williams, on pages 1611–1614.)

Background. Little is known about human bocavirus (HBoV) persistence and shedding and the association between HBoV detection and the onset and resolution of respiratory symptoms.

Methods. We performed HBoV testing on nasal swab samples from a prospective, longitudinal study of respiratory illness in 119 children who attended daycare.

Results. HBoV was detected in 70 children (59%) and in 106 (33%) of the 318 cases of illness. Another virus was detected in 76 (72%) of 106 HBoV-positive cases. Extended and intermittent shedding was observed, with consistent HBoV detection documented for up to 75 days. HBoV was detected in 20 (44%) of 45 asymptomatic enrollment samples, and HBoV prevalence and viral load did not differ significantly between children with and children without symptoms at enrollment. HBoV-positive illnesses were longer than HBoV-negative illnesses (odds ratio for duration of symptoms >7 days, 2.44; 95% confidence interval, 1.41–4.22), and illnesses with HBoV load $\geqslant 4 \log_{10}$ copies/mL required a visit to a health care provider more often than did HBoV-negative illnesses (odds ratio, 1.64; 95% confidence interval, 1.02–2.64).

Conclusion. HBoV was more common in illnesses with greater severity. However, detection of HBoV was not associated with the presence of respiratory illness or with specific respiratory symptoms in this prospective study of infants and toddlers attending daycare centers.

Respiratory infections are the leading infectious causes of death worldwide [1]. In addition to causing clinical morbidity, respiratory illnesses in children have been implicated in increased school absenteeism and parental work absenteeism [2, 3]. New viruses that potentially play a role in infectious respiratory illness are increasingly being recognized through sensitive methods of viral detection.

Since the initial detection of human bocavirus (HBoV) in 2005 [4], numerous studies have been published that document the detection of HBoV in nasal samples. Seroprevalence studies have also shown that HBoV is widespread among young children [5]. However, most studies of the role of HBoV in respiratory illness are cross-sectional in nature or focus on moderate-to-severe illness, and more specific evidence is needed to define the role that HBoV plays in respiratory illness [6, 7]. Current information has not proven that HBoV is a respiratory pathogen, rather than a colonizer. Little is known about the temporal relationship between HBoV detection and the onset and resolution of illness, and the relationship of viral load to disease severity is unclear. Few studies have used regular, repeated, longitudinal sampling; a longitudinal study involving a birth cohort of Danish children found frequent detection of HBoV in both symptomatic and asymptomatic infants [8]. To address these questions, we have performed HBoV testing on samples from a prospective,

longitudinal study of respiratory illness in young children who attended daycare.

METHODS

Study design. Nasal swab samples and symptom data were collected as part of a longitudinal study of respiratory illness at 3 large daycare centers on Fort Lewis Army Base in Washington State.

Study population and surveillance methods. Parents of daycare enrollees were recruited through flyers at the beginning of the study and when new children were enrolled at the daycare center. Inclusion criteria were being aged 6 weeks through 24 months at enrollment and attending ⩾20 h of daycare at one of the Fort Lewis daycare centers from February 2006 through February 2008. Informed consent was obtained in a consent conference with the on-site study nurse before enrollment. Children were enrolled before the initiation of respiratory virus surveillance and regardless of whether the child was symptomatic at the time of enrollment.

The age distribution of children in the study varied by daycare classroom. Individual classroom age ranges included 6 weeks through 12 months, 12–24 months, and 24–36 months at Site 1; 6 weeks through 24 months, 18–36 months, and 24–42 months at Site 2; and 6 weeks through 12 months and 12–24 months at Site 3. There were 9–18 children per classroom. The mean percentage of children in each classroom who were enrolled in the study was 56% (range, 35%–71%).

After informed consent was obtained from the parents, nasal swab samples were collected from children at study enrollment (for children enrolled after 1 May 2006) and with each new respiratory illness for up to 2 years. Respiratory surveillance was conducted from February 2006 through April 2008. A new respiratory illness was defined as the onset of at least 2 of 5 symptoms: cough, rhinorrhea, wheezing, fever, and nasal congestion. Study staff were notified of new or worsening respiratory illness by parents or by daycare staff. Active surveillance for respiratory illness was also conducted by the on-site study nurse.

At the onset of a new or worsened illness, data on the presence and duration of illness symptoms were collected by parent interviews performed by the study nurse and by daily symptom diaries completed by the parent for 10 days. Symptom data beyond day 10 were not collected. Medical record review and physician surveys were performed to collect data on health care visits related to study illnesses.

HBoV and respiratory virus detection. Nasal swab samples were collected by deep nasal swab using flocked-tip Copan swabs, which were immediately submerged into a vial containing 0.5 mL lysis buffer and then stored at room temperature. Samples were tested during the study using nucleic acid ex-

traction and polymerase chain reaction (PCR) for 14 common respiratory viruses: respiratory syncytial virus (RSV) A and B subtypes; human metapneumovirus (HMPV); influenza (Flu) A and B; parainfluenza (PIV) types 1, 2, 3, and 4; adenovirus (AdV); rhinovirus (HRV); and coronavirus (CoV) subtypes 229E, HKU1, NL63, and OC43 [9–13]. Results were available to study staff within 1 week. Repeat samples were collected weekly during respiratory illnesses until the child had results negative for all viruses in the respiratory virus panel and illness was beginning to resolve.

Study samples were tested retrospectively for HBoV using a real-time PCR assay targeting the NP1 gene [14] that provided semiquantitative cycle threshold results only. HBoV-positive specimens with sufficient volume ($n = 120$) were tested again, and specimen PCR threshold cycle values were compared with those of a standard curve generated by amplifying known copy numbers of a plasmid containing the HBoV amplicon. Quantitative results are reported as copies of virus per mL of lysis buffer. One-fifth of specimens positive by the qualitative assay had no HBoV detected by the quantitative assay. Cycle thresholds of these samples ranged from 36.4 to 39.7 in the initial assay, which indicated that HBoV copy number in these samples was low.

Statistical analysis. Data were analyzed using Stata, version 10.1 (Stata). Incidence and 95% jackknife confidence interval (CI) of HBoV-related illness was calculated overall and individually for age and sex subgroups. Cox regression models were used to determine independent risk factors for HBoV-related illness. An indicator term was included to adjust for respiratory season, and robust standard errors were used to account for multiple HBoV-related illnesses per child. Children were included in this model beginning at the time of their first HBoV-negative sample.

Differences in the presence and duration of symptoms between illnesses with and illnesses without detectable HBoV were evaluated using generalized estimating equations with a robust estimator to appropriately account for the association between measures on the same subject [15]. To minimize length bias, only illnesses in which HBoV was detected in the first 2 samples obtained (within ∼1 week of illness onset) were considered to be HBoV-positive illnesses. In response to a number of low-copy HBoV-positive samples that were detectable by only 1 of 2 PCR assays, a sensitivity analysis was performed that required a quantitative value of at least 4 log-copies per mL to determine positivity. This cutoff value was determined by rounding the 25th quartile quantity in our results to the nearest log. A corresponding cutoff of a cycle threshold value of 35.3 was used for samples with insufficient volume for quantitative testing; this cutoff value was selected because it linearly corresponds to 4 log-copies/mL, based on our data. As a secondary analysis,

Table 1. Characteristics of the Study Population

Variable	Children with no HBoV illness (n = 62)[a]	Children with any HBoV illness (n = 57)	Season-adjusted HR[b] of HBoV acquisition (95% CI)
Male sex	28 (45)	28 (49)	1.25 (0.62–2.52)
Age at enrollment			
0–6 Months	20 (32)	19 (33)	Reference
7–12 Months	14 (23)	19 (33)	1.01 (0.45–2.27)
>12 Months	28 (45)	19 (33)	0.61 (0.26–1.40)
Race			
White	27 (44)	30 (53)	Reference
Black	23 (37)	15 (26)	0.65 (0.32–1.34)
Multiple	8 (13)	5 (9)	0.54 (0.12–2.37)
Other	4 (6)	7 (12)	0.91 (0.34–2.46)
Hispanic	8 (13)	15 (26)	2.13 (0.93–4.87)
Siblings			
0	27 (44)	25 (44)	Reference
1	22 (35)	19 (33)	1.18 (0.52–2.69)
≥2	13 (21)	13 (23)	1.22 (0.51–2.90)
Breastmilk in diet	8 (13)	15 (26)	1.92 (0.84–4.41)
Single parent	8 (13)	3 (5)	0.31 (0.08–1.13)
Monthly pretax income, US $			1.11 (0.84–1.49)
0–999	1 (2)	0 (0)	…
1000–1999	1 (2)	2 (4)	…
2000–2999	8 (13)	15 (26)	…
3000–3999	18 (29)	11 (19)	…
4000–4999	13 (21)	10 (18)	…
≥5000	21 (34)	19 (33)	…
Civilian parents	4 (6)	2 (4)	0.21 (0.03–1.40)
Deployed parent	7 (11)	14 (25)	0.91 (0.42–1.96)
Gestational age <36 weeks	4 (6)	5 (9)	3.90 (0.49–30.8)
Study follow-up time, mean days (IQR)	335 (147–475)	363 (155–539)	…

NOTE. Data are no. (%) of subjects, unless otherwise indicated. CI, confidence interval; HBoV, human bocavirus; HR, hazard ratio; IQR, interquartile range.

[a] Thirteen of 62 children had HBoV detected only during asymptomatic periods.

[b] HR calculated using Cox regression models that included an indicator term to adjust for respiratory season.

characteristics of HBoV-positive illnesses were compared with the characteristics of HBoV-negative illnesses immediately preceding and following the case event in each individual with use of a bidirectional case-crossover analysis design. This comparison was analyzed with generalized estimating equations with robust variance. Because this analysis required both an HBoV-positive illness and a separate HBoV-negative illness for the inclusion of each child, the available dataset was markedly smaller than the full study analysis described above.

HBoV results for baseline enrollment swab samples were compared between children with respiratory symptoms and children without symptoms with use of a χ^2 test for differences in prevalence and a Student's t test for differences in log viral load. HBoV detection in asymptomatic children was also compared with the next consecutive illness sample in each child using McNemar's test.

RESULTS

A total of 119 subjects were enrolled in the study, with a total follow-up time of 115.3 child-years and a mean follow-up of 1 year (range, 11 days to 2 years). The mean age at enrollment was 10 months (range, 1.6–24.9 months), and 56 (47%) of the enrollees were female (Table 1).

A total of 318 illnesses in 93 children were reported during illness surveillance, including 45 illnesses reported at enrollment. The overall annual incidence of respiratory tract illnesses was 2.8 illnesses per child-year (95% CI, 2.3–3.4 illnesses per child). Annualized incidence in individual children ranged from 0 to 30 respiratory tract illnesses per year. A total of 495 weekly samples were collected during the 318 illnesses, and daily symptom diaries were available for 313 illnesses. Illnesses with missing diaries were excluded from analyses of symptom duration.
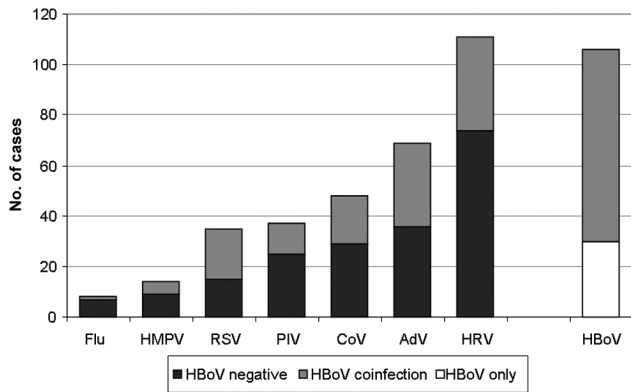
Figure 1. Viruses detected in 318 cases of illness. AdV, adenovirus; CoV, coronavirus; Flu, influenza; HBoV, human bocavirus; HMPV, human metapneumovirus; HRV, rhinovirus; PIV, parainfluenza virus; RSV, respiratory syncytial virus.

Forty-one additional swab samples were collected from children who were asymptomatic at the time of enrollment (48% of 86 total enrollment swab samples). A total of 536 total nasal swab samples were available for retrospective HBoV testing.

Of these, 152 samples (28%) had test results that were positive for HBoV, and the virus was detected in 106 (33%) of 318 study illnesses. Seventy (59%) of 119 enrolled children had an HBoV-positive sample obtained during the study, although 13 of these children had HBoV detected only from an enrollment sample that was obtained when they were asymptomatic.

Among illnesses with any virus detected, HBoV was detected significantly more often than were any of the 14 respiratory viruses but rhinovirus (Figure 1). This was also true among illnesses associated with fever and among illnesses that required a health care visit.

Quantitative HBoV results were available for 95 HBoV-positive specimens. Median HBoV load was 15,500 copies/mL (range, 1160–1.8 × 10^{10} copies/mL; IQR, 5210–408,000 copies/ mL). No relationship was identified between viral load and age at illness ($P = .941$). HBoV-positive specimens with other viruses identified had a lower median log viral load (3.9 $\log_{10}$ copies/mL; $n = 47$), compared with specimens with only HBoV detected (4.5 $\log_{10}$ copies/mL; $n = 48$) ($P = .04$, by Mann-Whitney U test). However, the comparison of the maximum HBoV log viral load between illnesses associated with HBoV plus other viruses versus illnesses associated with HBoV alone was not statistically significant (mean difference, -0.2 $\log_{10}$ copies/mL; $P = .602$, by generalized estimating equation).

Another virus was detected in 76 (72%) of the 106 HBoV-positive illnesses. This rate was significantly higher than the percentage of HBoV-negative illnesses associated with the presence of multiple viruses (24%; $P < .001$). The number of coinfecting viruses in addition to HBoV ranged from 1 (in 40 illnesses) through 4 (in 2 illnesses); 2 viruses were present in 23 illnesses, and 3 viruses were present in 11 illnesses. HRV and AdV were the most common coinfecting viruses (in 37 and 33 illnesses, respectively) (Figure 1). However, the rate of
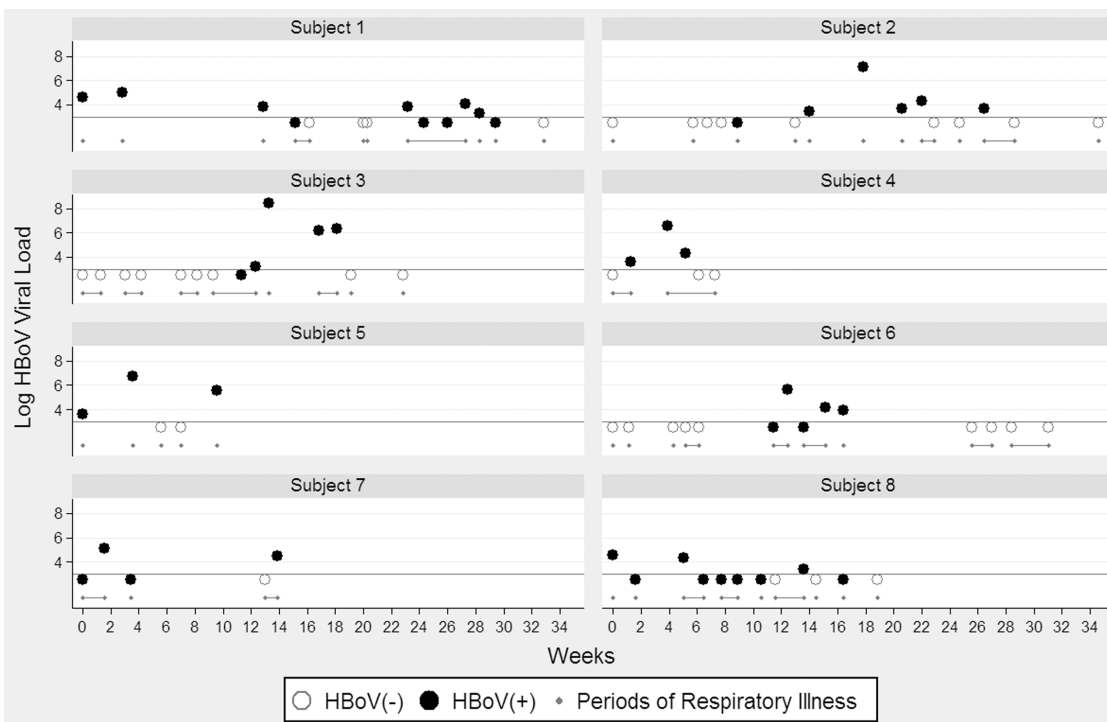

Figure 2. Extended human bocavirus (HBoV) shedding in 8 children.

Table 2. Human Bocavirus Incidence by Subgroup

Variable	Incidence, cases per 100 child-years (95% CI)
Sex	
Male	102 (69–156)
Female	83 (58–123)
Age	
0–6 Months	103 (60–188)
7–12 Months	121 (81–187)
>12 Months	67 (44–104)

HRV-specific coinfection was significantly lower in HBoV-positive illnesses than it was in HBoV-negative illnesses (33% vs 54%; $P = .003$).

HBoV shedding and temporal association with respiratory illness. The initial detection of HBoV and the duration of HBoV detection did not consistently correspond with the timing of respiratory illnesses. Of the 106 HBoV-positive respiratory illnesses, HBoV was not identified until the second weekly swab sample was obtained (during the second week of illness) or later in 22 (21%) of 106 cases of HBoV-positive respiratory illness. Thirty-two of 70 children with HBoV-positive swab samples had HBoV detected in >1 sample. Extended shedding with swab samples at most 1 month apart was observed for up to 75 days. Twenty HBoV-positive shedding events with consecutive HBoV-positive swab samples spanned the onset and resolution of multiple respiratory illnesses. Some illustrative patterns of HBoV shedding are presented in Figure 2. Recurrent detection of HBoV was documented in 18 children with multiple HBoV-positive events separated by at least 1 HBoV-negative swab sample. Altogether, recurrent detection was seen in individual children 2 times (in 13 children), 3 times (in 3 children), or 4 times (in 1 child).

Incidence of HBoV-positive respiratory illness. The incidence of all HBoV-positive illnesses was 93 illnesses per 100 child-years (95% CI, 71–123 illnesses). Age- and sex-specific incidence rates (Table 2) were not significantly different after accounting for correlated illnesses among individuals. No statistically significant difference in HBoV incidence was observed between daycare centers. The seasonal distribution of HBoV-positive illnesses closely resembled the distribution of all respiratory illnesses during the study period (Figure 3).

Lack of association between HBoV and the presence of respiratory illness symptoms. HBoV detection did not correlate with the presence of respiratory illness symptoms. We detected HBoV in 44% of asymptomatic enrollment samples and found no statistically significant difference in the prevalence of HBoV at enrollment between children with and children without respiratory symptoms (Table 3). There was no statistically significant difference in the mean HBoV load found in enrollment samples from children with and children without respiratory symptoms ($P = .694$; Table 3). These findings were consistent when restricting the analysis to those illnesses with HBoV detected alone. No association was found at enrollment between age and the detection or quantity of HBoV, regardless of whether symptoms were present. A case-crossover comparison of asymptomatic baseline samples with the next subsequent respiratory illness in each child ($n = 25$) found no statistically significant difference in HBoV prevalence between samples obtained from asymptomatic children and samples obtained from children with illness (52% and 32%, respectively; $P = .059$).

Association between HBoV and the duration and severity of respiratory illness. HBoV was detected in the first 2 samples obtained (within ~1 week of illness onset) in a total of 101 illnesses. No association was found between HBoV detection and the presence of the following symptoms: fever, wheezing, cough, rhinorrhea, congestion, vomiting, diarrhea, decreased appetite or activity, earache, fatigue, myalgia, malaise, rash, or difficulty breathing. However, extended respiratory illness, defined as symptoms other than rhinorrhea lasting longer than 7 days, was more common among children with HBoV-positive illnesses than it was among children with HBoV-negative illnesses (odds ratio [OR], 2.44; 95% CI, 1.41–4.22; $P = .001$). Specifically, HBoV-positive illnesses had higher odds of being associated with a cough lasting >7 days (OR, 2.09; 95% CI, 1.29–3.39; $P = .003$). Illness due to HBoV alone (ie, with no coinfecting viruses) was not associated with any outcomes (including duration of cough, duration of illness, and number of health care provider visits), compared with HBoV-negative illness; however, the number of HBoV monoinfections was small (30 cases). Our results were not affected by controlling for age. One child with HBoV infection was admitted to the hospital for severe respiratory illness; this child was also infected with RSV, CoV, HRV, and AdV during the course of the illness.

HBoV load did not correlate with severity of illness. In a sensitivity analysis that required at least 4 $\log_{10}$ copies/mL or a cycle threshold ≤35.3 to determine positivity, the results for
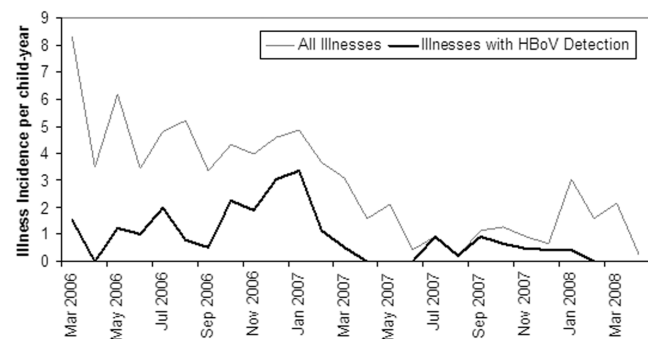

Figure 3. Seasonal distribution of illnesses. HBoV, human bocavirus.

Table 3. Human Bocavirus (HBoV) Prevalence at Enrollment

Symptoms at enrollment	No. (%) of subjects		Total
	HBoV negative	HBoV positive	
Yes	27 (66)	14 (34)[a]	41
No	25 (56)	20 (44)[b]	45
Total	52 (60)	34 (40)	86

[a] Mean viral load, 4.9 $\log_{10}$ copies/mL.
[b] Mean viral load, 4.6 $\log_{10}$ copies/mL.

all illness characteristics were similar to those of the previous analysis, with the exception of number of health care visits. HBoV-positive illnesses with load above the cutoff (47 cases) required more visits a health care provider (OR, 1.64; 95% CI, 1.02–2.64; $P = .042$). We did not identify a continuous effect between increasing HBoV load and greater duration of cough or illness or increased number of health care provider visits.

Case-crossover analysis of HBoV and the duration and severity of illness. No statistically significant difference in reported symptoms was found when comparing HBoV-positive (30 cases) and HBoV-negative illnesses (37 cases) that were matched by individual. Duration of illness was significantly longer for those with HBoV-positive illnesses than it was for those with HBoV-negative illnesses (mean difference, 0.93 days; 95% CI, 0.17–1.69 days; $P = .016$), as was duration of cough (mean difference, 1.20 days; 95% CI, 0.06–2.34 days; $P = .038$).

DISCUSSION

HBoV was the second most commonly detected virus in our prospective, longitudinal study of children in daycare and was detected in 33% of respiratory illnesses. The virus was frequently redetected in individual children over extended periods of time. Our study calls into question the role of HBoV as a respiratory pathogen, in light of the high frequency of co-detection of other viruses, the detection of asymptomatic carriage, the lack of correlation with illness onset, and the lack of correlation of viral load with severity of illness.

Coinfections with respiratory viruses were present in 72% of HBoV-positive illnesses. This estimate is within the range of previous findings that up to 83% of HBoV-positive children have coinfection with respiratory viruses [16]. The high percentage of HBoV detections that are concurrent with detections of known respiratory viruses calls into question the specificity of observed associations between HBoV and disease. We also found that HBoV detection did not have a distinct seasonal pattern, compared with the overall distribution of all respiratory illnesses over a 2-year period, in contrast with other reports [8, 17–19]. This pattern indicates that HBoV may be detected with a relatively consistent prevalence in individuals with respiratory illnesses caused by other viruses.

In our study, the prevalence of HBoV in samples from asymptomatic children was surprisingly high and was very similar to the findings of a Canadian study, which reported detection of HBoV in samples from 43 (43%) of 100 asymptomatic children [20]. Von Linstow et al [8] have also reported an HBoV rate in samples from asymptomatic children above that found in samples from symptomatic children. We did not find a difference in HBoV prevalence between asymptomatic samples and the next illness sample within each child. This analysis design has the advantage of allowing for the control of confounding by individual factors, such as age. Several studies have evaluated the association between HBoV and illness with comparisons to asymptomatic control groups and have found a low prevalence of HBoV detection in children without respiratory illness symptoms [16, 21–24]. Our results contradict these findings.

HBoV detection did not consistently correspond with the onset of respiratory illness. One-fifth of HBoV-positive illnesses occurred in children who had an HBoV-negative swab sample obtained at illness onset. We identified HBoV infections that spanned the incidence and resolution of multiple respiratory illnesses. Several HBoV infections persisted for an extended period of time. We documented shedding for up to 75 days with regular sampling. This reflects reports of extended shedding for at least 2 months in 32% of children with HBoV-positive respiratory illness who underwent monthly testing [8]. We also described extended shedding events that spanned the onset and resolution of multiple consecutive respiratory illnesses. The persistence of HBoV shedding beyond the resolution of single respiratory illnesses illustrates the difficulty in correlating an isolated HBoV-positive result with an incident illness in a cross-sectional study. Given that we have demonstrated that multiple HBoV-related events separated by periods in which HBoV-negative swab samples are obtained are possible in individual children, it is possible that persistent HBoV detection reported here and elsewhere [8, 17, 25] may be attributable to repeated infections, rather than to a single extended period of shedding. More frequent sample collection is needed to fully investigate this possibility.

Viral load was not significantly lower during asymptomatic periods than during periods of respiratory illness, and we did not see increased illness severity with increased HBoV load. We found increased viral load in individual specimens from children with HBoV monoinfection, similar to other reports [17, 23], but this comparison was not statistically significant at the illness level. Other groups have also been unable to establish a link between HBoV load and disease severity [26–28].

Although we did not identify any differences with respect to reported symptoms between HBoV-positive and HBoV-negative illnesses, HBoV did appear to contribute to the severity of respiratory illness. Illnesses with HBoV detection, alone or in

combination with other viruses, were more likely to last >7 days and, specifically, were more likely to have cough present for >7 days. HBoV infections with a viral load of at least 10,000 copies/mL were also associated with an increased number of visits to a health care provider. These findings indicate that HBoV may exacerbate respiratory illnesses caused by other pathogens or that more-severe illness may initiate HBoV shedding events.

The identification of HBoV shedding in the stool of children with vomiting and diarrhea [29] has led to speculation that HBoV may be a causative agent of enteric disease [6]. We did not find any association between HBoV detection and vomiting or diarrhea as reported by parental diaries and interviews. However, our study was designed for surveillance of respiratory diseases, and illnesses with vomiting or diarrhea without respiratory symptoms may have been missed.

Our rate of HBoV detection in respiratory illness is higher than other published estimates of 2.7%–21.7% [30, 31]. In a more direct comparison to published cross-sectional prevalence estimates, HBoV prevalence in our study among first incident respiratory illnesses was 35%. Notably, more than one-fifth of the children with HBoV-related illnesses did not have detectable HBoV until 1 week after illness onset. Studies that only collected a sample at illness onset may have missed such cases. To our knowledge, this study is the first longitudinal study to test for HBoV in a daycare setting. We evaluated all respiratory illnesses, including mild illnesses that are often missed by studies of hospital or primary care patients. It is possible that our high rate may be attributable to the very young age of our study participants (from 6 weeks through 24 months of age at enrollment). We do not know if this high prevalence is universally reflected among young children in other regions or among children not attending daycare.

Our reported duration of shedding may be an underestimation. HBoV testing was performed retrospectively, and weekly swabbing for respiratory viruses may have been discontinued before the resolution of the HBoV shedding. Second, testing began at the onset of respiratory symptoms. We would have missed the onset of HBoV infection if it preceded the start of illness. Our HBoV incidence may also be an underestimation if respiratory illnesses were missed during the surveillance by study staff and parents. However, we believe that very few illnesses were missed, because of the regular contact with the daycare providers and the on-site study nurse who promptly identified illnesses. By focusing our study on the daycare population, we may have missed primary HBoV infections in very young children. The symptoms of primary HBoV infection, if any, are unknown and merit additional study in young infants.

Few studies have longitudinally studied HBoV in children with mild illness symptoms [8]. To our knowledge, no longitudinal studies to date have used weekly sampling during respiratory illnesses and paired analysis to compare asymptomatic with symptomatic periods. HBoV was not independently associated with particular respiratory symptoms; however, our findings indicate that HBoV shedding may increase the duration of respiratory symptoms caused by other pathogens. We also documented long periods of persistent HBoV shedding, independent of the onset and resolution of respiratory illness. Overall, detection of HBoV was not associated with the presence of respiratory illness or with specific respiratory symptoms in this prospective study of infants and toddlers attending daycare centers.

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