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Heterotypic Infection and Spread of Rhinovirus A, B, and C among Childcare Attendees

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(See the Editorial Commentary by Ottolini and Malloy, on pages 845–7.)

Background. Despite the frequency of human rhinovirus (HRV), data describing the molecular epidemiology of HRV in the community are limited. Childcare centers are optimal settings to characterize heterotypic HRV cocirculation.

Methods. HRV specimens were prospectively obtained from a cohort of childcare attendees at enrollment and weekly during respiratory illness. The 5′ noncoding region sequences were used to determine HRV species (A, B, C) and genotypes.

Results. Among 225 children followed, sequence data were available for 92 HRV infections: HRV-A (n = 80; 59%) was most common, followed by HRV-C (n = 52, 39%), and HRV-B (n = 3, 2%). Forty-one genotypes were identified and cocirculation was common. Frequent spread between classrooms occurred with 2 HRV-A genotypes. Repeated detections within single illnesses were a combination of persistent (n = 7) and distinct (n = 7) genotypes. Prevalence of HRV among asymptomatic children was 41%. HRV-C was clinically similar to HRV-A and HRV-B.

Conclusions. HRV epidemiology in childcare consists of heterotypic cocirculation of genotypes with periodic spread within and among classrooms. Based on our finding of multiple genotypes evident during the course of single illnesses, the use of sequence-based HRV type determination is critical in longitudinal studies of HRV epidemiology and transmission.

Keywords. rhinovirus; childcare; rhinovirus C; genotypes.

Human rhinovirus (HRV) is one of the most frequently reported viruses in childcare centers. The importance of HRV in this setting has been a consistent finding over time in studies using culture or molecular methods [1–3]. Overall reported prevalence of HRV infections in children, an important cause of pediatric outpatient and inpatient disease, has been markedly higher in the last decade, due to the use of sensitive molecular methods and the recent identification of HRV-C, a species of HRV previously missed by culture-based studies [4]. The clinical significance of HRV, including newly identified HRV-C species, has been a source of ongoing debate. HRV is often regarded as a cause of mild, self-limited illness. Previously reported detection of HRV in high proportions of asymptomatic individuals [5–8] also presents challenges in determining the role of this virus in causing acute infection. Repeated sample collection is needed to determine if asymptomatic HRV detections are from subclinical illness or due to continued shedding from a previous, resolved illness.

HRV is notable for a high degree of genetic variation with over 100 known HRV-A and HRV-B subtypes and over 50 HRV-C subtypes [9, 10]. Multiple subtypes circulate in a given season, with little cross-immunity among types. This variation leads to frequent heterotypic reinfection in individuals, even within the span of a single respiratory illness season [3, 11, 12]. The significance of detecting HRV on clinical respiratory polymerase chain reaction (PCR) panels is hampered by the heterogeneity of this virus. Repeated detections using PCR panels do not distinguish between reinfection with a new subtype versus prolonged shedding. Longitudinal studies of the occurrence of HRV shedding and reinfection in children require determination of HRV strain or genotype to fully describe HRV epidemiology in individual children over time.

Much of the described molecular epidemiology of HRV in children has been focused on studies in outpatient and hospital settings using a single clinical sample collected from symptomatic children on presentation for medical care. Our objective was to longitudinally characterize the molecular epidemiology of HRV illnesses in community-based childcare attendees with both mild and moderate symptomatic disease, followed over multiple seasons, as well as asymptomatic detection.

MATERIALS AND METHODS

Study Population

Children between the ages of 5 weeks and 30 months were prospectively enrolled from 3 large childcare centers on a

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military base in Washington State, after obtaining informed parental consent, as previously reported [2, 13]. All children who attended childcare at least 20 hours per week were eligible for the study. Informed consent was obtained from parents or guardians prior to enrollment of participants. Children were continuously enrolled from February 2006 through April 2008 and from October 2008 through June 2009 and were followed for up to 2 years after enrollment, or up to age 40 months [13]. Children who left the center within 3 months after enrollment were excluded from analysis. Parent interviews and participant medical records were used to obtain baseline demographics, household characteristics, and medical histories at the time of enrollment. Institutional review board approval was obtained prior to all study procedures.

Symptomatic Specimen and Data Collection

Throughout the study period, children were followed for incident respiratory illness, defined as a new onset of at least 2 of 5 symptoms including cough, rhinorrhea, wheezing, fever (tympanic, axillary, or rectal temperature of ≥38°C), and nasal congestion. At the onset of illness, parents and/or the child's guardian or childcare personnel contacted the study nurse who collected a posterior nasal swab within 48–72 hours of illness onset and reviewed illness symptoms with parents. Swabs were repeated every 7 to 10 days until illness was stable or improving, unless swabs continued to be positive by PCR. PCR results were returned to the study team within 1 week to prompt continued swabbing following positive results. For children requiring a visit with a healthcare provider, a standardized form detailing clinical characteristics, physical exam findings, diagnosis, and treatment was completed by 1 of 2 study physicians. The study nurse conducted weekly phone follow-ups until symptom resolution.

Asymptomatic Specimen Collection

During the enrollment visit, study nurses collected posterior nasal swabs in all subjects regardless of the presence of clinical respiratory symptoms. If asymptomatic enrollment swabs were found to be positive by viral PCR, collection of subsequent postenrollment swabs was attempted every 7–10 days until PCR results were negative to determine duration of asymptomatic HRV detection.

Laboratory Methods

Respiratory secretions were collected from the posterior nasopharynx as previously described [2, 14]. Separate real-time PCR and reverse transcriptase PCR (RT-PCR) assays were used to test for human metapneumovirus (HMPV), respiratory syncytial virus (RSV), parainfluenza types 1 through 4 (PIV1-4), influenza viruses (Flu A and Flu B), rhinovirus (HRV), human coronavirus (HCoV) group 1 (229E and NL63) and group 2 (OC43 and HKU1), human bocavirus clade 1 (HBoV), and adenovirus (AdV). If HRV was identified, cDNA was generated from HRV RNA, amplification of a 175 base-pair fragment of the 5′ noncoding region was performed, and the DNA sequences were determined by Sanger sequencing as previously described [15]. Resulting sequences were compared to representative reference sequences of standard HRV genotypes [10, 16] with FASTA [17] using a 95% homology cutoff for genotype determination. Remaining sequences that were unmatched under this definition were then compared with each other to determine sets of sequences with 100% homology but no defined matching genotype. These sequence groups were sequentially numbered as "undetermined genotype" clusters 1 through 6 for HRV-A (Au1 through Au6) and 1 through 4 for HRV-C (Cu1 through Cu6). All remaining sequences were noted as having no available genotype and sequentially numbered within each species.

Statistical Methods

Cross-sectional HRV prevalence at enrollment was compared between children with and without respiratory symptoms by *t* test overall and by Fisher exact test for comparison of HRV species. Semiquantitative cycle threshold values were compared using Wilcoxon rank-sum tests. Comparison of participant and illness characteristics were made between illnesses with HRV-C versus HRV-A and HRV-B combined using generalized estimating equations with exchangeable correlation to account for the occurrence of multiple HRV illnesses by child.

RESULTS

During the study period, 225 children were enrolled, as previously reported [2, 13]. Participating children were an average of 10 months (range 5 weeks to 25 months) of age at enrollment. Forty-five children (37%) had at least 1 sibling [2, 13]. Overall, 455 illnesses were identified over 2 seasons, with HRV the most common respiratory virus detected. HRV was detected during 223 incident acute respiratory illnesses (49%) in 123 children (55%) [13]. Demographic and household characteristics of children with HRV were similar to those without HRV, as has been reported previously [2]. Children commonly experienced more than 1 HRV illness during the study period. Sixty-four children (52%) had multiple illnesses with HRV detected during study enrollment (36 [29%] with 2; 23 [19%] with 3; 3 [2%] with 4; 1 [1%] with 5; and 1 [1%] with 6 infections).

HRV at Study Enrollment

Swabs were collected at enrollment for 85% of enrolled children (n = 191) (Figure 1); 127 (66%) of these enrollment swabs were collected from children with no respiratory illness symptoms. HRV was detected in 41% of the asymptomatic enrollment swabs (n = 52) compared to 30% of symptomatic enrollment swabs (19 of 64). HRV sequence data were available for 32 asymptomatic enrollees and 13 symptomatic enrollees. Ten (31%) of the asymptomatic rhinovirus detections were HRV-A,

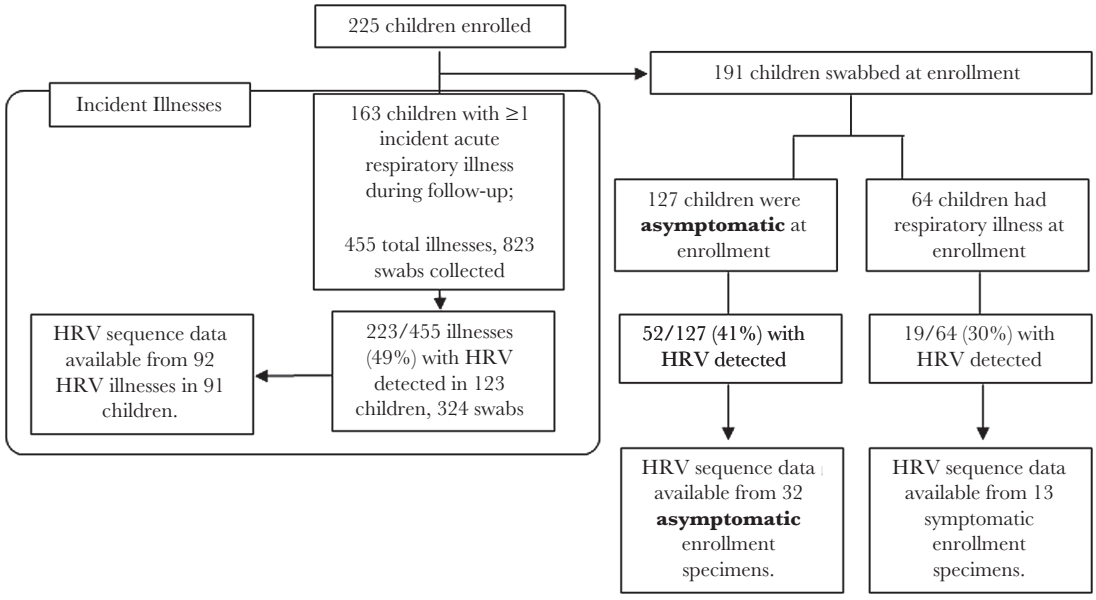

Figure 1. Flow chart of enrollment, sample collection, and testing. Abbreviation: HRV, human rhinovirus.

10 (31%) were HRV-B, and 12 (38%) were HRV-C; compared to 6 (46%) HRV-A, zero HRV-B, and 7 (54%) HRV-C in the symptomatic enrollees (P = .06 by Fisher exact test) (Supplementary Figure 1). Fourteen children had a subsequent weekly swab collected 7–10 days later; 9 (64%) of these children had HRV detected again. Three had HRV subsequently at a third consecutive swab, which occurred 18–19 days after the first swab. Genotype data were available for 1 case of extended asymptomatic HRV detection, where HRV-B17 was detected in 2 swabs 9 days apart. Additionally, 1 asymptomatic child had HRV-C02 detected at enrollment with a HBoV coinfection, AdV and HBoV detected at day 7, and HRV-A12 detected at day 18.

HRV in Incident Illnesses

Of the 823 nasal swabs collected during illness onset and follow-up, 324 (39%) were positive for HRV, collected from a total of 123 children during 223 HRV illnesses. Median cycle threshold (Ct) of detection by RT-PCR was 28.2 (interquartile range, 24.7–32.6; range: 16.6–40). Over half (n = 183; 56%) of the swabs with HRV detected had another respiratory virus detected concurrently. The most common virus codetected with HRV was AdV (n = 80; 25%), followed by HBoV (n = 63; 19%); PIV3 (n = 32; 10%), HCoV (n = 31; 10%), HMPV (n = 15; 5%), RSV (n = 14; 4%), PIV1 (n = 2; 0.6%), Flu A (n = 2; 0.6%), and Flu B, and PIV2 and 4 (n = 1; 0.3%, respectively). There was no difference in Ct by whether other viruses were detected in the same swab (28.1 with codetection vs 28.2 without; P = .71).

Demographic and clinical characteristics of HRV illnesses were compared based on HRV species present at the onset of illness. Of the 223 acute HRV illnesses identified, HRV species were determined for specimens collected at onset for 92 (41%) illnesses in 91 children (Table 1). HRV-A was detected at onset of symptoms for 49 illnesses (53%), HRV-B for 3 illnesses (3%), and HRV-C for 40 illnesses (43%). HRV-C illnesses were associated with decreased activity compared with normal behavior, determined by parent report (Table 1); all other illness symptoms were comparable between species. Mean reported days of missed childcare or work were 1.2 and 1.0, respectively, for HRV-A illnesses, 1.3 for both for HRV-B, and 1.0 for both for HRV-C. Prevalence of virus codetection did not significantly differ by species (P = .74). Days of missed childcare or parental absenteeism from work were not statistically associated with HRV species.

Molecular Epidemiology of HRV in Classrooms

Forty-one unique rhinovirus genotypes were identified in total, 30 of which had >95% homology to genotype reference sequences [10]. The most commonly identified genotypes were A12 (n = 10) and A78 (n = 8). HRV types varied greatly from week to week (Figure 2). Eleven clusters of sequences had 100% similarity to each other but not to any reference type; the largest of these clusters included 19 identical HRV-C sequences and 12 identical HRV-A sequences (Au6). Specimens with the HRV-A sequence of undetermined genotype 6 (Au6) were collected from children in multiple childcare rooms within the center (Figure 3). The sequenced segment of these specimens were identical between individuals, with 93% homology to the reference strain for genotype A20 (NCBI Accession FJ445120). Cocirculation of additional HRV genotypes occurred simultaneously during the circulation of this individual strain (Figure 3).

Table 1. Participant and Illness Characteristics by Rhinovirus Type at Onset of Acute Respiratory Illness

	HRV-A n = 49	HRV-B n = 3	HRV-C n = 40	HRV-C vs HRV-A/B (OR; 95% CI)
Male, n (%[a])	30 (61)	1 (33)	26 (65)	0.96; .69–1.33
Age at illness, mean months (SD)	13 (7.6)	9 (6.1)	13 (5.8)	1.01; .95–1.08
Tobacco use in home, n (%)	2 (4)	0 (0)	0 (0)	…
40+ hours per week in childcare, n (%)	43 (88)	3 (100)	34 (85)	1.00; .39–2.59
Missed childcare, mean days (SD)	1.2 (1.6)	1.3 (1.5)	1.0 (1.6)	0.93; .70–1.25
Parental missed work, mean days (SD)	1.0 (1.4)	1.3 (1.5)	1.0 (1.6)	0.96; .70–1.32
Fever, n (%)	20 (41)	1 (33)	17 (43)	1.09; .46–2.57
Wheeze, n (%)	20 (41)	0 (0)	10 (25)	0.54; .23–1.30
Healthcare visit, n (%)	20 (43)	0 (0)	17 (46)	1.03; .46–2.33
ARI > 7 d, n (%)	36 (75)	1 (33)	27 (69)	0.82; .32–2.10
Rhinorrhea, n (%)	48 (98)	3 (100)	38 (95)	0.38; .03–4.19
Cough, n (%)	43 (88)	2 (67)	33 (83)	0.75; .24–2.38
Fatigue, n (%)	16 (33)	0 (0)	17 (43)	1.77; .75–4.16
Congestion, n (%)	43 (88)	2 (67)	38 (95)	2.93; .58–14.9
Decreased activity, n (%)	14 (29)	0 (0)	16 (40)	**3.02; 1.08–8.47**
Earache, n (%)	5 (10)	0 (0)	6 (15)	1.57; .50–4.94
Decreased appetite, n (%)	15 (31)	0 (0)	11 (28)	0.95; .38–2.36
Vomiting, n (%)	9 (18)	1 (33)	3 (8)	0.36; .11–1.23

Abbreviations: ARI, acute respiratory illness; CI, confidence interval; HRV, human rhinovirus; OR, odds ratio; SD, standard deviation.

[a]Valid percents excluding missing data (2 for healthcare visit, 2 for ARI > 7 days, 1 for missed childcare and missed parental work).

Molecular Epidemiology of HRV in Individual Children

In 67 incident illness cases, HRV was detected in at least 1 weekly follow-up swab prior to illness resolution. HRV detection ranged from 3 to 40 days (median 15 days for illnesses with multiple HRV swabs). HRV sequencing revealed that repeated detections were a combination of persistent (repeated detections of the same genotype) and transient (sequential detections of different genotypes) HRV infections. Six children had repeated detection of the same genotype of HRV in 2 consecutive swabs, approximately 1 week apart, and 1 child had the same HRV genotype (HRV-A78) detected in 3 consecutive swabs spanning 19 days. We identified 7 illnesses with repeated HRV detection in which 2 different HRV genotypes were detected prior to resolution of the illness; the number of days between distinct genotypes ranged from 7 to 24 days.

DISCUSSION

In our study of the molecular epidemiology of HRV among prospectively followed childcare attendees, we documented that HRV circulation during the study period was largely comprised of numerous single occurrences of cocirculating HRV genotypes, along with a series of more common strains, sequentially affecting multiple children across classrooms. We also identified frequent infections with the recently characterized HRV-C and a high prevalence of all HRV species among asymptomatic children. This study adds to previous literature describing HRV spread among children based on culture-confirmed infection by providing more sensitive detection and molecular subtyping of HRV in this population. Based on our finding of multiple

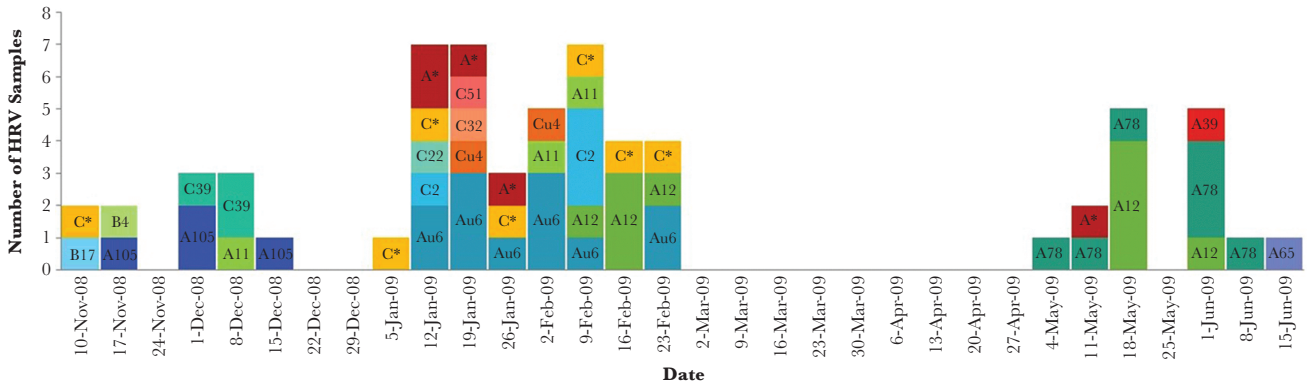

Figure 2. Rhinovirus genotypes identified from November 2008 through June 2009. Frequency of human rhinovirus (HRV) genotypes is labeled over time. * Species identified, no genotype identified within 95% homology to the 5′ noncoding region.

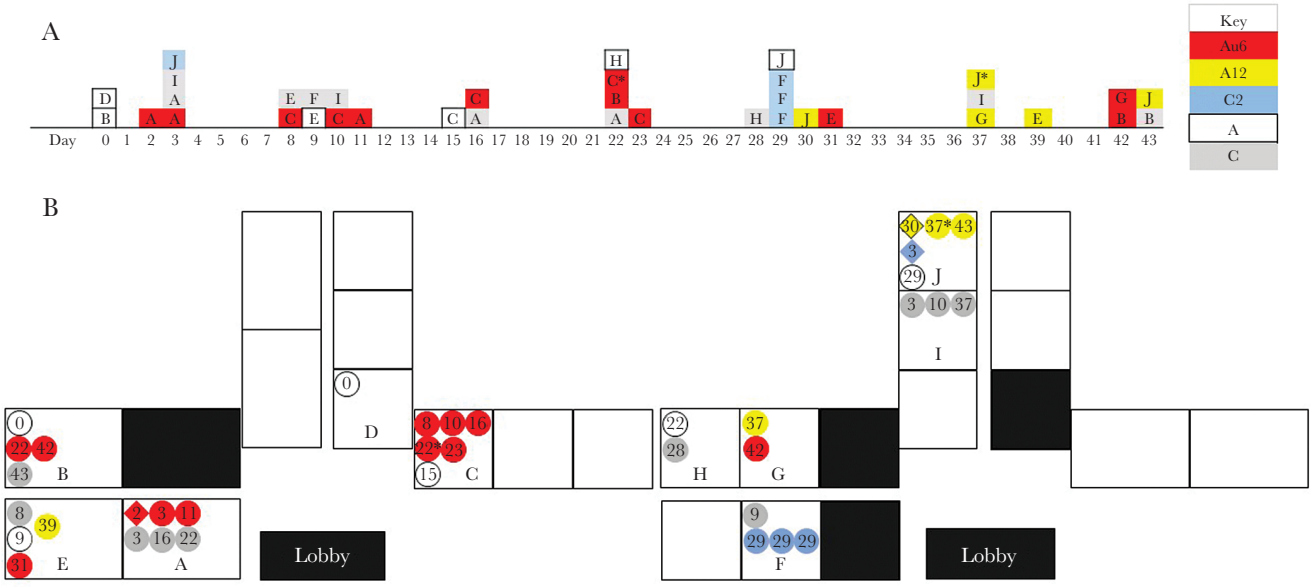

Figure 3. Rhinovirus outbreak from 12 January to 24 February 2009. *A*, Epidemiologic curve of human rhinovirus (HRV) detection. Day 0 is January 12. Letter in box indicates child's room (see *B*) and color indicates genotype. Repeat detections of a genotype in a single child were observed twice in this period, marked with asterisk (*). *B*, Location of genotypes is indicated on the classroom map, with each classroom labeled A through J. Each HRV detection (small circle or diamond) is labeled by day of sample (number) and genotype (color). The first occurrences of common genotypes (Au6, A12, C2) are marked with a diamond. Repeat detections of a genotype in a single child were observed twice in this period, marked with asterisk (*). Black boxes indicate rooms not used for childcare.

HRV genotypes evident during the course of a single respiratory illness, the use of sequence-based HRV type determination is critical in longitudinal studies of HRV epidemiology and transmission.

HRV-C cannot be detected by culture and for that reason has only been described in recent years following the introduction of molecular detection methods. Infection with HRV-C has been associated with more-severe lower respiratory tract illness in children compared to HRV-A [15, 18]; however, this finding has not been consistent across studies [19, 20]. The majority of previously conducted comparative studies of HRV-C are among children or adults presenting for ambulatory or hospital-based medical care [15, 18, 20, 21]. In contrast, our study used prospective, active surveillance in a community childcare setting to examine instances of mild to moderate respiratory illness. In this context, we found few differences by HRV species in our analysis, including no differences in fever or healthcare visits and no evidence of increased cough or wheezing among children with HRV-C. A substantial proportion of children with HRV-C were reported to have decreased activity levels during their illness compared to children with HRV-A (40% vs 27%, respectively); however, this did not result in increases in missed childcare or parental absenteeism from work.

In contrast to HRV-A or HRV-C, HRV-B was infrequently identified, with only 3 HRV-B illnesses out of 91 (3%). We found no evidence of room-to-room spread of distinct HRV-B genotypes as we did with HRV-A. HRV-B prevalence among asymptomatic children was notably higher (10/32; 31%) than among symptomatic children. This may indicate that HRV-B is less likely to cause clinically significant respiratory illness than HRV-A or -C infections. This finding corresponds with other studies, including a study in patients with HRV illness that found no cases of HRV-B among 22 children [22]. However, given the single time point for asymptomatic swab collection at enrollment, we cannot rule out the impact of larger HRV-B circulation patterns on this difference.

HRV was detected in 41% of samples from asymptomatic children. Our results agree with previous studies detecting HRV through RT-PCR, which have similarly detected HRV in 12%–35% of asymptomatic children [5, 23, 24]. Our high rate is unsurprising in this setting given the frequent detection of HRV overall, and the fact that we have documented year-round exposure to HRV in the childcare centers included in the study. Previous studies have shown high rates of asymptomatic HRV transmission between individuals with previous exposure to HRV such as older children and adults [25]. It is likely, given the frequent overall exposure to HRV in childcare attendees, that rates of asymptomatic HRV transmission are high in this setting as well. This high rate of detection of HRV among asymptomatic children, coupled with the frequent coinfection with other viruses observed here, highlights the difficulties with determining the role of HRV in causing the common cold. Even though decades of human challenge studies have demonstrated that experimentally administered rhinovirus results in upper

respiratory symptoms and transmissible disease, it is nevertheless difficult in observational studies to determine causation following a molecularly detected HRV. While not possible in the context of our study, evaluations of host response [26–28] to both HRV infection and especially HRV coinfections may present a way forward in understanding this complex system, as it has with studies of the microbiome.

We were able to assess the extent to which persistent HRV shedding may be present in children following illness resolution through our sampling collection protocol. In this protocol, collection continued until symptoms were no longer worsening and until swabs were negative by PCR, allowing us to follow HRV infections to resolution. From a total of 92 illnesses in 91 children with HRV sequence data available, only 4 children had repeated detections of the same genotype for longer than 2 consecutive weeks. In contrast, we identified 7 illnesses in which 2 different HRV genotypes were detected within the span of a single illness episode. Similar to other studies, we found that rhinovirus RNA was no longer detected in the nasal tract within 2–5 weeks following onset of infection.

There was a highly variable, heterotypic distribution of HRV genotypes observed to cocirculate even within small groups of children in single classrooms. However, in 2 specific instances, continued circulation of individual strains with 100% sequence identity showed evidence of person-to-person transmission within and between individual classrooms. In our study, 1 repeatedly detected strain showed genetic similarity to HRV-A12, and 1 had an undefined genotype that was identical between infected children but not within 95% homology of any previously identified genotype in the database [10]. Interestingly, HRV-A12 has been previously identified as a long-shedding strain by a prospective study of HRV infections in healthy young children [29]. Similar patterns of a limited number of apparent highly transmissible strains responsible for a disproportionately high number of infections have been documented in historical studies of HRV epidemiology [3, 30–33]. However, even these high-prevalence strains do not remain consistent from year-to-year or between populations, complicating efforts to ultimately identify consistent targets for future vaccine efforts.

The effect of large groups of individuals in close contact with one another facilitating the spread of rhinovirus is readily apparent in childcare settings. Group childcare allows numerous opportunities for contact between children with contaminated fomites (ie, toys), providing many pathways for the transfer of aerosolized virus and respiratory secretions. Environmental surveillance studies have found rhinovirus on toys in childcare centers [34], but trials of environmental cleaning have had mixed success in reducing respiratory illness overall in childcare [35–39]. Despite the frequency of missed childcare and lost parental work incurred by all HRV types in this study, effective strategies to reduce transmission of this virus remain scarce.

Our findings are limited by a number of aspects inherent in the study design. Asymptomatic sampling was only conducted during and directly following enrollment. Given the high prevalence of asymptomatic HRV observed, more in-depth asymptomatic testing at regular intervals throughout the respiratory season would be of interest. Secondly, the weekly sampling schedule limited our ability to precisely define HRV shedding duration, given that most HRV infections cleared before the second testing point around day 7. Nevertheless, the duration of genotype-specific HRV detection observed here is notably shorter than other common, frequently asymptomatic respiratory viruses such as AdV and HBoV [5, 13, 40].

Presumed HRV serotype was determined through matching of HRV sequence with a database of established, characterized HRV genomes. This analysis was only possible for the 44% of HRV specimens for which sequencing was successful; of note, specimens that were sequenced had significantly lower cycle thresholds ($P < .01$; data not shown), indicating higher approximate viral quantity. Overall, we have likely underestimated the diversity of HRV types present, and potentially missed the identification of additional clusters, as a result of low-quality sequence data and few asymptomatic collections.

Our application of molecular detection and characterization of HRV in group childcare has created a more detailed view of the epidemiology of HRV infections, as well as greater opportunity to differentiate between the various rhinovirus species present. The use of RT-PCR also allowed for greater detection of all HRV species both in children with ARTI and in asymptomatic carriers. Although our use of weekly sampling allowed us to define the strain-specific duration of HRV in new detail, our finding that most HRV infections last 2 weeks or less indicates that studies with more frequent sampling are needed to fully define the dynamics of naturally occurring rhinovirus infection in young children. This finer level of detail will ultimately be required to describe the transmission networks of HRV in high-prevalence settings, such as childcare or group living environments. As efforts continue to develop HRV vaccines, molecularly informed data on HRV infection, persistence, and transmission will be needed to inform vaccine development and intervention strategies.

Supplementary Data

Supplementary materials are available at *The Journal of 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.

Notes

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