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RESEARCH ARTICLE

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# Phylogenetic characterization of rhinoviruses from infants in Sarlahi, Nepal

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## Abstract

**Problem:** Rhinoviruses (RVs), the most common causes of acute respiratory infections in young children and infants, are highly diverse genetically.

**Objective:** To characterize the RV types detected with respiratory illness episodes in infants in Nepal.

**Study methods:** Infants born to women enrolled in a randomized trial of maternal influenza immunization in rural, southern Nepal were followed with household-based weekly surveillance until 180 days of age. Infants with respiratory symptoms had nasal swabs tested for twelve respiratory viruses. A subset with RV alone was selected for sequencing of the VP4/2 gene to identify RV types.

**Results:** Among 547 RV-only positive illnesses detected from December 2012 to April 2014, 285 samples (52%) were sequenced. RV-A, B, and C species were detected in 193 (68%), 18 (6%), and 74 (26%) specimens, respectively. A total of 94 unique types were identified from the sequenced samples, including 52 RV-A, 11 RV-B, and 31 RV-C. Multiple species and types circulated simultaneously throughout the study period. No seasonality was observed. The median ages at illness onset were 88, 104, and 88 days for RV-A, B, and C, respectively. The median polymerase chain reaction cycle threshold values did not differ between RV species. No differences between RV species were observed for reported respiratory symptoms, including pneumonia, or for medical care-seeking.

**Conclusions:** Among very young, symptomatic infants in rural Nepal, all three species and many types of RV were identified; RV-A was detected most frequently. There was no association between RV species and disease severity.

KEYWORDS
genotypes, infants, respiratory illness, rhinoviruses

## 1 | INTRODUCTION

Rhinoviruses (RVs) are the most common cause of acute respiratory infections in young children and infants.[1] RV infections are highly prevalent and associated with both mild upper respiratory tract and more severe lower respiratory tract (LRT) illnesses, including bronchiolitis, wheezing, and pneumonia.[2] RV are highly diverse genetically, with over 160 types, classified into three species, RV-A, RV-B, and RV-C.[3] Multiple species and types have been shown to circulate simultaneously in different populations including childcare facilities,[4] pediatric emergency department,[5] immunocompromised persons,[6] and hospitalized patients.[7] Individuals can be infected

  

multiple times with different RV types.[4,5] Some individuals have been shown to shed RV for long periods of time and detection of RV in asymptomatic persons is common.[2,8] The diversity of RV species and types and the wide range of clinical symptoms seen in RV illnesses have led to the suggestion that specific species or types may cause more serious illness than other types. Some investigators have reported that RV-C caused more serious illness, especially wheezing and exacerbation of asthma, in some populations[5,9-13] compared with illnesses caused by RV-A and B. However, no differences in illness severity between RV species were found in other studies.[14-19]

The epidemiology and clinical significance of specific RV types have not been analyzed for very young infants in a rural community setting. The molecular characterization of RV infections is limited for RV circulating in developing countries, including countries in Asia, such as Nepal. The detection and characterization of RV in settings with limited electricity, running water, or laboratory facilities have been difficult in the past but newer methods of specimen storage, transport, and molecular detection have permitted enhanced detection of RV and enabled the study of RV types. The aim of our study is to identify the RV species and types causing respiratory symptoms in a unique population of young infants in rural, southern Nepal. A secondary aim is to determine if there are relationships between RV species and types in demographics and illness severity in these very young infants in Nepal.

## 2 | METHODS

### 2.1 | Specimen collection and respiratory virus detection

Infants born to women enrolled in a randomized trial of maternal influenza immunization in the Sarlahi District of southern Nepal were followed with household-based weekly surveillance from birth until 180 days of age. Detailed methods for and results of the trial have been published.[20,21] This study was approved by the institutional review boards (IRBs) of the Cincinnati Children's Medical Center, Johns Hopkins Bloomberg School of Public Health (JHBSPH), the Institute of Medicine at Tribhuvan University, Kathmandu, and the Nepal Health Research Council. IRBs at Seattle Children's Hospital, the University of Washington, and George Washington University granted oversight to the IRB at JHBSPH.

Mid-nasal nylon flocked swabs and clinical data were collected from infants if they had any respiratory symptom reported in the previous 7 days (any one or more of fever, cough, wheeze, difficulty breathing, or draining ear). Swabs were placed in PrimeStore Molecular Transport Medium (Longhorn Diagnostics LLC, Bethesda, MD) and refrigerated until the shipment at room temperature to the testing laboratory where they were frozen at −80°C until testing. The swabs were tested for 12 respiratory viruses by laboratory-developed, real-time reverse transcription polymerase chain reaction (RT-PCR) assays, including respiratory syncytial virus, human metapneumovirus, influenza viruses, parainfluenza viruses, coronaviruses, adenoviruses, bocavirus, and RV.[5,22-25]

### 2.2 | RV typing

A subset of RV-positive samples collected from December 2012 to April 2014 were analyzed. Samples were sequenced if only RV and no other respiratory virus was detected by RT-PCR and the RV PCR cycle threshold ($C_t$) value was less than 30. Only one sample from each RV illness episode was sequenced. An RV illness episode was defined as a respiratory illness with an RV-positive swab. Unique RV illness episodes were defined as RV-positive swabs separated by greater than 21 days without an RV-positive swab.

A 543 base pair fragment of the RV VP4/2 gene was amplified in a semi-nested RT-PCR reaction.[26] PCR amplicons were purified after gel electrophoresis using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and sequenced with the PCR inner primers in both directions in an ABI 3730xl DNA Analyzer using the BigDye Terminator Kit (Applied Biosystems, NJ). Sanger sequencing was performed by GENEWIZ (Seattle, WA), which uses a Quality Score to assess sequence quality, similar to a Phred score, that is the average of the quality values (QVs) for each base in the sequence. To analyze the sequence, we used a QV cutoff of 30 when manually assessing the chromatogram quality, which equals a 0.001 error (or 1/1000) of probability in base calls. Sequences were compared with RV sequences in the NCBI GenBank database using BLAST to identify the RV type with greater than 90% identity. Sequences that did not match a known RV type in GenBank with at least 90% identity were identified as the RV species and the designation U (unknown type). Sequences were submitted to GenBank (accession numbers: MK059474, MK69085-MK69402, and MK078047-MK078053).

Sequences were manually trimmed to a partial RV VP4/2 amplicon of 420 bases. Multiple sequence alignments were performed on ClustlX 2.1. Parameters included a gap opening value of 15 and a gap extension of 6.66. Phylogenetic trees were generated with the maximum likelihood method and branch supported with 1000 bootstrap iterations using PhyML software. Substitution model Tamura-Nei (TN93) was utilized for evolutionary reconstruction. Sequences from GenBank were included in the tree construction to identify RV types. Trees were visualized in FigTree (tree.bio.ed.ac.uk).

### 2.3 | Illness severity

At each illness episode, the presence and duration (number of days) of signs and symptoms were recorded, including cough, fever, wheezing, difficulty breathing, and draining the ear. For each illness episode, the signs and symptoms were combined into a symptom score (each symptom was multiplied by the number of days for that symptom and the sum of all symptoms for all days was determined) and an episode duration (the total duration of any symptom). Care seeking was recorded and combined into a care rank score (no care = 0, nonmedical care = 1, medical care = 2, and doctor or hospital care = 3). Clinical characteristics were compared between infants infected with RV-A and B and those infected with RV-C using Wilcoxon rank-sum testing for medians and the $\chi^2$ test of categorical variables. Associations between specific RV types that were detected

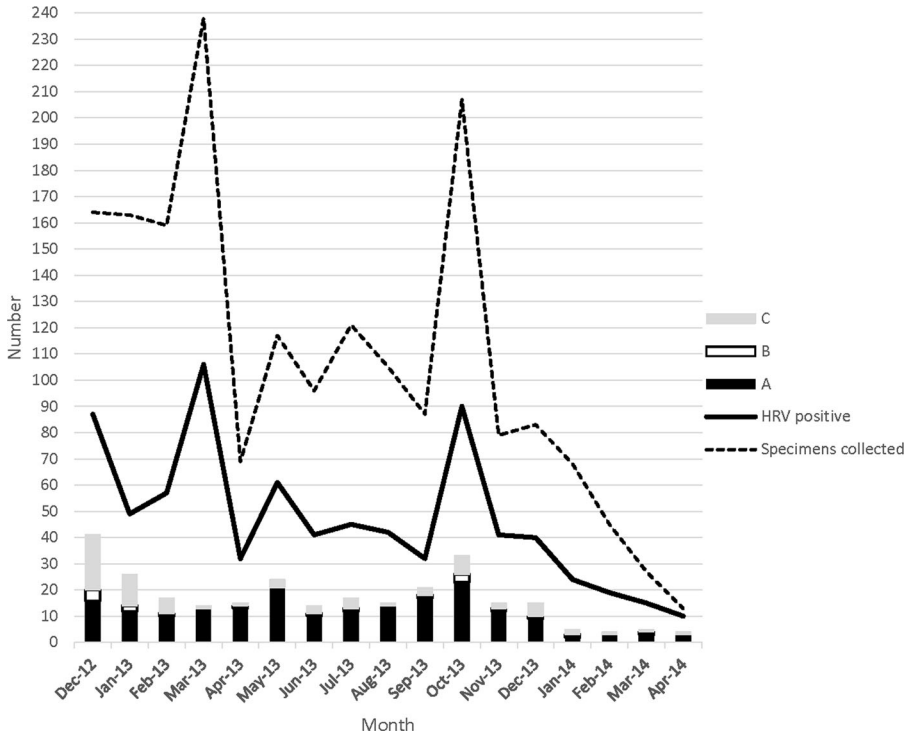


**FIGURE 1** Number of samples collected during the study period, number positive for any RV, and the number of sequenced samples positive for RV-A, B, and C by a month of specimen collection. HRV, human rhinovirus; RV, rhinovirus

in four or more illnesses and reports of LRT symptoms (wheeze, difficulty breathing, and/or pneumonia) were estimated by logistic regression models with and without adjustment for the monsoon season (June-September) and preterm birth. Statistical testing was performed using Stata 13.0 (College Station, TX) and R (R Core Team 2018).

## 3 | RESULTS

### 3.1 | RV detection and typing

Between 1 December 2012 and 30 April 2014, 791 RV-positive illness episodes were detected in 605 infants; 157 (26%) infants had more than one RV illness. RV alone was detected in 609 (77%) of RV-positive illnesses. Of these 609, 285 (47%) samples from 256 infants were sequenced; 25 (10%) infants had RV sequences from more than one illness. The median PCR $C_t$ value of sequenced samples was 26.0 (range: 14.7-29.9).

The number of samples collected, the number positive for any RV, and the number of sequenced samples with each RV species in each month of the study are shown in Figure 1. RV was detected in every month of the study. Monthly prevalence ranged from 30% to 77%, with no apparent seasonality. All three species were detected throughout the study. The majority of RV were species. RV-A, B, and C were identified in 193 (68%), 18 (6.3%), and 74 (26%) of 285 samples, respectively (Table 1). A large number of types was detected for each species. Many RV-A and C types were detected four or more times. Total 51% of RV-A and C illnesses were in infants less than 90 days old compared with 39% of RV-B illnesses. However, no differences were seen between RV species in median infant ages at detection or in median PCR $C_t$ values (Table 1).

The phylogenetic trees generated from the RV-A, B, and C study sequences and reference sequences from GenBank are shown in Figure 2. One RV-A sequence (detected in four illness episodes) and three RV-C sequences (detected in five, one, and one illness episodes, respectively) did not match any known RV types in GenBank within 90% identity. Figure 3 shows the 94 unique RV types identified in the

**TABLE 1** Number of RV-positive samples with sequencing results by RV species type, PCR $C_t$ value, and age at detection

| | RV species | | | |
|---|---|---|---|---|
| Specimen | Any | A | B | C |
| Number of samples, n (%) | 285 (100) | 193 (68) | 18 (6.3) | 74 (26) |
| Number of types | 94 | 52 | 11 | 31 |
| Number of types with >4 detections | 27 | 22 | 0 | 5 |
| Median PCR $C_t$ value (range) | 26.0 (14.7-29.9) | 25.8 (14.7-29.8) | 26.7 (20.9-29.9) | 26.1 (19.3-29.9) |
| Median age at detection, d (range) | 89 (0-179) | 88 (6-179) | 104 (34-169) | 88 (0-177) |

Abbreviations: $C_t$, cycle threshold; PCR, polymerase chain reaction; RV, rhinovirus.

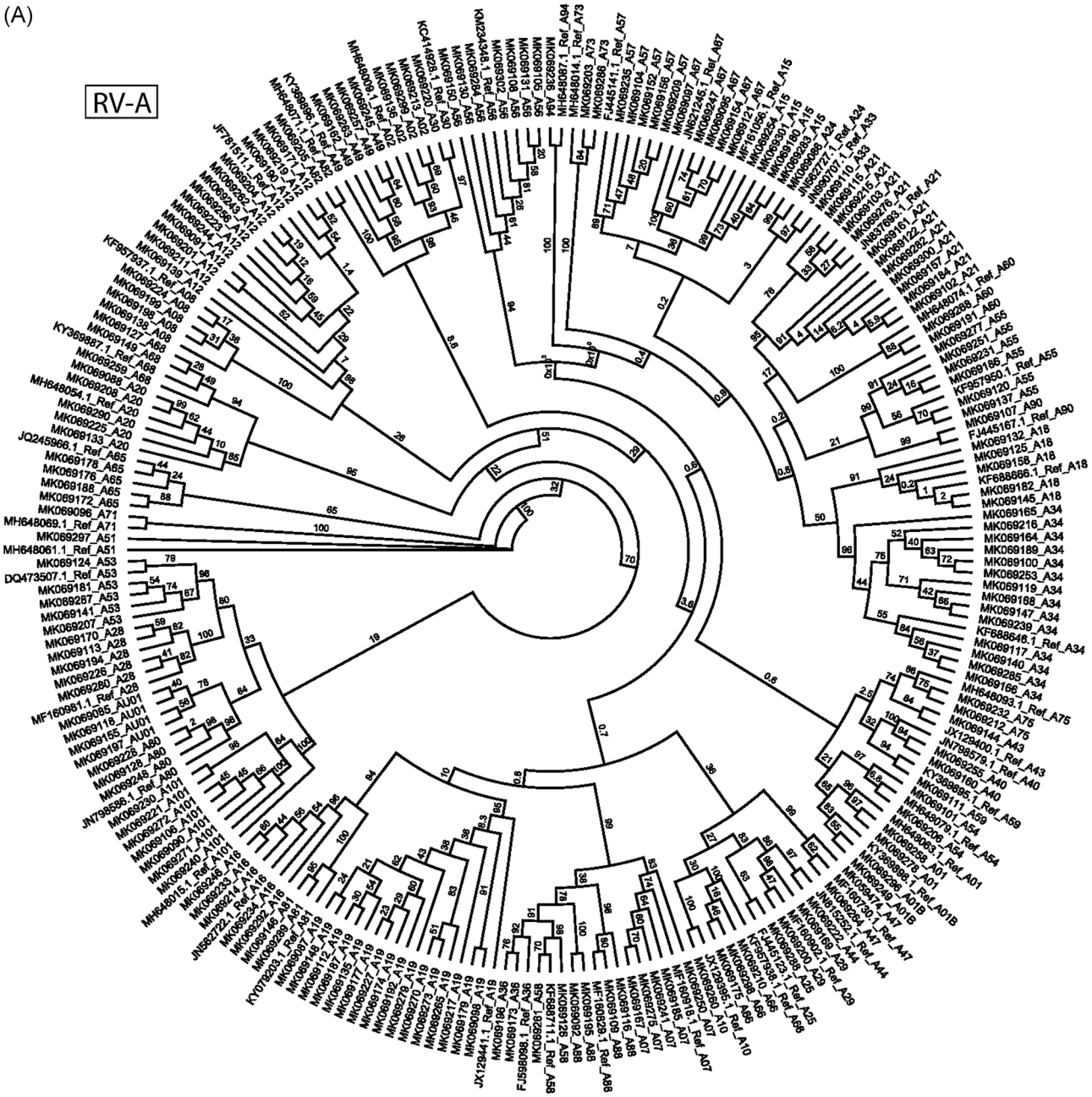


**FIGURE 2** Phylogenetic trees of RV partial VP4/2 gene sequences by RV species. A, RV-A types. B, RV-B types, C, RV-C types. Phylogenetic trees were generated from manually trimmed 420 base fragments using the maximum likelihood method and branch supported with 1000 bootstrap iterations using PhyML software. Bootstrap values are shown on tree nodes. Study sequences are identified by accession number and RV type. Reference sequences from GenBank are identified by accession number_Ref_RV type. RV, rhinovirus

study samples by the RV species and the week each sample was collected. The types are sorted by the date of their first detection. Multiple species and types (94 unique types overall) circulated simultaneously throughout the study period. Up to 27 types were detected in a single month. Some types were detected in only one illness episode, while several types were detected multiple times, either clustered during a short period or detected intermittently over the study period. The 25 infants with more than one RV illness (21 with two, 3 with three, and 1 with four) had different RV types identified in each illness. The median time between RV illness episodes was 63 days (range: 22-142 days).

## 3.2 | Illness severity

An LRT symptom was reported in 84 (72%) of 116 RV illnesses in infants less than 90 days old compared with 78 (56%) of 139 RV


**FIGURE 2** Continued

illnesses in infants 90 days or older ($P < .01$). We did not find any differences between illnesses caused by RV species A and B and those caused by RV-C for any reported respiratory symptom, including pneumonia, or for medical care-seeking (Table 2).

We also evaluated whether illnesses with some RV types were associated with LRT symptoms compared with other types, regardless of species. Symptoms reported during illnesses with 20 RV types (17 RV-A and 3 RV-C) that were detected at least four times (range: 4-13) were differentiated into upper and lower tract symptoms. Table 3 shows the number and percent of illnesses with any LRT symptom for each of the 20 types. Twelve of the 20 RV-A and C types detected in multiple infants were associated with reports of LRT symptoms in 75% to 100% of illnesses; eight types were associated with reports of LRT symptoms in 23% to 60% of illnesses. A logistic regression model comparing the proportion of illnesses with LRT symptoms for each type using type A34 (83% LRT illnesses) as the reference group indicated that specific types were not associated with LRT symptoms (Table 3).

## 4 | DISCUSSION

Among very young, symptomatic infants in rural Nepal, all three species and many types of RV were identified. Multiple types circulated simultaneously (up to 27 types in a month) and throughout the 17 month study period, with 94 unique types detected overall. Some types were detected only once. Others were detected repeatedly in multiple infants, occurring during a short time span

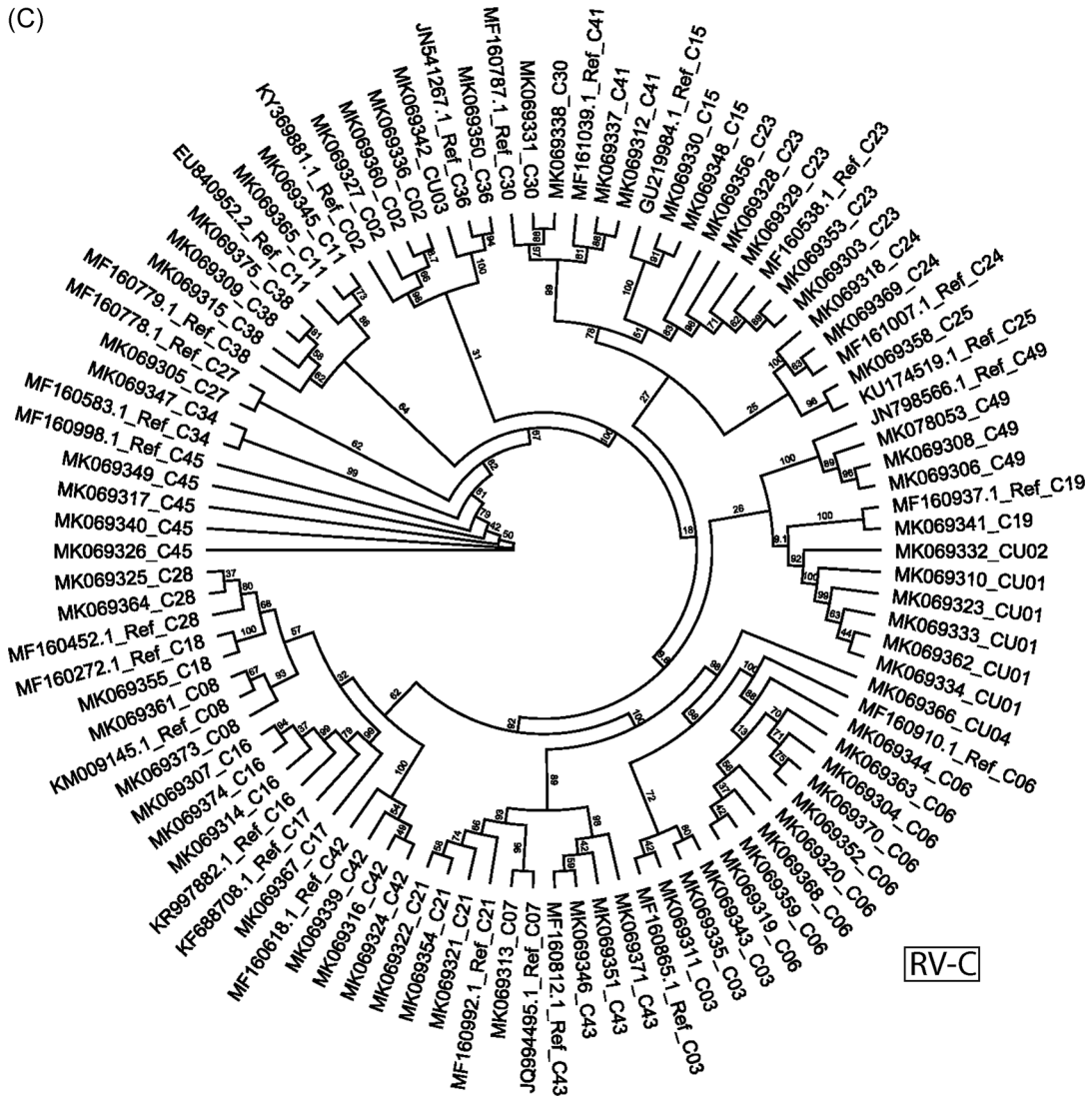


**FIGURE 2** Continued

or over the entire study period. Our findings confirm those of other investigators who examined RV types detected in samples from different populations in diverse settings and geographical locations.[5,9,12,27-32] The proportions of RV species and the high genetic diversity that we detected are similar to what many other studies have found, with RV-A being detected most frequently and RV-B being detected most infrequently. The majority of studies conducted in many parts of the world have consistently reported that RV-A, B, and C are detected in 48% to 65%, 2% to 12%, and 26% to 44% of samples, respectively.[4,5,7,9,12,14-19,26,27,30,32-35] Our study is unique to many other studies in that we assessed viral types in a community-based rural setting, using prospective surveillance, compared with the clinic or hospital-based surveillance in more urban settings. Nonetheless, this study, in agreement with previous studies, has found high genetic diversity and rapid turnover of types, with few types being detected in consecutive seasons or over long periods of time. This simultaneous and successive circulation of different RV types may be one reason for the high incidence of RV infections throughout the year.[28]

A previous report has described the risk factors for RV infections in infants in this Nepal study and provided more details on illness severity in the RV-positive infants.[36] In this report, analyses were limited to illnesses in which the only RV was detected to avoid confounding caused by coinfections with other respiratory viruses. LRT symptoms were more frequently reported in younger infants (<90 days old) compared with older infants (90-180 days old). We examined the association of RV species and types with age at infection, PCR $C_t$ value, and reported respiratory symptoms. RV species was not associated with age or PCR $C_t$ value, a surrogate for viral load. However, we tested infants only from birth to 6 months of age and were only able to sequence RV-positive samples with $C_t$ values less than 30. We did not observe any associations between RV species A and C and clinical signs or symptoms, pneumonia, or care-seeking in these infants. We previously did not observe an association between RV species in RV-positive febrile pregnant women in

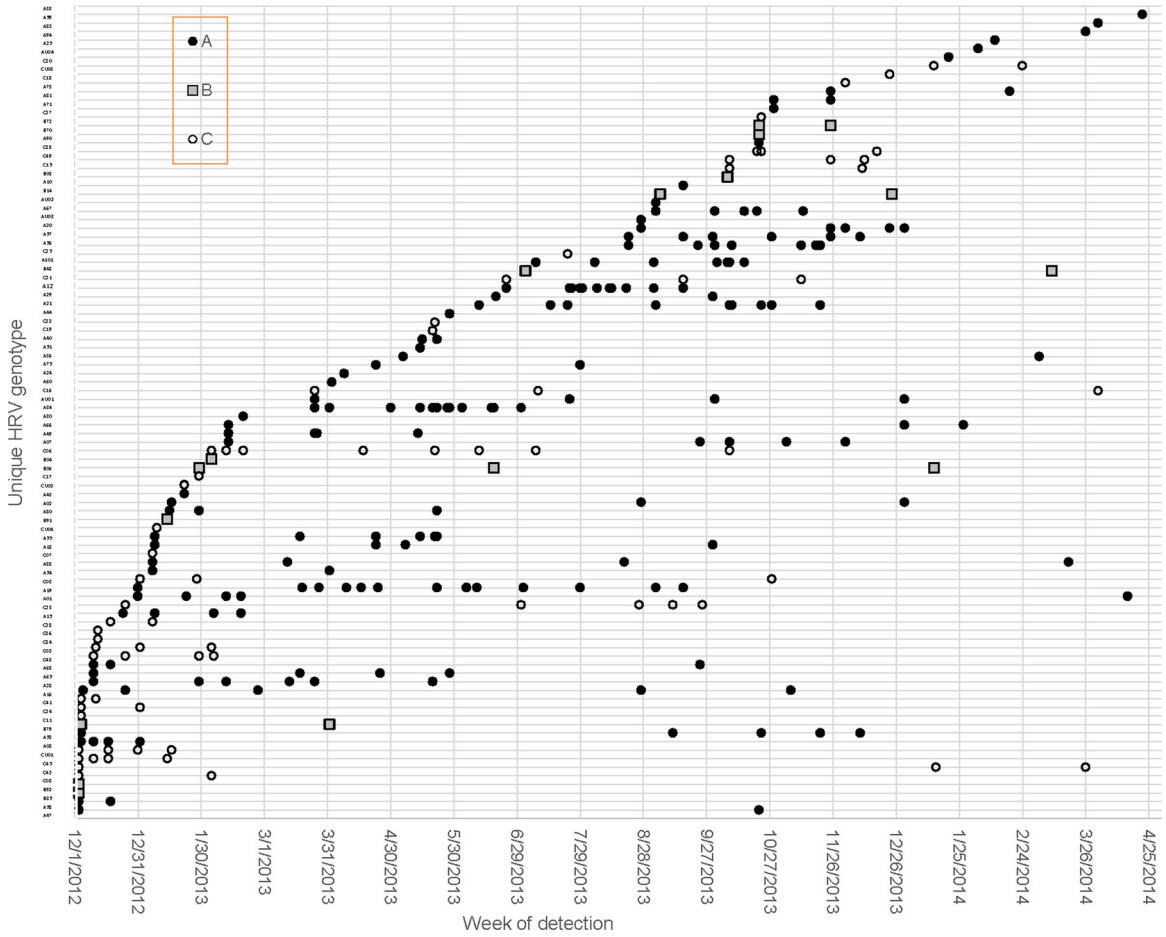


**FIGURE 3** A total of 94 unique RV types by a week of specimen collection. RV types are sorted by the date of their first detection. RV-A, B, and C types are indicated by filled circles, shaded squares, and open circles, respectively. RV, rhinovirus

**TABLE 2** Clinical characteristics of RV illness episodes by RV species

| | RV species | | | |
|---|---|---|---|---|
| **Clinical characteristic median (range) or n (%)** | **A (n = 171)** | **B (n = 17)** | **C (n = 67)** | ***P* value[a]** |
| Episode duration, d | 5 (1-104) | 10 (3-80) | 5 (2-104) | .34 |
| Symptom score | 7 (1-119) | 9 (3-23) | 6 (1-119) | .52 |
| Care rank score | 1 (0-3) | 1 (0-3) | 1 (0-3) | ND |
| Cough duration, d | 3 (0-56) | 3 (0-16) | 2 (0-28) | .18 |
| Fever duration, d | 1 (0-18) | 1 (0-7) | 0 (0-13) | .10 |
| Wheeze duration, d | 1 (0-41) | 0 (0-6) | 2 (0-32) | .32 |
| Difficulty breathing duration, d | 0 (0-53) | 0 (0-8) | 2 (0-53) | .14 |
| Cough | 116 (68) | 14 (82) | 42 (63) | .33 |
| Fever | 95 (56) | 9 (53) | 30 (45) | .14 |
| Wheeze | 91 (53) | 7 (41) | 41 (61) | .20 |
| Difficulty breathing | 74 (43) | 6 (35) | 38 (57) | .05 |
| Pneumonia | 88 (58) | 8 (53) | 39 (65) | ND |
| Any care | 113 (66) | 10 (59) | 42 (63) | .69 |
| Medical doctor or hospital | 19 (11) | 1 (6) | 6 (9) | .70 |

Abbreviations: ND, not defined; RV, rhinovirus.

[a]Rank-sum testing of medians compares RV-C to RV-A and B; $\chi^2$ tests of categorical variables compare RV-C to RV-A and B.

**TABLE 3** Twenty RV types detected in four or more illnesses and the proportion of illnesses with reported LRT symptoms (wheeze, difficulty breathing, and pneumonia)

| RV type | Number of LRT illnesses/number of illnesses (% LRT illnesses) | Odds ratio (95% CI) | *P* value |
|---|---|---|---|
| A08 | 4/4 (100) | 1.2 e7 (0-∞) | .99 |
| A18 | 5/5 (100) | 1.1 (0.1-12.6) | .95 |
| A28 | 5/5 (100) | 4.0 (0.3-102.4) | .30 |
| A101 | 7/7 (100) | 0.8 (0.1-5.9) | .80 |
| A12 | 10/12 (83) | … | … |
| A19 | 10/12 (83) | 0.3 (0.1-1.6) | .18 |
| A16 | 4/5 (80) | 1.1 (0.1-12.6) | .95 |
| A56 | 4/5 (80) | 2.3 (0.2-55.5) | .52 |
| A57 | 4/5 (80) | 2.3 (0.2-55.9) | .52 |
| C06 | 7/9 (78) | 1.4 (0.2-11.5) | .73 |
| A01 | 3/4 (75) | 2.4 (0.2-64.8) | .53 |
| C (NT01) | 3/4 (75) | 0.5 (0.04-5.6) | .60 |
| A20 | 3/5 (60) | 0.5 (0.04-4.7) | .52 |
| A55 | 3/5 (60) | 0.4 (0.03-3.8) | .44 |
| A21 | 4/7 (57) | 0.4 (0.1-2.6) | .34 |
| A53 | 2/4 (50) | 0.7 (0.1-8.7) | .77 |
| A65 | 2/4 (50) | 0.8 (0.1-10.7) | .86 |
| C45 | 2/4 (50) | 0.8 (0.1-10.5) | .86 |
| A67 | 1/4 (25) | 0.2 (0.01-2.3) | .22 |
| A34 | 3/13 (23) | 0.2 (0.03-1.2) | .09 |

Abbreviations: CI, confidence interval; LRT, lower respiratory tract; RV, rhinovirus.

the Nepal study and low birth weight in their infants.[37] No associations between RV species and illness severity have been reported by others.[4,14-16,18,19,30,38] However, some studies have shown that RV-C species are more likely to cause LRT illnesses than RV-A[5,9] including specific clinical findings, such as wheezing,[12,39] asthma,[31] rhonchi, vomiting,[13] and viremia.[26] The discrepancies between these studies may be due to the patients that were tested. The majority of studies that showed an association between RV-C and more severe illnesses were conducted in children with LRT symptoms.

In addition to a lack of association between symptoms and RV species, we also did not see an association between specific RV-A and RV-C types and LRT symptoms. Our results are in contrast to those reported by Martin et al[5] and Delano et al,[27] in which specific types of RV were associated with more severe illness compared with other types. The number of infections with RV-B was too small to include in our analyses; no RV-B types were detected in four or more illnesses.

A limitation of the study was the inability to sequence samples with RV PCR $C_t$ values of 30 or higher. The number of viral copies in these samples was too low to provide adequate sequencing data. If some RV types are more likely to be present in low viral loads, their prevalence would be underestimated by our sequencing method. Also, we relied on symptoms reported by parents at weekly interviews and some episodes could have been missed due to limited recall. A total of 25 infants had multiple RV illnesses, in which different RV types were detected. However, we may have missed sequential infections caused by different RV types during single illness episodes because we defined an episode as having more than 21 days between RV-positive nasal swabs and we sequenced only one sample per illness episode. Consequently, for illnesses with symptoms and RV-positive swabs over a long period of time, we were not able to distinguish between prolonged shedding of a single RV type and possible infection with a new type. because we did not test asymptomatic infants, RV types circulating during the study period in asymptomatic infants would also have been underestimated.

In conclusion, in very young infants in rural, southern Nepal, with generally mild respiratory illnesses, we detected multiple RV types circulating simultaneously and throughout the study period. However, we did not find differences between RV species in infant demographics or symptoms.

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## CONFLICT OF INTERESTS

JK, GAP, KLN, JK, JMT, SKK, SLC, and KRJ have no conflict of interests. HYC has received research support from Sanofi Pasteur. JAE has been a consultant for Pfizer, a member of a Data Safety Monitoring Board for GSK influenza antiviral studies, and her institution has received research support for clinical studies from GSK, Gilead, Chimerix, and Roche.

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