

Molecular characterization of human rhinovirus strains detected in pediatric acute respiratory tract infections

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Received: September 2025, Accepted: January 2026

ABSTRACT

Background and Objectives: Acute respiratory tract infections (ARTI) are a leading cause of morbidity and mortality in children worldwide, accounting for approximately 18% of deaths in those <5 years of age. Viruses cause 50-90% of pediatric ARTI cases. Human rhinovirus (HRV) is increasingly associated with lower respiratory tract infections (LRTIs). This study aimed to detect HRV in pediatric ARTI cases and characterize circulating genotypes.

Materials and Methods: Nasopharyngeal swabs from 154 children (≤5 years) presenting with ARTI were screened for HRV using real-time PCR. Thirteen samples with a cycle threshold ≤30 were sequenced. Phylogenetic analysis was performed using 41 global reference sequences representing different geographical regions and HRV types.

Results: HRV was detected in 34.41% (53/154) of the samples. In children aged >1 month to 1 year, HRV positivity was significantly associated with severe acute respiratory infection (SARI) compared with influenza-like illness (ILI). Phylogenetic analysis revealed a predominance of HRV-C strains (n = 7), followed by HRV-A (n = 5) and HRV-B (n = 1).

Conclusion: HRV was detected in a significant proportion of pediatric ARTI cases, with HRV-C as the predominant strain. Infants aged >1 month to 1 year showed a higher association with severe illness, underscoring the need for closer clinical monitoring in this age group.

Keywords: Rhinovirus; Respiratory tract infections; Molecular characterization; Phylogeny; Acute disease; Real-time polymerase chain reaction

INTRODUCTION

Acute respiratory tract infections (ARTI) are a leading cause of mortality in young children glob-

ally. Of the approximately 13 million annual deaths in children under five, ARTI are responsible for an estimated 3.9 million, with 95% of these occurring in developing countries (1). In India, pneumonia re-

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mains a major contributor (15.9%) to mortality in children <5 years, despite a substantial decline in acute respiratory infection prevalence from 5.6% in 2005-2006 to 2.7% in 2015-2016 (2). In contrast, lower respiratory infections attributable to second-hand smoke accounted for 16.6% and 11.18% deaths in <5 years age group in Central Asia and South Asia, respectively (3). Further, underlying risk factors like overcrowding, malnutrition, immature immunity, high pathogen diversity, limited access to healthcare, antibiotic resistance, and low birth weight might be responsible for 40% of ARTI mortality in countries like India, Bangladesh, and Nepal (4). Among the ARTI-associated pathogens in children, viruses account for a substantial proportion, ranging from 50% to 90% of cases (5). Influenza and human Respiratory Syncytial viruses (hRSV) are the major causes of lower respiratory tract infection (LRTI), followed by human rhinovirus (HRV), human metapneumovirus (hMPV), and Parainfluenza viruses.

Human rhinovirus (HRV) belongs to the genus Enterovirus under the family Picornaviridae (6). Since its identification in the 1950s, it has been associated with the common cold and circulates in nature as HRV-A, HRV-B, and HRV-C genotypes with 83, 32, and 55 subtypes, respectively (7, 8). A study by Sonawane et al. (9) on infants aged 1-12 months with lower respiratory tract infection (LRTI) revealed that 35.4% and 25.6% of cases were due to RSV-A/B and HRV, respectively. Thus, identifying viral agents in LRTI and monitoring their trends is needed to provide important inputs for the proper management of patients. In the absence of rapid diagnostics, it would be more appropriate to rely on molecular methods for their detection and genotyping. As there is a paucity of data regarding the circulating rhinovirus strains, this study aimed to understand the epidemiology of HRV in the pediatric population. Keeping in view the futuristic approach towards better management of cases of ARTI, identifying strains as vaccine candidates or for the development of a drug target, the present study aimed to detect the HRV types in respiratory samples from pediatric patients with ARTI (<5 years of age), through molecular and phylogenetic analysis.

MATERIALS AND METHODS

Study subjects. The present study was a retrospective, hospital-based, observational study on the

pediatric patients (<5 years of age) admitted with ARTI between October and December 2020 in the Advanced Pediatric Centre, Postgraduate Institute of Medical Education and Research, Chandigarh. Children presenting with fever, signs and/or symptoms of respiratory tract infection, and/or with a chest radiograph consistent with community-acquired pneumonia (CAP), were screened for SARS-CoV-2 RNA using the ICMR-recommended TRUPCR SARS-CoV-2 RT-PCR kit (3B BlackBio Biotech, India) in their nasopharyngeal swabs (NPS) samples. The assay targets the E gene for screening and the *RdRp* gene for confirmation, with a cycle threshold (Ct) value of <35 for both target genes being considered positive. Children who tested negative for SARS-CoV-2 RNA in NPS samples were included in the study. The study was carried out after approval from the Institute Ethics Committee vide no. INT/IEC/2020/SPL-1619, dated 04/12/2020. Waiver for consent of the patients to participate in the study was approved with vide no. INT/IEC/2022/SPL-107, dated: 12-2-2022; with reference no. NK/6792/MD/407.

Real-Time PCR for HRV detection. 200 µl of nasopharyngeal samples was used for the RNA extraction using NUCLISENS® easyMAG® automated nucleic acid extraction platform (bioMérieux, NC). TaqMan chemistry-based primers and probes (Biosearch Technologies, Inc., Novato, CA) targeting the 5'UTR conserved region of HRV were used for the target amplification using the Real-Time PCR System (Applied Biosystems, US) following standard procedure (Table 1). The probe was labeled with a reporter molecule 6-carboxy fluorescein (6-FAM) at the 5'end and with the quencher Black Hole Quencher 1 (BHQ-1) at the 3'end (Biosearch Technologies, Inc., Novato, CA) (10). Locked nucleic acid (Exiqon A/S, Vedbaek, Denmark) analogs (LNA) were introduced during oligonucleotide synthesis, where the last base at 3' for-

Table 1. Primers and probe used for TaqMan real-time PCR detection of human rhinovirus

| Primer/Probe | Sequence (5'-3') |
|--------------|--------------------------------|
| HRV Forward | CYA (LNA-A)GCCT(LNA-T)GCGTG GY |
| HRV Reverse | GAAACACGGACACCCAAAGTA |
| HRV Probe | TCCTCCGGCCCTGAATGYGGC |

LNA: Locked nucleic acid. Degenerate bases follow IUPAC nomenclature (Y=C/T)

ward primer was changed to "Y (C or T)" degenerate base from single base "C" to accommodate recently published HRV C strains.

The Real-Time RT-PCR assay was performed using the one-step RT-PCR reagents (Applied Biosystems, CA, US). Each 25µL reaction mixture contained 1.25 µl of each forward and reverse primers (40 µM), 1.25 µl probe (10 µM), 1 µl enzyme mix, 12.5 µl 2X master mix, 2.75 µl nuclease-free water, and 5 µL of extracted RNA. The amplification was performed using the following thermal conditions: 30 min at 50°C for reverse transcription, followed by 15 min Taq activation at 95°C, and then amplification for 45 cycles of 15 sec at 95°C and 1 min at 56°C. Real-time PCR data were analyzed using Applied Biosystems SDS software v1.4. Each run included a template (HRV positive) and a non-template (water) control. Samples showing a cycle threshold (Ct) value ≤36 were considered positive for HRV.

HRV serotyping by nested reverse transcriptase PCR. Thirteen representative HRV-positive samples with a Ct value of ≤30 were subjected to the amplification of the VP4/VP2 region of the HRV genome by serotype nested RT-PCR (Table 2). The first outer cycle of the nested PCR was done in a 20 µl reaction mixture containing 2 µl 10X reaction buffer, 2 µl dNTPs (2 mM), 0.5 µl MgCl₂ (50 mM), 1.2 µl each of 458OS, and 1125OAS (10 µM) (11), 0.3 µl Hotstart Taq polymerase (5U/ml), 9.8 µl water and 3µl of cDNA to amplify a 667-bp product with following thermal conditions: initial denaturation at 94°C for 2 minutes, followed by 35 cycles of amplification with denaturation at 94°C for 18 seconds, primer annealing at 50°C for 21 seconds, and extension at 72°C for 90 seconds, with the final extension step at 72°C for 7 minutes. This was followed by a second internal cycle of the nested PCR with inner primers as 547IS, and 1087IAS at the same concentration (11), 3 µl 10X

reaction buffer, 3 µl dNTPs (2 mM), 2 µl MgCl₂ (50 mM), 0.4 µl Hotstart Taq polymerase (5U/ml), 15.2 µl water with 4 µl of first cycle amplified product. The primer annealing temperature was raised to 55°C; however, other thermal conditions were the same. The amplified product was loaded and resolved on a 1.5% agarose gel, resulting in an expected 540 bp band, which was further excised and purified using a commercial gel extraction kit (Qiagen, GmbH, Germany) following the manufacturer's instructions.

Sanger sequencing and phylogenetic analysis. The extracted amplified products were subjected to sequencing PCR using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA) using the IS and IAS primers, followed by sequencing on an ABI 3500xl Dx Genetic Analyzer (Applied Biosystems, USA). Sequences were analyzed using Finch TV version 1.4.0 to determine the quality of sequences. 41 global sequences were selected from different geographical regions, covering all the different types of HRV strains. Multiple sequence alignment was carried out using the ClustalW algorithm in MEGA (Molecular Evolutionary Genetics Analysis) version 11.0.11.

The nucleotide sequences (positions 623-1012; 390bp) of the partial VP4/VP2 coding region were analyzed phylogenetically, and evolutionary distances were estimated by the neighbor-joining method. The reliability of the tree was estimated with 1000 bootstrap replications.

Ethics approval. The study was approved by the Institute Ethics Committee, PGIMER, Chandigarh vide no. INT/IEC/2020/SPL-1619, dated: 04-12-2020. The waiver for consent of the patients to participate in the study was approved with vide no. INT/IEC/2022/SPL-107, dated: 12-2-2022 with reference no. NK/6792/MD/407.

Table 2. Primers used for serotyping of human rhinovirus by nested PCR targeting the VP4-VP2 region

| Target gene | Primer orientation | Position | Sequence (5'-3') |
|-------------|-----------------------|----------|---------------------------|
| VP4-VP2 | Outer sense (OS) | 458 | CCGGCCCCTGAATGYGGCTAA |
| VP4-VP2 | Inner sense (IS) | 547 | ACCRACACTTTGGGTGTCCGTG |
| VP4-VP2 | Inner antisense (IAS) | 1087 | TCWGGHARYTTCCAMCACCANCC |
| VP4-VP2 | Outer antisense (OAS) | 1125 | ACATRTTYTSNCCAAANAYDCCCAT |

Degenerate bases are indicated according to IUPAC codes

RESULTS

A total of 154 patients, including males (74.03%, 114/154) and females (25.97%, 40/154), who presented with respiratory illness from October 2020 to December 2020 were included in the study. The study subjects belonged to neighboring states of Punjab (n=65), Haryana (n=36), Chandigarh (n=12), Himachal Pradesh (n=12), Uttar Pradesh (n=11), Jammu and Kashmir (n=6), Rajasthan (n=4), Uttarakhand (n=3), West Bengal and Bihar (n=2 each), and Maharashtra (n=1). These patients were categorized into three age groups, namely ≤ 1 month (26.62%, 41/154), 1 month to 1 year (48.05%, 74/154), and 1 year to < 5 years (25.3%, 39/154). 87 of 154 (56.49%) patients presented with symptoms of fever, sore throat, and coryza were grouped as Influenza-like illness (ILI). In comparison, sixty-seven patients (43.20%, 67/154) who had severe respiratory distress due to bronchiolitis, pneumonia, or wheezing illness were grouped as severe acute respiratory infection (SARI). Both ILI (48.28%, 42/87) and SARI (47.76%, 32/67) cases were more commonly observed in the 1-month to 1-year age group. Of the 154 ARTI patients (children ≤ 5 yr. age), HRV RNA was detected in 53 (34.41%) patients (Fig. 1A).

Most of the HRV-positive children, i.e., 58.49% (31/53), belonged to the age group of 1 month to one year. Overall, 56.6% (30/53) presented with ILI, whereas 43.39% (23/53) had SARI (Fig. 1B). Further, 28 of 154 patients (18%) were born prematurely with low birth weight, of whom 16 presented with SARI (57%), and 10 (35%) tested positive for HRV. Among the 17 patients with congenital heart disease, HRV was detected in only two cases.

Phylogenetic analysis and evolutionary relationship. A total of thirteen nucleotide sequences of the VP4/VP2 gene (HRV) were aligned with forty-one global nucleotide sequences using CLUSTAL W in MEGA-11 software. Phylogenetic analysis showed predominance with HRV-C strains (HRV-C3, C42, C3, C36, and C45) being detected in two SARI and five ILI patients, followed by HRV-A strains (HRV-A21, A1, A2, and A31) detected in two SARI and three ILI patients, and HRV-B72 in one SARI patient (Fig. 2). Our sequences showed $\sim 94.67\%$ sequence homology (%) with reference strains (Table 3).

Risk of SARI / ILI with HRV infection. When the

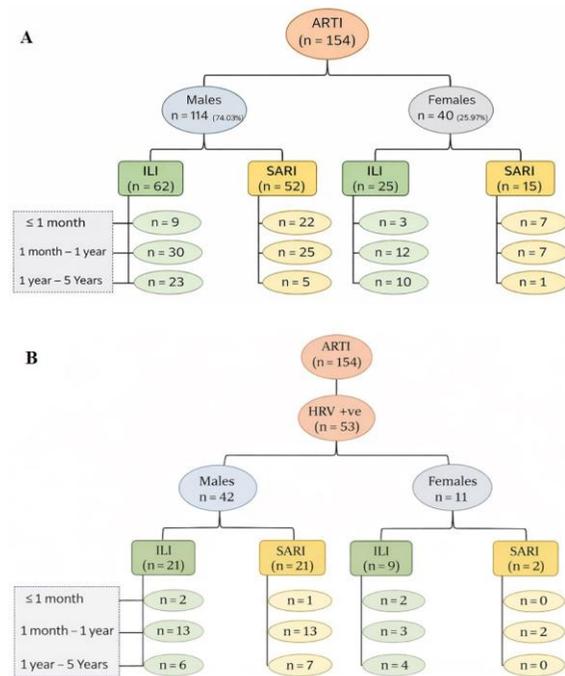


Fig. 1. A) Distribution of patients with ILI and SARI among males and females in different age groups. Among male patients with ILI (n=62), 48.38% (30/62) and those with SARI, 48.07% (25/52) were in >1 month to 1-year age group. Among female patients with ILI (n=25), 48% (12/25) were in the > 1 month to 1 year age group, and those with SARI (n=15), 46.6% (7/15) fell in the ≤ 1 month age and >1 month to 1 year age group. B) Age-wise distribution of ILI and SARI among male and female HRV-positive patients. Among HRV-positive male patients with ILI, 61.9% (13/21) belonged to the > 1 month to 1 year age group, and those with SARI 61.9% (13/21) belonged to the >1 month to one year age group. Among human rhinovirus-positive female patients with ILI, 44.44% (4/9) each belonged to >1 year to five years age group, and those with SARI, 100% (2/2) fell into >1 month to one year age group.

whole population of children <5 years was evaluated, there was no statistically significant risk of developing SARI [Odds Risk (OR = 0.99, 95% Confidence Interval (CI): 0.51-1.94) when testing positive for HRV. However, in the sub-group aged > 1 month to 1 year, HRV PCR positivity was associated with a higher likelihood of SARI diagnosis compared with ILI (OR = 0.14, 95% CI: 0.04-0.47). Such findings were not seen with the sub-group population of <1 month or 1 year to 5 years. There was no gender predisposition for risk of SARI in children diagnosed with HRV (OR = 1.40, 95% CI: 0.67-2.92) (Table 4).

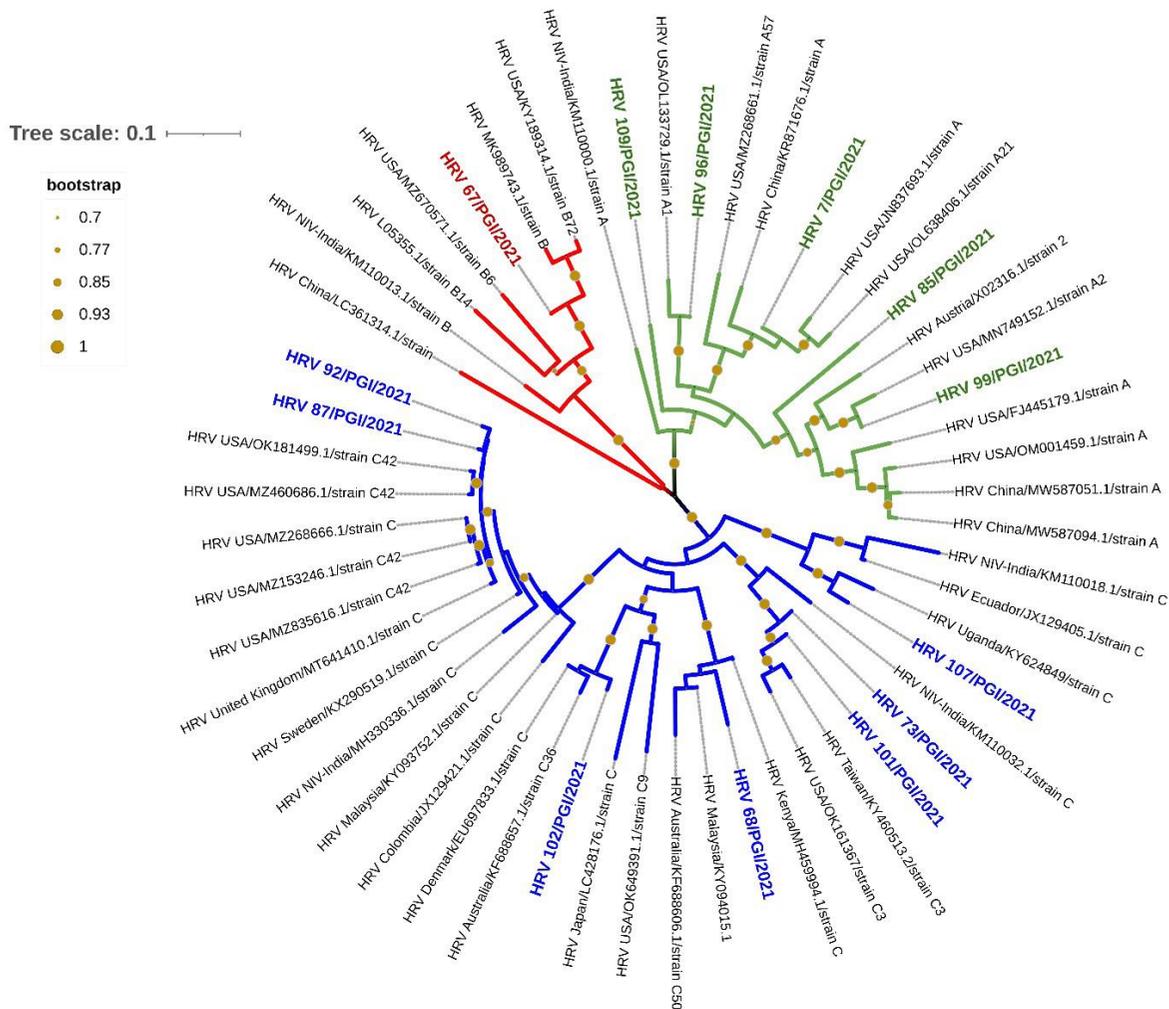


Fig. 2. Neighbor-Joining phylogenetic tree of HRV sequences constructed using the Tamura-Nei nucleotide substitution model. Tree topology was assessed using bootstrap analysis with 1000 replicates, and bootstrap values $\geq 70\%$ are displayed as internal node labels (as shown in iTOL). Branch lengths are proportional to the number of nucleotide substitutions per site. Study isolates are indicated in bold, while reference sequences were retrieved from GenBank. The tree shows clear segregation of HRV sequences into three clades: HRV-A (green), HRV-B (red), and HRV-C (blue).

DISCUSSION

In our study, 74.03% of patients were males and 25.97% were females, reflecting a male preponderance consistent with other studies (12, 13). Cultural factors in South Asia, potentially smaller airway size (diameter), and obstructing airflow which results in inflammation and mucus build-up in young boys may contribute to this gender specificity for a higher risk of infection (13). In our study, HRV RNA was detected in 34.41% of ARTI in children ≤ 5 years, in concordance with reported HRV prevalence ranging from 21% to 49% (14, 15). Slight difference in preva-

lence compared to studies by Sonawane et al. (25.6%) and Mishra et al. (31.1%) may be explained by the inclusion of different age groups (9, 12).

Studies from India have reported considerable heterogeneity in the viral etiology of acute and lower respiratory tract infections among children. Yeolekar et al. (16) and Maitreyi et al. (17) identified RSV as the predominant virus in children with ARTI (26% and 17%, respectively), and Singh et al. (18) found RSV to be the most common virus (21.3%) in children aged 0-13 years with LRTI. None of the above studies evaluated samples for HRV, and recent studies reporting a higher prevalence of HRV as a

Table 3. Sequence homology homology (%) with reference strains

| Strains identified in this study | Homology (%) | Named subtypes | Scientific name | GenBank Accession number |
|----------------------------------|--------------|---|-----------------|--------------------------|
| HRV 07/PGI/2021 | 98.02 | Rhinovirus A21 strain RvA21/USA/2021/4BVZBR | Rhinovirus A21 | OL638406.1 |
| HRV 67/PGI/2021 | 98.07 | Rhinovirus B72 strain SCH-101 polyprotein gene | Rhinovirus B72 | KY189314.1 |
| HRV 68/PGI/2021 | 94.67 | Rhinovirus C50 strain hu-man/Australia/SG1/2008 | Rhinovirus C50 | KF688606.1 |
| HRV 73/PGI/2021 | 96.92 | Rhinovirus C3 strain RvC3/USA/2021/2DEQES | Rhinovirus C3 | OK161367.1 |
| HRV 85/PGI/2021 | 96.28 | Rhinovirus A isolate S0937XLQ polyprotein gene | Rhinovirus A | MW587089.1 |
| HRV 87/PGI/2021 | 98.22 | Rhinovirus C strain CLI-B3-21-Rhino C polyprotein gene | Rhinovirus C | MT641410.1 |
| HRV 92/PGI/2021 | 98.67 | Rhinovirus C4 strain RvC42/USA/2021/BZ2L22 | Rhinovirus C42 | MZ460686.1 |
| HRV 96/PGI/2021 | 98.48 | Rhinovirus A1 strain RvA1/USA/2021/5Q6L7V | Rhinovirus A1 | OL133729.1 |
| HRV 99/PGI/2021 | 97.76 | Rhinovirus A2 strain 12L5 | Rhinovirus A2 | MN749152.1 |
| HRV 101/PGI/2021 | 97.58 | Rhinovirus C3 strain RvC3/USA/2021/2DEQES | Rhinovirus C3 | OK161367.1 |
| HRV 102/PGI/2021 | 96.68 | Human rhinovirus C36 strain HRV-C36QRS_092_2001 VP4VP2 gene | Rhinovirus C36 | KF688657.1 |
| HRV 107/PGI/2021 | 94.8 | Rhinovirus C isolate RV-C45-cpzl-2013 polyprotein gene | Rhinovirus C45 | KY624849.1 |
| HRV 109/PGI/2021 | 98.77 | Rhinovirus A31 strain RvA31/USA/2021/4XWP9R | Rhinovirus A31 | MZ322911.1 |

Table 4. Odd ratios (ORs) and 95% confidence intervals (CIs) for risk of SARI associated with HRV infection.

| Subgroup | Outcome | OR | 95% CI |
|-------------------------|-------------|------|-------------|
| All children <5 years | SARI | 0.99 | 0.51 – 1.94 |
| Age >1 month to 1 year | SARI vs ILI | 0.14 | 0.04 – 0.47 |
| Gender (male vs female) | SARI | 1.40 | 0.67 – 2.92 |

cause of ARTI / LRTI in children highlight the need to include HRV in future viral panels. Multi-country studies have reported HRV as a common virus in children with ILI or LRTI: Taylor et al. (41.5%, 6 months to <10 years) (19), Xiao et al. (23%) (20), Guerrier et al. (34%) (21), and Fujitsuka et al. (22) found HRV (31.3%) alongside RSV (40.9%) as major contributors. Variations in HRV prevalence between studies may result from differences in age groups, geographical location, climate, disease severity, co-morbidities such as chronic diseases or malnutrition, and adherence to preventive measures, including hand hygiene and social distancing.

Our study was conducted from October to December 2020, coinciding with fall and early winter. The severity of HRV illness may vary with the seasonal preponderance of HRV-C during winter and spring. Yan et al. (23) reported HRV infections year-round in their hospitalized children (<14 years of age), peaking in summer and fall, while Lau et al. (24) found HRV-C (<18 years of age) most prevalent in fall

and winter, often associated with febrile wheezing or asthma (76% cases). Since our study was a single-season design, seasonal variation, incidence, and severity could not be fully assessed.

In our study, 56.49% had ILI and 43.5% had SARI, whereas Mishra et al. (12) reported LRTI in 58% and URTI in 42%. Lower SARI incidence in neonates may reflect age-related differences, with most HRV-positive cases occurring in infants >1 month to 1 year. Yan et al. (23), Daleno et al. (25), and Louie et al. (26) reported higher HRV-related LRTI in children >1 year, likely due to inclusion of older age groups (up to 14 years).

Our findings suggest that in infants aged 1 month to 1 year, HRV positivity is associated with decreased ILI risk (OR = 0.14, 95% CI: 0.04-0.47), implying a higher risk of SARI. Such findings were not seen with the sub-group population of <1 month or 1 year to 5 years. The lower risk of SARI in HRV-positive neonates (<1 month) may be due to protective maternal IgG antibodies passed from mothers previously exposed to HRV (12). Baillie et al. (27) reported HRV prevalence of 13%-59% in pediatric LRTI, but its frequent detection in asymptomatic individuals (6%-50%) makes causation uncertain. No gender predisposition was observed for the SARI risk in HRV-diagnosed children.

Phylogenetic analysis of our 13 representative samples revealed HRV-A in two SARI and three ILI cases, HRV-B in one SARI case, and HRV-C in

two SARI and five ILI cases. Previous studies associate pediatric CAP with HRV-A (A12, A78) and HRV-C, with HRV-B less commonly implicated (25, 28). HRV-C has been frequently linked to acute asthma exacerbations and bronchiolitis (24, 29-31), although Jin et al. (32) reported lower hospitalization rates with HRV-C compared to HRV-A/B in the ≤ 14 years of pediatric population. The limited sample size for phylogenetic analysis precludes definitive conclusions on genotype virulence, highlighting the need for larger molecular studies to establish the relationship of different genotypes with clinical severity.

Among the 154 patients, 28 (18%) were born prematurely with low birth weight. Of these, 16 (57%) developed SARI, and 10 (35%) tested positive for HRV. This is consistent with finding of Piggelen et al. (33) who reported HRV in 82% of preterm infants (9/11). In contrast, among 17 patients with congenital heart disease, HRV was detected in only two cases. Ozdemir Sahan et al. (34) reported 24% cyanotic and 76% acyanotic congenital heart diseases among children with lower respiratory tract infections. HRV co-infections with other viruses have been reported in various studies. Fujitsuka et al. (22) reported 12.2% co-infection with RSV, and a South African study reported HRV co-infection in 37.3% H1N1 and in 40.4% H3N2 patients (35). In Cambodia, HRV and RSV alone contributed to 34% of ALRI infections, with co-infections in 6% (21). In our study, co-infections were not evaluated, representing a limitation, as they may influence disease severity.

CONCLUSION

Overall, HRV remains a prevalent cause of ARTI/LRTI in children < 5 years, and the incidence varies by age, season, geography, and population characteristics. Infants aged 1 month to 1 year appear particularly vulnerable to severe HRV infection. HRV-C predominates among genotypes, frequently associated with wheezing and asthma exacerbations. Year-round molecular surveillance with follow-up studies, including different age groups of children from diverse geographical and climatic conditions, with and without co-morbidities, is warranted to understand HRV epidemiology, genotype-related virulence, and clinical outcomes in pediatric ARTI.

ACKNOWLEDGEMENTS

The authors are grateful to the DHR/ICMR-funded Regional VRDL, Department of Virology, PGIMER, Chandigarh, for providing instruments and reagents support for carrying out this study.

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