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# Evaluation Of Real-Time PCR For Early Diagnosis of Acute Respiratory Infections Among Pediatric Population at A Tertiary Care Setting

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## ABSTRACT

Respiratory infections are triggered by a variety of viruses, and there is a scarcity of information regarding viral coexistence, comparative symptoms, and overall burden of illness. This observational study was conducted to identify the etiological agents responsible for acute respiratory tract infections (ARI) in children under five years of age using the TRUPCR multiplex real-time respiratory pathogen panel. Samples were collected from pediatric patients with respiratory infections admitted to the Department of Pediatrics at Bundelkhand Medical College in Sagar, Madhya Pradesh, between June 2023 and January 2025. In total, 417 samples were subjected to real-time PCR analysis. RT-PCR detected 332 of 417 positive samples, resulting in an overall positivity rate of 79.6%. Family history of similar illnesses, breastfeeding, and nutritional status were significantly associated with acute respiratory infections.The highest prevalence of ARI (51.55%) was observed in children aged 1–12 months. The most common respiratory infections were noted in the 1 to 5 year age group (44.7%). The human respiratory syncytial virus (hRSV) was the most frequently identified virus (35.76%), followed by human rhinovirus (hRV) (27.81%), human adenovirus (hAdV) (6.29%), and influenza B virus (Inf B) (3.6%). No significant differences were noted in clinical presentation; however, fever, cough, breathlessness, and stridor were significantly associated with viral respiratory infections. These results enhance our understanding of viral etiology and distribution, which can improve the management and treatment of respiratory infections. Nevertheless, for a more comprehensive clinicopathological correlation, further studies should include a complete evaluation of all etiological agents, the clinical profiles of patients, and treatment outcomes. Therefore, multiplex real-time PCR may play a crucial role in the rapid and precise diagnosis of respiratory viruses, potentially improving patient outcome.

## 1. INTRODUCTION

Acute respiratory infections (ARIs) have a profound effect on children's health globally. Despite regional and seasonal variations in the pathogens that cause ARIs, viruses remain the predominant cause of ARIs on a global scale [1,2,3]. A recent systematic review identified the most prevalent respiratory viruses responsible for acute lower respiratory tract infections (ALRI) in children under five years of age, including human respiratory syncytial virus (hRSV), influenza virus (InfV), human parainfluenza virus (hPIV), human metapneumovirus (hMPV), and rhinovirus (RV) [1]. Additionally, 10–50% of children with ARI develop secondary bacterial infections such as acute otitis media, sinusitis, or pneumonia [4]. Viruses are the leading pathogens linked to severe respiratory illnesses, including bronchiolitis, asthma exacerbations, and pneumonia in early childhood, and are a major cause of hospitalization in children under two [5,6,7]. While the viral causes of ARIs and their impact on healthcare are well-researched in developed countries, there is a lack of information in developing countries, including India [8]. From a public health perspective, it is crucial to identify the most common viral agents causing ARIs, their symptoms, the frequency of severe cases, and methods to

prevent severe ARIs. This study aimed to explore the viral spectrum and patterns of lower ARIs in children under five years

of age in central India.

The diagnosis of viral respiratory infections has been based on the use of conventional methods, such as isolation by cell culture and antigen detection. Although these methods are effective and often complementary, they have certain limitations. Cell culture is considered to be the "gold standard" for virus detection, but it is too laborious and time consuming. Antigen detection is not sufficiently sensitive and/or specific [9].

The accuracy and reliability of respiratory virus detection have significantly improved with the introduction of nucleic acid amplification tests (NATs) [10,11,12,13,14]. For a swift response to potential outbreaks, it is crucial to have diagnostic methods that are not only fast and precise, but also capable of simultaneously detecting and sub typing viruses. The emergence of real-time PCR and associated genome amplification methods as precise and flexible diagnostic tools has revolutionized our understanding of the causes and clinical manifestations of viral respiratory tract infections (RTIs) [15].

However, virus-specific polymerase chain reaction (PCR) assays are expensive. Several research groups have developed multiplex RT-PCRs to identify respiratory viruses in clinical samples [10,16,18,19,20,21,22,23,24,25]. These diagnostic tests are affordable, swift, sensitive, and can simultaneously assess respiratory samples for 15 or more pathogens [26]. This advancement has encouraged clinicians to submit more samples for virus detection than previously anticipated. Important insights gained from recent years of structured molecular diagnosis of respiratory viruses have revealed that determining an etiologic diagnosis based solely on clinical observations is more complex than initially thought, even for experienced clinicians, and that positive samples frequently contain multiple respiratory viruses [27]. This implies that several viruses may coexist and replicate in the respiratory tract, potentially leading to various symptoms [28] . The choice between different tests is influenced by their specificity, sensitivity, turnaround time, and cost, particularly in resource-limited settings. This study aimed to detect circulating respiratory viruses in pediatric patients < 5 years of age using qrt-pcr and to investigate the clinical conditions associated with ARTI

## 2. MATERIALS & METHODS

### Study site and study population

This prospective observational study was conducted over a period of 16 months from August 2023 to January 2024 at the Virology Lab, Department of Microbiology, Bundelkhand Medical College Sagar, which is the largest tertiary care hospital in the Sagar Division in the state of Madhya Pradesh. A total of 444 subjects below 5 years of age presented with acute respiratory Illness (ARI) and were admitted to the inpatient ward (IPD) While, patients with severe acute respiratory infection (SARI) were admitted from the emergency room, pediatric intensive care unit (PICU) and high-dependency unit (HDU) ward, Department of Pediatrics Bundelkhand Medical College Sagar. Among these individuals, 27 samples were rejected because of not fulfilling inclusion criteria and inappropriate sample handling. Moreover, real-time PCR analysis was done for the remaining 417 samples. The study protocol was approved by the Institutional Ethics Committee of the Bundelkhand Medical College located in Sagar M.P. (EC ID: IECBMC/EC/2023/125). As the participants were children under the age of five years, their parents were informed about the study in their native language and provided with a patient information sheet in the same language. Prior to enrollment of the children in the study, written consent was obtained from their parents, ensuring adherence to ethical standards and safeguarding the rights of the participants. All identifiers were anonymized for analysis and the samples were collected by qualified medical professionals.

### Inclusion and Exclusion criteria

Study subjects were recruited within the age group of 1-60 months, parents who provided informed consent for study participation. The study excluded any prior instances of respiratory infections to avoid the identification of residual nucleic acid. Children aged > 60 months with a history of respiratory infections or other chronic conditions, including HIV, Tuberculosis, Cardiac failure, primary cardiac failure, severe metabolic acidosis without signs of respiratory tract infection (RTI), empyema, hydropneumothorax, or tuberculosis, non-respiratory causes of respiratory distress, underlying chronic conditions, hospitalization exceeding 7 days, and lack of written consent from parents were excluded from the study.

### Sample collection:

All swabs were collected in compliance with WHO guidelines using a Plastic-shaft, rayon-budded swab in a transport tube with a foam pad reservoir soaked with a viral transport medium HiViral Transport Kit (HiMedia, Cat. No: MS2760A-50NO) the skilled clinical staff obtained nasal and or throat respiratory specimens from enrolled ARTI cases after that these samples were delivered with the cold chain intact to the VRDL lab at Department of Microbiology to maintain their integrity and stored at -80°C until further analysis.VTM samples were batch-tested for 18 viruses and 14 bacteria using validated real-time PCR assays.

### Case Defination of ARI, Case Enrollment and Data Collection

The World Health Organization (WHO) classification of acute respiratory tract infection (ARTI) in children presenting with cough, difficult breathing, or both is as follows: pneumonia, respiratory rate per minute > 50 breaths (2-11 months of age) 0r > 40 breaths (12-59 months of age) ; no lower chest in drawing; severe pneumonia - symptoms of pneumonia, and lower chest in drawing with or without rapid breathing; very severe disease- symptoms of severe pneumonia, inability to drink, convulsions, central cyanosis, abnormal sleepiness or difficulty to wake, stridor in calm child, or clinically severe malnutrition (29)

**Extraction and purification of Viral nucleic acid.**

Viral Nucleic acids (DNA/RNA) were extracted and purified from 200µl of VTM sample collected viral RNA using a DNA Nucleospin Virus kit (MACHEREY-NAGEL GmbH & Co, Germany). Briefly, 5 µL of liquid proteinase K was added to 200 µL of sample and mixed moderately, and then 200 µL of VL Buffer was added to the 1.5 ml micro centrifuge tubes and vortexed for 15 s. 5.6 µl of Carrier RNA was added to the mixture and vortexed for 15 s. The suspension was incubated at room temperature (15-25oC) for 3 min, followed by brief centrifugation to remove droplets from the inner side of the lid. Absolute ethanol (200 µL) was added to the sample and mixed by vortexing for 15 s, followed by brief centrifugation of the tube to remove droplets from the inner side of the lid. Carefully 610µl of the solution was applied to the nucleospin virus column with 2 ml of collection tube without wetting the rim. The cap was closed and centrifuged at 4000 g for 3 min. The Nucleospin virus column was placed into a fresh 2 ml collection tube, and the tube containing the filtrate was discarded. The Nucleospin virus column was opened carefully and 400µl of VW1 Buffer was added to the Nucleospin virus column and centrifuged at 11000 g for 30 s. This was followed by replacement with a fresh 2 ml collection tube, and the tube containing the filtrate was discarded. 400µl of the VW2 Buffer was added to the column and centrifuged at 11000 g for 30 s. This was followed by replacement with a fresh 2 ml collection tube, the tube containing the filtrate was discarded, the Nucleospin virus column was opened carefully and 400µl of VW2 Buffer was added to the Nucleospin virus column and centrifuged at 20000 g for 5 min. Finally, the Nucleospin virus column was placed in a sterile, DNAase, RNAase, free 1.5 ml microcentrifuge tube. The old collecting tube containing the filtrate was discarded and replaced with fresh 1.5 ml MCT. 30µl of RNase-free water was added to the nucleospin virus column and equilibrated to room temperature for 3 min, followed by centrifugation at 20,000 × g for 3 min. The eluate which was collected into the MCT consisted of DNA/RNA and was stored at -80°C until further use.

**Multiplex Real Time PCR for Respiratory Pathogens**

The purified and extracted nucleic acid were amplified using the TRUPCR Respiratory Pathogen Panel kit (3B BlackBio Biotech India Ltd., Cat. No. version 1.0) on the CFX 96 Biorad Real time PCR for The detection of 18 respiratory viruses and 14 respiratory bacterial pathogens was achieved through real-time PCR, which included Human Parechovirus, Human coronavirus (both alpha and beta strains), Human Parainfluenza viruses 1, 2, 3, and 4, (hPIV-1,23,4) Influenza A virus (Inf A), Enterovirus, Influenza A (H3N2), Human Metapneumovirus (A/B) (hmPV), Pandemic H1N1 Influenza virus (InfV), Influenza B virus (Inf B), Influenza C virus (Inf C), Human Adenovirus (hAdv), Human Respiratory Syncytial Virus (A/B) (hRSV), Human Rhinovirus (Hrv), and Human Bocavirus (hBoV), *Staphylococcus aureus*, *Streptococcus pneumonia*, *Klebsiella pneumonia*, *Mycoplasma pneumonia*, *Salmonella spp.*, *Streptococcus pyogenes*, *Bordetella spp.*, *Chlamydia pneumonia*, *Streptococcus agalactiae*, *Acinobacter baumannii*, *Psudomonas aeroginosa*, *Legionella pneumophila*, *Haemophilus influenza (A-F)*, *Morexella catarrhalis* using the TRUPCR Respiratory Pathogen Panel kit (3B BlackBio Biotech India Ltd., Cat. No. version 1.0). The assay incorporated RNase P as an endogenous internal control.

The reaction mixture comprised 9.65 µl of primer-probe mix, 10 µL of master mix buffer, 0.35 µl of SuperScript III enzyme, and 5 µL of nucleic acid templates, culminating in a total volume of 25 µL for each PCR reaction. Thermal cycling was performed under the following conditions: reverse transcription at 50 °C for 20 min, initial denaturation at 94 °C for 10 min, followed by 40 cycles consisting of two stages (10 s at 94 °C and 60 s at 60 °C, with data acquisition) on a Quantstudio 5 Thermo Scientific. For the detection of SARS-CoV-2, the Viral Detect –II Multiplex Real-Time PCR kit for COVID-19 (GENES2ME, India) was used. Specifically, 9 µl of RNA was combined with 11 µl of reaction mix according to the kit's protocol. Real-time PCR was performed on the Quantstudio 5 Thermo Scientific, adhering to the recommended cycling conditions, with a cycle threshold (Ct) value of 35 used for the interpretation of results.

**Quality Control**

endogenous internal control gene (RNase p gene) included in the assay to verify the quality of the extracted nucleic acid, amplification procedure, and possible presence of inhibitors, which may cause false-negative results. In addition, Ct values were used as semi quantitative markers of viral load as in real-time PCR assays they are inversely proportional to the amount of specific virus nucleic acid present in the specimen.

| Step | Time | Temperature (°C) | Cycles | Fluorescence Acquisition |
|---|---|---|---|---|
| Initial Step | 5 min | 37 | 1 | |
| Reverse Transcription | 20 min | 50 | 1 | |
| Taq Polymerase Activation | 10 min | 94 | 1 | |
| Amplification – Denaturation | 10 sec | 94 | 40 | |
| Amplification – Annealing | 60 sec | 60 | 40 | Yes |

**Table 1Thermal Profile for Multiplex Real-Time PCR – Respiratory Pathogens**

| **Step** | **Time** | **Temperature (°C)** | **Cycles** | **Fluorescence Acquisition** |
|---|---|---|---|---|
| **Reverse Transcription** | 10 min | 55 | 1 | |
| **Taq Polymerase Activation** | 03 min | 95 | 1 | |
| **Amplification – Denaturation** | 15 sec | 95 | 40 | |
| **Amplification – Annealing** | 60 sec | 60 | 40 | Yes |

**Table 2 Thermal Profile for SARS-Cov-2 real-time PCR**

**Data Analysis**

The data that was obtained from the patients was analysed with the help of using Epi-info 7.2. Different types of statistical tests including the Chi-square test or Fisher's exact test were used for categorical data. Descriptive statistics, including percentages, means, and standard deviations, were computed for continuous data. Inferential statistical tests, such as the Chi-squared test and Fisher's exact test, were used to examine factors associated with ARI in children aged under five. A p-value of less than 0.05 was regarded as statistically significant. The findings were conveyed through relevant tables and graphs.

| **Aetiological Agent** | **Reporter Dye** |
|---|---|
| Staphylococcus aureus | FAM |
| Streptococcus pneumoniae | HEX |
| Klebsiella pneumonae | TEXAS RED |
| Mycoplasma pneumoniae | CY5 |
| Salmonella spp. | FAM |
| Streptococcus pyogenes | HEX |
| Bordetella spp | TEXAS RED |
| Chlamydia pneumoniae | CY5 |

| | |
|---|---|
| Streptococcus agalactiae | FAM |
| Acinetobacter baumannii | HEX |
| Pseudomonas aeroginosa | TEAS RED |
| Legionella pneumophila | CY5 |
| Haemophilus influenza (A-F) | FAM |
| Moraxella catarrhalis | HEX |
| Human parechovirus | TEXAS RED |
| Human corona virus (alpha & beta) | CY5 |
| Human parainfluenza virus 1 | FAM |
| Human parainfluenza virus 2 | HEX |
| Human parainfluenza virus 3 | TEXAS RED |
| Human parainfluenza virus 4 | CY5 |
| Influenza A virus | FAM |
| Enterovirus | HEX |
| Influenza A (H3N2) virus | TEXAS RED |
| Human metaneumovirus (A/B) | CY5 |
| Pandemic H1N1 influenza virus (pdm H1N1) | FAM |
| RnaseP gene | HEX |
| Influenza B virus | TEXAS RED |
| Influenza C virus | CY5 |
| Human adenovirus | FAM |
| Human respiratory syncitial virus (A/B) | HEX |
| Human Rhinovirus | TEXAS RED |
| Human bocavirus | CY5 |

**Table 3: Common Aetiological agents of RTIs and their corresponding probe targets identified by multiplex Real time PCR.**

## 3. RESULTS

Among 417 samples tested for real-time PCR 287 (68.82%) were identified as male and 130 (31.17%) as female. The ages of the patients ranged from 1month to 5 years, with the largest group (51.55%) being aged between 1 to 12 months. The socio-demographic and clinical profiles of the ARI (n = 417) patients are outlined in Table 3.

Analysis of the socioeconomic characteristics of the participants indicated that younger children, specifically those aged 1 - 12 months, had a notably high prevalence of Acute Respiratory Infections (ARTI) at 51.55%. In terms of maternal age, it was found that mothers younger than 25 years and those who were unemployed had higher ARI rates of 72% and 70%, respectively. The study's results also demonstrated that ARTI rates were similar and comparable across the three socioeconomic classes. Moreover, an increase in household size, particularly with more than 10 family members, corresponded with a rise in ARI prevalence to 90.9% (Table 3).

Children born with an extremely low birth weight exhibited a higher prevalence of ARI at 83.3%, while those with a birth

weight exceeding 4 kg had a prevalence of 72.7%. Among children with a birth spacing of 1–2 years, 80.6% experienced ARI. The likelihood of developing ARI was increased for second-born children, with a prevalence of 72.5%. Additionally, families with more than two children under five years old showed a prevalence of ARI at 77.4%. A comparable illness present within the family or community alongside viral positivity was statistically significant ($p < 0.05$) as indicated in Table 3.

| | | ARI condition | | |
|---|---|---|---|---|
| Variables | | present | Absent | p-value |
| Study cohort, n=417 (100 %) | - | 405 | 12 | - |
| Age (Years) | 1-12 | 204 (34) | 23 (11) | 0.345 |
| | 13-24 | 115 (84) | 21 (16) | |
| | 25-36 | 48 (82) | 10 (18) | |
| | > 36 | 7 (87) | 1 (15) | |
| Gender | Male | 206 (72) | 80 (28) | 0.482 |
| | Female | 98 (76) | 31 (24) | |
| Mothers age | ≤ 25 years | 157 (72) | 62 (28) | 0.246 |
| | 26-30 years | 97 (67) | 47 (33) | |
| | > 30 years | 32 (58) | 24 (42) | |
| Mothers education | Illiterate | 16 (48.4) | 13 (39.3) | 0.334 |
| | Literate | 265 (68.29) | 123 (31.70) | |
| Mothers occupation | Unemployed | 208 (70) | 88 (30) | 0.168 |
| | Employed | 63 (52) | 58 (47) | |
| Socioeconomic status | Lower class | 68 (65.38) | 36 (34.61) | 0.762 |
| | Middle class | 147 (63.36) | 67 (28.87) | |
| | Upper class | 54 (66.6) | 27 (33.3) | |
| Type of Family | Joint family | 287 (74.2) | 100 (25.83) | 0.579 |
| | Nuclear Family | 21 (72.6) | 9 (30) | |
| Birth space between children | < 1 | 12 (60) | 8 (40) | 0.408 |
| | 1-2 yrs | 27 (71) | 11 (29) | |
| | > 2 years | 81 (72) | 32 (28) | |
| | None | 163 (67) | 83 (33) | |
| Siblings | Present | 159 (75.71) | 51 (24.28) | 0.079 |
| | Absent | 129 (61.42) | 78 (37.14) | |
| Number of under five children | 1 | 14 (73) | 5 (26) | **0.050** |
| | 2 | 244 (90) | 28 (10) | |
| | > 2 | 98 (78) | 28 (22) | |
| History of smoking | Present | 66 (67) | 32 (33) | 0.548 |

| | | | | |
|---|---|---|---|---|
| in household | Absent | 203 (64) | 114 (317) | |
| Breastfeeding | Yes | 210 (67) | 102 (32) | **<0.05** |
| | No | 44 (42) | 61 (58) | |
| Nutritional status | Under Nourished | 12 (41) | 17 (58) | **<0.05** |
| | Normal | 323 (83) | 65 (17) | |
| Birth weight | ≥ 2.5 | 215 (68) | 102 (32) | 0.865 |
| | ≤ 2.5 | 64 (70) | 28 (30) | |
| | < 2 | 7 (88) | 1 (12) | |
| Family history of similar illness | Present | 140 (63) | 79 (36) | **<0.05** |
| | Absent | 23 (12) | 175 (88) | |

*Chi-square, *Fischer's exact test

**Table 4 Socio-demographic characteristics associated with ARI**

| **Clinical Features** | **Total Cases (%) n=417** | **Total Positives (%) n=332** | ***p* value** |
|---|---|---|---|
| Fever | 386 (92.6) | 308 (92.77) | **0.010** |
| Cough | 414 (99.5) | 329 (99.09) | **< 0.05** |
| Breathlessness | 381 (91.6) | 307 (92.46) | **0.020** |
| Stridor | 336 (80.6) | 308 (92.77) | 0.241 |
| Nasal Congestion | 241 (5.1) | 269 (81.02) | **0.001** |
| Diarrhoea | 55 (13.2) | 195 (58.73) | 0.342 |
| Earache | 5 (1.2) | 5 (1.5) | 0.641 |
| Rash | 17 (4.2) | 6 (1.8) | 0.343 |
| Tachypnea | 292 (70.2) | 237 (71.38) | 0.211 |
| Wheeze | 9 (2.2) | 9 (2.71) | 0.473 |
| Respiratory Distress | 311 (74.6) | 252 (75.90) | 0.322 |
| Lethargy | 303 (72.8) | 246 (74.09) | 0.510 |
| Sore Throat | 43 (10.5) | 242 (72.89) | 0.147 |
| Rhinorrhoea | 352 (84.6) | 258 (77.7) | 0.818 |
| Vomitting | 127 (30.6) | 134 (40.36) | 0.319 |
| Seizures | 8 (2.1) | 24 (7.22) | 0.122 |
| Nasal Flaring | 211 (50.6) | 250 (75.30) | 0.234 |
| Chills/Rigors | 27 (6.6) | 73 (21.98) | 0.450 |
| Nasal Discharge | 206 (49.6) | 201 (60.54) | 0.393 |

**Table 5: Clinical characteristics of the study population**

**Viral Etiologies in ARI**

Real-time PCR identified 332 positive samples, representing 79.6% of the total. The most frequently detected pathogen was the HRSV influenza virus, found in 108 out of 417 cases. The qRT-PCR method detected single as well as coinfections. The most commonly identified viruses were hRSV (35.76%), HRV (27.81%), hAdV (6.29%), and Inf B (3.6%), which together made up one-third of the positive results. The median age of hRSV, hRV, H1N1, H3N2 and hAdV were 12, 14, 15, 20 and 26 months respectively. The bacteria identified included *Streptococcus pneumoniae* [n = 8 (32%)], *Klebsiella pneumonia* [n= 6 (24%)] *Staphylococcus aureus* [n = 3 (12%)], and *Haemophilus influenzae* [n = 2 (8%)], *Pseudomonas aeroginosa* 3 (12%) *Acenitobacter baumanii* 3 (12%), *Haemofilus influenza* 2 (8%) (Table I). 225 (53.95%) samples showed the presence of single virus, 49 samples (11.7%) showed the presence of more than one virus, Whereas, 28 (6.71%) samples were positive for virus and bacterial co-infections. all in children under two, with RSV accounting for half of these cases. There was one instance of co-detection involving three respiratory viruses. Bacterial pathogens were found in 27 co-detection cases, with at least one infectious agent from either the viral or bacterial groups.

The diagnosis of viral respiratory tract infections in the lab is typically achieved using traditional methods like culture or antigen detection tests. These methods have limitations, such as delays in obtaining results and the need for monoclonal antibodies for newly discovered viruses [30]. Multiplex PCR assays have emerged as a crucial tool for identifying the causative agents of ARIs [31]. The updated version of the multiplex real-time PCR assay used in this study has expanded its pathogen detection capability to include 18 viral and 14 bacterial species simultaneously, thereby enhancing its diagnostic potential.

**Clinical Features of Acute Respiratory Infection Cases Compared Across Various Respiratory Viral Pathogens**

The results indicate that the majority of acute respiratory infection (ARTI) symptoms reported by participants were cough, fever and breathlessness. Majority of symptoms, including cough, fever and breathlessness and Nasal congestion, showed a statistically significant correlation (Table 4). However, symptoms including stridor, diarrhea, earache, wheeze, Rhinorrhea and vomiting are not statistically significant. In patients diagnosed with ARTI, those who tested positive for RSV positive cases exhibited fever (100%), cough (100%), and sore throat (95.6%) as the most common symptoms, with nasal discharge reported in 48.5% of cases. Some patients also presented with breathlessness (30.3%), and vomiting (8.5%). For patients positive for human HRV, common symptoms include fever, cough, sore throat, and breathlessness, with tachypnea occurring in 47.2% and nasal flaring in 42% of cases. Similarly, in cases positive for InfA (H3N2) fever and sore throat was universally present, while cough was reported in 59.9% of cases with vomiting diarrhea and in 20.3% and 17.8% respectively . In cases positive for PIV-1, fever (100%), cough (96.8%), and sore throat (97.9%) were prevalent symptoms. However, nasal discharge (46.7%), breathlessness (23.1%), apnea (4.8%), and chills/rigors (2.8%) were observed in only a minority of cases. Symptoms such as vomiting, seizures, diarrhea, wheezing, and rash were absent in all PIV-1 positive cases. HAdV-positive cases, cough and fever were frequently reported, with nasal discharge in 37.7%, breathlessness in 28.9%, nasal flaring in 41.3%, and tachypnea in 10.7%. In the study of PIV-3 positive cases, it was observed that fever and cough were common among all patients, with nasal discharge noted in 34.8%, breathlessness in 19.9%, nasal flaring in 34.3%, and tachypnea in 13.7%. In PIV-4 positive cases, the typical symptoms included sore throat, fever, and cough, while nasal discharge was present in 37%, chills/rigors in 17.7%, breathlessness in 47%, nasal flaring in 34%, and tachypnea in 14.7% (Table 5).

## 4. DISCUSSION

This study is primarily focused on pediatric population below 5 years reflecting the prevalence of ARIs in the community which is the leading cause of under-5 morbidity worldwide, with nearly 156 million new episodes annually [32]. Severe ALRI cases place a substantial burden on healthcare systems worldwide and are a major cause of pediatric referrals and hospital admissions [33] The pediatric age group is primarily affected by ARIs, which occur three to eight times annually in infants and young children, with incidence rates decreasing with age [34,35].

Although significant inter-regional variations are expected in a large country with diverse climates, these variations may also result from differences in study design. All viruses screened in this study were more frequently identified in children, except for influenza viruses, which were more commonly found in adults (8.6%) than in pediatric patients (6.8%), as documented in a recent study [36]. The second most prevalent viral agent was HRV (8%): 77% of HRV-positive samples were identified in children less than two years of age. This finding underscores the significance of this virus in infants, as hRV has been linked to recurrent respiratory illnesses and wheezing in this age group [37]. Other picorna viruses may cause ARIs; however, hRV is the primary virus detected in common cold cases [38]. In a multicenter study, rhinoviruses were frequently associated with ARIs, even during peak influenza season [39]. In Brazil, according to Arruda et al. (1991), HRV was the most common viral agent (46%) detected in children with ARIs [40].

In India, acute respiratory tract infections (ARTI) constitute 69% of all communicable diseases, with severe acute respiratory infection (SARI) being a significant cause of mortality in children under five years of age [41]. There is a

scarcity of studies focusing on pediatric RTI in India, particularly in terms of prevalence, surveillance, disease burden, and diagnostics for viral respiratory illnesses. This research aims to examine the clinical and viral profiles of ARTI in children under five at a tertiary care center in central India. A statistically significant difference was noted in the number of male versus female participants in the study ($p < 0.05$), aligning with findings from earlier studies [42]. Our results indicate that in the study region, human respiratory syncytial virus (hRSV) and human rhinovirus (hRV) are the most frequently identified viral causes of RTI in children up to five years old. Other respiratory pathogens includimg Influenza A (H3N2), Human Adenovirus (hAdV), influenza B (Inf B), human parainfluenza virus type 1 (hPIV-1), human parainfluenza virus type 3 (hPIV-3), influenza A/H1N1pdm09, human parainfluenza virus type 4 (hPIV-4) and a few cases of human bocavirus (hBoV), , human metapneumovirus (hMPV),mono-infections, ranked by occurrence. A study involving a community-based cohort of rural children under ten years in northern India reported hRSV as the most commonly detected pathogen, followed by parainfluenza virus (hPIV), hMPV, and various influenza viruses [43]. A report from Bangalore India indicated that hRSV was the most frequently detected pathogen among hospitalized children under five diagnosed with RTI [44]. Furthermore, a study conducted in Rajasthan from 2012 to 2013 identified hMPV as the primary cause of SARI in children less than five years of age [45]. In eastern India, hRSV and influenza B viruses are reportedly the leading pathogens responsible for ARTI in children under five [42]. hRSV exhibits a higher prevalence in India compared to other respiratory pathogens. Currently, RV infections are being reported more frequently due to the availability of sensitive detection methods. Globally, RV is the most prevalent respiratory pathogen causing RTI, followed by RSV. In India, the prevalence of hMPV and Inf B is higher than that observed worldwide; while the prevalence of Inf A is lower [41].

This research utilizes a commercial multiplex real-time PCR assay which is capable of identifying 18 viral and 14 bacterial respiratory pathogens. The testing is conducted in a sequential manner through eight RT-PCR real-time assays, each targeting different pathogens. Each assay has the capacity to detect up to four targets. However, larger studies are necessary to gather additional data for the design of multiplexing panels aimed at diagnosing pediatric respiratory tract infections (RTI) in India. Our findings suggest that for children aged 5 years and younger, an initial multiplexing of RSV, RV, InfluA (H3N2), and HPIV1, followed by a panel including HAdV, InfB, HPIV3, Inf A(H1N1 pdm09), HPIV4, SARS-CoV-2, HPIV3, would enhance turnaround time (TAT) and reduce expenses. However, larger studies are necessary to gather additional data for the design of multiplexing panels aimed at diagnosing pediatric respiratory tract infections (ARTI) in India.

The incidence of viral acute lower respiratory tract infections (ALRIs) in infants in central India is not well understood, primarily due to the limited number of published studies. This communication employs cutting-edge technology for the identification of viral agents, aiming to address this knowledge deficiency. The research revealed that as many as 65% of samples from infants with respiratory infection symptoms tested positive for single or viral coinfections, with hRSV, hRV, hADV, Inf B (H3N2), hPIV-1, Had being the most commonly identified viruses. hRSV (A/B) was detected in 35.5% of the samples as mono-infection which is consistent with previously reported rates of 10–58% [16,46,47,48,49], . This indicates that maternal antibodies may not be sufficient to prevent hRSV infections in infants, leading to considerable morbidity. hRV, the second most frequently detected virus, was present in 25.7% of infants, falling within the 0.5–33% range reported by other studies [47,48,50,51]. Once thought to be primarily a cause of upper respiratory tract infections, hRV has now been recognized as a significant contributor to LRTIs in infants [48]. Human Adenovirus was found in 6.6% of virus-positive samples, traditionally regarded as a major cause of ALRI in older children and adults, but not in infants, suggesting a need for a reassessment of this understanding [52]. Human bocavirus was detected in 0.88% of infants with positive samples, frequently identified in this age group, although its role in disease causation remains uncertain. Longtin et al., reported that prevalence rates are higher in children under two years of age and decrease with age, indicating that antibody protection against hBoV is acquired early in life [53]. Furthermore, the study predominantly identified the Inf A (H3N2) in 8% of infants whereas novel Influenza A (H1N1) pdm 09 virus in 2.6 % of infants perhaps because of the predominance of H3N2 during seasonal outbreak in 2023. While H1N1 outbreaks occur more frequently than those of H3N2, recent outbreaks in countries such as the United States, Australia, and several European nations have been primarily caused by H3N2. Significantly, during the 2017–2018 flu seasons, H3N2 was the dominant flu virus strain in the United States, leading to a higher incidence of hospitalizations and fatalities compared to earlier seasons. [54]

The rate of *H. influenzae* type b (Hib) in developed countries has seen a decline, primarily due to the deployment of the Hib-containing pentavalent vaccine. However, the absence of any Hib positive case may be due to the universal immunization program from Mohfw across multiple Indian states (MohfW) [55]. Previous studies have indicated a co-infection rate of 2% to 82% [16,46,47,50,56]. In this study, multiple pathogens were identified in 23.1 % (77/322) of the positive samples, with HRSV being the most frequently detected virus among these. The incidence of co-infections in infants has been increasingly documented [56]. However, the clinical implications of these co-infections remain insufficiently understood. For viruses such as hAdV and hBoV, lymphoid tissues are known to act as reservoirs, which may lead to prolonged shedding in asymptomatic individuals and the transmission of these viruses to other susceptible individuals [57]. It is well established that certain viruses, including hCoV-NL63, hRV, and hBoV, can continue to shed for a considerable duration after the onset of symptoms [58]. Additionally, the duration of viral shedding may depend on

whether the virus was the sole pathogen or if it was present alongside other co-pathogens [50].

Likewise, the presence of pathogenic bacteria such as *M. pneumoniae* and Hib in nasal carriage may lead to misleading results from nasopharyngeal swabs. Nevertheless, hRV should not be overlooked as a persistent factor, as it has been linked to adverse outcomes, likely due to its capacity to impair the proliferation and self-repair of bronchial epithelial cells [59]. Therefore, any positive result should be interpreted with caution and aligned with the clinical symptoms of the patient.

The disparity in positivity rates observed between the previously mentioned studies focused on virus and bacteria detection and the current study may be attributed to several factors, including the varying age demographics of the patients, the types of samples collected, the diagnostic techniques employed, and significant inter-regional differences influenced by diverse climatic conditions. Furthermore, the relationship between the presence of risk factors or multiple pathogens and mortality remains undetermined due to the limited sample size available, which presents an opportunity for future investigation.

The study's strength lies in its application of a sensitive and innovative method for detecting viral agents. The preferred specimens for identifying respiratory viruses are nasopharyngeal aspirates (NPA) or nasal washes [60,61]. However, both methods present certain drawbacks, including the discomfort associated with the procedures, the necessity of a suction device for NPA, and the risks of aspiration and aerosol generation during nasal wash collection. Consequently, these techniques are not practical for widespread clinical application. In contrast, nasopharyngeal swabs offer a straightforward, painless alternative that can be performed in various settings without the need for specialized equipment. Furthermore, the newly developed flocked swabs utilized in this study are not only more sensitive but also less traumatic for pediatric patients, making them more acceptable to parents [56].

The lack of a comprehensive test to identify all etiological agents presents a significant challenge in diagnosing the causative agent in cases of lower respiratory tract infections (ALRI). Multiplex real-time PCR has made progress in overcoming this obstacle as it possesses the ability to lower the overall antibiotic usage and to refine the targeted application of antibiotics, which can subsequently assist in controlling the transmission of viruses in healthcare settings. Conducting a case-control study would be beneficial to ascertain the presence of these viruses and bacteria in infants who are asymptomatic. This could serve as a promising direction for future research. The study has confirmed that Multiplex real-time PCR can be effectively utilized for identifying viral agents in nasopharyngeal swabs. Furthermore, it has provided foundational data on the likely viruses detected in the nasopharynx of infants with clinical symptoms of ALRI.

Respiratory Syncytial Virus (hRSV) and other respiratory pathogens significantly contribute to acute lower respiratory infections (ALRI) in pediatric populations in India. ALRI is a leading cause of mortality among children under five in developing nations [1,62] with hRSV identified as a critical factor in pediatric ALRI cases [1,63]. Studies conducted in India have revealed a high incidence of ALRI among children under five, with male being 3.6 times more affected than female [64]. The peak incidence of ALRI typically occurs during the first year of life. In young infants, hRSV infections can lead to severe complications, including respiratory failure, extended hospital stays, and mortality rates comparable to those seen with seasonal influenza. Previous hospital-based research in India has reported hRSV prevalence rates ranging from 11.4% to 26.0% [16,46,63,65,66] although, the variability among these studies limits direct comparisons with current findings. Incidence rates of RSV-related ALRI have been documented between 2.4% and 21.2% in various countries [67]. Studies conducted in hospitals have established a notable correlation between male gender and the occurrence of hRSV-associated ALRI [68]. A recent meta-analysis by Nair et al. concluded that hRSV is the predominant cause of lower respiratory tract infections (LRTI) in children and a significant reason for hospital admissions, with 99.0% of hRSV-related fatalities occurring in resource-limited settings [69]. The findings revealed that south east asia and Africa including India, China, Pakistan, Indonesia and Nigeria collectively account for approximately 16 million hRSV infection cases, representing half of the global childhood mortality rate for children under five [70].

In India, the routine diagnosis of viral ALRI in laboratories is both unavailable and under-researched, even within tertiary care centers. The viral origins of ALRI frequently remain undetermined, leading to the empirical use of antibiotics in most cases. Although viral isolation and real-time PCR are acknowledged for their high sensitivity as the gold standard, these tests are seldom conducted in clinical diagnostic labs in low and middle Income Countries (LMICs) due to their significant cost [71]. The early identification of conditions allows for swift management and plays a crucial role in combating ALRIs. In India, the administration of antibiotics for pediatric ALRI is frequently observed, even when a viral etiology is suspected. A Cochrane review focused on the effectiveness of antibiotics in children under two years diagnosed with bronchiolitis found inadequate evidence to support their use [72]. Antibiotics may be appropriate only when there is clear, documented evidence of secondary bacterial infections [1]. Early diagnosis of viral respiratory infections could help curtail the excessive use of antibiotics.

Understanding epidemiology enhances awareness of pathogens, facilitates accurate diagnosis, and ensures timely management. Our findings indicate that around 16% of specimens were coinfected with multiple viruses. Nevertheless, no significant differences in clinical presentations or laboratory results were observed between individual viral infections and

coinfections; the clinical implications of coinfection remain unclear.

The prompt and precise identification of respiratory viruses is becoming highly crucial in clinical settings. The provision of rapid diagnostic tests is vital for enhancing the efforts of infection control teams to mitigate the spread of virulent or resistant pathogens within hospitals [73]. Nucleic acid amplification tests have emerged as the new benchmark for diagnosing respiratory viruses. Our research indicates a high detectability of PCR for these viruses, implying that PCR-based diagnostic methods could be effective for identifying a broad spectrum of respiratory viruses. Viral infections can be life-threatening, particularly for premature infants and those with congenital heart conditions [74]. In both adult and pediatric populations, the significant effects of respiratory viral infections in patients with hematologic malignancies, those undergoing hematopoietic stem cell transplants, and individuals with solid organ transplants have been acknowledged over the last ten years [74,75]. The use of multiplex real-time PCR for detecting respiratory viruses in high-risk populations has proven to be beneficial. Our findings demonstrate a high detectability of PCR for respiratory viruses, indicating that PCR-based diagnostic tools may assist in identifying a broader array of respiratory viruses.

An earlier research study in Taiwan indicated that hRSV was the leading pathogen, accounting for 41.7%, followed by hMPV at 27.1%, hBoV at 6.3%, and Entero Virus at 6.3% [76]. While hRSV remains the most frequently identified pathogen in young children around the world, the accompanying pathogens can differ [77,78,79,80].

An additional significant observation from this research is that *K. pneumoniae* (20/58, 34.48%), *P. aeruginosa* (7/58, 12.06%), *S.aureus* (9/58 15.51%) and *S. pneumoniae* (17/58, 29.31%) emerged as the predominant bacterial pathogens among patients with laboratory-confirmed viral-bacterial co-infection. It is noteworthy that all co-infections in this group were likely community-acquired, as samples were collected within 48 hours of hospital admission, making the prevalence of *P. aeruginosa* as a co-pathogen particularly unexpected. Historically, *P. aeruginosa* has been infrequently associated with community-acquired respiratory infections (0.8%–1.9%) [81,82,83]. Nevertheless, recent investigations have indicated a rising incidence of P. aeruginosa co-infection alongside influenza [84]. Noteworthy certain pathogens such as, human parechovirus, human coronavirus, InfC, Mycoplasma pneumonia, Chlamydia pneumonia were absent in either gender during the study.

## 5. CONCLUSION

The implementation of PCR has demonstrated a superior ability to detect respiratory viruses compared to conventional rapid antigen tests and viral cultures. More than fifty percent of respiratory specimens that tested negative in initial assessments were subsequently identified as positive through the PCR detection method. The further utilization of PCR presents substantial potential for prompt and accurate diagnosis, which will be beneficial in pediatric primary care settings.. Moreover, RSV and RV were identified as the primary pathogens in our pediatric respiratory samples; approximately 16% of the positive respiratory specimens were found to be co-infected with two or more viruses, although no significant differences in clinical presentations or laboratory findings were observed between single infections and co-infections. Additional studies are needed to explore the accuracy, feasibility, accessibility, and cost of PCR in detecting respiratory viruses, as well as to clarify the clinical relevance of co-infection.

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