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Original Research Article

Leveraging real-time PCR for early diagnosis of respiratory conditions in the Indian pediatric population

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ABSTRACT

Background: Respiratory tract infections (RTIs) caused by various viruses and microbial agents present clinical challenges due to their diverse presentations. This retrospective study aimed to identify epidemiological trends and clinical characteristics of RTIs in pediatric populations using molecular diagnosis.
Methods: The study cohort comprised 100 paediatric individuals, predominantly male (70%) compared to female (30%), distributed across different age brackets. Real Time PCR targeting 32 microbial pathogens includes bacteria (15) and viruses (17).
Results: The majority of participants were aged 1 to 12 months (38%), followed by 1 to 3 years (29%), and 3 to 10 years (25%), with the smallest group being infants under 1 month (8%). A total of 134 pathogens were detected in 100 patients, with a higher prevalence in males (80%) compared to females (20%). Human respiratory syncytial viruses A and B were the most common, with 17 cases in males and 5 in females. *Klebsiella pneumonia* followed, with 19 cases in males and 5 in females. Influenza A and B viruses were also notable, with 7 and 9 cases in males, and 4 and 7 cases in females, respectively. Other significant microbes included human parainfluenza virus 3, human adenovirus, and *Streptococcus pneumoniae*. Notably, SARS-CoV-2 and *Acinetobacter baumannii* were not detected in either gender during the study period.
Conclusions: Our study shows that nucleic acid-based tests, especially multiplex PCR, effectively detect respiratory viruses in pediatric RTI patients, reducing inappropriate antibacterial use and mortality/morbidity.

Keywords: Bacterial pathogens, Clinical patterns, Multiplex real-time PCR, Paediatric respiratory infections, Viral pathogens

INTRODUCTION

Respiratory viruses and microbial pathogens are widely distributed and result in a diverse range of clinical symptoms. Respiratory tract infections (RTIs) are highly prevalent, and identifying the causative pathogen is crucial for proper management.[1] Besides well-known respiratory viruses like respiratory syncytial virus (RSV) and influenza virus, human metapneumovirus (MPV) was discovered in 2001, followed by the identification of other respiratory viruses.[2,3] The disease burden of respiratory viruses is currently not fully understood. These viruses have been found in over two-thirds of children with radiographically confirmed community-acquired pneumonia (CAP). Similarly, in the United States, molecular diagnostics revealed viral infection in 43%-67% of pediatric CAP cases.[2] Respiratory viruses also play a significant role in adult pneumonia, being

detected in 15%-56% of adult CAP cases.[2,4] They are responsible for the majority of respiratory infectious diseases in both children and adults, leading to a substantial disease burden.[5,6] Additionally, identifying the causative viruses allows for accurate diagnosis of respiratory infections, prescription of specific antiviral agents against certain viruses (e.g., oseltamivir for influenza viruses), and improves prognostic evaluation Recognizing causative viruses can also inform appropriate infection control measures, potentially reducing unnecessary hospital stays and enabling the discontinuation of unnecessary antibiotics.[2,7-9] In summary, respiratory virus infections are common, and testing for respiratory pathogens can enhance understanding of their roles in respiratory diseases and contribute to improved clinical management.[10]

The timely and accurate diagnosis of viral infections can be challenging. Rapid antigen tests are used worldwide to detect influenza virus infection, but concerns exist regarding the sensitivity of currently available viral antigen tests.[11] Technological advancements have improved the sensitivity, accessibility, and utility of viral diagnostic tools.[12,13] Molecular assays have been developed and progressively multiplexed to diagnose numerous respiratory viruses in a single assay with excellent sensitivity and specificity.[14] The importance of molecular-based diagnostic modalities is increasing, with polymerase chain reaction (PCR) technology being increasingly utilized in clinics to rapidly diagnose respiratory infections[15]. This study aims to detect respiratory viruses in children using PCR and to compare the detection power of this technique against that of traditional antigen tests. The clinical conditions were also investigated.

METHODS

Study type

This study employed a descriptive approach to analyse respiratory pathogens in children presenting with respiratory symptoms suspected of viral or bacterial infections.

Study place

The study was conducted at Lifecell International Pvt Ltd., a clinical laboratory located in Chennai, India.

Study period

This study was conducted from August 2023 to February 2024.

Sampling technique/selection criteria of patients

Non-probability sampling: Children with respiratory symptoms and clinical suspicion of viral or bacterial infections are referred to Lifecell International Pvt Ltd. by pediatricians for routine diagnostic testing. This method suggests that participants are selected based on their availability and the clinical judgment of referring pediatricians, rather than through random selection from a larger population.

Table 1: The list of pathogens and their corresponding probe targets as identified by the multiplex real-time PCR method.

Detection	Reporter
Bacteria	
Acinetobacter baumannii	VIC
Escherichia coli	FAM
Haemophilus influenzae (AF)	FAM
Klebsiella pneumoniae	ROX
Moraxella catarrhalis	VIC
Pseudomonas aeruginosa	ROX
Staphylococcus aureus	FAM
Streptococcus agalactiae	FAM
Streptococcus pneumoniae	VIC
Bordetella spp.	ROX
Salmonella spp.	FAM
Streptococcus pyogenes	VIC
Atypical bacteria	
Chlamydia pneumoniae	CY5
Legionella pneumophila	CY5
Mycoplasma pneumoniae	CY5
Viruses	
Adenovirus (DNA)	FAM
Coronavirus (RNA)	CY5
Metapneumovirus (RNA	CY5
Rhinovirus/Enterovirus (RNA)	ROX
Pandemic H1N1 influenza virus (PDM H1N1) (RNA)	FAM
Influenza A (RNA)	FAM
Influenza A (H3N2) virus (RNA)	ROX
Influenza B (RNA)	ROX
Influenza C (RNA)	CY5
Parainfluenza virus 1(RNA)	FAM
Parainfluenza virus 2 (RNA)	VIC
Parainfluenza virus 3 (RNA)	ROX
Parainfluenza virus 4 (RNA)	CY5
Bocavirus (DNA)	CY5
Parechovirus (RNA)	ROX
Respiratory Syncytial Virus A/B (RNA)	VIC
SARS-CoV-2 (ORF1ab)	FAM

Procedure

Nasopharyngeal swabs were collected from each participant by pediatricians using sterile swabs and placed in 2.5 ml viral transport medium. Specimens were processed to extract nucleic acids using the Applied Biosystems MagMAX Viral/Pathogen II Nucleic Acid Extraction Kit and the Kingfisher Flex automation instrument.

Nucleic acid extraction and real-time PCR

Nucleic acids were extracted from nasopharyngeal swabs using the Applied Biosystems MagMAX Viral/Pathogen II Nucleic Acid Extraction Kit and the Kingfisher Flex automation instrument. Real-time PCR, based on TaqMan technology, was employed to detect 32 respiratory pathogens, including:

Bacteria: *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Mycoplasma pneumoniae*, *Salmonella* spp., *Streptococcus pyogenes*, *Bordetella* spp., *Chlamydia pneumoniae*, *Streptococcus agalactiae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Legionella pneumophila*, *Haemophilus influenzae* (AF), *Moraxella catarrhalis.*

Viruses: Human parechovirus, SARS-CoV-2, Human parainfluenza virus 1-4, Influenza A virus, Pandemic H1N1 influenza virus (PDM H1N1), Influenza A (H3N2) virus, Influenza B virus, Influenza C virus, Enterovirus, Human metapneumoviruses (A/B), Human adenovirus, Human respiratory syncytial viruses (A/B), Human rhinovirus, Human bocavirus (Table 1).

Ethical approval

This study was approved by the Institutional Review Board of Lifecell International Pvt Ltd., Chennai.

RESULTS

The study included a total of 100 paediatric participants, comprising 70 males and 30 females, distributed across various age groups (Table 2). Table 3 presents the age-wise distribution of participants.

Table 2: Presents the gender-wise distribution of participants.

Gender wise	Total
Male child	70
Female child	30
Total	100

Table 3: Presents the distribution of positive cases for a specific condition among different age groups and genders.

Age wise	Total	Male	Female
Below 1 month	8	7	1
Between 1 month-12 months	38	29	9
Between 1-3 years	29	18	11
Above 3- 10 years	25	16	9
Total	100	70	30

The majority of participants were in the age group of 1 month to 12 months (38%), followed by those between 1 and 3 years (29%), and above 3 to 10 years (25%). Infants below 1 month of age constituted the smallest proportion (8%) of the study population (Table 3).

Table 4: Presents the distribution of microbial pathogens detected among the study group.

Microbes	Male	Female
Influenza A virus	7	4
Influenza B virus	9	7
Influenza A(H1N1) virus	0	0
H3N2 virus	2	0
Influenza C virus	0	0
Human coronavirus alpha and beta (229E, NL63, HKU1, OC43)	1	0
SARS-CoV-2	0	0
Human parainfluenza virus 1	1	1
Human parainfluenza virus 2	1	0
Human parainfluenza virus 3	8	2
Human parainfluenza virus 4	1	0
Human metapneumo viruses A and B	4	1
Human bocavirus	2	3
Human respiratory syncytial viruses A and B	17	5
Human rhinovirus	7	2
Human parechovirus	1	1
Enterovirus	5	2
Human adenovirus	12	6
Mycoplasma pneumoniae	2	0
Staphylococcus aureus	2	2
Chlamydophila pneumoniae	0	0
***Haemophilus influenzae* (A-F)**	22	3
Streptococcus pneumoniae	10	6
Klebsiella pneumoniae	19	5
Acinetobacter baumannii	0	0
Escherichia coli	0	0
Moraxella catarrhalis	0	4
Pseudomonas aeruginosa	0	0
Streptococcus agalactiae	0	0
***Bordetella* spp.**	1	0
***Salmonella* spp.**	0	0
Streptococcus pyogenes	0	0
Chlamydia pneumoniae	0	0
Legionella pneumophila	0	0
Total	134	54

As multiple pathogens were identified in some samples, the total count of pathogens reached 188 despites only 100 samples being studied.

Among males, the highest number of positive cases was observed in the age group below 1 month, with 7 out of 7 cases (100%), followed by the age group between 1 month to 12 months, where 28 out of 29 total cases were positive (96%). Similarly, the age group between 1 and 3 years had 100% positivity with 18 positive cases out of

18 total cases. In older age groups, all males above 3-10 years of age tested positive (100%). For females, all age groups studied showed a 100% positive rate except for the 1-12 months age group, which had a 96% positive rate. The distribution of positive cases for a specific condition among different age groups and genders is detailed in Table 3.

In total, 134 pathogens were identified across 100 patients, exhibiting a predominant occurrence in males (80%) relative to females (20%). Among the diverse microbial entities identified, Human respiratory syncytial viruses A and B emerged as the most prevalent, constituting 17 cases in males and 5 in females. Following closely, *Klebsiella pneumoniae* ranked as the second most prevalent pathogen, with 19 cases detected in males and 5 in females. Noteworthy prevalence was also observed for Influenza A virus and Influenza B virus, with 7 and 9 cases respectively in males, and 4 and 7 cases in females. Additional notable pathogens included Human parainfluenza virus 3, Human adenovirus, and *Streptococcus pneumoniae*, each demonstrating considerable occurrence across both genders. It is noteworthy that certain pathogens, such as SARS-CoV-2 and *Acinetobacter baumannii*, were absent in either gender throughout the observation period. These findings underscore the diversity of microbial infections and underscore the significance of gender-sensitive analysis in elucidating their epidemiology and implications for public health. Table 4 presents a comprehensive summary of microbial infections discerned among male and female individuals.

Table 5: Presents the co-infection pathogens detected among the study group, indicating the presence of more than one pathogen in a single sample.

Coinfection - more than 2 pathogens detected	Total	Male	Female
Influenza A virus/human respiratory syncytial viruses A and B	5	4	1
Influenza B virus/human adenovirus/Human rhinovirus/*Haemophilus influenzae* (A-F)	2	0	2
Influenza B virus/*Haemophilus influenzae* (A-F)	4	3	1
Human parainfluenza virus 3/human rhinovirus/enterovirus	2	2	0
Human parainfluenza virus 3/*Haemophilus influenzae* (A-F)	5	5	0
Human parainfluenza virus 3/human adenovirus	2	2	0
Human parainfluenza virus 3/*Streptococcus pneumoniae*	2	1	1
Human metapneumo viruses A & B/*Haemophilus influenzae* (A-F)	2	2	0
Human metapneumo viruses A & B/human adenovirus	2	2	0
Human respiratory syncytial viruses A and B/*Staphylococcus aureus*	3	2	1
Human respiratory syncytial viruses A and B/*Klebsiella pneumoniae*	7	5	2
Enterovirus/*Klebsiella pneumoniae*	2	1	1
Total	38	29	9

Male samples exhibited a higher rate of co-infection compared to female samples.

Statistical analysis reveals that out of the total 38 cases, 29 (76.3%) were in males and 9 (23.7%) were in females. Among the various coinfection combinations, the most prevalent was Human respiratory syncytial viruses A and B coexisting with *Klebsiella pneumoniae*, with 7 cases recorded. This was followed by coinfections involving Human parainfluenza virus 3 and Haemophilus influenzae (A-F), as well as *Human metapneumo* viruses A & B and *Haemophilus influenzae* (A-F), each with 5 cases. Notably, coinfections involving Influenza B virus and Haemophilus influenzae (A-F) exhibited gender disparity, with 3 cases in males and 1 case in females. Additionally, coinfections involving *Enterovirus* and *Klebsiella pneumoniae* showed equal distribution between males and females, with 1 case each. These findings underscore the complexity and diversity of coinfections, highlighting the need for further research to understand their clinical implications and management strategies. The table 5 provides a detailed breakdown of coinfections involving more than two pathogens detected in individuals, categorized by pathogen combinations and gender.

DISCUSSION

In contemporary clinical environments, the swift and precise diagnosis of respiratory viruses has become paramount. Not only does this aid infection control teams in hospitals to mitigate the transmission of highly virulent or resistant pathogens, but it also ensures timely interventions for vulnerable populations such as premature infants and individuals with underlying health conditions.

Our study demonstrates that PCR significantly outperforms traditional diagnosis. Utilizing PCR-based viral detection allows physicians to make more informed decisions, reducing unnecessary antibiotic or antiviral usage. Accurate identification of specific viruses enables prompt antiviral treatments, such as oseltamivir for RSV infections, and appropriate antibiotic therapies, like cefotaxime for *Haemophilus influenzae*. Utilizing TaqMan probe assays covering a panel of 32 pathogens, we identified 188 microbial pathogens, including viruses,

bacteria, and atypical bacteria. This comprehensive approach facilitated enhanced pathogen detection

Our research found RSV and *Haemophilus influenzae* to be the most prevalent pathogens among pediatric patients, especially in neonates and children aged 6 days to 3 years, with higher incidences in males (24.29% for RSV and 31.43% for *Haemophilus influenzae*) compared to females (16.7% for RSV and 10% for *Haemophilus influenzae*). Rapid diagnosis and early treatment of RSV with palivizumab or ribavirin, and *Haemophilus influenzae* with antibiotics, are essential. Timely identification also aids in implementing effective infection control measures.

Pathogen distribution varies by age, with children generally experiencing higher rates of viral infections than adults. Research indicates that in hospitalized adults with community-acquired pneumonia (CAP), rhinovirus (9%) and influenza viruses (6%) are predominant, while in hospitalized CAP children, respiratory syncytial virus (28%), rhinovirus (27%), and *metapneumovirus* (13%) are most common.

A study conducted by Yu Lin et al in 2020 investigated both symptomatic and asymptomatic premature infants in a neonatal ICU through prospective screening using multiplex PCR twice weekly. This screening identified respiratory viruses in 52% of the infants during their hospitalization. Infected infants experienced significantly longer hospital stays (70 days versus 35 days) and a higher incidence of bronchopulmonary diseases.[2]

Over the past decade, the substantial impact of respiratory viral infections on adult and pediatric patients, particularly those with hematologic malignancies, hematopoietic stem cell transplants, and solid organ transplants, has gained recognition. In these highly immunocompromised groups, respiratory viruses frequently progress to pneumonia, with mortality rates ranging from 30% to 50%. Several studies have demonstrated the utility of multiplex PCR in detecting respiratory viruses in these high-risk populations.[16-19]

In our investigation, respiratory syncytial virus (RSV) was the most commonly detected pathogen in both males and females, followed by adenovirus and influenza B virus. These findings align with prior research, such as a study in Spain where RSV was the predominant pathogen. Our research further emphasizes the crucial role of PCR in identifying respiratory viruses, particularly in vulnerable populations like premature infants and immunocompromised patients. In the neonatal ICU, PCR screening revealed viral infections in more than half of prematurely born infants, leading to prolonged hospital stays and increased incidences of bronchopulmonary diseases.[20]

Respiratory viral infections, often presenting with fever and respiratory symptoms, are prevalent and can be challenging to differentiate from bacterial infections, resulting in unnecessary antibiotic use. This misuse exacerbates the global challenge of antimicrobial resistance (AMR), necessitating accurate and prompt diagnosis to mitigate the risks associated with inappropriate antibiotic use.[7, 21-26]

Our study underscores the effectiveness of nucleic acid-based tests, especially multiplex PCR, in detecting respiratory viruses in pediatric patients with respiratory tract infections (RTIs). A retrospective analysis revealed a male predominance across various age groups, highlighting the significant impact of respiratory viral infections in this demographic.

Expanded pathogen coverage led to a significantly higher rate of positive test results, with pathogens like *Haemophilus influenzae* (*A-F*), *RSV, adenovirus,* and *Klebsiella pneumoniae* frequently identified across all age groups. Male patients exhibited a higher prevalence of infections, with distinct pathogen profiles observed across different age categories. Additionally, coinfections involving multiple microbial pathogens were more prevalent in males, particularly in the 0-10 year's age group, underscoring the importance of considering coinfections in clinical management.

India is among the 15 countries with the highest burden of pneumonia cases and related childhood mortality. Approximately 400,000 children under the age of five die each year in India due to acute respiratory infection (ARI) diseases, accounting for 13-16% of all child deaths among pediatric hospital admissions.[27,28] In children, exposure to *Haemophilus influenzae*, particularly type b infections, poses a risk of secondary infection, with approximately a 2.1% likelihood among contacts. This highlights the importance of vigilance and preventive measures, as adults in close contact with infected children must be aware of the potential for transmission. Preventive strategies such as vaccination are crucial in reducing the spread of *H. influenzae* infections and protecting vulnerable populations, including children, from severe outcomes associated with this pathogen.[29]

A study of children aged 4-5 years reported a 47.3% prevalence rate of ARI, while we observed a higher prevalence among infants. Community-based studies in coastal Karnataka and urban West Tripura, India, also reported a higher incidence of pneumonia among infants.[30-32] ARI prevalence was highest among infants aged 0-12 months (63.2%), followed by children aged 25-60 months (59.5%), and was lower in those aged 13-24 months (52.6%). Boys (62.9%) had a higher prevalence of ARI than girls (55.1%). Higher ARI rates were also observed in children with birth weights under 2.5 kg, those whose mothers had education levels between 1st and 7th grade, those with two or more siblings, and those living in overcrowded conditions.[33]

A comparison between rural Kenya and North America highlights differing healthcare challenges. In rural Kenya, infant and child mortality rates remain high at 60 and 20 per 1000 live births, respectively, despite a nearly 50% reduction in infant mortality from 2003 to 2010, largely due to decreased deaths from respiratory infections. By 2010, malaria had surpassed respiratory infections as the leading cause of infant mortality. In contrast, a U.S. study reported hospitalization rates for radiologically proven pneumonia at 6.2 per 1000 infants and 1.6 per 1000 children, with only three deaths among 2358 admitted children. Notably, 33% of these children were diagnosed with asthma or reactive airways disease. The low mortality rates in developed countries are likely due to factors such as vaccination, access to antibiotics, good nutrition, low biofuel exposure, and improved sanitation.[34-37]

In the study by Jain et al bacterial pathogens were identified in a minority of cases, with viruses being more common. Respiratory syncytial virus (RSV) was prevalent in younger children, while rhinovirus was more common in older children. In 15-30% of cases, more than one virus or a combination of virus and bacteria were identified, varying by age. While identifying a "respiratory pathogen" in acutely ill subjects might suggest a causal relationship, it is known that bacteria like *Streptococcus pneumoniae*, *Moraxella catarrhalis*, and *Haemophilus influenzae* are often found in asymptomatic individuals. Similarly, some studies show that certain respiratory viruses are more frequently detected in asymptomatic children than in those with symptoms.[35]

Principi et al conducted weekly upper airway sampling in 88 infants, finding that 64% of the 326 rhinovirus-positive samples were from asymptomatic infants. Symptom likelihood was not significantly affected by rhinovirus type (A, B, or C), and no infants were hospitalized.[38] Chonmaitree et al identified viruses in 39% of samples, with rhinovirus being most common; 28% of asymptomatic infants had viruses, accounting for 53% of positive samples. The odds of viral identification in symptomatic subjects were 11.7 times higher than in asymptomatic infants with RSV.[39] Rhedin et al found RSV, hMPV, influenza, and adenovirus were more common in community-acquired pneumonia than in healthy controls, unlike rhinovirus and bocavirus.[40] Shi et al.'s systematic review confirmed these findings, showing strong causal links for RSV (OR 9.8), influenza (OR 5.0), parainfluenza (OR 3.4), and hMPV (OR 3.8).[41]

Though the study underscores the necessity of molecular diagnosis for pediatric respiratory illnesses, it has several limitations. The small sample size of 100 pediatric participants restricts the generalizability of the findings. Additionally, the use of non-probability sampling and reliance on clinical suspicion for participant inclusion may introduce selection bias, impacting the representativeness of the study population. To enhance the validity and sensitivity of the findings, further research with a larger sample size is required.

CONCLUSION

In conclusion, our study highlights the evolving landscape of respiratory virus detection and emphasizes the importance of PCR-based methods in improving patient outcomes and guiding infection control measures. Continued research efforts are necessary to elucidate the clinical significance of viral coinfections and optimize diagnostic and management strategies for respiratory tract infections in pediatric populations.

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