Prevalence of different respiratory viruses detected by multiplex real time PCR in a tertiary care hospital, Kolkata

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Abstract: *Background:* Worldwide, respiratory viral infection is a leading cause of morbidity and mortality throughout the year. It is a serious cause of concern, particularly for older adults and those in the lower age group. *Aim of the study:* To detect a wide range of respiratory viruses by using multiplex Real Time PCR and also to corroborate epidemiological and clinical findings. *Material and methods:* Nasopharyngeal and oropharyngeal swabs from 487 patients with respiratory tract infections were collected from January 2023 to December 2024 and tested for respiratory viral panel by multiplex Real Time PCR. *Results:* Majority of the patients affected from urban areas and within lower age group. Infection by respiratory viruses are found throughout the year but high infection rate was found in the month of March and April. It was found that human adenovirus was most prevalent (32.84%) followed by human rhinovirus, influenza A/H3N2, human parainfluenza and human influenza A virus among 414 positive samples by Multiplex Real Time PCR.Majority of patients had elevated CRP levels, which indicates the patients were suffering from an infection. *Conclusion:* As per our study, infected patients are mostly children below 10 years of age and predominantly infected with Adenovirus. The gold standard Multiplex Real-Time PCR method has revolutionized laboratory diagnosis with high detection sensitivity and simultaneous detection of multiple viruses in a short period of time.

Keywords: Respiratory viral infection, Multiplex Real Time PCR, Rapid diagnosis, Adenovirus, Outbreak.

Introduction

Respiratory viruses are indeed among the most common causes of disease in humans, leading to significant morbidity and mortality [1]. These viruses primarily infect the upper and lower respiratory tract, causing a range of illnesses as acute respiratory infections (ARIs) and Severe acute respiratory infections (SARI). Many respiratory viruses from various families are highly adapted to efficient transmission between people and can spread globally, often with significant impact on public health [1]. These viruses typically spread through respiratory droplets when an infected person coughs, sneezes, talks, or breathes, but can also spread via surface contact in some cases. The increasing frequency and severity of global warming driven by extreme environment, climate change, migration of people and changing habitats of animals are playing a significant role in exacerbating respiratory diseases [2]. Over the past several decades, numerous community-based studies have provided substantial evidences that respiratory viruses such as human adenovirus, parainfluenza, influenza A, and respiratory syncytial virus (RSV) are among the leading causes of both ARIs and SARI [3-6]. Influenza viruses, particularly the influenza A/H1N1 pandemic (which caused the 2009 swine flu pandemic) and other novel strains, are known for their ability to undergo antigenic shift (major changes in the virus) and cause pandemics [6].

Respiratory viral infected patient's data in different parts of our country is very limited and scattered. Pan-India influenza surveillance conducted by ICMR during 2021-2022, found that beside COVID-19, influenza A/H3N2 and influenza B/Victoria were prevalent in 2021 and influenza A/H1N1 pandemic was

dominant in 2022 [7]. In another study in South India, adenovirus and RSV were found major circulating virus during 2021-2022 [8]. In the year 2023, a potential Adenoviruses outbreaks was observed in West Bengal [9].

Approximately one-fifth of all childhood deaths worldwide are related to acute respiratory infections (ARIs), particularly in impoverished populations of tropical regions, where ARI case-to-fatality ratios can be remarkably higher than in temperate regions of the world [1]. Respiratory viruses such as adenovirus, influenza A viruses often circulate commonly in all age groups and are recognized as adapted to efficient person-to-person transmission very frequently [1]. In addition to all these respiratory viruses, COVID-19, influenza A/H5N1 and avian influenza virus have emerged in recent years as threats to public health [10]. Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO), and several other national agencies have identified many unconventional viral pathogens emerging globally with increasing frequency [11]. Although vaccines and effective antiviral drugs are available for some of these viruses only, much progress has been made in vaccine development and target based antiviral therapies for the other viruses [12].

Historically, Asia is believed to be the epicenter of several emerging viral diseases that are of significant global public health importance [13]. Approximately 11.5% of all lower respiratory tract infection (LRTI) episodes occurring in 2017 were attributable to the influenza virus globally [14]. This corresponds to about 54 million episodes of illness, including 9.4 million hospitalizations [13-15]. A recent worldwide COVID-19 outbreak happened in 2020 to 2021, which corresponds to increase number of cases with high mortality rate worldwide [16]. More number of surveillance and research is required to understand the possible causes of respiratory viral outbreak and also prompt diagnosis to detect the exact pathogen by sensitive molecular methods.

The aim of the study to overview of the prevalence of the respiratory viral pathogens in a tertiary care level hospital, Kolkata, India. We corroborate epidemiological and clinical findings of the infected patients and also detected a wide range of respiratory viruses by using multiplex

Real Time PCR. We will also discuss about the seasonal viral surge throughout the year and the viruses which are mostly circulating in this region during 2023-2024.

Material and Methods

This study protocol was approved by the Institutional ethical committee of Institute of Post Graduate Medical Education and Research (IPGME&R), Kolkata. Nasopharyngeal and Oropharyngeal swabs from 487 patients with suspected respiratory tract infection (of them 405 patients, i.e, 83.16%were SARI cases and 82 patients i.e,16.83% were ARI) were collected from different wards of the hospital during January 2023 to December 2024.

All the ARI samples came from various respiratory and pulmonary outdoor patients and SARI samples from hospitalized patients. All the patients signed the consent form which was approved by the ethical committee of the hospital. Respiratory viral panel by Real Time PCR were performed at Virus Research and Diagnostic Laboratory (VRDL), unit of Microbiology department of the IPGME&R. Out of the total number of cases, we have tested samples from neonate aged 13th day of life to older person aged 83 years old. We have also collected the clinical data of each patient from respective case sheets who were admitted in the various in-patient-departments (IPD) and also from the clinician's prescriptions of out-patient-departments (OPD).

RNA Extraction: Under biosafety level-IIB2 cabinet, all the VTM tubes containing suspected patient samples (Nasopharyngeal and Oropharyngeal swab) were sorted out serially. RNA extraction process was performed by MagMAX Viral/Pathogen II (MVP II) nucleic acid isolation kit using King Fisher automated extraction system (Thermo Fisher Scientific) according to manufacturer's protocol [17].

Detection of respiratory viral panel by multiplex Real Time PCR: Isolated RNA was then subjected to multiplex Real Time PCR using TRUPCR respiratory viral panel RT PCR Kit [18]. This kit simultaneous detect 17

different viruses with subtypes and internal control (IC) qualitatively in BIORAD CFX 96 Real Time PCR system. 20 microlitre (μL) reaction was standardized as follows: 10 μL of master mix, 0.35 μL of enzyme mix,9.65 μL and 5 μL of primer-probe mix and extracted RNA for each tube. The thermally cycled condition as follows: 20 minutes of RT at 50 °C, 10 minutes of initial denaturation at 94 °C, then 7 cycles (denaturation at 94 °C for 15 s, annealing and extension at 66 °C for 30 secs, 72 °C for 15 secs), 37 cycles (94 °C for 10 secs, 66 °C for 60 secs for data acquisition). Four different detector florescent channels (FAM, Tex Red, Cy5, HEX) were used to detect different virus qualitatively and IC. Every fluorescent dye's cycle threshold (Ct) value was evaluated separately and the result was analyzed as per respective kit manual [18].

Results

A total of 487 patients with upper and lower respiratory tract infections presented with clinical features like fever, cough, body ache and occasionally shortness of breath (sob) were screened from January, 2023 to December, 2024. Out of the 487 number of cases, 269 (55.23%) were female and rest were male 218 (44.76%). Their age ranged from 13th day of life to 83 years old adult. Out of the total 487 patients, 336 were from urban areas (68.99%) and rest were from rural areas (31.01%). We also observed that from January onwards, the various respiratory viral pathogen prevalence increases gradually and the peak rises in the month of March and April in both the year 2023 and 2024 (Fig 1).

Fig-1: Month wise affected patients (%) during 2023-2024

Month wise data of affected individuals were also presented in Fig.1. As per age wise distribution of affected positive patients (n=414) by multiplex Real Time PCR, 135 were found between the age of 13 days to 5 years, 86 cases in the age group between >5-10 years, 53 cases in age groups >10-15 years, 19 cases in age groups >15-20 years and

121cases were age groups >20 to 85 years. Age wise presentation were depicted in Fig 2.

Fig-2: The bar diagram represents the respiratory virus prevalence in different age groups of during 2023-2024
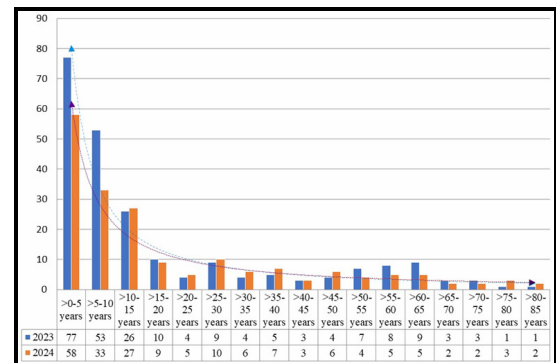
After performing the gold standard multiplex Real Time PCR, 414 samples were found positive to various respiratory virus out of 487 suspected samples. It was found that human adenovirus was most prevalent among all the respiratory viruses tested, which corresponds to 32.84% followed by human rhinovirus (21.01%), influenza A (18.35%), human influenza A/H3N2 (10.14%) and human parainfluenza (9. 17%).Other viruses found in small percentage i.e, influenza A/H1N1, boca virus, RSV and corona alpha/beta viruseswith 4.34%,1.69%,1.44% and 0.96% respectively (Fig.3). Out of 414 positive cases, 29 (7%) were found coinfected with more than one virus.

Fig-3: The prevalence of various respiratory virus along with their percentages among infected patients
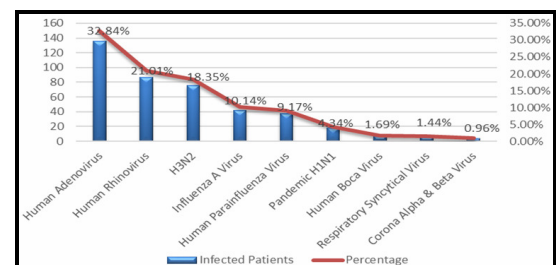
Clinical features and Lab test parameter of the positive cases infected by different respiratory viruses are summarized Table-1 and Table-2 respectively. Hemoglobin, WBC, platelets count were found to be in relatively lower range and AST, ALT, Creatinine found to be

on higher range in the positive cases. It was also noted that elevated level of CRP were found high in most of the positive patients. We have also found that most of the patients were infected with rhinoviruus, influenza A/H3N2, adenovirus and influenza A viruses showed relatively higher level of CRP. We also noted that there is no significant difference in clinical outcome between co-infected patients in comparison with patients infected with single virus.

Table-1: Clinical features of the positive cases infected by different respiratory viruses									
Clinical features of positive cases	Adenovirus (n=136)	Rhinovirus (n=87)	Influenza A/H3N2 (n=76)	Parainfluenza A (n=42)	Influenza A (n=38)	Influenza A/Pdm H1N1 (n=18)	Bocavirus (n=7)	RSV (n=6)	Corona alpha & beta (n=4)
Fever	128	79	74	34	38	18	6	5	3
Body ache	88	81	73	22	38	17	4	5	1
Cough	82	64	71	18	33	17	3	2	1
Shortness of breath (SOB)	68	41	68	10	13	12	1	3	0
Malaise	48	38	52	8	29	14	2	4	2
Rhinorrhea	42	28	11	7	32	16	4	3	1
Diarrhea	26	11	38	5	9	6	1	0	1
Altered Mental Status	43	21	16	2	6	4	1	0	0

Table-2: Lab test data (in average) of the positive cases infected by different respiratory viruses									
Lab test parameter of positive cases	Adenovirus (n=136)	Rhinovirus (n=87)	Influenza A/H3N2 (n=76)	Parainfluenza A (n=42)	Influenza A (n=38)	Influenza A/Pdm H1N1 (n=18)	Bocavirus (n=7)	RSV (n=6)	Corona alpha & beta (n=4)
Hemoglobin (gm%) (Below 10 gm%)	53	36	25	10	8	6	1	1	0
WBC (cells /mm3) (More than 11000/mm3)	62	30	21	8	6	5	1	1	1
Platelet (mm3) (<100000 mm3)	12	8	6	2	4	1	0	0	0
ALT (u/l) (>50 u/l)	18	12	7	6	2	1	0	0	0
AST (u/l) (>50 u/l)	18	11	7	6	2	1	0	0	0
CRP (mg/dl) (>6 mg/dl)	121	81	73	32	32	12	4	2	1
Creatinine (mg/dl) (>1.5 mg/dl)	4	2	3	2	2	1	0	0	0

Discussion

The prevalence of respiratory viruses is a consequential public health concern, especially owing to their potential to cause widespread morbidity and mortality. This study aims to elucidate the seasonal patterns and prevalence of key respiratory viruses within a suspected lower and upper respiratory tract infected patients and also provide a comprehensive analysis of their impact across different seasons and age groups. Our study was a hospital-based study from January 2023 to December 2024 with a sample size of 487 suspected patients. We have found that most positive patients were from urban areas (66.95%) than rural areas.

The high positivity rate in urban areas due to the combination of high population density, extensive public transportation networks, air pollution, and global connectivity which creates an environment where respiratory viruses can spread more easily and rapidly [16].

The infected cases had the most underlying clinical manifestations which include the fever, cough and severe headache. Many patients develop shortness of breath (SOB). Altered Mental Status is mainly found in some patients infected with adenovirus. Initial presenting symptoms were more or less common in all the patients infected with different viruses, but complications if appeared are of different types. We have noticed that, from the month of January onwards the various respiratory viral pathogen prevalence increases gradually and the peak rises in the month of March and April in both the year 2023 and 2024. It can be influenced by several factors related to seasonal patterns, and environmental conditions. The transition from winter to spring often involves fluctuating temperatures and changes in humidity which is indirectly responsible for the viral replication and transmission [19]. Some respiratory viruses, like the flu, tend to peak in colder months, while others may thrive in the milder conditions of spring [20].

Our Study also noticed that the individuals under the lower age group are mostly affected compare to the higher age groups. Most of the children under <5 years age group were mostly affected. Due to their low immunity, the immune response of young infants is dampened or altered, resulting in increased susceptibility and prone to infection. The school going kids are often seen affected with respiratory illness due to viral transmission expelled when an individual coughs or sneezes. Few studies also found that the burden of viral pathogens was higher among infants and children who made up more than half of the viral detections [3,5,8,21]. These finding emphasizes the need for vaccination in pediatric population.

We have performed the multiplex Real time PCR to detect the various respiratory virus panel in a single PCR run. Our investigation reveals that, out of the total 414 positive cases, adenovirus was responsible for the highest number (32.84%) of infection, followed by human rhino virus (21.01%), influenza A/H3N2 (18.35%). In another study in Kolkata, they have also found that there is a sharp rise in the positivity rate of respiratory adenovirus which ranged from 22.1% in early December 2022 to 52.6% in mid-March 2023 [21]. After analyzing the result, it was also noticed that 24 individuals are co-infected with more than one virus for eg. rhinovirus with parainfluenza and in another coinfected patient had rhinovirus with adenovirus and parainfluenza virus.

While dealing with the lab parameters, we have found high CRP level in most of the positive cases which indicate high levels of inflammation in the host body due to the infection but significant correlation was not found. High CRP levels were detected in majority of respiratory virus infected patients in another study also [22]. Significant changes in the liver function tests and kidney function tests were detected only in few cases.

Conclusion

Nowadays respiratory virus is one of the leading and most common infectious pathogen affecting mainly in the lower age groups. These viruses can lead to various respiratory illnesses, ranging from mild colds to more severe conditions. Due to the intense climatic change, the respiratory viruses are present roughly all throughout the year but the major outbreak is noticed in the month of March and April.

Multiplex Real Time PCR is the gold standard method to detect the respiratory viruses in a very short period of time which will be of great help to all the clinicians to give the treatment accordingly. The accurate molecular diagnosis of the causative agent can avert unnecessary treatment with antibiotics and thus prevent development of antibiotic resistance in future. It will also give the information to the policy makers, clinicians and healthcare authority to take necessary steps to prevent the viral outbreaks and apply modern molecular methods for prompt diagnosis.

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