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Prevalence of Viral Respiratory Pathogens with their Emerging Co-Infection by QIAstat Dx Respiratory Panel during COVID-19 Pandemic in a Tertiary Care Hospital, Bangladesh: 2020-2022

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Abstract: ***Background**: Acute respiratory infection (ARI) is a major cause of morbidity and mortality worldwide, particularly among children. Viruses are responsible for a large proportion of ARIs, of which Influenza, Parainfluenza, Rhinovirus/Enterovirus (RV/EV) and Respiratory Syncytial Virus (RSV) are the leading cause. Co-infections have a potential role in increase severity of viral illness during COVID-19 pandemic. Human Metapneumovirus, Adenovirus and Bocavirus emerge as single and in co-infection during this period. However, local data on the etiologic diagnosis of ARIs are limited. QIAstat Dx Multiplexed Respiratory panel have recently been added to the diagnostic work-up that can simultaneously detect multiple pathogens of respiratory infections in a single run. **Objectives**: To determine the prevalence of respiratory viral and atypical bacterial pathogens by rapid multiplex PCR assay, known as the Respiratory Panel, among suspected ARIs patients during COVID-19 pandemic. This study also assesses viral co-infection rates with both COVID-19 and other respiratory viruses. **Methods:** This retrospective study was conducted at molecular laboratory of SQUARE Hospital Ltd, Dhaka, Bangladesh from March 2020 to October 2022. A total of 1557 respiratory samples were tested by QIAstat Dx Respiratory panel and results were analyzed by software. **Results**: Of total 1557 tested samples, 458 (29.4%) were positive, 457 respiratory viruses and one Legionella pneumophila. Among total positives, 28.2% were from children and 71.8% from adults with highest numbers found in less than 15 years and more than 60 years of age. Except pandemic virus; SARS-CoV-2 (27.1%), Influenza virus was the most commonly detected pathogen (21.8%) followed by RV/EV (15.9%), RSV(11.6%) and other human Coronaviridae (7.9%). Parainfluenza, Human Metapneumovirus, Adenovirus and Bocavirus were also found, between 2-5%. FLU virus were prevalent among both below15 years and above 60 years but predominance of RV/EV and RSV can be seen in under 15 years. A total of 40 (8.7%) patients were co-infected; 14(35%) with COVID-19 and 26 (65%) between other respiratory viruses, of which the most co-infection was Rhinovirus/Enterovirus plus Bocavirus (15%). **Conclusion**: Respiratory panel significantly improve etiological diagnosis of multiple respiratory infections because of viruses and atypical bacteria which enhance patient care with more rational antimicrobial use and improving infection control measures during pandemic.***

Keywords: Acute respiratory infection, Respiratory panel, Respiratory virus, Co-infection, COVID-19-pandemic

1. Introduction

Acute respiratory infection (ARI) is a serious healthcare burden and the major cause of outpatient visits and hospitalizations in all age categories worldwide. [1],[2] Influenza virus A and B, the most clinically important types, continues to be one of the greatest public health challenges in the world. It is responsible for recurrent annual epidemics, causing one billion cases of acute respiratory infection per year ranging from mild symptoms to viral pneumonia with fatal complications. [3]Current estimates found that annually Influenza cause up to 100,000 deaths in children under five years and 650,000 deaths in low-resource settings as well people over 75 years. [4],[5]Rhinovirus/ Enterovirus (RV/EV) and Respiratory Syncytial Virus (RSV) are the most common causes of respiratory tract infection (RTI) in children. [6] RV/EV can be both asymptomatic and symptomatic with common cold, acute otitis media (AOM), pneumonia, bronchiolitis, recurrent wheezing in children. Young children, the elderly and adults with chronic medical conditions are at the greater risk for severe RSV infections.[7] Globally an estimated 33.1 million episodes of RSV associated ARI occur per year in children under 5 years of age. [8]A study suggests RSV infection to occur in 3-7% of elderly people, and in 4-10% of high-risk adults. [9]

By the end of 2019, infections with the new pandemic virus SARS-CoV-2 emerged which till now infected over one billion people, causing over two million deaths globally. [10]With the COVID-19 pandemic still ongoing, the annual season of influenza and other respiratory virus epidemics has arrived. [11]Several case studies showed that viral co-infection with SARS-CoV-2and/or other viruses influence infection dynamics. [12]

Clinical diagnosis of viral respiratory diseases remains a challenge for clinicians, due to the overlapping sign-symptoms caused by different virus. Bacteria also cause respiratory illness with non-specific presentations where empiric antibiotics are frequently commenced to cover possible bacterial pneumonia, leading to overuse of inappropriate antibiotics. [13], [14]The rapid, accurate diagnosis of respiratory viruses is essential for the appropriate selection of treatment, which helps in improving patient care and prevent overuse/misuse of antibiotics. In addition, the early detection plays an important role in minimizing cost and effective implementation of infection control as well as antimicrobial stewardship programs. [15]

Conventional microbiological and immunological diagnostic tests for virus have limitations in comprehensiveness, accuracy and timeliness. [16]Though applied in diagnostic field for many years, traditional PCR is not completely satisfactory because of its incapability to target multiple pathogen at a time and high risk of contamination during laboratory procedures. The development of new molecular technologies like multiplexed respiratory viral panel assay has improved the capability for simultaneous detection of nucleic acids of two or more viral and/or bacterial pathogens in a single reaction within an easy workflow. [17], [18]

Respiratory Panel assay is a fully automated, highly sensitive, faster multiplex PCR system. It does not require advanced equipment or expertise in molecular diagnostics, making it a useful point-of care test for acute respiratory infections of single or multiple organisms. [19]This test has improved turn-around time, reduced number of manual steps in the laboratory, increasing sensitivity and specificity and the multiplexing of several pathogens within a single panel that finally reducing time to clinical treatment. [20], [21]

The aim of this study was to evaluate the prevalence of viral respiratory pathogens in Dhaka, Bangladesh during the COVID-19 pandemic with their age and time associated distribution and frequency of viral co-infection.

2. Methods

This retrospective, observational study was conducted in Molecular Laboratory of Square Hospital Ltd (SHL). Nasopharyngeal/Oropharyngeal swabs were collected from patients with symptomatic respiratory tract infection, attended and/or admitted in Emergency, Critical care, Out-patient department (OPD) and In-Patient department (IPD) of SHL. These samples were processed by QIAstat-Dx Respiratory Panel for detection of 19 viruses including SARS-CoV-2 and 3 atypical bacteria between March 2020 and October 2022.The study also analyzed some general information on number of viruses according to age and months of the year during the study period.

The Respiratory Panel is a multiplexed nucleic acid real-time PCR test intended for the qualitative detection of nucleic acid from samples containing multiple respiratory viral and bacterial organism types and subtypes. The 300µl samples were analyzed in a single-use cartridge that includes all reagents needed for automated nucleic acid extraction, amplification and detection of 22 viruses and bacteria. Diagnostic tests with the Respiratory Panel are performed on the QIAstat-Dx Analyzer 1.0 according to the manufacturer's instructions and following Standard Operating Procedures. A qualitative result for each target was automatically interpreted by the software. Amplification curves of detected pathogens viewed with corresponding cycle threshold (CT) and endpoint fluorescence (EP) values. The possible results of each target in a valid run were reported as detected (CT=23-35) or not detected(CT>35). The overall procedures took approximately one hour (1 h) for a single test. [22]

Pathogens that can be detected with the QIAstat-Dx Respiratory Panel are listed in Table 1.

Table 1: Pathogens detected by QIAstat-Dx Respiratory Panel

Pathogen	Classification (genome type)
Viruses	
Influenza A	Orthomyxovirus (RNA)
Influenza A H1	Orthomyxovirus (RNA)
Influenza A H1N1 pdm 09	Orthomyxovirus (RNA)
Influenza A H3	Orthomyxovirus (RNA)
Influenza B	Orthomyxovirus (RNA)
Coronavirus 229E	Coronavirus (RNA)
Coronavirus HKU1	Coronavirus (RNA)
Coronavirus NL63	Coronavirus (RNA)
Coronavirus OC43	Coronavirus (RNA)
SARS-coV-2	Coronavirus (RNA)
Para Influenza virus 1	Paramyxovirus (RNA)
Para Influenza virus 2	Paramyxovirus (RNA)
Para Influenza virus 3	Paramyxovirus (RNA)
Para Influenza virus 4	Paramyxovirus (RNA)
Respiratory syncytial virus A+B	Paramyxovirus (RNA)
Human Metapneumovirus A+B	Paramyxovirus (RNA)
Adenovirus	Adenovirus (DNA)
Rhinovirus/Enterovirus	Picornavirus (RNA)
Bocavirus	Parvovirus (DNA)
Bacteria	
Bordetellapertussia	Bacterium (DNA)
Legionella pneumophila	Bacterium (DNA)
Mycoplasma pneumoniae	Bacterium (DNA)

3. Results

During the study period of two and a half-year from March, 2020 to October, 2022 a total of 1557 respiratory samples were tested by QIAstat Dx Respiratory panel. Of 1557, 458(29.4%) were found detected with single/multiple of listed virus/bacteria in the panel and 1099(70.6%) were negative for any pathogens. (Figure 1)

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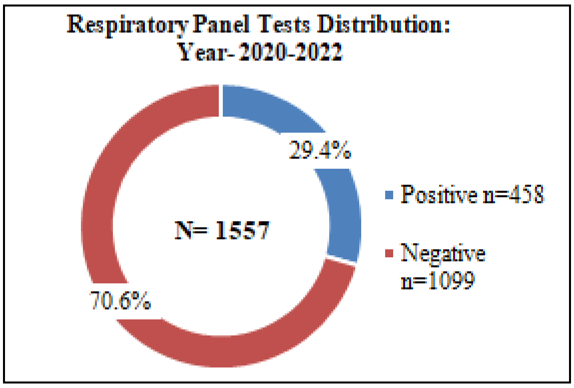

Figure 1: Distribution of Respiratory Panel Tests: March, 2020- October, 2022

Table 2 shows the distribution of isolated viruses and bacteria by the age and gender. Among 458 detected respiratory pathogens, 129/458 (28.2%) were from children and 329/458 (71.8%) were from adults. Of the adult population, 124/329 (37.7%) contributed from over 60 years of age. However highest numbers of positive isolated respiratory pathogens were found among extremes of age group like between 0-15 years (129) and above 60 years (124) of age. No gender variability can be seen.

Table 2: Demographic distribution of isolated Respiratory viruses by age and gender

Age	Male	Female	Total
0-15	73	56	129
15-30	8	38	46
30-45	33	64	97
45-60	32	30	62
>60	77	47	124
Total	**223** (48.7%)	**235** (51.3%)	**458** (100%)

The Respiratory panel test detected 458 pathogens: 457 (99.8%) viruses and only 1 (0.2%) atypical bacteria, known as Legionella pneumophila. As the study was done in COVID pandemic, SARS-CoV-2 were found highest (27.1%). Coronaviruses of other types, Influenza virus(A, B subtype), Parainfluenza virus [*1,2,3,4 subtype*], Rhinovirus/Entero virus (RV/EV), Respiratory SyncytialVirus A+B (RSV), Human Metapneumovirus A+B (HMPV), Adenovirus (AdV) and Bocavirus (BoV) were other respiratory viruses isolated in this study period. (Table 3).

Table 3: Frequency of Isolated Viruses: 2020-2022

Name of the VIRUSES	Virus Isolates (n=458)	Virus Isolates (%)
SARS-CoV-2	124	27.1
Coronavirus NL63	10	2.2
Coronavirus HKU1	17	3.7
Coronavirus OC43	5	1.1
Coronavirus 229E	4	0.9
Rhinovirus/Enterovirus	73	15.9
Respiratory syncytial virus (RSV) A+B	53	11.6
Influenza virus A	88	19.2
Influenza virus B	12	2.6
Parainfluenza virus 1,3,4	20	4.4
Adenovirus	16	3.5
Bocavirus	13	2.8
Human Metapneumovirus A+B	22	4.8
Legionella pneumophila	1	0.2
Total	**458**	**100**

Figure 2 shows that pandemic SARS-CoV-2 virus isolated much higher (27.1%) than other human Coronaviridae (7.9%) by Respiratory panel assay.

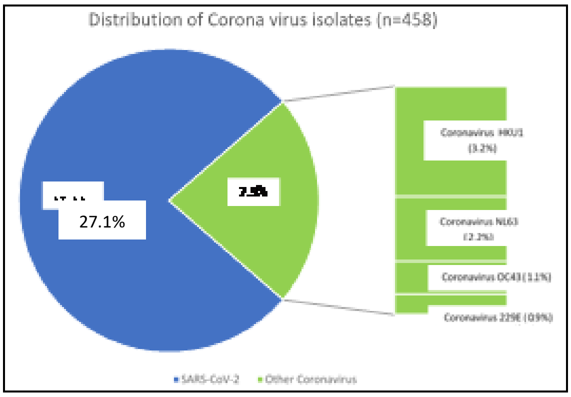

Figure 2: Comparison of SARS-CoV-2 and other Coronaviridae

Apart from pandemic virus SARS-CoV-2 (27.1%), Influenza viruses (A,B) was the most prevalent(21.8%) followed by Rhinovirus/Enterovirus (15.9%) and Respiratory SyncytialVirus A+B (11.6%).The detection rates of other respiratory pathogens were as follows: other Coronavirus [*NL63, HKU1, OC43, 229E subtypes*] (7.9%), HumanMetapneumovirus A+B (4.8%), Parainfluenza virus [*1,2,3,4 subtypes*] (4.4%), Adenovirus (3.5%) and Bocavirus (2.8%). (Figure 3)

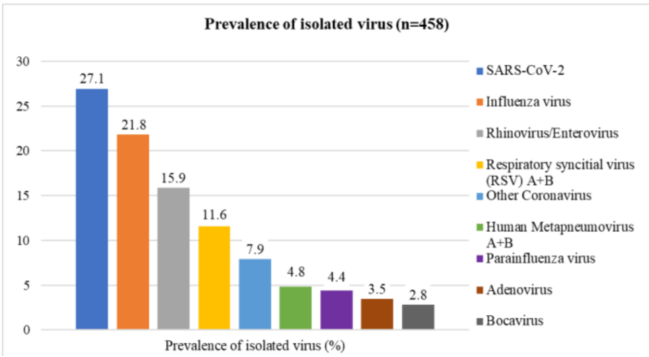

Figure 3: Prevalence of Respiratory viruses (n=458)

Detection of more than one respiratory pathogen was found in 40/458(8.7%) of the positive samples, with a higher co-detection rate in the children's group (25/40; 62.5%) than in the adult group (15/40; 37.5%) (Figure 4)

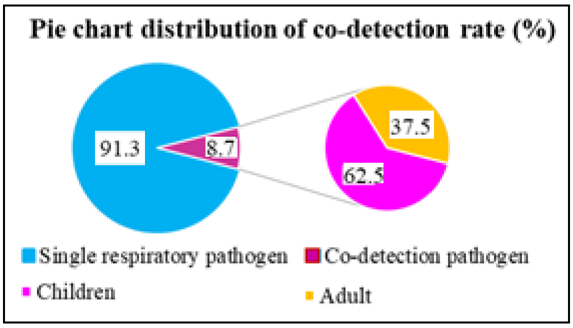

Figure 4: Frequency of co-detected pathogen among children and adult (n=458)

The most commonly observed co-infection was Rhinovirus/Enterovirus plus Bocavirus with 6 cases out of 40 (15%), followed by both Rhinovirus/Enterovirus plus Adenovirus and Rhino/Enterovirus plus SARS-CoV-2 (5/40; 12.5%) and SARS-CoV-2 plus Adenovirus (4/40; 10%). The combination of RSV plus Parainfluenza 1, RSV plus RV/EV, RSV plus Influenza A virus and SARS-Cov-2 plus Influenza A virus were the fourth most common types (3/40; 7.5%), as shown in Table 4. Rhinovirus/Enterovirus were found in 4 cases; 2 with Influenza A virus and 2 with Human Metapneumovirus. Human Bocavirus also co-infected with SARS-CoV-2 virus for 2 cases while with Influenza A virus in one case. A single combination of Respiratory Syncytial Virus and Adenovirus was detected.

Table 4: Summary of multi-organism positive samples

Co-infection of viruses (Pathogens)	Percentage (%)
Rhinovirus/Enterovirus + Bocavirus	15% (6/40)
Rhinovirus/Enterovirus + Adenovirus	12.5% (5/40)
SARS-CoV-2 + Rhinovirus/Enterovirus	12.5% (5/40)
SARS-CoV-2 + Adenovirus	10% (4/40)
Respiratory Syncytial virus (RSV) A+B + Parainfluenza virus 1	7.5% (3/40)
Respiratory Syncytial virus (RSV)A+B+ Rhinovirus/Enterovirus	7.5% (3/40)
Respiratory Syncytial virus (RSV) A+B+ Influenza A virus	7.5% (3/40)
SARS-CoV-2 + Influenza A virus	7.5% (3/40)
Rhinovirus/Enterovirus + Influenza A virus	5% (2/40)
Rhinovirus/Enterovirus + Human Metapneumovirus A+B	5% (2/40)
SARS-CoV-2 + Bocavirus	5% (2/40)
Respiratory Syncytial virus (RSV)A+B +Adenovirus	2.5% (1/40)
Influenza A virus + Bocavirus	2.5% (1/40)

SARS-CoV-2 were found highest in number (51) among over 60 years' population and lowest in 0-15 years (08). Influenza and Parainfluenza viruses has highest isolation among extremes of age like 0-15 years (24) and above 60 years (49). But the other two important respiratory viruses namely Rhinovirus/Enterovirus (26) and Respiratory Syncytial Virus A+B (37) has highest predilection for under 15 years' population. (Figure 5)

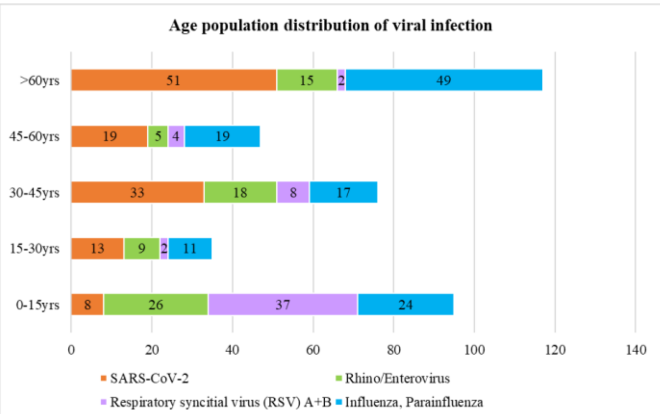

Figure 5: Distribution of Viruses according to age

During peak of COVID pandemic in 2020, Influenza and Parainfluenza virus were less detected. Their infection has started rising since May, 2021 became highest in 2022(78). Particularly higher number of influenza and parainfluenza virus were found between May to August of 2022 and September to December of 2021.

Rhinovirus/Enterovirus were found lowest in 2020 and highest between September, 2021 to December, 2022. Respiratory Syncytial Virus started rising since May 2020, remain high through all seasons of 2021, particularly between September to December. It showed decline in isolation in 2022. (Figure 6)

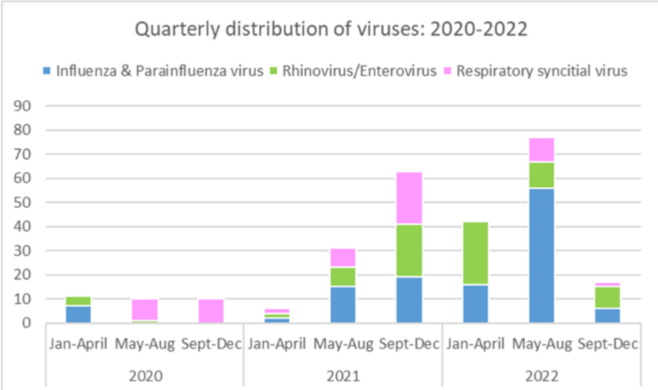

Figure 6: Quarterly distribution of Respiratory viruses of the year: 2020-2022

4. Discussion

The majority of acute respiratory tract infections (ARIs) are caused by viruses, leading to morbidity and mortality particularly in paediatric populations. [23], [24] Primary infections with viral pathogens can predispose to secondary bacterial infections. [25] Co-infection with multiple pathogens (viral-viral/viral-bacterial) are found with same clinical presentations. Use of inappropriate, empiric antibiotic in these patients with ARIs is a problem that concerns of all ages. [26]A single multiplexed panel test is required to identify nucleic acids of viral or bacterial pathogens simultaneously that improve the detection rate of multiple respiratory pathogens and reduce unnecessary antibiotic use. [27] This is crucial for the diagnosis of ARIs because the pathogens can be present at low levels in clinical samples and their abundance quickly drops over time despite symptoms being present.[28]

QIAstat Dx Respiratory panel can detect 22 viruses/bacteria in a single run with higher specificity, sensitivity and faster turn-around time compared to both conventional methods and traditional PCR, therefore showed an increase in diagnostic yield. [25]

Our retrospective study for ARIs tested with Respiratory panel revealed 99.8% respiratory viruses and only 0.2% bacteria. A previous study similarly identified higher proportion of viruses (27.2%) than bacterial agents (10.7%). [29] Along with highest detection of SARS-CoV-2, this study also provides new insights into the distribution of other viral respiratory infections during the pandemic.

Influenza viruses and RSV are well-known respiratory pathogens, while RV/EV become increasingly responsible for ARIs. Apart from SARS-CoV-2, our study showed Influenza A/B virus was the most prevalent pathogen

(21.8%) followed by RV/EV (15.9%), RSV (11.6%), Other Coronaviridae (7.9%), HMPV (4.8%), Parainfluenza virus (PIV; 4.4%), AdV (3.5%) and BoV (2.8%). Influenza A/B, EV/RV and RSV were most commonly detected in Singapore during the COVID-19 pandemic. [30] The results of our study is also comparable to several studies done abroad, [2], [13-14] but different for RV/EV which appeared as dominant (61.8%) in a study done by Chen AP et al.[31]

Influenza A and B are the most common cause (55.7%) of respiratory illness in all age groups, especially among adults. [32], [33] Our study found 19.2% Influenza A virus and 2.6% Influenza B virus which are in line with surveillance studies done by the Centers for Disease Control and Prevention (CDC), where they found 99.8% and 0.2% Influenza A and B respectively. [34]In our observation, RV/EV was the second identified virus in almost all age groups, but especially among under 15 years. This predominance is in accordance with previously published data. [35-37] The increasing availability of multiplex Respiratory panel allows rapid detection of RV/EV and provides the opportunity for timely treatment and early recognition of outbreaks.[38] Based on our results, RV/EV should certainly be included in the differential diagnosis of ARIs in older patients, too. RSV is a regular winter visitor and highly contagious among persons of all ages. [39] The reported rates of hospitalization for RSV varies,[40-41] which highlights the challenges in accurately understanding the respiratory disease burden of RSV each year. Though not confirmed by our study, previous limited findings showed that both young children and older adults with RSV are at higher risk for severe infection. [42]

Human Metapneumovirus (HMPV) was first identified in 2001 [43] has been detected in all continents and accounted for approximately 5-15% of all respiratory tract infections. We found 4.8% HMPV in our study which was little higher than a study done in Israel where it was 3.7%. [44]Parainfluenza virus (PIV) predominantly cause lower respiratory tract infection in infants and young children [45] but the significance of this virus has been underappreciated. [46] In the present investigation, prevalence of PIVs was 4.4% which was similar like China (4.8%), [47] lower than Cameroon (7.5%) [48] and slightly higher than Latin America (3.2%).[49]The overall prevalence of Adenovirus(AdV) was 13% in a meta-analytic review, 9.8% in individual studies in the Eastern Mediterranean region [50]and 8.8% in India. [51] All were higher than we found, about 3.5%. More than a decade ago, human Bocavirus (HBoV) were discovered in respiratory samples. Since then, it's detection in different geographic regions, suggesting a worldwide distribution as a causative agent of ARIs.[52]The HBoV prevalence varies worldwide, between 1.5-18.3% at different ages and diverse regions of the country. [53]We estimated the prevalence of the Bocavirus as 2.8% using Respiratory panel assay which was almost similar to Kuwait (1.9%)[54] and lower than India (6.6%),[55]United Kingdom (98.6%)[56]and China (18.2%).[57] Multiplex Respiratory Panel assay could be used for the accurate diagnosis and detection of HMPV, PIV, ADV and Bocavirus along with FLU virus, RSV, RV/EV, human coronaviridae and COVID-19.

Multiple studies have shown that patients with respiratory-tract diseases commonly have more than one virus detected. [58] Co-infections has been found in 31%,36%,37%,42%, and 51.8% [59-63] of positive respiratory samples, all are higher than our results. In this study, dual viral infections were observed in 40 patients; 8.7% of the total positive samples. However, the predominance of RV/EV in our single (51) and multiple (22) detections is similar with previously published data. [64] Our finding is also in agreement with many other studies, where most frequent co-infections can be seen with RV/EV, AdV, RSV, Influenza A, HMPV together with human Bocavirus. [65] Adults have been reported to have co-infections at a much lower rate, we observed same in this study(Adult:37.5% and Children:62.5%). We found 35% co-infection of respiratory viruses with COVID-19(14/40) which is in line to some recent studies. [66] Glass EL et al. reported more frequent SARS-CoV-2 co-infection with RV/EV, [67] whereas Motta JC et al. showed with adenovirus, [68] all these were revealed in our result also. In contrast to our study that indicated SARS-CoV-2-infected patients co-infected with Influenza A and Bocavirus, but no RSV, other studies reported RSV as the commonest virus among COVID-19 patients. [69]

This study has several limitations. First, it was retrospective and little data about patients' clinical conditions are available. Second, it was a single-center study with a relatively small sample size that cannot represent a large population accurately and might cause sampling errors. Third, the cost of Respiratory panel is very high in compare to other tests necessary for diagnosis and management of atypical pneumonia and other ARIs. Fourth, we assessed the presence or absence of viruses, not their viral load, so disease severity remains unknown. Lastly, results can be false positive due to cross reactivity and nonspecific amplification by multiple targets/primers in one reaction.

An important strength of our study was the inclusion of patients from emergency, outpatients and admitted personnel. Therefore, random sampling ensured an unbiased selection of samples tested by Respiratory panel (RP) assays. Our study demonstrates a useful retrospective application of clinically tested respiratory specimens, which provide new insight into future exploration and also enhance our understanding as well interpretation of co-infections.

5. Conclusions

In conclusion, the prevalence of respiratory pathogens in our institution during the study period showed 29.4% of samples tested positive with Respiratory Panel, all of which were viruses except one bacteria. Pandemic virus COVID-19 was the most prevalent. However, our results corroborate the data which indicate that Influenza virus, RV/EV, RSV, human Coronaviridae, HMPV, Parainfluenza virus, Adenovirus and Bocavirus were among the other most important agents of ARIs, mostly in children but adult populations are also quite significant. The clinical impact of Influenza virus, RV/EV and RSV infections made them considered as highly significant respiratory pathogens. Viral co-infections were detected in 8.7%. Due to similarities of viral and bacterial ARIs with inconclusive laboratory findings, a diagnostic dilemma appears that needs treatment with empiric antibiotic and might lead to antibiotic misuse.

Respiratory Panel is highly suggested for the simultaneous detection of different pathogens, provides rapid and high-yield results which can guide diagnosis, therapy and infection control measures. Further investigation about viral co-infection with SARS-CoV-2 is an urgent need. Future study is needed for measuring viral loads and genotyping analysis, its epidemiology, co-infections and re-infections. Lastly, future prospective studies to further assess the impact of Respiratory Panel on outcomes including correct, timely diagnosis, use or misuse of antibiotics, minimizing other diagnostic tests, length of hospital stay and clinical course is recommended.

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