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Pattern of Respiratory Pathogens with their Co-Infection Detected by Multiplex Real-Time PCR at a Tertiary Care Hospital in Dhaka City of Bangladesh

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Abstract

Background: Acute respiratory infections caused by a multitude of microorganisms, including viruses and bacteria are wide spread and are characterized by overlapping clinical presentations. **Objective:** This study utilized a multiplex real-time PCR Respiratory Panel for early, accurate and simultaneous detection of multiple respiratory pathogens in a single test that plays a crucial role in guiding antibacterial and/or antiviral therapy. **Methodology:** This retrospective study was conducted in molecular laboratory of Square Hospitals Ltd. Nasopharyngeal and/or oropharyngeal swabs were collected from patients of all age groups with suspected acute respiratory infections from each setting like IPD, OPD and critical care of the hospital from March 2023 to October 2023. Both viral and bacterial detection were performed following DNA/RNA extraction and Multiplex real-time PCR using VIASURE Respiratory Panel III kit for the period of 8 months. Results were analyzed by software according to manufacturer instructions. **Results:** Among 569 respiratory samples tested, 442(77.7%) samples were positive and total 784 pathogens were detected including virus (42.1%) and bacteria (57.9%), represented as single or co-infection. Of total positives, 70.2% cases were from adults and 29.8% cases from children. Isolation of bacteria was more than virus in both age group with highest prevalence of Streptococcus pneumoniae (53%; 38%) followed by Haemophilus influenzae and Moraxella catarrhalis. The most common detection was Influenza A virus (30%) followed by Respiratory Syncytial Virus (17%), Rhino Virus (15.0%) and Influenza B virus (13%). The overall positive rate for respiratory pathogens in ICU was 48.5%. The results indicated 50% co-infected patient samples, more in children, of which the largest proportion were Influenza A, RSV, Streptococcus pneumoniae and Haemophilus influenzae among 2, 3, 4, 5 different combinations. **Conclusion:** Respiratory panel significantly improve etiological diagnosis of multiple respiratory pathogens which enhance patient care with more rational antimicrobial and/or antiviral use. [Bangladesh

Keywords: Acute respiratory infection; respiratory panel; multiplex PCR; co-infection; atypical pathogen

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Introduction

Acute Respiratory Infection (ARI), ranging from common cold to severe pneumonia is the major cause of outpatient visits and hospitalizations in all age categories worldwide, many of which are associated with significant morbidity and mortality particularly in children, elderly and immunocompromised individual[1]. A large heterogeneous group of typical or atypical bacteria and viruses are responsible for these ARIs that produce similar clinical presentations and put physicians into a diagnostic dilemma. Viral infections, however accounts for approximate 80.0% of ARIs with influenza virus, respiratory syncytial virus, rhinovirus and respiratory adenovirus being the

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most common pathogens[2-4]. Pneumonia is the most common nosocomial infection in the intensive care unit (ICU) and half of deaths from pneumonia are attributable to bacterial infections[5-6]. Highly infectious pathogenic typical bacteria include *Streptococcus pneumoniae*, Haemophilus influenzae and Moraxella catarrhalis. In addition, atypical bacteria specifically *Mycoplasma pneumoniae* is responsible for 10.0 to 30.0% of community-acquired pneumonia[7-9].

The human respiratory tract hosts co-circulating pathogens that causes ARIs with combination of virus/virus, bacteria/bacteria and virus/bacteria, leading to patient's poor outcome[10]. The accurate and rapid detection of causative organisms is helpful in selecting appropriate antiviral or anti-bacterial treatment in time, that improve patient prognosis, prevent overuse of antibiotics and control the outbreak of contagious pathogens. Due to the diversity and complexity of infectious pathogens and overlapping sign-symptoms, specific diagnosis of ARI relies almost entirely on laboratory investigation.

Conventional diagnostic methods such as bacterial culture, targeted polymerase-chain reaction (PCR) assays, rapid viral antigen tests, viral culture and direct fluorescent antibody test have some limitations in comprehensiveness, accuracy, and/or timeliness of results to guide clinical decisions while fail to establish an etiological diagnosis for more than50.0% of patients[11]. Among new molecular technologies, Multiplex RT-PCR assays are fast, accurate, able to detect non-cultivable organisms, can identify multiple respiratory viruses and bacteria in a single run within few hours[12]. Thus, it helps clinicians to distinguish ARI due to viral and/or bacterial origin and assist in delivering proper treatment to patients. A multiplex RT-PCR could simultaneously detect a panel of 22 different respiratory pathogens that included influenza virus A (FluA), influenza virus B (FluB), parainfluenza virus (PIV-1,2,3,4), human respiratory syncytial viruses (RSV), human adenoviruses (AdVs), human metapneumoviruses (HMPV), human coronaviruses (HCoVs-NL63, HKU 1, 229E, OC43), human enteroviruses (EVs), human rhinoviruses (RVs) and bocaviruses (BoV) as well as the fastidious respiratory bacteria including *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, *Chlamydia pneumoniae*, *Mycoplasma pneumoniae* and *Legionella pneumophila* with their co-infections in a single respiratory sample[13-14].

This study was aimed to evaluate efficacy of multiplex RT-PCR Respiratory Panel, as an important tool to detect multiple respiratory pathogens in a single test among all age groups of patients that improve etiological diagnosis of ARI in both hospitalized and outpatient setting in a tertiary care hospital, Bangladesh, rather doing a bundle of routine microbiological diagnostic assays.

Methodology

Study Settings and Population: This retrospective study was conducted in Molecular Laboratory unit of diagnostic Laboratory services of Square Hospital Ltd (SHL), Dhaka, Bangladesh from March to October, 2023 for a period of 8 months. Nasopharyngeal or Oropharyngeal swabs were collected in a sterile screw capped Centrifuge tube containing sterile Normal saline(NS) or Universal transport media (UTM), from patients with symptomatic respiratory tract infection who were visited or admitted in Critical care, Out-patient department (OPD) and In-Patient department (IPD) of Square Hospital Ltd., Dhaka, Bangladesh.

Study Procedure: In this study, the multiplex PCR assay was done by using detection kit (VIASURE Respiratory Panel III Real Time PCR Detection Kit,

Table1: Pathogens detected by VIASURE Respiratory Panel kit

Pathogen	Types(RNA/DNA)
Viruses	
Influenza A (FLU A)	Orthomyxovirus (RNA)
Influenza B (FLU B)	Orthomyxovirus (RNA)
Adenovirus (AdV)	Adenovirus (DNA)
Bocavirus (BoV)	Parvovirus (DNA)
Human Metapneumovirus (HMPV)	Paramyxovirus (RNA)
Enterovirus (EV)	Picornavirus (RNA)
Rhinovirus (RV)	Picornavirus (RNA)
Respiratory syncitial virus (RSV)	Paramyxovirus (RNA)
Parainfluenza virus (PIV 1)	Paramyxovirus (RNA)
Parainfluenza virus (PIV 2)	Paramyxovirus (RNA)
Parainfluenza virus (PIV 3)	Paramyxovirus (RNA)
Parainfluenza virus (PIV 4)	Paramyxovirus (RNA)
Coronavirus NL63	Coronavirus (RNA)
Coronavirus HKU1	Coronavirus (RNA)
Coronavirus OC43	Coronavirus (RNA)
Coronavirus 229E	Coronavirus (RNA)
Bacteria	
Streptococcus pneumoniae (SPN)	Bacterium (DNA)
Haemophilus influenzae (HI)	Bacterium (DNA)
Moraxella catarrhalis (MC)	Bacterium (DNA)
Chlamydia pneumoniae (CP)	Bacterium (DNA)
Legionella pneumophila (LP)	Bacterium (DNA)
Mycoplasma pneumoniae (MP)	Bacterium (DNA)

BIOTEC; Genome Dx; Spain) which is designed for the specific and qualitative detection of DNA/RNA from 16 Viruses and 6 Bacteria in respiratory samples from patients with signs and symptoms of respiratory infection. Pathogens were detected with the VIASURE Respiratory Panel (Table1).

Respiratory Panel Detection Kit: VIASURE Respiratory Panel III Real Time PCR Detection Kit contained all the components necessary for real time PCR assay (A mix of enzymes, primers, probes, buffer, dNTPs, stabilizers and internal control in stabilized format) in each well. Rehydration buffer is used to reconstitute the stabilized product, positive control and negative control. RNAse/DNAse free water and Optical caps for sealing wells during thermal cycling were included in the kit also.

PCR Technique: Nucleic acid (RNA/DNA) extraction was performed from samples either by using nucleic acid extraction kit manually or by auto-extraction machine according to manufacturer instructions[13]. Nucleic acids (RNA/DNA) were isolated by amplification of a conserved region of the specific genes using specific primers and fluorescent–labelled probes, which then used for Respiratory pathogens identification. To reconstitute the number of wells

Table 2: Programming for thermal cycling

Cycles	Steps	Time	Temp
1	Reverse transcription	15 min	45°C
1	Initial denaturation	2 min	95°C
45	Denaturation	10 second	95°C
	Annealing/ Extension	50 second	60°C

Table 3: Findings Interpretation

Reaction Mix	Pathogens	Channels			
		FAM	HEX	ROX	Cy5
ABR	Influenza A	+ve			
	Internal Control		+ve/-ve		
	Influenza B			+ve	
	Respiratory Syncytial Virus				+ve
PAC	Parainfluenza virus 3	+ve			
	Internal Control		+ve/-ve		
	Parainfluenza virus 1				+ve
PBD	Parainfluenza virus 4	+ve			
	Internal Control		+ve/-ve		
	Parainfluenza virus 2				+ve
AMB	Adenovirus	+ve			
	Internal Control		+ve/-ve		
	Human Metapneumovirus			+ve	
	Bocavirus				+ve
RHE	Rhinovirus	+ve			
	Enterovirus		+ve		
	Internal Control				+ve/-ve
COR	Corona virus 229E	+ve			
	Corona virus HKU 1		+ve		
	Corona virus NL63			+ve	
	Corona virus OC43				+ve
CML	Legionella pneumophila	+ve			
	Internal Control		+ve/-ve		
	Chlamydia pneumoniae			+ve	
	Mycoplasma pneumoniae				+ve
HSM	Haemophilus influenzae	+ve			
	Internal Control		+ve/-ve		
	Streptococcus pneumoniae			+ve	
	Moraxella catarrhalis				+ve

needed, 15µL of Rehydration Buffer is added into each well. Then, 5µL of RNA/DNA sample, reconstituted Respiratory Panel III Positive Control or Negative Control were added in different wells and closed them with the provided caps. Finally, the plate or the strips were loaded in the thermal cycler for amplification. The thermocycler (either Lightcycler, Cobas Z480, RotorGeneQ 6000) was programmed for 40 cycles for DNA amplification (Table 2).

Finally, the amplified RNA/DNA was determined using fluorescent signals of the samples and observed in Real Time on the FAM, ROX, Cy5 and/or HEX (JOE or VIC) channels. The analysis of the samples and controls are done by the software used by the real time PCR equipment itself following manufacturer´s manual[13].

Findings Interpretation: Amplification curves of detected pathogens were viewed with corresponding cycle threshold (CT) and endpoint fluorescence (EP) values. The overall procedures took approximately four hours (4h) for a single test[13]. Results were read, analyzed and interpreted according to manufacturer instruction (Table 3).

Statistical Analysis: Statistical analysis was performed by Windows based software named as Statistical Package for Social Science (SPSS), versions 22.0 (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.). Categorical data were summarized in terms of frequency counts and percentages. Continuous data were expressed as mean, standard deviation, minimum and maximum.

Ethical Clearance: Ethical approval for the study was obtained from the Square Hospitals Ltd., Dhaka, Bangladesh Medical Review Board. All methods were performed in accordance with the relevant guidelines and regulations. All procedures of the present study were carried out in accordance with the principles for human investigations (i.e., Helsinki Declaration 2013) and also with the ethical guidelines of the Institutional research ethics. Participants in the study were informed about the procedure and purpose of the study and confidentiality of information provided. All participants consented willingly to be a part of the study during the data collection periods. All data were collected anonymously and were analyzed using the coding system.

Results

A total of 569 Nasopharyngeal or Oropharyngeal swab samples were collected from patients with Respiratory Tract Infection (RTI) during our eight (8) months of study period from March to October, 2023. Of the 569 samples tested; 442(77.7%) had single/multiple of listed virus/bacteria in the panel and 127(22.3%) samples were negative for any pathogens (Figure I).

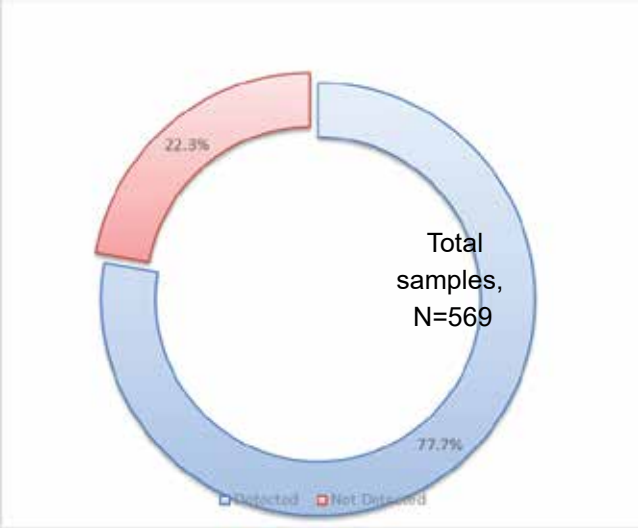


Figure I: Distribution of Respiratory samples by Panel Tests (N=569): March-October, 2023 *(Values represent the percentage of samples studied)*

Over the entire study period, total 784 respiratory pathogens including viruses and bacteria either single or in-combination were detected from 442 positive samples. The results vary between children and adults, as shown in (Table 4). Both in adult and children group, bacteria were isolated more than viruses. *Streptococcus pneumoniae* (SPN) was the highest in number among >18 years (53%) of age compared to <18 years (38%). *Haemophilus influenzae* (HI) and *Moraxella catarrhalis* (MC) were also higher than any virus in children but less than Influenza A virus in adult. *Mycoplasma pneumoniae* (MP), represented as an atypical bacteria causing pneumonia was found more among adult than in children. Study showed Respiratory syncytial virus (RSV) more prevalent in children (20.0%) than in adult (15.0%), whereas Influenza A and Influenza B virus higher among adult (36.5%; 14.0%) compared to children (18.0%; 11.0%). Isolation of all other viruses found higher in children than in adult except Human Metapneumovirus (HMPV) and different types of Coronavirus. Of the positive isolates, detection of more than one respiratory pathogen was found in 531/784 (68.0%) with a higher co-detection rate in the children's group (75.2%) than in the adult group (64.5%).

Respiratory panel test detected total 330 viruses in this

Table 4: Detection of Respiratory Organisms Tested Among Adults and Children

Pathogens (N=784)	**Age Groups**			
	<18 Years		**>18 Years**	
	Positive	**Co-detected**	**Positive**	**Co-detected**
Virus Panel				
Respiratory syncytial virus (RSV)	22 (20)	17 (81)	34 (15)	18 (53)
Influenza A	19 (17)	16 (80)	81(36.5)	54 (67)
Rhinovirus (RV)	20 (18)	17 (85)	29(13)	19 (66)
Adenovirus (AdV)	14 (13)	12 (86)	4 (2)	00 (0)
Influenza B	12 (11)	06 (50)	31 (14)	16 (52)
Enterovirus (EV)	6 (6)	00 (0)	10 (4)	05 (50)
Human Metapneumovirus (HMPV)	4 (4)	00 (0)	9 (4)	02 (22)
Parainfluenza virus (PIV 3)	3 (3)	02 (67)	6 (3)	00 (0)
Bocavirus (BoV)	3 (3)	00 (0)	2 (1)	00 (0)
Parainfluenza virus (PIV 1)	2 (2)	00 (0)	2 (1)	01 (50)
Parainfluenza virus (PIV 4)	2 (2)	01 (50)	2 (1)	00 (0)
Parainfluenza virus (PIV 2)	1 (1)	00 (0)	1 (0.5)	00 (0)
Coronaviruses [NL63, HKU1,OC43, 229E]	0 (0)	00 (0)	11 (5)	10 (91)
Total Virus	**108(46.2%)**		**222(40.4%)**	
Bacterial Panel				
Streptococcus pneumoniae (SPN)	48 (38)	40 (83)	173 (53)	117(68)
Haemophilus influenzae (HI)	42 (34)	38 (91)	72 (22)	55(76)
Moraxella catarrhalis (MC)	28 (22)	27 (97)	69 (21)	51(74)
Mycoplasma pneumoniae (MP)	8 (6)	00 (0)	14 (4)	07(50)
Total Bacteria Detected	**126(53.8%)**		**328(59.6%)**	
Total Detected Pathogens	234(29.8%)	176/234; 75.2%	550(70.2%)	355/550; 64.5%

study. The most frequently reported respiratory viruses were influenza A (100) followed by Respiratory Syncytial Virus A/B (56), Rhinovirus (49), Influenza B (43), Adenovirus (18), Enterovirus (16) and Human Metapneumovirus (13) respectively. The detection rates of other respiratory viruses were less than 10 in number (Figure III).

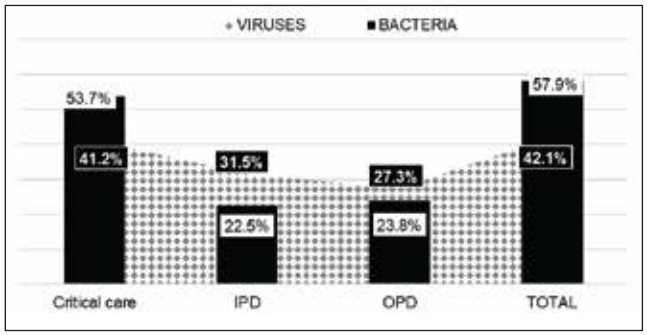
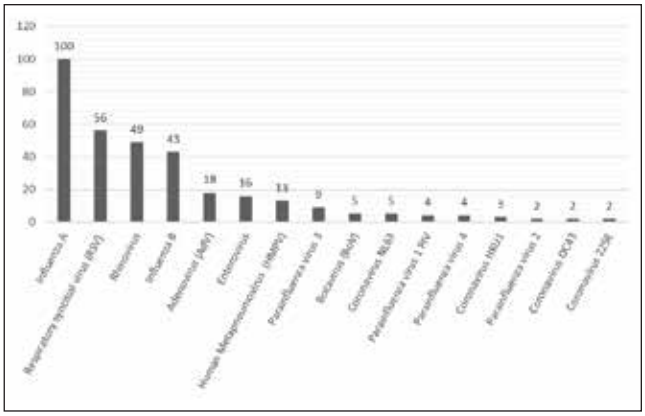


Figure II: Isolation rate of respiratory pathogens (N=784) in different units of hospital *(Values represent the percentage of isolates studied)*



FigureIII: Frequency of Isolated Viruses (N=330) *(Values represent the number of isolates studied)*

VIASURE multiplex real-time RT-PCR assay was applied to detect 3 typical and 3 atypical bacteria simultaneously. A total of 454 (57.9%) bacteria were identified, of which 3 typical and only one atypical bacteria, specifically Mycoplasma pneumoniae (4.0%). Among typical ones, most prevalent was Streptococcus

pneumoniae (48%) followed by Haemophilus influenzae (26.0%) and Moraxella catarrhalis (22.0%). Other two atypical bacteria like Chlamydia pneumoniae (CP) and Legionella pneumophila (LP) were not found in our study (Figure IV).

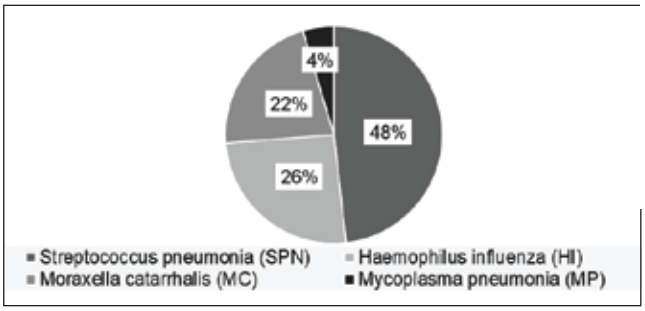


Figure IV: Prevalence of Respiratory Bacteria(N=454) *(Values represent the percentage of isolates studied)*

The assay detected 49.5% (219) positive cases with single bacterial or viral infection, while more than one respiratory pathogen in 50.5% (223) of the detected samples. Bacterial-viral co-detections were found most prevalent (167) followed by bacterial-bacterial (46) and Viral co-infections (10) (Figure V).

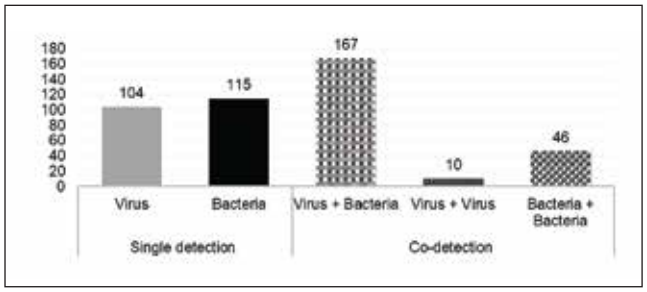


Figure V: Comparison of Single and Co-Isolation among Detected Samples (Values represent the number of samples studied)

A total of 46bacterial co-infections were detected by multiplex RT-PCR used in this study, mostly involving *Streptococcus pneumoniae* and H*aemophilus influenzae*. The most commonly prevailed co-infection was *Streptococcus pneumoniae* combined with *Moraxella catarrhalis* (39%), followed by *Streptococcus pneumoniae* plus Haemophilus influenzae (31.0%). A triple combination of *Streptococcus pneumoniae, Moraxella catarrhalis* and *Haemophilus influenzae* took third position (11%). *Mycoplasma pneumoniae* also being part of double and triple co-infections (Figure VI).

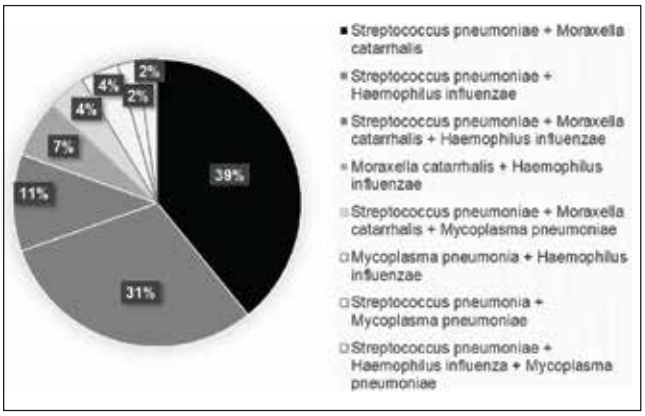


Figure VI: gure VI: Distribution of Co-Detected Bacteria among study samples *(Values represent the percentage of isolates studied)*

Table 6: Summary of Double organisms among Viral-Bacterial co-infection positive samples

Co-infection of Pathogens (Virus + Bacteria)	**N=167**
Double infections	93(55.6%)
Influenza A virus Plus Streptococcus pneumoniae	25
Respiratory Syncytial virus (RSV) A+B Plus Streptococcus pneumoniae	10
Influenza A virus Plus Haemophilus influenzae	7
Influenza B virus Plus Streptococcus pneumoniae	6
Rhinovirus Plus Streptococcus pneumoniae	6
Influenza A virus Plus Moraxella catarrhalis	5
Influenza B virus Plus Moraxella catarrhalis	5
Respiratory Syncytial virus (RSV) A+B Plus Haemophilus influenzae	5
Parainfluenza virus (PIV 3) Plus Streptococcus pneumonia/ RSV Plus Moraxella catarrhalis	3
Respiratory Syncytial virus(RSV) A+B	3
Influenza B virus plus Haemophilus influenzae	2
Parainfluenza virus (PIV 3) + Haemophilus influenzae	2
Adenovirus plus Haemophilus influenzae	2
Rhinovirus plus Haemophilus influenzae	2
Rhinovirus plus Moraxella catarrhalis	2
Influenza A virus + Mycoplasma pneumoniae	1
Parainfluenza virus (PIV 2)Plus Streptococcus pneumoniae	1
Parainfluenza virus (PIV 4)Plus Streptococcus pneumoniae	1
Human Metapneumovirus Plus Streptococcus pneumoniae	1
Human Metapneumovirus Plus Moraxella catarrhalis	1
Coronavirus OC43 Plus Streptococcus pneumoniae	1
Coronavirus NL63 Plus Moraxella catarrhalis	1
Coronavirus 229E Plus Mycoplasma pneumoniae	1

We found total 10 viral co-detections, of which 7 were dual, 2 triple and 1 quadruple infections, one each in all combinations (Table 5).

The complexity of 167 virus-bacteria co-infections can be subdivided into 93 cases of dual infections, 53 triple infections, 15 quadruple infections and 6 quintuple Infections. Among double combinations, 25 cases has Influenza A combined with Streptococcus pneumoniae, 10 cases of RSV with Streptococcus pneumoniae, 7 cases of Influenza A with Haemophilus influenzae, 06 cases of each Streptococcus pneumoniae combined

Table 5: Viral Combination among Co-Detected Positive Samples

Co-infection of Pathogens(Virus+Virus) (N=10)	Frequency
Influenza A virus + Adenovirus +Bocavirus+ Coronavirus HKU 1	1
Adenovirus +Bocavirus+ Respiratory Syncytial virus (RSV) A+B	1
Adenovirus +Bocavirus+ Rhinovirus	1
Influenza A virus + Influenza B virus	1
Influenza A virus + Respiratory Syncytial virus (RSV) A+B	1
Respiratory Syncytial virus (RSV) A+B + Rhinovirus	1
Respiratory Syncytial virus (RSV) A+B + Enterovirus	1
Enterovirus +Adenovirus	1
Enterovirus + Rhinovirus	1
Parainfluenzae virus (PIV 3) plus Bocavirus	1

Table 7: Summary of Viral-Bacterial Co-Infections Containing Triple Organisms

Co-infection of Pathogens (Virus+Bacteria)	N=167
Triple Infections	53(31.7%)
Influenza A virus +Streptococcus pneumoniae + Haemophilus influenzae	9
Influenza A virus +Streptococcus pneumoniae + Moraxella catarrhalis	4
Influenza B virus +Streptococcus pneumoniae + Moraxella catarrhalis	4
Adenovirus + Respiratory Syncytial virus (RSV) A+B + Streptococcus pneumoniae	2
Rhinovirus + Parainfluenza virus (PIV 1)+ Streptococcus pneumoniae	2
Rhinovirus + Streptococcus pneumoniae + Haemophilus influenzae	2
Adenovirus + Streptococcus pneumoniae + Haemophilus influenzae	2
Adenovirus + Streptococcus pneumoniae + Moraxella catarrhalis	2
Influenza A virus + Rhinovirus +Moraxella catarrhalis	1
Influenza A virus + Parainfluenza virus (PIV 3)+ Moraxella catarrhalis	1
Influenza A virus + Rhinovirus +Haemophilus influenzae	1
Influenza A virus + Enterovirus + Haemophilus influenzae	1
Influenza A virus +Streptococcus pneumoniae + Mycoplasma pneumoniae	1
Influenza A virus + Haemophilus influenzae + Moraxella catarrhalis	1
Influenza B virus + Enterovirus + Streptococcus pneumoniae	1
Influenza B virus +Streptococcus pneumoniae +Haemophilus influenzae	1
Parainfluenza virus (PIV 1)+ Rhinovirus +Haemophilus influenzae	1
Parainfluenza virus (PIV 3) +Streptococcus pneumoniae + Haemophilus influenzae	1
Parainfluenza virus (PIV 4)+ Streptococcus pneumoniae + Moraxella catarrhalis	1
Respiratory Syncytial virus (RSV) A+B + Rhinovirus +Haemophilus influenzae	1
Respiratory Syncytial virus (RSV) A+B + Streptococcus pneumoniae+ Haemophilus influenzae	1
Respiratory Syncytial virus (RSV) A+B + Streptococcus pneumoniae+ Mycoplasma pneumoniae	1
Respiratory Syncytial virus (RSV) A+B + Moraxella catarrhalis + Haemophilus influenzae	1
Adenovirus + Respiratory Syncytial virus (RSV) A+B + Haemophilus influenzae	1
Adenovirus + Rhinovirus + Moraxella catarrhalis	1
Rhinovirus + Coronavirus OC43 + Streptococcus pneumoniae	1
Rhinovirus + Enterovirus + Mycoplasma pneumoniae	1
Rhinovirus + Streptococcus pneumonia + Mycoplasma pneumoniae	1
Rhinovirus + Haemophilus influenzae + Moraxella catarrhalis	1
Human Metapneumovirus + Streptococcus pneumoniae + Moraxella catarrhalis	1
Human Metapneumovirus + Streptococcus pneumoniae + Haemophilus influenzae	1
Human Metapneumovirus + Moraxella catarrhalis + Haemophilus influenzae	1
Coronavirus NL63 + Streptococcus pneumoniae + Haemophilus influenzae	1
Coronavirus 229E + Streptococcus pneumoniae + Haemophilus influenzae	1

Table 8: Summary of Quadruple Co-Infections (Virus plus Bacteria) Positive Samples

Co-Infection of Pathogens (Virus Plus Bacteria)	**N=167**
Quadruple Infections	15(8.9%)
Influenza A virus +Streptococcus pneumoniae + Moraxella catarrhalis + Haemophilus influenzae	3
Respiratory Syncytial virus (RSV) + Enterovirus + Streptococcus pneumoniae + Moraxella catarrhalis	2
Influenza A virus + Rhinovirus + Streptococcus pneumoniae + Moraxella catarrhalis	1
Influenza A virus + Enterovirus + Streptococcus pneumoniae + Haemophilus influenzae	1
Influenza A virus + Influenza B virus + Coronavirus NL63 + Streptococcus pneumoniae	1
Influenza B virus + Adenovirus + Rhinovirus + Haemophilus influenzae	1
Influenza B virus + Streptococcus pneumoniae + Moraxella catarrhalis + Haemophilus influenzae	1
Influenza A virus + Rhinovirus + Streptococcus pneumoniae + Moraxella catarrhalis	1
Influenza A virus + Enterovirus + Streptococcus pneumoniae + Haemophilus influenzae	1
Influenza A virus + Influenza B virus + Coronavirus NL63 + Streptococcus pneumoniae	1
Influenza B virus + Adenovirus + Rhinovirus + Haemophilus influenzae	1
Influenza B virus + Streptococcus pneumoniae + Moraxella catarrhalis + Haemophilus influenzae	1

Table 9: Summary of quintuple co-infections (Virus plus Bacteria) positive samples

Co-infection of Pathogens (Virus plus Bacteria)	**N=167**
Quintuple Infections	6(3.6%)
Influenza A virus + Rhinovirus + Streptococcus pneumoniae + Moraxella catarrhalis + Haemophilus influenzae	2
Influenza A virus + Respiratory Syncytial virus (RSV) A+B + Streptococcus pneumoniae +Moraxella catarrhalis + Haemophilus influenzae	1
Influenza B virus + Enterovirus + Bocavirus + Coronavirus HKU1+Streptococcus pneumoniae	1
Rhinovirus + Human Metapneumovirus + Streptococcus pneumoniae + Haemophilus influenzae + Mycoplasma pneumoniae	1
Rhinovirus + Respiratory Syncytial virus (RSV) A+B + Coronavirus NL63 + Streptococcus pneumoniae + hilus influenzae	1

with either Influenza B or Rhinovirus and 05 cases of each found Influenza A and Moraxella catarrhalis, Influenza B and Moraxella catarrhalis, RSV and Haemophilus influenzae (Table 6).

Of total 53 triple infections, most prevalent co-infection found Influenza A combined with Haemophilus influenzae and Streptococcus pneumoniae (9). Other 26 combinations have highest isolation of Streptococcus pneumoniae (Table 7).

We detected 15 cases of quadruple combinations, of which Streptococcus pneumoniae and Influenza A virus were found in most cases (Table 8).

Among 6 cases with five pathogens, Streptococcus pneumoniae, Moraxella catarrhalis, Haemophilus influenzae and Influenza A combined with RV in 2 cases and RSV in one case. Other three also has Streptococcus pneumoniae with other different viruses and bacteria (Table 9).

Discussion

Respiratory infections due to various causes was found in Bangladesh. This study provides a glimpse of such infections from a substantial number of samples from patients with suspected pulmonary infection. A Multiplex RT-PCR method is used to detect Respiratory viruses and bacteria among adults and children. The outbreak of SARS, the threat of human avian H5N1 influenza virus cases, presence of H1N1 influenza A and the recent 2020 Covid-19 pandemic has heighten the need for improved diagnostic tests for respiratory pathogens[15].

Molecular assays for identification and simultaneous detection of common respiratory pathogens are now widely used, replacing many conventional diagnostic methods. The main impact of this multiplex PCR was its broader spectrum of detection[16]. In the current study, we can detect 22 selected respiratory pathogens causing acute respiratory infections in clinical practice

and provided the widest spectrum ever reported. Multiplex RT-PCR assays display a variety of benefits, including a significant reduction in the turnaround time with results being available within the same day of specimen collection and become more economical as multiple pathogens can be detected in a single assay[17]. A nucleic acid extraction kit was used to extract both DNA and RNA simultaneously and the study can be done with small amount of samples, along with minimizing time, labor, and materials involved in nucleic acid extraction. Our reporting time of 4 hour was consistent with outcomes reported in previous studies where reporting mean time was 3.1 hour[18]. The present study results showed an increase in diagnosis within designated TAT that help in timely implementation of isolation precaution and judicious use of antimicrobials.

Acute respiratory infections are caused by a complex array of pathogens, most commonly viruses and bacteria, as well as atypical microorganisms like Mycoplasma and Chlamydia[18,19]. Furthermore, clinical diagnosis is complicated with co-infection of several pathogens leading to treatment difficulty of acute respiratory infection[20]. In this study, 569 nasopharyngeal and oropharyngeal specimens were collected and analyzed with VIASURE Multiplex Respiratory panel over a period of eight months. The overall results revealed a positive rate of 77.7%, in agreement with the range of positive rates (33.39 to 65.2%) reported in other studies[1,21]. Both virus and bacteria are identified among these positive samples either single or in combination, leads to total detection of 784 organisms responsible for ARIs. Of them, 70% were from adult and 30% belong to children which is contrast to other studies where positivity rate was more in pediatric patients than in adult[22,23]. Respiratory tract infection accounts for a majority of the admissions in acute care hospitals and like other studies we also found more than half of both respiratory bacteria and virus in critical care than in wards and outpatient department[17].

Virus has found at a rate of 42.1% which is higher than the reported previous studies[23,24]. This can be at least partially explained by the use of a sensitive multiplex RT-PCR, which can detect 16 different viruses and improve the diagnostic yield. Of them, approximately one-third were Influenza A (33%) virus in all age groups. Other important viruses detected are RSV in 19.0% of positive samples followed by Rhinovirus in 16.0% of positive samples and Influenza B which represented 14.0% of positive cases. These results are in line with surveillance studies done by the Centers for Disease Control and Prevention (CDC), which indicated that 99.8% of influenza viruses isolated were type A and 0.2% were type B[25]. The findings of the present study did not agree with studies by Brittain-Long et al[26] where they found Rhinovirus as highest isolation followed by Influenza A virus, and RSV. Avcu et al[27] also reported that viral agents were detected in 83.3% of patients by molecular methods and RSV has been reported as the most common agent. Rapid molecular diagnostic tests for relevant bacterial pathogens in addition to viral targets can limit antibacterial therapy[28]. More bacteria were isolated than virus in both age group with highest prevalence of Streptococcus pneumonia in this study. Haemophilus influenzae and Moraxella catarrhalis were more than any virus in children but less than Influenza A virus in adult. These results are in line with former report[29]. We detect Mycoplasma pneumoniae at a rate of 2.8%. Previous studies have reported varying detection rates of Mycoplasma pneumoniae: 1.7% (adults and children in U.S.) to 7.1% (adults in Shanghai, China) which might be attributed to the difference in collection sites[30,31].

Various studies found that at least one viral or bacterial respiratory pathogen detection rate ranged from 41.8% to 78.6%, which is in accordance with our findings of 49.5% positivity. Co-infection has been found in 31.0% to 51.8% of positive respiratory samples in previous studies[19,20,22,32,33]. In our study, a higher rate of 68.0% co-infection was detected. Of them, children belong the highest which is almost similar with previously published data[34-35]. We detect three types of co-infection including Viral/viral (10), Bacterial/bacterial (46) and viral/bacterial (167) with 2, 3, 4, and up to 5 different combinations. The specific and sensitive detection of this study revealed that, of 167 mixed infections; 93 were dual, 53 triple, 15 quadruple and 6 quintuple positive samples. Only dual and triple combinations are found among viral/viral and bacterial/bacterial co-detected samples. The largest proportion of co-detected pathogens was Influenza A combined with Streptococcus pneumonia (25) followed by Streptococcus pneumonia and Moraxella catarrhalis (18), Streptococcus pneumonia and Haemophilus influenzae (14) and RSV combined with Streptococcus pneumonia (10), similar evidence were provided by other independent studies[7,8,10,12,17,36]. This study found three patients with quadruple

infections detected as Influenza A combined with *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis*; two cases of quintuple infections included Influenza A/RV and *Streptococcus pneumonia* / *Haemophilus influenzae* / Moraxella catarrhalis. Such findings constitute strong evidence to support that hospitalized patients with acute respiratory infections are likely to be infected by more than one pathogen. However, the predominance of bacteria include *Streptococcus pneumonia*, *Haemophilus influenzae* and viruses include Influenza, RV and RSV in our single and multiple detections, all these findings are in line with previously published data[9-11,17,24]. There is no doubt that this multiplex real-time PCR assay will significantly expand the diagnostic potential for a careful evaluation of concomitant bacterial infection to patients with positive results of viral examination, thus help clinicians to adopt rapid and accurate antibiotic treatment regimens.

There were several limitations to our study. First, the retrospective design may have led to an inevitable selection bias. Second, our specimens are collected from a single location over eight months which may limit the general applicability of the findings and seasonal variation cannot be detected. Third, analyzing the clinical feature was beyond the scope of this study and thus the demographic data, clinical presentation with severity and length of hospitalization were not provided in our results. Fourth, commercial assays used in this study are invariably expensive and difficult to implement in resource poor settings, also cannot be quickly modified when new pathogens or new strains of known pathogens emerge which are missed by the existing assays.

Conclusion

In conclusion, the prevalence of respiratory pathogens in this institution is high with Respiratory Panel, of which majority is bacteria. Influenza virus, RSV and RV/EV are the most important viral agents of ARIs, while among bacteria, *Streptococcus pneumoniae* is the highest isolated followed by *Haemophilus influenzae* and *Moraxella catarrhalis*. The only atypical bacteria identified is *Mycoplasma pneumoniae*. Present study has detected in half of co-infection of which higher rate is in children. Bacterial-viral co-detections has found most prevalent followed by bacterial-bacterial and viral co-infections. Due to similarities of viral and bacterial ARIs with inconclusive laboratory findings, a diagnostic dilemma appears. Implementation of Panel test detect different respiratory pathogens simultaneously, provides rapid and high-yield results which can guide diagnosis and enhance a more rational use of antibiotics and/or antivirals. Future prospective studies for further assessing 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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Conflict of Interest

The authors have no financial or other conflicts of interest involved in producing this manuscript for publication

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Authors' contributions

Nurun Nahar Mawla conceived and designed the study, analyzed the data, interpreted the results, and wrote up the draft manuscript. Ms. Marynatun Nessa contributed to the analysis of the data. Prakash Nandi, Md. Mustafizur Rahman, Binod Saha and Md. Omar Faruk helped in data collection. Md Anowar Hossain critically reviewed and edit the manuscript. Shagufta Mahmood involved in the manuscript review. All authors read and approved the final manuscript.

Data Availability

Any inquiries regarding supporting data availability of this study should be directed to the corresponding author and are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate

Ethical approval for the study was obtained from the Institutional Review Board. As this was a prospective study the written informed consent was obtained from all study participants. All methods were performed in accordance with the relevant guidelines and regulations.

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