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Research Article

The Demographic and Clinical Characteristics of the Co-infection for the Detection of SARS-COV-2 and Influenza A Virus by rRT-PCR

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ABSTRACT

The potential for co-infection with COVID-19 and other respiratory infections raises the possibility that a new severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) mimics the influenza A virus regarding methods and modes of transmission, clinical features, related immune responses, and seasonal coincidence. This study aimed to investigate the presence of SARS-CoV-2 and influenza A virus coinfections in admitted patients of a tertiary care hospital. In this study, the total included 589 admitted patients in our tertiary care hospital. The detection of co-infection between SARS-CoV-2 and influenza A virus by real-time reverse-transcriptase polymerase chain reaction (rRT-PCR) in a patient during the second wave of the COVID-19 pandemic. There were 207 (35.1%) patients infected with SARS-CoV-2 and 43 (7.3%) patients infected with the influenza A virus. Only 6 (1.0%) patients were infected with SARS-CoV-2 and influenza A viruses. The females were more likely to be infected with SARS-CoV-2 than non-infected SARS-CoV-2 case-patients (60.9% (n = 126) vs. 31.4% (n = 120), and also with the influenza A virus compared with influenza-negative patients (40.9% (n = 223) *vs.* 55.9% (n = 24). In conclusion, our results strongly suggest that influenza A co-infects with SARS-CoV-2. The patients with SARS-CoV-2 and influenza A co-infection had similar clinical characteristics as those with SARS-CoV-2 infection alone. Comorbidities, like hypertension and diabetes, and increasing age make patients more susceptible to SARS-CoV-2 and influenza A coinfections.

Keywords: COVID-19, Co-infection, Influenza A virus, SARS-CoV-2 infection, Real-time reverse-transcriptase polymerase chain reaction.

INTRODUCTION

There currently exists a worldwide epidemic as a consequence of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) outbreak that ceased in January of 2019. Over 90 million confirmed virus cases and 1.9 million deaths have occurred worldwide as of January 13, 2021 [1]. It is still unknown whether the current COVID-19 pandemic wave will come to an end as well as how severe the situation will ultimately become. In the meantime, the current pandemic and the influenza season are combining to potentially cause more difficulties and higher risks to public health [2,3]. There has been extensive discussion on whether vaccination against influenza is required for the upcoming winter, as well as if seasonal flu will affect the COVID-19 pandemic's intensity. Nevertheless, there is currently no laboratory evidence of co-infection between IAV and SARS-CoV-2 [4–7, 9–13].

It is commonly known that the symptoms of an IAV infection, such as fever, cough, pneumonia, and acute respiratory distress syndrome, are quite similar to those of a SARS-CoV-2 infection [8]. Furthermore, the respiratory tract as well as nasal, bronchial, and alveolar epithelial cultures are infected by both SARS-CoV-2 and IAV, which are airborne pathogens [14]. Moreover, alveolar type II cells, or AT2 pneumocytes, seem to be the primary target of SARS-CoV-2 infection and IAV replication. Hence, a sizable population may be at a heightened risk of contracting both the COVID-19 pandemic and seasonal influenza concurrently due to their overlap.

A relatively low IAV rate of infection resulting from social distancing probably led to the inadequacy of epidemiological evidence gathered during the most severe winter flu season in the southern hemisphere on the interaction between COVID-19 and flu. According to a case report, the respiratory health of three out of four individuals who are coinfected with SARS-CoV-2 and IAV rapidly deteriorates [15–17, 19–22]. Another study, however, only found modest symptoms in outpatients with limited coinfection. According to a retrospective investigation, 307 COVID-19 patients in a single-centered study conducted during the Wuhan outbreak period had a coinfection rate of up to 57.3% between the influenza virus and SARS-CoV-2. Therefore, when considering the hazards of both viruses, the high likelihood of coinfection and the uncertain nature of clinical results present serious issues [18, 22-24]. Therefore, our aim was to investigate the presence of SARS-CoV-2 and influenza A virus coinfections in admitted patients of a tertiary care hospital.

Table 1: Primers and probe sequences for Influenza A and SARS-CoV-2 by real-time RT-PCR

Specificity	*Primer*	*Sequence 5'-3'*	*References*
(H1N1) 2009 Matrix Gene	M(76)-For	TCAGGCCCCCTCAAAGCCGA	15
	M(99)-Probe	FAMa-CGCGCAGAGACTGGAAAGTGTC-TAMRAb	
	M(234)-Rev	GGGCACGGTGAGCGTGAACA	
RdRp gene / nCoV_IP2	nCoV_IP2-12669Fw	ATGAGCTTAGTCCTGTTG	5
	nCoV_IP2-12759Rv	CTCCCTTTGTTGTGTTGT	
	nCoV_IP2-12696bProbe(+)	AGATGTCTTGTGCTGCCGGTA [5']Hex [3']BHQ-1	
RdRp gene / nCoV_IP4	nCoV_IP4-14059Fw	GGTAACTGGTATGATTTCG	
	nCoV_IP4-14146Rv	CTGGTCAAGGTTAATATAGG	
	nCoV_IP4-14084Probe(+)	TCATACAAACCACGCCAGG [5']Fam [3']BHQ-1	
E gene / E_Sarbeco	(CoVE) E_Sarbeco_F1	ACAGGTACGTTAATAGTTAATAGCGT	
	E_Sarbeco_R2	ATATTGCAGCAGTACGCACACA	
	E_Sarbeco_P1	ACACTAGCCATCCTTACTGCGCTTCG [5']Fam [3']BHQ-1	

MATERIAL AND METHODS

Patient's Demography

Patients with high suspicion of SARS-CoV-2/Influenza A infection were admitted to a center's COVID ward in May and June 2021. Written, informed consent was obtained from patients of varying severity, age, and gender.

Oligonucleotide Design

Gene sequences from endemic influenza A virus strains were retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/index.html). The TaqMan probe was dual-labeled with 6-carboxyfluorescein (FAM) at the 5' end and with tetramethylrhodamine (TAMRA) at the 3' end [15].

The GISAID database released the first SARS-CoV-2 sequences on January 11, 2020, leading to the design of primers and probes targeting the RdRp+ N gene. Primer sets nCoV_IP2 and nCoV_IP4 can be multiplexed in Table 1.

Extraction of Viral RNA

The handling of respiratory samples and the preparation of aliquots were performed in accordance with relevant guidelines and regulations (ICMR-NIV). SARS-CoV-2 and H1N1 RNA were extracted using the Insta NX Viral RNA purification kit with the help of an automated nucleic acid extractor. RT-PCR was carried out using the Insta Q 96 Plus LA1012 instrument using the TRUPCR SARS-CoV-2 RT qPCR Kit and the TRUPCR H1N1 detection kit (3B BlackBio Biotech India) with internal, positive, and negative controls.

Real-time PCR Confirmation of SARS-CoV-2 and Influenza A Virus

The detection of SARS-CoV-2 by RT-PCR assay targeting the E gene and RdRp with the real-time PCR-based single-tube detection of the SARS-CoV-2 virus RNA kit. Thermal cycling conditions include reverse transcription at 50°C for 15 minutes, followed by 95°C for 5 minutes, 95°C for 5 seconds, 60°C for 40 seconds, and lastly 72°C for 15 seconds.

The H1N1RT-PCR assay included four positive controls and four negative controls for each primer-probe, such as infA, infApdm, H1N1 pdm, and RNAsP. In 15 μL of the prepared reaction mix is transferred to PCR tubes. Add 10 μL of extracted RNA samples or 5 μL of positive or negative control to each 0.2 mL PCR tube. This brought the total volume to 25 μL. Thermal cycling conditions include reverse transcription for 20 minutes at 50°C, denaturation for 10 minutes at 94°C, followed by 45 cycles of primers' annealing for 15 seconds at 94°C, 30 seconds of template extension at 55°C, and lastly 72°C for 30 seconds. The positivity or negativity of the results was determined according to the manufacturer's interpretation guide.

Statistical Analysis

The data was anonymized and presented in percentages and figures.

Ethics Statement

The study was approved by the Ethics Committee of Vedantaa Institute of Medical Sciences, Palghar (approval number: EC/03/2021).

RESULTS AND DISCUSSION

Demographic and Clinical Characteristics

Demographic and clinical information concerning the SARS-CoV-2 virus and influenza A virus was reported in Table 2. There were 207 (35.1%) patients infected with SARS-CoV-2 and 43 (7.3%) patients infected with the influenza A virus. Only 6 (1.0%) patients were infected with SARS-CoV-2 and influenza A viruses.

Zang *et al.*[22], Kim *et al.*[12], and Khodamoradi *et al.* [10] reported that the USA (0.9%) and China (0.4%) showed relatively low rates of influenza-related coinfection. However, another study by Ma *et al.* [14] using larger cohorts showed that influenza A was significantly higher (52%) among SARS-CoV-2 laboratory-confirmed patients and that 20% of individuals in China and the USA also had other respiratory infections.

Patients of age group >60 were more likely to be infected with SARS-CoV-2 (20.2% (n = 42) vs. 12.3% (n = 47)), but less likely if age is <60 years (79.8% (n = 165) vs. 87.7% (n = 335)). Alternatively,

those aged >60 years were more likely to be infected with the influenza A virus than non-influenza case patients (16.2% (n = 7) vs. 15.8% (n = 86)).

Age has been identified as a major risk factor for COVID-19 disease, according to research from Li *et al.* [13] in China. In our study, females were more likely to be infected with SARS-CoV-2 than non-infected SARS-CoV-2 case-patients (60.9% (n = 126) vs. 31.4% (n = 120)) and also with the influenza A virus compared with influenza-negative patients (40.9% (n = 223) vs. 55.9% (n = 24)). Males were reported to be more infected than females, according to Li *et al.*'s findings [13]. Female participants outnumbered male participants in one region of our study. Five (83.3%) female patients were coinfected with the SARS-CoV-2 and influenza A viruses.

During the early pandemic period, Chinese researchers reported fever and cough to be the most prevalent symptoms studied by Yang *et al.* [21]. Clinical symptoms were more likely to be present among SARS-CoV-2-infected patients compared with those without any SARS-CoV-2: difficulty breathing (n = 169) vs. 75.1% (n = 287) and sore throat (n = 57) vs. 16.8% (n = 64). Difficulty breathing was less likely among Influenza A virus-infected patients than those without 65.1% (n = 28) vs. 46.6% (n = 418). Also, a meta-analysis found that breathing problems were the most common symptom during the early pandemic era, according to Rodriguez-Morales *et al.* [17]; this was followed by fever (89%) and cough (58%). Runny nose was less common in SARS-CoV-2-infected patients than in those without any SARS-CoV-2 (n = 62) vs. 48.7% (n = 186), while it was more common in Influenza A virus-infected patients compared to those without 67.4% (n = 29) vs. 44.9% (n = 245). Compared to patients without any SARS-CoV-2 infection, individuals with underlying chronic diseases had higher rates of SARS-CoV-2 infection: diabetes 15.4% (n = 32) vs. 6.6% (n = 25), and hypertension 27.0% (n = 56) vs. 12.3% (n = 47). On the other hand, those with underlying chronic diseases had lower rates of influenza A virus infection than those without such symptoms: hypertension 6.9% (n = 3), vs. 14.5% (n = 79), and diabetes 2.3% (n = 1), vs. 7.7% (n = 42). According to Akhtar *et al.* [1], those infected with the influenza virus were more likely to have a runny nose. SARI case patients were significantly less likely to be diagnosed with SARS-CoV-2 infection than those who were not infected with any (28.4% (n = 81) vs. 49.9% (n = 849), p<0.001). However, SARI case patients reported being more likely than those without (62.9% (n = 110) vs. 45.3% (n = 820), p < 0.001).

In comparison to those without any SARS-CoV-2 infection, SARI case-patients with underlying chronic diseases had higher rates of SARS-CoV-2 infection: hypertension (26.7%; n = 76) vs. 11.5% (n = 195); diabetes (14.0%; n = 40) vs. 5.9% (n = 101); (p < 0.001). Conversely, SARI case-patients with underlying chronic

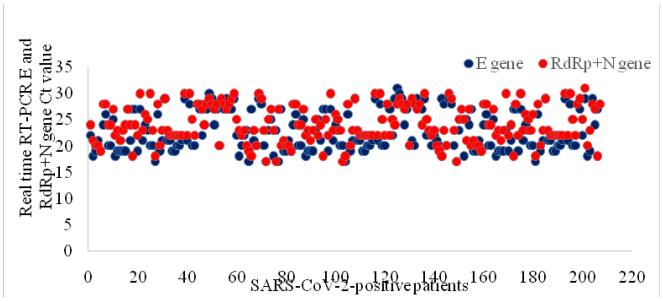

Fig. 1: Real-time RT-PCR E and RdRp+N gene cyclic threshold (Ct) values in the sense of expression

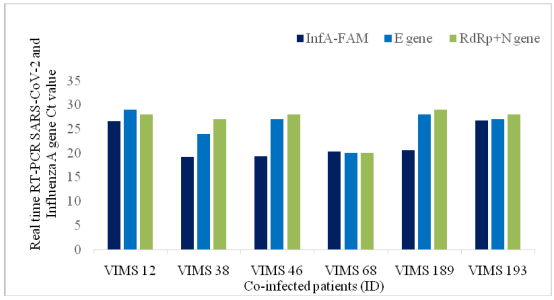

Fig. 2: Coinfection of influenza A and SARS-CoV-2 patients by detecting real-time RT-PCR

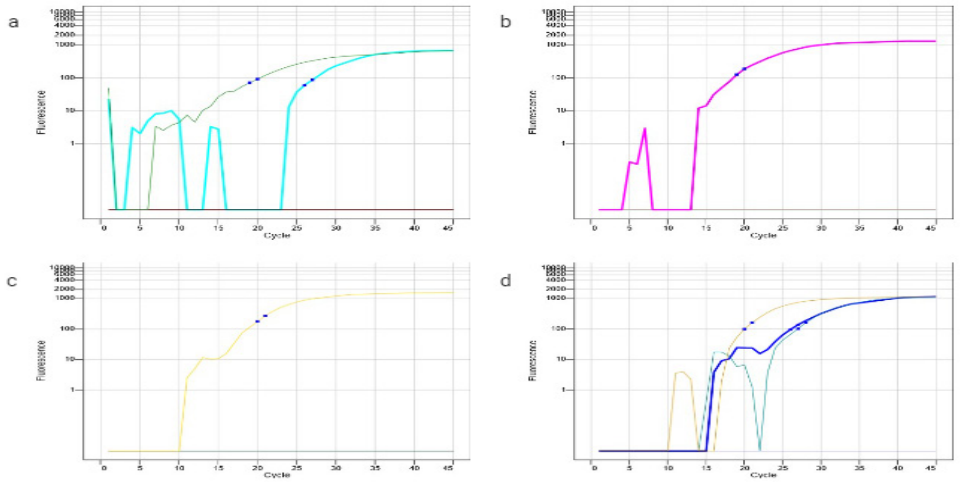

Fig. 3: Representative overlay plots showing a) infA gene (ct value=26.58) and infA gene positive control (green) b) infApdm gene absent and infApdm gene positive control (pink) c) H1N1 pdm gene absent and H1N1 pdm gene positive control (yellow) d) RNAsP gene present (the human RNAse P transcript, was positively detected in all samples tested, thereby confirming the reliability of sample collection and the consistency of the tested genetic material) and RNAsP gene positive control (yellow).

Table 2: Demographic and clinical characteristics of SARS-CoV-2 and influenza A virus infection during May and June 2021 (n = 589)

Characteristics	*SARS-CoV-2 infection*		*Influenza A virus infection*		*Coinfection with influenza A and SARS-CoV-2*	
	Positive	Negative	Positive	Negative	Positive	Negative
	n = 207	n = 382	n = 43	n = 546	n = 06	n = 583
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Age						
0–60 years	165 (79.8)	335 (87.7)	36 (83.8)	463 (84.8)	6 (100)	524 (89.9)
> 60 years	42 (20.2)	47 (12.3)	7 (16.2)	86 (15.8)	0	59 (10.1)
Gender						
Male	81 (39.1)	262 (68.5)	19 (44.1)	323 (59.1)	1 (16.7)	356 (61.0)
Female	126 (60.9)	120 (31.4)	24 (55.9)	223 (40.9)	5 (83.3)	227 (38.9)
Clinical features (symptoms)						
Runny nose	62 (29.9)	186 (48.7)	29 (67.4)	245 (44.9)	2 (33.3)	218 (37.3)
Headache	103 (49.8)	124 (32.4)	13 (30.2)	194 (35.6)	2 (33.3)	171 (29.3)
Sore throat	57 (27.6)	64 (16.8)	9 (21.0)	94 (17.2)	0	112 (19.2)
Difficulty of breathing	169 (81.7)	287 (75.1)	28 (65.1)	418 (46.6)	5 (83.3)	464 (79.6)
Variables						
Asthma	16 (7.8)	18 (4.8)	2 (4.7)	27 (5.0)	1 (16.7)	21 (3.6)
Diabetes	32 (15.4)	25 (6.6)	1 (2.3)	42 (7.7)	0	43 (7.3)
Hypertension	56 (27.0)	47 (12.3)	3 (6.9)	79 (14.5)	1 (16.7)	67 (11.4)

illnesses had lower influenza virus infection rates than those without such symptoms: hypertension (5.7% (n = 10), vs. 14.4% (n = 261), $p < 0.001$), and diabetes (4.0% (n = 7), vs. 7.4% (n = 134), $p = 0.047$). Huang discovered a study that was similar. A similar study was found by Huang *et al.* [9] and Garg *et al.* [7].

Influenza A Virus and SARS-CoV-2 Detection

From the total 589 RNA samples, 207 (35.1%) patients were infected with SARS-CoV-2, and 43 (7.3%) patients were infected with the influenza A virus. Result interpretation for RT-PCR testing was done by differentiating positive and negative samples based on a threshold Ct value obtained in comparison with control reactions for the confirmatory of E and RdRp+ N gene and severity of COVID-19 disease. The tested specimen was considered positive for SARS-CoV-2 for the cycle threshold (Ct) value less than or equal to 35 for E gene and both RdRp+ N gene Fig. 1. In Fig. 2 strongly suggests that influenza A co-infection with SARS-CoV-2.

The human RNAse P transcript gene (internal control) was positive for all samples in both SARS-CoV-2 and influenza A viruses, thereby confirming the reliability of sample collection and the consistency of the tested genetic material reported in Fig. 3 (d). This was based on the fluorescence capture read-out of the specific DNA probe hybridized with the RT-PCR-amplified DNA. The detection of influenza A virus by RT-PCR in Fig. 3 reported the presence of RNAseP and infA gene amplification signals. The sample was positive for type A influenza viruses. All of the samples were devoid of infApdm and H1N1 pdm. All positive samples were found by electrophoresis visualization of the DNA pattern in the amplified RT-PCR nucleic acid products, indicating the quality of the cDNA.

CONCLUSION

In conclusion, RT-PCR was the most widely used diagnostic method for SARS-CoV-2 and influenza A, respectively. Our results strongly suggest that influenza A co-infection with SARS-CoV-2. Our study found that patients with SARS-CoV-2 and influenza A co-infection had similar clinical characteristics as those with SARS-CoV-2 infection alone. Comorbidities, like hypertension and diabetes, and increasing age make patients more susceptible to SARS-CoV-2 and influenza A coinfections. Also, co-infection could lead to a more severe condition. Our findings strongly highlight the importance of screening for influenza A co-infection with SARS-CoV-2 patients. Consequently, physicians should focus more on COVID-19 patients who also have IFV-A infections. In order to completely protect themselves from disease, the susceptible population should take the necessary mitigating actions, such as vaccination. The coinfection with SARS-CoV-2 and influenza A viruses was not very common and had less disease severity considering mortality in tertiary care hospitals. There was no circulating influenza A virus during the influenza peak season during the COVID-19 pandemic in 2021. Studies exploring possible causes of the marked decrease in influenza A virus infections during the COVID-19 pandemic are warranted to better understand the association between the COVID-19 pandemic and the reduction of influenza virus infection.

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CONFLICTS OF INTEREST

The authors declared that there is no conflict of interest.

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