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Detection of influenza virus by real-time polymerase chain reaction (RT-PCR) on archived COVID-19 respiratory specimens in Ibadan, Nigeria

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Abstract:

Background: Every year, influenza virus affects millions of people, causing morbidity and mortality among adults and children prompting many to seek outpatient care. Influenza is largely undetected among infected individuals in lower-middle-income settings. Influenza co-infection with other respiratory viral infections is associated with a severe clinical presentation and a worse clinical outcome. The current study aimed to detect influenza A and B viruses from respiratory samples of patients with suspected CoVID-19, in order to establish possible co-infection, and its implications.
Methodology: Archived respiratory samples from a total of 230 patients with suspected COVID-19 (73 confirmed COVID-19 and 157 without COVID-19) at the Biorepository Clinical Virology Laboratory (BCVL), College of Medicine, University of Ibadan, were included in the study. RNA extraction was carried out on the samples, and then amplified by real-time PCR (RT-PCR) for specific genes of influenza virus. Data obtained were sorted out and analyzed. Descriptive statistics such as percentages were used to summarize variables while inferential statistics such as the Chi-square test was used to compare categorical variables. SPSS version 21.0 was used for statistical computation, R version 4.1.1 was used for graphical illustration.
Results: Of the 73 SARS-COV-2 positive specimens, no influenza virus gene was detected by RT-PCR. However, of the 157 SARS-COV-2 negative specimens, 2 (1.3%) were positive for influenza A virus while 3 (1.9%) for influenza B virus. Co-infection of SARS-COV-2 and influenza virus was not detected.
Conclusion: In this study, the frequency of influenza virus detection in patients with suspected COVID-19 was low for both influenza A and B viruses, but no co-infection of the virus with SARS-CoV-2 was observed. The study emphasizes the need for continuous surveillance for respiratory viral pathogens among individuals presenting with respiratory symptoms in the study setting.

Keywords: Influenza viruses, SARS-CoV-2, COVID-19, Real-Time PCR, Archived samples

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Détection du virus de la grippe par réaction en chaîne par polymérase en temps réel (RT-PCR) sur des échantillons respiratoires COVID-19 archivés à Ibadan, Nigéria

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Résumé:

Contexte: Chaque année, le virus de la grippe touche des millions de personnes, entraînant morbidité et mortalité chez les adultes et les enfants, ce qui incite nombre d'entre elles à consulter en ambulatoire. La grippe passe largement inaperçue chez les personnes infectées dans les milieux à revenu intermédiaire de la tranche inférieure.

Français La co-infection grippale avec d'autres infections virales respiratoires est associée à une présentation clinique sévère et à un pronostic clinique plus défavorable. L'étude actuelle visait à détecter les virus grippaux A et B à partir d'échantillons respiratoires de patients suspectés de COVID-19, afin d'établir une éventuelle co-infection et ses implications.
Méthodologie: Des échantillons respiratoires archivés provenant d'un total de 230 patients suspectés de COVID-19 (73 COVID-19 confirmés et 157 sans COVID-19) au Laboratoire de Virologie Clinique de Biodépositaire (BCVL), Collège de Médecine, Université d'Ibadan, ont été inclus dans l'étude. L'extraction d'ARN a été réalisée sur les échantillons, puis amplifiée par PCR en temps réel (RT-PCR) pour des gènes spécifiques du virus de la grippe. Les données obtenues ont été triées et analysées. Des statistiques descriptives telles que les pourcentages ont été utilisées pour résumer les variables tandis que des statistiques inférentielles telles que le test du Chi carré ont été utilisées pour comparer les variables catégorielles. SPSS version 21.0 a été utilisé pour le calcul statistique, R version 4.1.1 a été utilisé pour l'illustration graphique.
Résultats: Sur les 73 échantillons positifs au SARS-CoV-2, aucun gène du virus de la grippe n'a été détecté par RT-PCR. Cependant, sur les 157 échantillons négatifs au SARS-CoV-2, 2 (1,3%) étaient positifs au virus de la grippe A et 3 (1,9%) au virus de la grippe B. Aucune co-infection par le SARS-CoV-2 et le virus de la grippe n'a été détectée.
Conclusion: Dans cette étude, la fréquence de détection du virus de la grippe chez les patients suspectés d'être atteints de la COVID-19 était faible, tant pour les virus de la grippe A que pour les virus de la grippe B, mais aucune co-infection du virus par le SARS-CoV-2 n'a été observée. L'étude souligne la nécessité d'une surveillance continue des agents pathogènes viraux respiratoires chez les personnes présentant des symptômes respiratoires dans le cadre de l'étude.

Mots-clés: Virus de la grippe, SARS-CoV-2, COVID-19, PCR en temps réel, Échantillons archivés

Introduction:

Members of the *Orthomyxoviridae* family are responsible for the acute viral illness known as influenza. There are three primary types of influenza viruses; A, B, and C. Among these, influenza A virus is particularly significant due to its association with major pandemics, including four global outbreaks in the 20th century. The most catastrophic of these was the "Spanish flu" pandemic of 1918–1919, which claimed approximately 20 million lives worldwide (1,2).

Both influenza A and B viruses are principal contributors to seasonal influenza epidemics (3, 4). Influenza A virus infects a wide range of hosts including humans, birds, pigs, and horses, whereas influenza B and C viruses are predominantly human pathogens. However, influenza type C viruses have occasionally been isolated from pigs and dogs (3). Subtype classification of Influenza A viruses is based on their surface glycoproteins; haemagglutinin (HA) and neuraminidase (NA). The subtypes are denoted using the letters "H" and "N," such as H1N1 and H3N2, which have been the most common circulating strains in humans since the late 1970s (4).

According to the World Health Organization (WHO), influenza affects around 1 billion individuals annually, with 3–5 million cases progressing to severe illness and approximately 290,000 to 650,000 influenza-related respiratory deaths each year (5). Influenza affects all age groups, but accurate incidence estimates are hindered by the fact that many individuals do not seek medical attention or receive formal diagnoses. A meta-analysis incorporating statistical extrapolation from United States hospitalization data estimates the annual incidence at around 8% (range 3–11%) (6). Additionally, several other respiratory viruses, including parainfluenza, adenoviruses, enteroviruses, and paramyxoviruses, can mimic influenza-like illnesses, complicating clinical diagnosis (7).

Historically, viral culture has served as the 'gold standard' for influenza diagnosis. However, this method is time-consuming, technically demanding, and has limited sensitivity, especially during widespread outbreaks. Modern diagnostic approaches include non-culture-based techniques such as immunofluorescence, enzyme immunoassays, and molecular assays. Among these, real-time reverse transcription polymerase chain reaction (RT-PCR), including one-step and multiplex RT-PCR formats, provides superior sensitivity and specificity, along with rapid turnaround time (8). Real-time RT-PCR has thus become the diagnostic method of choice, especially during outbreaks and pandemics.

During the COVID-19 pandemic, reports of co-infection with influenza A virus (IAV) and SARS-CoV-2 varied significantly across different regions and studies (9). This highlights the need for continued surveillance and accurate diagnostic tools to differentiate between respiratory pathogens with overlapping clinical presentations.

Materials and method:

Study site and sample:

This laboratory-based study was conducted at the Biorepository and Clinical Virology Laboratory (BCVL), College of Medicine, University of Ibadan. The laboratory is accredited by the Nigerian Centre for Disease Control (NCDC) and enrolled for external quality assurance with the WHO-accredited organization. To date, the BCVL has tested and archived over 40,000 nasopharyngeal and oropharyngeal swab samples from suspected COVID-19 cases.

The laboratory maintains a collabora-

tive quality control network with Northwestern University and the African Centre of Excellence for Genomics of Infectious Diseases (ACEGID). Samples are stored at –80°C. Archived nasopharyngeal specimens confirmed as SARS-CoV-2 positive and negative were retrieved for this study.

Viral RNA Extraction and RT-PCR:

RNA extraction was performed in a Class II biosafety cabinet using the Da An Gene extraction kit (Da An Gene Co., Ltd., Sun Yat-Sen University) following the manufacturer's instructions. RT-PCR amplification was carried out using the detection kit for influenza A, influenza B, and respiratory syncytial virus (RSV) RNA from Guangdong Huayin Medicine Science Co. (Catalog No. HY06128). The RT-PCR protocol involved reverse transcription at 50°C for 10 minutes, pre-denaturation at 95°C for 2 minutes, followed by 45 amplification cycles with 5 seconds at 95°C for denaturation and 35 seconds at 95°C for annealing and extension.

Data analysis:

Descriptive statistics (percentages) were used to summarize variables. Inferential statistics, including Chi-square tests, were used to compare categorical variables. Statistical computations were performed using SPSS version 21, while graphical illustrations were created using R version 4.1.1.

Results:

The demographic distribution of the COVID-19 patients whose archived samples were retrieved, and the sample collection locations is summarized in Tables 1 and 2. A total of 230 patient samples were analyzed, consisting of 73 samples from patients with COVID-19 and 157 from individuals without COVID-19.

Among patients with COVID-19, 50 (68.5%) were males and 23 (31.5%) were females. Most of the samples (n=167, 72.6%) were collected from patients in the community site at Agodi, Ibadan (Health is Wealth Clinic), while 1 (0.4%) and 62 (27.0%) were collected from hospital-based sources in BCVL and University College Hospital, respectively.

Table 1: Age and gender distribution of the study participants

Characteristics	No of patients with COVID-19 (n=73)	No of patients without COVID-19 (n=157)	Total (n=230)
Age group (years)			
≤ 19 years	3 (4.1)	15 (9.6)	18 (7.8)
20-39 years	44 (60.3)	72 (45.9)	116 (50.4)
40-59 years	10 (13.7)	50 (31.8)	60 (26.1)
≥60 years	16 (21.9)	20 (12.7)	36 (15.7)
Sex			
Male	50 (68.5)	70 (44.6)	120 (52.2)
Female	23 (31.5)	87 (55.4)	110 (47.8)

Table 2: Distribution of respiratory samples by collection sites

COVID-19 sample collection areas	Frequency (%)
Hospital	
Biorepository & Clinical Virology Laboratory, College of Medicine, University of Ibadan	1 (0.4)
University College Hospital	62 (27.0)
Subtotal	63 (27.4)
Community	
Health is Wealth, Agodi-Ibadan	167 (72.6)
Total	230 (100.0)

Regarding patient status, 7 (4.5%) of the patients without COVID-19 were in-patients and 150 (95.5%) were out-patients, while among patients with COVID-19, 6 (8.2%) were in-patients and 67 (91.8%) were out-patients (Fig 1). No significant difference was observed between the groups with respect to inpatient or outpatient location (p=0.356). In terms of clinical symptoms, all the patients with COVID-19, presented with fever. Cough and headache were also common, occurring in 48 (65.8%) and 41 (56.2%) of patients, respectively. Similarly, all the patients without COVID-19 experienced fever (Table 3). Tiredness and cough were reported in 103 (65.6%) and 96 (61.1%) of patients without COVID-19 respectively.

Importantly, no co-infection of SARS-CoV-2 with influenza A or B virus was detected. Of the 157 SARS-CoV-2 negative patients, 2 (1.3%) tested positive for influenza A virus (Ct values 23.37 and 34.57), and 3 (1.9%) tested positive for influenza B virus (Ct values 36.1, 36.03, and 17.79). All influenza virus-positive patients had fever; some also experienced cough, runny nose, and headache (Table 3).

Table 3: Comparative symptom distribution between patients with confirmed COVID-19 and those without COVID-19

Clinical symptoms	**COVID-19 status**	
	Positive (%)	**Negative (%)**
Fever	73 (100.0)	157 (100.0)
Sore throat	26 (35.6)	43 (27.4)
Running nose	23 (31.5)	51 (32.5)
Cough	48 (65.8)	96 (61.1)
Shortness of breath	6 (8.2)	25 (15.9)
Headache	41 (56.2)	87 (55.4)
Vomiting	3 (4.1)	16 (10.2)
Nausea	12 (16.4)	18 (11.5)
Diarrhea	6 (8.2)	14 (8.9)
Tiredness	35 (47.9)	103 (65.6)
Chest pain	6 (8.2)	40 (25.5)
Red eyes	5 (6.8)	19 (12.1)
Loss of taste	14 (19.2)	44 (28.0)
Loss of smell	9 (12.3)	30 (19.1)

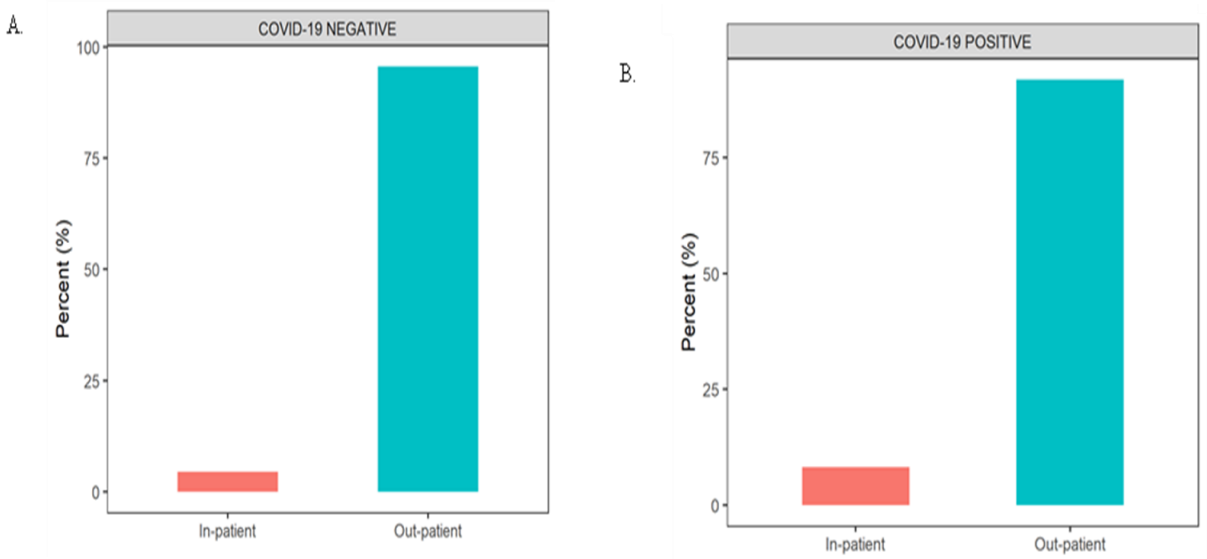

Fig 1: Frequency (%) of patients with and without COVID-19 with respect to in-patient and out-patient locations

Discussion:

This study employed a highly sensitive and specific real-time RT-PCR assay to detect influenza A and B viruses in archived SARS-CoV-2 respiratory specimens. Our findings demonstrated the absence of influenza virus in all SARS-CoV-2 positive samples (n=73). Among the 157 SARS-CoV-2 negative samples, 2 (1.3%) tested positive for influenza A virus, and 3 (1.9%) tested positive for influenza B virus. These findings are consistent with prior surveillance reports in Nigeria and neighboring countries.

A nationwide surveillance program in Nigeria initiated in 2008 and published in 2012 reported a 7.7% influenza virus positivity rate, predominantly influenza A virus (9). In Ghana, monthly influenza virus positivity in co-infection surveillance programs was reported as low as 0.3%, although higher rates were observed in specific age groups, especially in children aged 0–10 years, where the positivity rate was 20% (10).

Reports of co-infection rate of influenza virus with SARS-CoV-2 vary by region. Lansbury et al., (11) estimated a 3.0% co-infection rate with other respiratory viruses among hospitalized COVID-19 patients, with influenza A virus being a frequent co-pathogen. Alotaibi et al., (12) in 2023 reported concurrent detection of influenza A and B viruses in SARS-CoV-2 patients, while a study in Burkina Faso also documented such co-infections (13). Wu et al., (14) in 2020 noted that influenza A can complicate SARS-CoV-2 diagnosis. Case reports suggest that dual infections with SARS-CoV-2 and other viruses, including influenza, may exacerbate disease severity and increase mortality (15). Therefore, accurate and timely identification of such co-infections is vital for improving clinical outcomes.

While our RT-PCR assays for influenza virus were not conducted immediately following COVID-19 diagnosis, prior research confirms that delayed processing (even up to 105 hours) does not compromise RNA integrity for influenza detection. The absence of influenza among SARS-CoV-2 positive patients and the low prevalence among SARS-CoV-2 negative patients may be influenced by sample size, study design, environmental or seasonal factors, and population characteristics. Possible contributing factors include lower viral shedding in older individuals, mucosal dryness, and the use of supplemental oxygen, as well as delayed sample collection beyond the early phase of illness when viral load peaks. Additionally, specimen site variability may influence detection sensitivity, though this remains underexplored.

Moreover, distinguishing COVID-19 from other viral respiratory illnesses during flu season is complicated by overlapping symptoms such as fever, cough, and shortness of breath. Both diseases can present with similar blood abnormalities (e.g., leukopenia and lymphopenia) and radiographic findings (e.g., ground-glass opacities), further complicating diagnosis. Hence, concurrent detection of both SARS-CoV-2 and influenza virus reinforces diagnostic challenges, particularly when SARS-CoV-2 is not detected but other viral infections are present.

Influenza and COVID-19 share overlapping transmission patterns, clinical symptoms, and seasonal peaks. Co-infections are especially concerning in high-risk populations, where they can worsen clinical presentation. Experimental studies suggest that prior influenza infection enhances SARS-CoV-2 entry and replication, potentially worsening disease in susceptible individuals (10,16). Recent studies from China observed frequent SARS-CoV-2/influenza co-infections during the early COVID-19 outbreak in Wuhan, China, associating them with poorer clinical outcomes (17). These findings, along with sporadic African data, motivated our investigation. Our results underscore the need for comprehensive respiratory pathogen surveillance to distinguish between co-circulating viruses. This is crucial for effective patient management, particularly during flu seasons and pandemic surges, where disease severity may be amplified by co-infection (18).

Influenza remains a highly contagious viral respiratory illness with a history of significant morbidity and mortality during epidemics and pandemics. Differentiating it from COVID-19 is essential, as the two are caused by distinct viruses and may require divergent management strategies.

Conclusion:

In this study, influenza virus was not detected in archived samples of patients with confirmed COVID-19. However, influenza virus was detected in samples of patients without COVID-19, albeit at a very low prevalence. This finding may suggest that various clinical mitigation measures such as adherence to patient care protocols, improved clinical conditions, and compliance with hospital policies might have influenced the reduced detection of influenza.

Concurrent infections involving multiple respiratory viruses are commonly observed in human populations and are associated with an increased risk of hospitalization, ICU admission, and mortality. In our study, influenza type B virus was more frequently detected than type A. These findings reinforce the importance of employing advanced molecular methods, beyond RT-PCR alone, for the detection of influenza viruses. It is also important to

note that the genetic variability of influenza viruses may compromise assay sensitivity and increase the risk of false-negative results. Therefore, improved molecular diagnostics and continued surveillance are essential to accurately assess the burden and dynamics of influenza virus infections, particularly during pandemics such as COVID-19.

Contributions of authors:

OJO was involved in the study conceptualization, design, methodological activities, and initial draft production; FA was involved in the study conceptualization, design, methodological activities, initial draft production and supervision, and OOA was involved in the critical review of the initial draft. All authors were involved in findings interpretation, critical review and approval of the final draft.

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Conflict of interest:

Authors declare no conflict of interest.

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