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ORIGINAL ARTICLE

Design and assessment of a multiplex real-time PCR method for simultaneous detection and differentiation of COVID-19 and Influenza A/B

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ABSTRACT

Background and Objectives: Viral infections of the respiratory system are a major public problem due to their ease of spread, pandemic potential, and significant rate of death. Diagnosing these infections requires laboratory testing, as clinical symptoms alone are often insufficient. Influenza A, Influenza B, and COVID-19 are common infections that burden the population, especially during winter. We developed a multiplex real-time PCR method to simultaneously detect Influenza A and B, as well as COVID-19. Compared to existing detection kits, it offers higher accuracy, lower costs, and faster results, making it an efficient diagnostic tool.

Materials and Methods: We designed primer/TaqMan probes for the M2 gene of Influenza A, N gene of SARS-CoV-2, and NS1 gene of Influenza B. Reaction components were optimized and functional parameters were tested using standard samples with known viral copy numbers.

Results: The method's detection limit is 10 copies for Influenza A and B, and 60 for SARS-CoV-2. Sensitivity and specificity for Influenza A are 88% and 100%, for Influenza B, 95.6% and 100%, and for SARS-CoV-2, 90.4% and 100%.

Conclusion: This multiplex real-time PCR method can accurately detect and distinguish SARS-CoV-2, Influenza B, and Influenza A infections.

Keywords: Multiplex; Real-time polymerase chain reaction; SARS-CoV-2; COVID-19; Influenza B; Influenza A

INTRODUCTION

Acute respiratory disease (ARD) is a significant health concern globally, causing a substantial number of acute illnesses and fatalities. Among the various causes of ARD, respiratory virus infections are the most prevalent, accounting for approximately 80% of the cases (1). During the COVID-19 pandem-

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ic, caused by the SARS-CoV-2 virus, the concurrent circulation of Influenza A virus (IAV) and Influenza B virus (IBV), as two common respiratory infections during the fall and winter seasons, had the potential to significantly increase the prevalence of respiratory illnesses within the population (2).

The novel coronavirus pneumonia pandemic began from China, at the end of December 2019 (3). SARS-CoV-2 is the ninth known coronavirus that specifically targets humans, and it is the seventh to be found within the last two decades (4). Influenza respiratory infection, commonly caused by IAV and IBV (two separate genera within the virus family Orthomyxoviridae) in humans, is considered as a significant contributor to mortality rates among the most severe infections that occur worldwide. Influenza virus infection varies in forms from mild upper respiratory tract symptoms to life-threatening pneumonia caused by the virus itself (5, 6). Influenza viruses are categorized as global pathogens because they have the potential to infect more than 20% of the world's population. Annually, they result in 3.5 million hospitalizations and significant mortality and morbidity (7). Given the overlap in the spread of the influenza viruses and COVID-19, along with the similarities in clinical presentations, it is crucial for healthcare providers to distinguish between these respiratory infections during the early stages. Additionally, co-infection with these respiratory viruses has more severe clinical consequences in patients and can quickly lead to acute respiratory distress syndrome (ARDS), ultimately increasing the mortality rate. Thus, early diagnosis for managing patients is critical (8, 9).

In the field of detection of respiratory virus infections, various diagnostic methods have been developed to identify viral antigens and the antibodies produced against them. These methods include viral culture, immunoassay techniques, and polymerase chain reaction (PCR) as a nucleic acid amplification test (10, 11). Molecular assays are now the preferred diagnostic methods for detecting and identifying viruses causing ARDS (12).

Although conventional PCR has already established itself as a vital technique in laboratories, real-time PCR is currently more widely embraced because of its enhanced speed, sensitivity, reproducibility, and decreased risk of cross-contamination (13). Multiplex real-time PCRs are reactions where two or more target fragments are amplified and identified simultaneously via the concurrent use of several sets of primer pairs and probes (14). The benefits of using this method include a decrease in the required sample volume, as well as savings in both time and cost (15, 16).

The present study aims to design and develop an in-house multiplex qRT-PCR technique based on TaqMan assay that can detect and distinguish three common respiratory pathogens, including IAV and IBV, as well as COVID-19, with high sensitivity and specificity.

MATERIALS AND METHODS

Primers and probes design. To design the probes and primers, sequences related to the highly conserved regions of viruses were selected from NCBI and GISAID. The sequences belonging to the genes encoding matrix protein 2 (M2) of IAV, nonstructural protein 1 (NS1) of IBV, and nucleocapsid (N) protein of COVID-19 were received from NCBI. Databanks were then created using Mega11 software for the gene sequences that were obtained, and the databanks were aligned using Clustal W analysis. We designed primers/probes for the most conserved regions of these genes using AlleleID 6. Finally, the Metabion Company synthesized the primers and probes. Probes were labeled with reporter 6-carboxyfluorescein (FAM) at the 5′ end for IVA, Yakima Yellow for IVB, and Texas Red for COVID-19. All sequences are shown in Table 1.

RNA extraction. Viral RNAs were extracted from validated specimen types, including throat swabs, nasal swabs, and nasopharyngeal swabs, using an RNA extraction kit (ROJE Technology, Iran). The samples were kept at -80°C for further analyses.

Reaction conditions. Initially, the real-time PCR assay for all three viruses was performed individually. The final reaction volume adjusted to 20 µl, which included 5 µl of 4X CAPITAL qRT-PCR one-step master mix (biotechrabbit, Germany), 9 µl double-distilled water, each primer (0.5 µM, forward- reverse), probe (0.25 µM, TaqMan), and extracted RNA(5 µl). ABI® StepOne™ real-time PCR system (Applied Biosystems, USA) was used to perform the real-time PCR reactions with thermal cycling conditions consisting of 10 minutes at 50°C for cDNA synthesis, 95°C for 3 minutes for enzyme activation, and 45 cy-

Table 1. Primers and probes used in the multiplex real-time PCR assay. Both the primers and the probes were custom-made, and different fluorescent reporter dyes (FAM, Yakima Yellow, and Texas Red) were used to label each probe.

Target	Primer/probe	Sequence 5′ > 3′
Influenza A	Fwd	CAA GAC CAA TCY TGT CAC CTC TGA C
Influenza A	Rev	GCA TTY TGG ACA AAV CGT CTA CG
Influenza A	Probe	5'-FAM-TGC AGT CCT CGC TCA CTG GGC ACG-BHQ1-3'
Influenza B	Fwd	TCC TCA AYT CAC TCT TCG AGC G
Influenza B	Rev	CGG TGC TCT TGA CCA AAT TGG
Influenza B	Probe	5'-Yakima Yellow-CCA ATT CGA GCA GCT GAA ACT GCG GTG-BHQ1-3'
SARS-COV-2	Fwd	CTG CAG ATT TGG ATG ATT TCT CC
SARS-COV-2	Rev	CCT TGT GTG GTC TGC ATG AGT TTA G
SARS-COV-2	Probe	5'-Texas Red-ATT GCA ACA ATC CAT GAG CAG TGC TGA CTC-BHQ2-3'

cles of 10 seconds at 95°C and 40 seconds at 55°C.

Evaluation of the functional parameters: Limit of Detection (LOD) and analytical sensitivity. The LOD as well as analytical sensitivity of developed method were evaluated using patients' samples of IVA, and IVB, as well as COVID-19, with predetermined viral loads. The samples were diluted serially in tenfold increments and tested for identification of the lowest detectable concentration using this method.

Analytical specificity. The analytical specificity of the method was assessed using in silico analyses. For this purpose, the accuracy of the specific binding of primers and probes to the target template was evaluated recruiting BLASTn (in NCBI) against the nr/nt database.

Clinical sensitivity. To evaluate the clinical sensitivity, 69 positive samples that had been tested with the commercial nucleic acid diagnostic kit were utilized. These recruited samples were also evaluated with the developed multiplex method, and the clinical sensitivity was computed.

Clinical specificity. Fifty pooled human genome samples as negative control and nucleic acids from various non-target pathogens were applied to determine the clinical specificity of the assay. These included Adenovirus, Hepatitis C virus (HCV), Hepatitis B virus (HBV), Herpes simplex 2 (HSV 2), Herpes simplex 1 (HSV 1), *Mycobacterium Tuberculosis*, Human papillomavirus (HPV), Human Immunodeficiency Viruses 1 (HIV 1), Epstein-Barr virus (EBV), Cytomegalovirus (CMV), Toxoplasma gondii, Brucella, BK virus, and JC virus.

Precision. Precision of the assay was evaluated using intra-assay as well as inter-assay. Intra-assay precision evaluates the percent of variations among various measurements of a single sample within a single test run, whereas inter-assay precision assesses the variation in the results of replicates of the same sample analyzed in different runs or on different days. In intra-assay analysis, we tested each dilution in triplicate in one run. We also performed inter-assay for each dilution in three runs of real-time PCR on three separate days.

Co-infections. For evaluation the effectiveness of RNA extraction and presence of co-infections on the results of this assay, various combinations of IVA, IVB, as well as COVID-19 viral loads were merged. Subsequently, all extracts underwent testing using the developed assay.

Clinical performance. To clinically evaluate the performance of developed multiplex method, 119 swab samples, including 50 negative samples and 69 positive samples, were utilized. Additionally, the samples were evaluated by the Viga SARS-CoV-2 and IVA/B kits (ROJE Technology Company, Iran).

RESULTS

Limit of detection (LOD). The lowest concentration of a sample that a technique can detect is known as analytical sensitivity (LOD). The assay's LOD was 10 copies/mL for IVB and IVA, and also 60 copies/mL

for COVID-19, according to obtained results.

Analytical specificity. The primer and probe sequences prepared for all three viruses were checked applying BLASTn. The results demonstrated that all designed primer pairs and probes were identical to the corresponding regions and had no cross-reactivity with other respiratory pathogens (Figs. 1-3). As a result, the analytical specificity of this developed method is 100%.

Clinical Specificity. In the clinical specificity evaluation of the developed method using the nucleic acids from the mentioned pathogens, no unspecific amplification was observed with these specimens, indicating the assay's high specificity.

Precision. Calculating the mean and also standard deviation of Ct values and then expressing them as a percentage of the coefficient of variation (CV%) is the method of choice for determining the precision of the developed assay. For the intra-assay, the highest and lowest CVs for the M2 gene were 1.4% and 0.4%; for the NS1 gene, they were 3.7% and 0.1%; and for the N gene, they were 3.3% and 0.1%, respectively. For the inter-assay, the highest and lowest CVs for the M2 gene were 10.4% and 6.9%; for the NS1 gene, they were 2.7% and 0.3%; and for the N gene, they were 5.4% and 0.6%, respectively. The CV for intra-assay should be below 5% and for inter-assay it should be below 10%, based on the guidelines.

Co-infections. The test results for combinations of different viral loads showed that, in all spiked samples, all three targets virus were detectable, even at low concentrations. This indicates that no competitive inhibition occurs in the detection of different viral loads.

Clinical performance of the multiplex real-time PCR assay. The developed assay was recruited to investigate a total of 69 RNA-positive swab samples (23 for IVB, 21 for COVID-19, and 25 for IVA viruses) and 50 RNA-negative samples that had been previously determined. According to the calculated results of 119 clinical samples that had been tested, it was found that this developed method has 100% negative

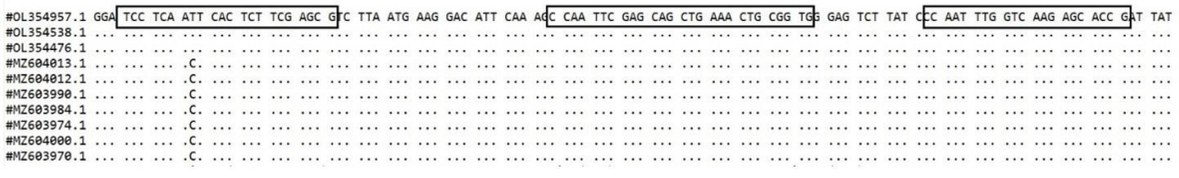

Fig. 1. Conserved sequences of Influenza B primers and probe. (A) Influenza B forward primer; (B) Influenza B probe sequence; (C) Influenza B reverse–complemented reverse primer.

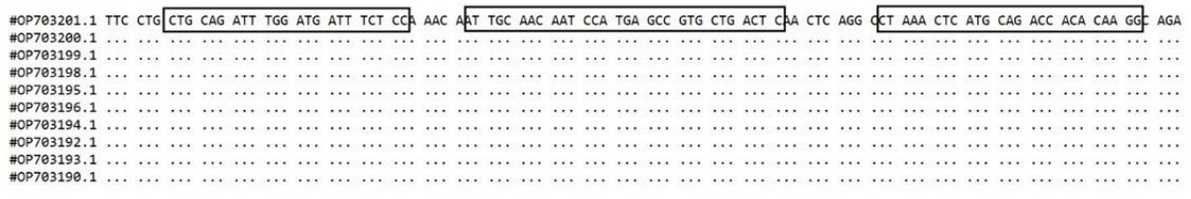

Fig. 2. Conserved positions of COVID-19 primers and probe. (A) COVID-19 forward primer; (B) COVID-19 probe sequence; (C) COVID-19 reverse–complemented reverse primer.

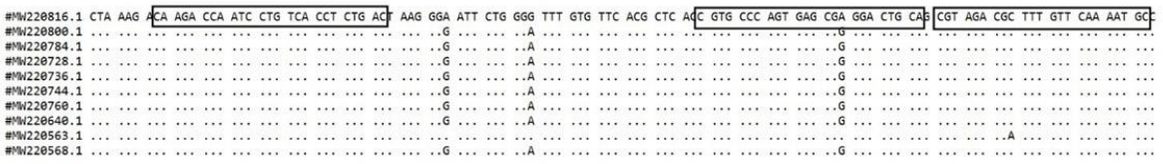

Fig. 3. Conserved sequences of Influenza A primers and probe. (A) Influenza A forward primer; (B) Influenza A probe sequence; (C) Influenza A reverse–complemented reverse primer.

percent agreement (NPA) for all three viruses (Table 2). Additionally, the positive percent agreement (PPA) for COVID-19, IVA, and IVB were 96.1%, 94.3%, and 98%, respectively. Ultimately, the overall rate of agreement (ORA) for COVID-19, IVA and IVB were 97.1%, 98.6% and 96% respectively (Table 2).

DISCUSSION

During cold seasons, COVID-19 spreads in the environment along with other respiratory viruses, especially influenza viruses. Management and treating of infections caused by IVA, IVB, and severe acute respiratory syndrome coronavirus 2 are distinct, yet patients infected with these viruses have similar clinical presentations. Diagnostic testing that provides rapid results for detecting infected patients can be a critical factor in the prevention of widespread outbreaks, the appropriate selection of treatment, and the effective control of disease (17, 18). While commercial kits like the Viga SARS-CoV-2 and IVA/B Molecular Diagnostic Kit (developed by ROJE Technologies, Tehran, Iran) are available, our proposed method, with its specifically designed primers and TaqMan probes, offers higher sensitivity, specificity, and precision.

Here, we developed a multiplex RT-PCR method based on TaqMan probes for the simultaneous detection of IVA, IVB, and COVID-19 genome. For this molecular method, we selected conserved gene regions to design primers and probes, and then we optimized the values of all reaction components. Since the samples had been previously diagnosed as positive in hospitals, and we compared the efficiency of our detection method with standard ones, we did not include an internal control. Ultimately, we validated the methodology by evaluating the functional parameters of molecular methods to verify the method's specificity, sensitivity, and precision.

Analytical sensitivity analysis showed that the LOD of this technique is 60 copies/mL and 10 copies/mL for COVID-19 and IVA/B respectively, which was comparable to the multiplex RT-PCR technique developed by Dominik Nörz for the purpose of detecting SARS-CoV-2 and IVA/B (19). The primers/probes were found to be 100% compatible with our target sequences through in-silico analysis. Additionally, they showed no cross-reactions with a variety of other viral and bacterial pathogens. Most importantly, this method is capable of sensitively detecting COVID-19 and accurately distinguishing influenza virus from COVID-19, particularly in the context of co-infections. In another investigation, the similar technique, which was created by B. Shu in order to detect the IVA, IVB, and COVID-19, had an analytical sensitivity of 5 copies/mL of RNA for all three pathogens. Similar to our method, this

Table 2. Clinical Performance of the multiplex real-time PCR assay using positive and negative samples. A total of 69 RNA-positive swab samples (21 for COVID-19, 25 for IVA, and 23 for IVB) and 50 RNA-negative samples were utilized. The results of negative percent agreement (NPA) for all three viruses were 100%, and positive percent agreement (PPA) for COVID-19, IVA, and IVB were 96.1%, 94.3%, and 98% respectively. ORA: overall rate of agreement.

		Positive	**Negative**	**Total**
	Positive	22	0	22
	Negative	3	50	53
Influenza A(IVA)	Total	25	50	75
	PPA= 88%, NPA= 100%, ORA= 96%			
		Positive	Negative	Total
	Positive	22	0	22
	Negative	1	50	51
Influenza B(IVB)	Total	23	50	73
	PPA= 95.6%, NPA=100%, ORA= 98.6%			
		Positive	Negative	Total
	Positive	19	0	19
	Negative	2	50	52
SARS-COV-2(COVID-19)	Total	21	50	71
	PPA= 90.4%, NPA= 100%, ORA= 97.1%			

technique exhibits no non-specific interaction and operates with very high specificity (20). The clinical sensitivity test for our approach yielded the following results: 88% sensitivity for IVA, 95.6% sensitivity for IVB, and 90.4% sensitivity for COVID-19, whereas Kanti Pabbaraju's Multiplex Reverse Transcriptase-PCR technique showed 99.8% specificity and 100% sensitivity for COVID-19, 98.9% specificity and 100% sensitivity for IVB, and 100% sensitivity and specificity for IVA(21). In comparison to our technique, it exhibited a greater degree of sensitivity; however, our technique maintained 100% specificity for all three viruses.

In accordance with the instructions, our method's intra-assay and inter-assay precision, like the multiplex assay developed by Fabiola Mancini (22), were within an acceptable range. The highest and lowest CVs for the intra-assay were 1.4% and 0.4% for the M2 gene, 3.7% and 0.1% for the NS1 gene, and 3.3% and 0.1% for the N gene. Furthermore, the highest and lowest CVs for the inter-assay were 10.4% and 6.9% for the M2 gene, 2.7% and 0.3% for the NS1 gene, and 5.4% and 0.6% for the N gene. This obtained value indicates good reproducibility of the test results, which means that it can be efficiently used for clinical performance.

CONCLUSION

Our in-house developed multiplex real-time PCR assay is a robust, reliable, and precise method for identifying influenza viruses as well as SARS-CoV-2 in clinical specimens. Throughout the seasonal influenza activity period, the test serves as a critical diagnostic tool because the treatment is effective when the disease is diagnosed quickly and accurately.

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