Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Journal of Virological Methods 293 (2021) 114151

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Development and validation of a multiplex reverse transcriptase-PCR assay for simultaneous testing of influenza A, influenza B and SARS-CoV-2

Kanti Pabbaraju [a,*], Anita A. Wong [a], Raymond Ma [a], Nathan Zelyas [b,c,d], Graham A. Tipples [b,c,d,e]

[a] Alberta Precision Laboratories, Public Health Laboratory, Calgary, Alberta, Canada
[b] Department of Microbiology, Immunology and Infectious Diseases, University of Calgary, Alberta, Canada
[c] Alberta Precision Laboratories, Public Health Laboratory, Edmonton, Alberta, Canada
[d] Department Laboratory Medicine and Pathology, University of Alberta, Edmonton, Alberta, Canada
[e] Li Ka Shing Institute of Virology, University of Alberta, Edmonton, Alberta, Canada

ARTICLE INFO

Keywords:
SARS-CoV-2
Influenza
COVID-19
multiplex
Co-infection

ABSTRACT

In the current pandemic of coronavirus disease (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the co-circulation of SARS-CoV-2 and other respiratory viruses during the upcoming fall and winter seasons may present an unprecedented burden of respiratory disease in the population. Important respiratory viruses that will need to be closely monitored during this time include SARS-CoV-2, influenza A and influenza B. The epidemiology of these viruses is very similar in terms of susceptible populations, mode of transmission, and the clinical syndromes, thus the etiological agent will be difficult to differentiate without target specific assays. The availability of a sensitive and specific multiplex assay that can simultaneously detect all these targets will be valuable. Here we report the validation of a real-time reverse transciptase-PCR assay for the simultaneous detection of SARS-CoV-2, influenza A and influenza B. This multiplex assay is comparable to its singleplex counterparts with a limit-of-detection being less than 5 copies/reaction, 100 % specificity, over seven logs of dynamic range, less than 1 % coefficientof variation showing high precision, and equivalent accuracy using patient samples. It also offers the added benefits of savings in reagents and technologist time while improving testing efficiency and turn-around-times in order to respond effectively to the ongoing pandemic.

1. Introduction

Since early 2020, the world has been grappling to contain the transmission of the SARS-CoV-2 virus, the causative agent of COVID-19 that has resulted in significant morbidity and mortality worldwide (Zhu et al., 2020; Wenjie et al., 2020) (World Health Organization. Novel coronavirus–China. Disease outbreak news: update 12 January [cited 2020 Feb 12] https://www.who.int/csr/don/12-january-2020-novel-coronavirus-china/en). The public health strategy that has proven to be vital in the containment of this virus has been extensive testing, early detection, contact tracing and isolation of cases (Kucharski et al., 2020; Reddy et al., 2021; Cheng et al., 2020; Kretzschmar et al., 2020). This will be made more challenging with the increased circulation of other respiratory viruses with similar symptoms during the winter months. Of these co-circulating viruses, influenza A and B cause outbreaks annually with a predictable seasonality beginning in late fall or early winter (Krammer et al., 2018). A comprehensive study to estimate the burden of seasonal influenza concluded that it has a significant annual effect on global health resulting from lower respiratory tract infections and other respiratory conditions, and highlights the importance of influenza prevention measures (Collaborators GBDI, 2019).

SARS-CoV-2 and influenza share several epidemiological features. The susceptible populations are similar and include children, elderly, immunocompromised, and individuals with chronic comorbidities such as asthma, chronic obstructive pulmonary disease, cardiac and renal failure, or diabetes (Krammer et al., 2018; Yang et al., 2020). The transmission routes are also similar including contact and respiratory droplets (Lansbury et al., 2020). In the setting of the current COVID-19 pandemic, the number of patients infected with one or more of these viruses may increase as the influenza season approaches (Lansbury

* Corresponding author at: Provincial Laboratory for Public Health, 3030 Hospital Drive, Calgary, Alberta, T2N 4W4, Canada.
E-mail address: kanti.pabbaraju@albertaprecisionlabs.ca (K. Pabbaraju).

https://doi.org/10.1016/j.jviromet.2021.114151
Received 28 October 2020; Received in revised form 15 March 2021; Accepted 6 April 2021
Available online 8 April 2021

et al., 2020). The ability to differentiate between these viruses will be helpful to determine appropriate public health and patient management strategies especially in symptomatic cases, outbreaks, vulnerable populations and critically ill patients. With the necessity for increased volume of testing, any efficiency in testing by increasing throughput, improving turnaround time (TAT), maximizing the use of available equipment, personnel and reagents would be very beneficial to restrict transmission and contribute to the control of the COVID-19 pandemic. In the province of Alberta, Canada, over a million tests have been conducted for the detection of SARS-CoV-2 RNA to date. During the current respiratory virus season, patients will need to be tested for influenza A and B, as well as SARS-CoV-2 to effectively control outbreaks and provide appropriate patient management. To address the necessity for high test volumes for these respiratory viruses, we report the validation of a sensitive and specific multiplex real-time reverse transcriptase-PCR (rtRT-PCR) assay for the simultaneous detection of influenza A, influenza B, SARS-CoV-2 and bacteriophage MS2 as an internal extraction and inhibition control (SC2/Flu assay).

An added benefit would be the ability to detect co-infections. In a study including 93 patients a co-infection rate as high as 50 % with influenza A/B and SARS-CoV-2 has been reported (Ma et al., 2020). No significant difference in rates of SARS-CoV-2 infection in patients with and without other pathogens has been reported. Thus the presence of other pathogens may not provide reassurance that a patient does not also have SARS-CoV-2 and it would be prudent to test for multiple pathogens simultaneously (Kim et al., 2020). A systematic review and meta-analysis show the pooled proportion of viral co-infections with SARS-CoV-2 is around 3 %, with respiratory syncytial virus and influenza A being the most common (Lansbury et al., 2020). Co-infection with SARS-CoV-2 and influenza virus may lead to a much earlier occurrence of a possible cytokine storm and organ damage in critically ill COVID-19 patients, supporting simultaneous testing for the two pathogens. Treatment strategies for influenza virus and dampening inflammatory responses may be helpful for critically ill patients co-infected with SARS-CoV-2 and influenza virus (Ma et al., 2020).

Simultaneous testing for SARS-CoV-2 and influenza A/B in a high-throughput way will significantly reduce the number of tests that need to be carried out to detect the respiratory viruses that require strong surveillance and can be managed with specific therapy.

2. Methods

2.1. Design of primers and probes

An in-house designed rtRT-PCR assay targeting the envelope (E) gene was used for the detection of SARS-CoV-2 (Pabbaraju et al., 2021). Detection of influenza A and B was performed using rtRT-PCR assays targeting the matrix (M) and non-structural protein 1 (NS1) genes of influenza A and B, respectively. These real-time RT-PCR assays were developed at the Centers For Disease Control (CDC, USA) to detect seasonal influenza A, B, H1, H3, and avian H5 serotypes. These assays have been approved by the Food and Drug Administration (FDA) and were distributed in December 2008 through the U.S. Public Health laboratories and the WHO's Global Influenza Surveillance Network. They were provided to the Provincial Public Health Laboratory in Alberta by the National Microbiology Laboratory (NML) at the Public Health Agency of CANADA (PHAC) (CDC USA, 2007). These assays were multiplexed along with the detection of MS2 phage (Dreier et al., 2005) used as a spiked extraction and inhibition control; this multiplex assay is hereafter referred to as the SC2/Flu (SARS-CoV-2/influenza A and B) assay. All primers and probes are summarized in Table 1. The SARS-CoV-2 E gene probe, with an MGB protein and NED reporter/non-flourescent quencher, and the influenza B probe, with a VIC reporter/QSY quencher, were purchased from Applied Biosystems (ABI, Foster City, California). The MS2 probe with an ATTO647 reporter/Iowa Black quencher, and influenza A probe, with FAM reporter/-Iowa Black terminal/ZEN internal quencher, were purchased from Integrated DNA Technologies (IDT, Iowa, USA). Primers were purchased either from IDT or LGC Biosearch Technologies (Petaluma, CA, USA).

2.2. Real-time RT-PCR assay

The SC2/Flu assay was performed using TaqMan® Fast Virus One-Step RT-PCR Master Mix (ABI), 0.8μM each of sense and antisense primers and 0.2μM of the probes for the SARS-CoV-2 and influenza targets; 0.2μM and 0.1μM of the primers and probe, respectively, for the MS2 target. Ten microliters of extracted RNA was combined with 10μL of master mix, primers and probes; the primer and probe concentrations were optimized for preferential amplification of the SARS-CoV-2, influenza A and influenza B target genes to prevent any competitive inhibition from MS2 amplification. The reverse-transcription step was performed at 50 °C for 5 min followed by incubation at 95 °C for 20 s. Amplification included 45 cycles of denaturation at 95 °C for 3 s, followed by annealing, extension and data acquisition at 60 °C for 30 s on the 7500 Fast Real-Time PCR system (ABI).

2.3. Preparation of RNA transcripts for sensitivity studies

Long oligonucleotide sequences (gblocks) including the detection region with flanking T7 and SP6 RNA polymerase promoter binding sites were designed and purchased for the E-gene of SARS-CoV-2 from IDT. Primers external to the real-time detection region were used for the amplification of a longer fragment of the M-gene of influenza A and NS1 region of influenza B. The PCR products were cloned using the TOPO® TA Cloning Dual Promoter Kit (Life Technologies, California, USA). The plasmid DNA was linearized using restriction enzymes and the T7 RiboMAX™ Express kit (Promega, Madison, WI, USA) was used for the transcription of the gblocks and plasmid DNA. The transcribed RNA was spectrophotometrically quantified for the calculation of copy numbers.

Table 1
Primers and probes for the detection of SARS-CoV-2, influenza A, influenza B and MS2 in the SC2/Flu assay.

Target	Primer/probe name	Primer/probe sequence (5'-3')	Source
Influenza A M-gene	CDC-M-F	GACCRATCCTGTCACCTCTGAC	https://www.fda.gov/media/134922/download
	CDC-M-R	AGGGCATTYTGGACAAAKCGTCTA	
	CDC-M-Probe	FAM –TGCAGTCCT /ZEN/ CGCTCACTGGGCACG-IABkFQ	
Influenza B NS1 gene	CDC-NS1-F	TCCTCAACTCACTCTTCGAGCG	
	CDC-NS1-R	CGGTGCTCTTGACCAAATTGG	
	INF B CDC NS1	VIC –CCAATTCGAGCAGCTGAAACTGCGGTG-QSY	
SARS-CoV-2 E gene	COVID19_E_For_V2	GAGACAGGTACGTTAATAGTTAATAGCG	Pabbaraju et al. (2021)
	COVID19_E_Rev_V2	CAATATTGCAGCAGTACGCACAC	
	COVID19_ E_MGB_NED	NED-CTAGCCATCCTTACTGCG -(MGB/NFQ)	
MS2	MS2-TM2-F	TGCTCGCGGATACCCG	Dreier et al. (2005)
	MS2-TM2-R	AACTTGCGTTCTCGAGCGAT	
	MS2-TM2_ATTO647	ATTO647-ACCTCGGGTTTCCGTCTTGCTCGT-Iowa Black	

2.4. Extraction of viral nucleic acid

Viral RNA from the different specimen types was extracted on one of three platforms using manufacturers' instructions: easyMAG® (BioMerieux, Quebec, Canada) with associated reagents; the MagMAX Express 96 or KingFisher Flex automated extraction and purification systems (Thermo Fisher Scientific) with either the MagMAX™-96 Viral RNA Isolation Kit (ABI) or the Maxwell HT Viral TNA custom Kit (Promega); or the Hamilton STARlet automated extractor (Hamilton, Reno, NV, USA) with the Maxwell HT Viral TNA Custom Kit. The validated specimen types included throat swab, nasal swab, nasopharyngeal swab and aspirate, auger suction, bronchoalveolar lavage, endotracheal secretion, and lung tissue. The sample input and output volumes were 200 µl and 110 µl for all the respiratory sample types, respectively, and 60 µl and 200 µl for the tissue samples, respectively.

2.5. Analytical sensitivity, analytical specificity, reproducibility and dynamic range of the SC2/Flu assay

The analytical sensitivity for the assay was determined by testing ten-fold serial dilutions of quantified in-vitro RNA in triplicate on three independent runs. The 95 % limits of detection (95 % LOD) were calculated by probit analysis. The range of viral loads tested in copies/reaction were 1.3E + 00–1.3E + 07 for the M gene of influenza A, 1.1E + 00–1.1E + 07 for the NS1 gene of influenza B, and 3.3E-01 to 3.3E + 07 for the E gene of SARS-CoV-2. All dilutions were tested in a total reaction volume of 10 µl and 20 µl respectively by the target-specific singleplex assays and the SC2/Flu assay. Quantitated viral RNA with the pfu/mL for SARS-CoV-2 was obtained from the National Microbiology Laboratory (NML, Winnipeg, Manitoba, Canada), serially diluted, and tested in triplicate by all the target specific singleplex and SC2/Flu assays to compare sensitivity.

Linear regression fitting of the log viral load versus cycle threshold (Ct) allowed for the calculation of PCR efficiency.

Ten-fold serial dilutions of two positive samples each for the SARS-CoV-2, influenza A and influenza B targets were tested in parallel by the target specific singleplex and SC2/Flu assays to compare the sensitivity of detection, all samples were tested in triplicate.

Specificity of the assay was determined by testing high viral load samples of several RNA and DNA viruses and bacteria with clinical symptoms overlapping those of influenza or COVID-19. The pathogens tested included coronaviruses (NL63, OC43, 229E, HKU1, MERS-CoV, SARS-CoV-1, SARS-CoV-2), influenza A (pdm09 H1N1, H3N2, H5N1, H7N9, H7N3), influenza B (Victoria and Yamagata lineages), respiratory syncytial virus (A and B), parainfluenza virus (1, 2, 3, 4a, 4b), rhinovirus, enterovirus, adenovirus, bocavirus, human metapneumovirus, cytomegalovirus, herpes simplex viruses (1 and 2), *Streptococcus pneumoniae, Mycoplasma pneumoniae, Chlamydia pneumoniae, Legionella pneumophila, Bordetella pertussis, Haemophilus influenzae,* and *Neisseria meningitidis*.

To assess the breadth of detection, the following influenza A and influenza B subtypes were tested: A/Kansas/14/2017-like (H3N2), A/Singapore/INFIMH-16-0019/2016-like (H3N2), A/Brisbane/02/2018-like (H1N1), A/Michigan/45/2015-like (H1N1), FluA/Panama UK/2007/99 (H3N2), A/Vietnam/1203/2004 (H5N1), A/Alberta/001/2014 (H5N1), A/Anhui/1/2013 (H7N9), A/Canada/504/04 (H7N3), B/Victoria V1A-3, B/Victoria V1A-3Del, B/Colorado/06/2017-like, B/Phuket/3073/13-like (H1N1), B/Brisbrane/60/2008-like, and several other influenza B Victoria and Yamagata strains available in the lab.

The intra- and inter-assay variability were calculated using two mixtures of all three viruses in equal proportions starting with amounts representing high (Ct values of 19.92, 19.49 and 18.43) and low (Ct values of 29.21, 31.29 and 27.65) viral loads for influenza A, influenza B and SARS-CoV-2 respectively. Both mixtures were tested in triplicate on three independent runs. The average Ct values, standard deviation and percent coefficients of variation (%CV) were calculated.

2.6. Specimens tested to determine accuracy

To assess the accuracy of influenza A detection by the SC2/Flu assay, a total of 128 samples (38 positives and 90 negatives for Influenza A) previously tested by diagnostic assays were tested by the SC2/Flu assay. These included nasal swabs (n = 4), nasopharyngeal swabs (n = 93) and throat swabs (n = 31). For influenza B, 132 previously tested samples (44 positive and 88 negative for influenza B) were tested by the SC2/Flu assay. The specimen types included were nasal swabs (n = 4), nasopharyngeal swabs (n = 98) and throat swabs (n = 30). For SARS-CoV-2, a total of 635 previously tested samples (53 positive and 582 negative for SARS-CoV-2) were tested by the SC2/Flu assay. These included nasal swabs (n = 2), nasopharyngeal swabs (n = 77), throat swabs (n = 550), tracheal secretions (n = 5) and one swab of unknown origin.

The majority of the validation was performed using upper respiratory specimens such as throat, nasal, and nasopharyngeal swabs. To validate other specimen types, three to five positive samples and five negative samples of auger suctions, bronchoalveolar lavages/bronchial washes, endotracheal secretions, nasopharyngeal aspirates, and lung tissues were tested by the SC2/Flu assay. Contrived samples, spiked with target nucleic acid, were used for specimen types with insufficient positive samples.

2.7. Co-infections

To assess the efficiency of target extraction and detection in cases with co-infections, spiked samples with combinations of high and low viral loads of the different viruses with Ct values ranging from about 19–35 were combined and tested.

3. Results

3.1. Assessment of the SC2/Flu assay performance: analytical sensitivity, analytical specificity, and reproducibility

The results for analytical sensitivity, dynamic range and assay efficiency are summarized in Table 2. The 95 % LOD for all targets were

Table 2
Real-time RT-PCR assay characteristics.

Target_Assay	95 % LOD (copies/reaction)	Dynamic range (copies/reaction)	Slope	Calculated efficiency (%)	R^2 value
Influenza A-Singleplex	4/10µl	6.4E + 00–6.4E+07	−3.4	96.4	0.9994
Influenza B-Singleplex	1/10µl	5.3E + 00–5.3E+07	−3.3	101.7	0.9995
SARS-CoV-2-Singleplex	2/10µl	1.7E + 00–1.7E+07	−3.3	102.8	0.9981
Influenza A-SC2/Flu	2/20µl	1.3E + 00–1.3E + 07	−3.4	96.2	0.9992
Influenza B-SC2/Flu	2/20µl	1.1E + 00–1.1E + 07	−3.2	101.6	0.9996
SARS-CoV-2-SC2/Flu	3/20µl	3.3E-01–3.3E + 07	−3.29	101.52	0.9999

The 95 % LOD for the real-time assays is reported as copies detected per reaction, the singleplex and SC2/Flu assays used 5µl and 10µl of template, respectively. The total reaction volume for the singleplex and SC2/Flu assays was 10µl and 20µl respectively Extraction input and output volumes were used to calculate the sensitivity per ml of patient sample.
Linear regression plots of the copy number and Ct values were used to calculate PCR efficiency.

Table 3
Comparison of sensitivity using diluted patient sample.

Sample (dilution)	Singleplex Ct	SC2/Flu Ct
SARS-CoV-2 positive samples		
Sample 1 (1:10)	37/36.03/38.18	37.43/38.24/38.25/37.24
Sample 1 (1:100)	38.18/neg/neg	neg/neg/41.58/neg
Sample 2 (1:10)	36.44/35.6/37.09	38.95/38.54/37.78/41.81
Sample 2 (1:100)	neg/38.06/neg	neg/neg/39.5/39.68
Influenza A positive samples		
Sample 1 (1:10)	34.77/36.55/35.12	38.56/38.92/38.36/37.87
Sample 1 (1:100)	neg/neg/neg	40.28/neg/neg/neg
Sample 2 (1:10)	34.74/35.58/36.93	41.84/neg/39.87/neg
Sample 2 (1:100)	neg/neg/neg	neg/neg/neg/neg
Influenza B positive samples		
Sample 1 (1:10)	34.75/35.59/neg	41.5/38.38/37.76/38.46
Sample 1 (1:100)	neg/neg/neg	neg/40.59/neg/neg
Sample 2 (1:10)	neg/neg/neg	40.29/39.66/neg/39.6
Sample 2 (1:100)	neg/neg/neg	39.49/neg/neg/neg

Cycle threshold (Ct) values for ten-fold serial dilutions of patient samples tested in triplicate and quadruplicate by the singleplex and SC2/Flu assays respectively.
5 µl of template was used for the singleplex assays and 10 µl was used for the SC2/Flu assay.

comparable by the singleplex and SC2/Flu assays, The analytical sensitivity for influenza A was 4 and 2 copies/reaction; influenza B was 1 and 2 copies/reaction; and SARS-CoV-2 was 2 and 3 copies/reaction by the singleplex and SC2/Flu assays, respectively. The total reaction volume was 10µl and 20µl respectively for the singleplex and SC2/Flu assays. Log linear amplification of target was obtained over seven logs of template concentration for each virus. Using these amplification plots, the efficiency of each rtRT-PCR assay was calculated for each of the viral targets and, as indicated in Table 2, these values ranged from 96.2 %–101.7 % for the SC2/Flu assay. Using quantitated RNA extracted from whole SARS-CoV-2 virus, the E gene singleplex assay detected 2/3 replicates (average Ct = 38.0) and the SC2/Flu assay detected 1/3 replicates (Ct = 38.0) at 1.2E-02pfu/mL. Both assays reproducibly detected 1.2E-01pfu/mL.

Comparison of end-point using ten-fold serial dilutions of two positive samples each for the SARS-CoV-2, influenza A and influenza B targets shows comparable sensitivity of detection by the singleplex and SC2/Flu assays as shown in Table 3.

None of the probes reacted non-specifically with the other pathogens tested in the specificity panel, though the E gene probe detected SARS-CoV-1 as expected. All the subtypes of influenza A and B were detected with equal efficiency by the singleplex and SC2/Flu assays.

The percent coefficient of variation (%CV) representing intra-assay variability based on testing mixtures of low (Ct values of 32.93, 34.4, 29.55) and high (Ct values of 23.48, 23.54, 19.72) viral loads for influenza A, influenza B and SARS-CoV-2, respectively, ranged from 0.1 % to 0.9 % (Table 4). The inter-assay variability between the three runs (Table 4) ranged from 0.02–0.4 % for all targets, showing reproducible detection at different viral loads.

Table 5
Accuracy using positive and negative samples.

Influenza A			
		Singleplex	
		Pos	Neg
SC2/Flu	Pos	38	0
	Neg	0	90

Sensitivity: 100 % (95 %CI: 90.8 %–100 %; Specificity: 100 % (95%CI: 94.03 %–99.97 %)

Influenza B			
		Singleplex	
		Pos	Neg
SC2/Flu	Pos	44	1*
	Neg	0	87

*Singleplex: 36.84/negative/negative; SC2/Flu: 41.42/38.99/negative
Sensitivity: 100 % (95 %CI: 91.96 %–100 %); Specificity: 98.86 % (95%CI: 93.8 %–99.97 %)

SARS-CoV-2			
		Egene/MS2 duplex	
		Pos	Neg
SC2/Flu	Pos	53	1*
	Neg	0	581

Sensitivity: 100 % (95 %CI: 93.3 %–100.00 %); Specificity: 99.83 % (95 % CI:99.1 %–100.00 %).
* Singleplex: negative/negative/negative; SC2/Flu: 41.32/37.93/41.55; CDC N1: negative and N2: 38.39.

3.2. Testing of clinical samples to determine accuracy

Results from all the retrospective positive and negative influenza A samples were concordant by the SC2/Flu assay for the 38 positive and 90 negative samples (Table 5). All 44 previously-tested influenza B positive samples tested positive by the SC2/Flu assay. Of the 88 influenza B negative samples, one tested positive two out of three times on the SC2/Flu assay (Ct values of 41.42/38.99/negative). This sample was repeated in triplicate by the singleplex assay and the results were 36.84/negative/negative (Table 5). All 53 previously-tested SARS-CoV-2 positive samples tested positive by the SC2/Flu assay. Of the 582 negative

Table 4
Assay variability.

Intra-assay variability								
Influenza A			Influenza B			SARS-CoV-2		
Average Ct	SD	%CV	Average Ct	SD	%CV	Average Ct	SD	%CV
32.84–33.19	0.14–0.20	0.1 % - 0.6 %	34.58–34.99	0.1–0.3	0.2 % to 0.9 %	30.72–30.79	0.06–0.13	0.2 % to 0.4 %
23.24–23.47	0.02–0.08	0.1 %-0.3 %	22.88–23.89	0.01–0.1	0.1 % to 0.2 %	21.47–21.60	0.01–0.02	0.05%-0.09 %

Inter-assay variability								
Influenza A			Influenza B			SARS-CoV-2		
Average Ct	SD	%CV	Average Ct	SD	%CV	Average Ct	SD	%CV
33.04	0.03	0.10	34.77	0.14	0.4	30.77	0.04	0.12
23.34	0.03	0.13	23.53	0.02	0.1	21.55	0.00	0.02

Intra- and inter-assay variability was calculated using spiked samples representing high and low viral loads with all three viruses. Shown here are the results from three independent runs with each sample tested in triplicate.
Ct: Cycle threshold value; SD: Standard deviation; %CV: coefficient of variation.

Table 6
Testing co-infections.

Target_Viral load	Singleplex			SC2/Flu		
	Flu A	Flu B	SARS-CoV-2	Flu A	Flu B	SARS-CoV-2
FluA_low/FluB_low/SARS-CoV-2_ low	32.93	34.4	29.55	33.19	34.58	30.72
FluA_high/FluB_high/SARS-CoV-2_high	23.48	23.54	19.72	23.24	22.88	21.47
FluA_low/FluB_high/SARS-CoV-2_high	32.52	23.46	34.64	36.21/39.22	23.34/24.38	neg/neg
FluA_high/FluB_high/SARS-CoV-2_low	21.53	23.46	34.45	21.12/21.37	23.26/24.07	neg/neg
FluA_high/FluB_low/SARS-CoV-2_high	23.37	31.8	23.63	22.92	30.48	25.01
FluA_high/FluB_high/SARS-CoV-2_high	23.37	23.54	19.72	23.57	23.82	21.57
FluA_high/FluB_high/SARS-CoV-2_low	23.48	23.54	29.68	23.32	23.97	31.03
FluA_low/FluB_high/SARS-CoV-2_high	32.93	23.54	19.72	43.53	24.15	21.93
FluA_low/FluB_high/SARS-CoV-2_high	30.65	23.46	23.63	33.68	23.4	24.74

High and low viral loads of influenza A, influenza B and SARS-CoV-2 were mixed at different concentrations to estimate the efficiency of detection for co-infections.

samples, one sample tested positive by the SC2/Flu assay with Ct values of 41.32/37.93/41.55 when tested in triplicate; this sample was tested in triplicate by the singleplex SARS-CoV-2 assay and all replicates were negative. This sample was also tested by the CDC assays and was negative by the PCR targeting the N1 gene but yielded a Ct of 38.39 by the assay targeting the N2 gene (Table 5) (https://www.fda.gov/media/134922/download).

Using the singleplex assays as the gold standard, the sensitivity and specificity with the corresponding 95 % confidence intervals (CI) by the SC2/Flu assay were calculated to be as follows; sensitivity: 100 % (95 % CI: 90.8 %–100 %) and specificity: 100 % (95 %CI: 94.03 %–99.97 %) for influenza A; sensitivity: 100 % (95 %CI: 91.96 %–100 %) and specificity: 98.9 % (95 %CI: 93.8 %–99.97 %) for influenza B; sensitivity: 100 % (95 %CI: 93.3 %–100 %) and specificity: 99.83 % (95 %CI: 99.1 %–100 %) for SARS-CoV-2. The numbers of positive and negative samples tested that were used for the calculation of sensitivity and specificity are indicated in Table 5.

All the positive and negative samples for each of the additional specimen types for influenza A, influenza B and SARS-CoV-2 (auger suctions, bronchoalveolar lavages/bronchial washes, endotracheal secretions, nasopharyngeal aspirates, and lung tissues) gave the expected results, suggesting that these specimen types are suitable for testing by the SC2/Flu assay.

3.3. Testing of co-infections

Results of testing spiked specimens with co-infections including the Ct values by the singleplex and SC2/Flu assays are indicated in Table 6. A shift in Ct values was noted by the SC2/Flu assay; two samples with SARS-CoV-2 target at Ct values of 34.64 and 34.45 tested negative by the SC2/Flu assay, however, the influenza A and B targets were detected in these samples. One sample with a Ct of 32.93 for influenza A gave a Ct of 43.53 by the SC2/Flu assay, the strong influenza B and SARS-CoV-2 targets in this sample tested positive.

4. Discussion

As of October 20, 2020, the World Health Organization reported that SARS-CoV-2 had caused 40,118,332 cases of COVID-19 worldwide with 1,114,749 deaths since the beginning of the pandemic (https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation-reports/). Many human respiratory viruses exhibit an annual increase in incidence each winter, with the cases of influenza peaking between November and April in northern temperate latitudes (Dowell and Ho, 2004). The WHO estimates that annual epidemics of influenza result in approximately 1 billion infections, 3–5 million cases of severe illness and 300,000–500,000 deaths globally each year (Krammer et al., 2018; Collaborators GBDI, 2019). Symptoms associated with influenza virus infection vary from a mild respiratory disease confined to the upper respiratory tract and characterized by fever, sore throat, runny nose, cough, headache, muscle pain and fatigue to severe pneumonia owing to influenza virus itself or secondary bacterial infections of the lower respiratory tract. Epidemiologic investigations and case reports indicate that influenza infection often results in diverse clinical presentations including involvement of organ systems other than the respiratory tract with extra-pulmonary complications of the heart and central nervous system causing viral myocarditis and encephalitis (Sellers et al., 2017; Malosh et al., 2018). Thus patients infected with SARS-CoV-2 or influenza viruses demonstrate similar clinical symptoms (Ma et al., 2020). In addition to the common clinical manifestations blood tests showing leucopenia and lymphopenia, and chest tomography scans showing ground-glass opacity and consolidation with bilateral lung involvement are shared by SARS-CoV-2, influenza and other respiratory viruses, making it difficult to differentiate COVID-19 from respiratory illness caused by other pathogens (Wu et al., 2020a). This shared clinical presentation of patients infected with a respiratory virus creates a diagnostic dilemma, which will be particularly emphasized during the upcoming influenza season. This issue can be resolved using nucleic acid tests for a definitive diagnosis, however, sequential testing for the different pathogens will delay a diagnosis and thus the mitigation strategies.

In addition co-infections with SARS-CoV-2 and other respiratory pathogens will be a concern in the upcoming respiratory season. A recent study including 1217 patient specimens tested for SARS-CoV-2 and other respiratory pathogens showed that 20.7 % and 26.7 % of the SARS-CoV-2 positive and negative specimens respectively were also positive for one or more additional respiratory pathogen suggesting that the presence of a non–SARS-CoV-2 pathogen will not provide reassurance that a patient does not also have SARS-CoV-2 (Kim et al., 2020). A study comparing the clinical characteristics and outcomes of 93 critically ill COVID-19 patients with or without co-infection with influenza virus (47.3 % with influenza A and 2.2 % with influenza B) showed that critically ill COVID-19 patients with influenza were more prone to cardiac injury than those without influenza. These patients also exhibited more severe inflammation, organ injury, substantially elevated serum levels of pro-inflammatory cytokines that mediate extensive pulmonary pathology with massive infiltration of neutrophils and macrophages indicating that co-infection with the influenza virus may induce an earlier and more frequently occurring cytokine storm (Ma et al., 2020). In addition high D-dimer levels associated with viral-infection induced cytokine storm cause local vascular injury, ischemia and thrombosis (Davidson and Warren-Gash, 2019) and have been reported as risk factors for death in COVID-19 patients (Wu et al., 2020b; Zhou et al., 2020). D-dimer levels were found to be substantially elevated among the non-survivors, especially so in patients co-infected with influenza (Ma et al., 2020). These results suggest that co-infection with the influenza virus may induce an earlier and more severe cytokine storm in critically ill COVID-19 patients, leading to serious complications such as shock, acute respiratory distress syndrome (ARDS), fulminant myocarditis, acute kidney injury or multiple organ failure (Ma et al., 2020).

Antiviral agents that target the neuraminidase enzyme of influenza A and B viruses have been developed for prophylaxis and therapy and

three drugs (oseltamivir, zanamivir and peramivir) are approved in Canada. Studies consistently report improved outcomes associated with neuraminidase inhibitor use, including reduced risk of pneumonia and hospitalization, and reduced risk of mortality in patients who have been hospitalized (Dobson et al., 2015). Thus, there is a clinical benefit to detecting influenza A and B as co-infecting pathogens since clinical management can be changed in appropriate patients.

For the simultaneous detection of SARS-CoV-2, influenza A, influenza B, and an internal extraction and inhibition control, we have developed a SC2/Flu rtRT-PCR assay; running this multiplex assay may require a higher level of training in molecular testing and close attention will need to be paid to the assay setup and analysis. This assay will be used in patients with influenza-like illness during the upcoming respiratory season. This assay will streamline testing for coinfections, increase testing throughput and improve the laboratory TAT. The analytical sensitivity of the SC2/Flu assay is comparable to the assay currently used to test patient samples in Alberta (Pabbaraju et al., 2021). A comparison of this current frontline assay to commonly used primer-probe sets for the detection of SARS-CoV-2 world-wide has been previously reported and shown to be highly comparable (Nalla et al., 2020; Vogels et al., 2020). Efficiency of the SC2/Flu assay for the different targets ranged from 96.24 %–101.61 %. All rtRT-PCR evaluated in this study showed 100 % analytical specificity as they did not cross-react with any viral or bacterial pathogens that would be included in the differential for COVID-19 (the exception being SARS-CoV-1 for the E gene target, which was expected based on in-silico analysis and is not a concern given that this virus is not currently circulating in humans).

The availability of this rtRT-PCR assay for the upcoming respiratory season will be valuable to differentiate influenza and SARS-CoV-2 simultaneously in patient samples and therefore will help to provide valuable time to the local health authorities to contain transmission and prepare appropriate response strategies against all three viruses.

Contributors

Conceptualization: KP, NZ.
Methodology: RM.
Formal Analysis: KP, AAW.
Data Curation: KP, AAW.
Writing-Original Draft: KP.
Writing – Review & Editing: KP, AAW, NZ, GAT.
Supervision: NZ, GAT.
Funding Acquisition: GAT.

Funding

This work did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Ethical approval

Not required.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to thank all technologists from the Molecular Diagnostics and Virology departments and staff at Alberta Precision Laboratories, Public Health Laboratory, Calgary and Edmonton, Alberta, Canada for their hard work and dedication.

References

CDC USA, 2007. CDC Realtime RTPCR (rRTPCR) Protocol for Detection and Characterization of Influenza (version 2007). CDC REF.# I-007-05. 23.
Cheng, H.Y., Jian, S.W., Liu, D.P., Ng, T.C., Huang, W.T., Lin, H.H., 2020. Contact tracing assessment of COVID-19 transmission dynamics in Taiwan and risk at different exposure periods before and after symptom onset. JAMA Intern. Med. 180 (9), 1156–1163.
Collaborators GBDI, 2019. Mortality, morbidity, and hospitalisations due to influenza lower respiratory tract infections, 2017: an analysis for the Global Burden of Disease Study 2017. Lancet Respir. Med. 7, 69–89.
Davidson, J.A., Warren-Gash, C., 2019. Cardiovascular complications of acute respiratory infections: current research and future directions. Expert Rev. Anti. Ther. 17, 939–942.
Dobson, J., Whitley, R.J., Pocock, S., Monto, A.S., 2015. Oseltamivir treatment for influenza in adults: a meta-analysis of randomised controlled trials. Lancet 385, 1729–1737.
Dowell, S.F., Ho, M.S., 2004. Seasonality of infectious diseases and severe acute respiratory syndrome-what we don't know can hurt us. Lancet Infect. Dis. 4, 704–708.
Dreier, J., Stormer, M., Kleesiek, K., 2005. Use of bacteriophage MS2 as an internal control in viral reverse transcription-PCR assays. J. Clin. Microbiol. 43 (9), 4551–4557.
Pabbaraju, K., Wong, A.A., Douesnard, M., Ma, R., Gill, K., Dieu, P., Fonseca, K., Zelyas, N., Tipples, G.A., 2021. Development and validation of RT-PCR assays for testing for SARS-CoV-2. JAMMI 6 (1). https://doi.org/10.3138/jammi-2020-0026.
Kim, D., Quinn, J., Pinsky, B., Shah, N.H., Brown, I., 2020. Rates of co-infection between SARS-CoV-2 and other respiratory pathogens. JAMA 323, 2085–2086.
Krammer, F., Smith, G.J.D., Fouchier, R.A.M., Peiris, M., Kedzierska, K., Doherty, P.C., Palese, P., Shaw, M.L., Treanor, J., Webster, R.G., Garcia-Sastre, A., 2018. Influenza. Nat. Rev. Dis. Primers 4, 3.
Kretzschmar, M.E., Rozhnova, G., Bootsma, M.C.J., Boven, M.V., van de Wijgert, J.H.H. M., Bonten, M.J.M., 2020. Impact of delays on effectiveness of contact tracing strategies for COVID-19: a modelling study. Lancet Public Health 5 (8), E452–E459.
Kucharski, A.J., Klepac, P., Conlan, A.J.K., Kissler, S.M., Tang, M.L., Fry, H., Gog, J.R., Edmunds, W.J., group CC-w, 2020. Effectiveness of isolation, testing, contact tracing, and physical distancing on reducing transmission of SARS-CoV-2 in different settings: a mathematical modelling study. Lancet Infect. Dis. 20, 1151–1160.
Lansbury, L., Lim, B., Baskaran, V., Lim, W.S., 2020. Co-infections in people with COVID-19: a systematic review and meta-analysis. J. Infect. 81, 266–275.
Ma, S., Lai, X., Chen, Z., Tu, S., Qin, K., 2020. Clinical characteristics of critically ill patients co-infected with SARS-CoV-2 and the influenza virus in Wuhan, China. Int. J. Infect. Dis. 96, 683–687.
Malosh, R.E., Martin, E.T., Ortiz, J.R., Monto, A.S., 2018. The risk of lower respiratory tract infection following influenza virus infection: a systematic and narrative review. Vaccine 36, 141–147.
Nalla, A.K., Casto, A.M., Huang, M.W., Perchetti, G.A., Sampoleo, R., Shrestha, L., Wei, Y., Zhu, H., Jerome, K.R., Greninger, A.L., 2020. Comparative performance of SARS-CoV-2 detection assays using seven different primer/probe sets and one assay kit. J. Clin. Microbiol. 58 (6), e00557–00520.
Reddy, K.P., Shebl, F.M., Foote, J.H.A., Harling, G., Scott, J.A., Panella, C., 2021. Cost-effectiveness of public health strategies for COVID-19 epidenic control in South Africa: a microsimulation modelling study. Lancet Glob. Health 9 (2), E120–E129.
Sellers, S.A., Hagan, R.S., Hayden, F.G., Fischer 2nd, W.A., 2017. The hidden burden of influenza: a review of the extra-pulmonary complications of influenza infection. Influenza Other Respir. Viruses 11, 372–393.
Vogels, C.B.F., Brito, A.F., Wyllie, A.L., Fauver, J.R., Ott, I.M., Kalinich, C.C., Petrone, M. E., Casanovas-Massana, A., Muenker, M.C., Moore, A.J., Klein, J., Lu, P., Lu-Culligan, A., Jiang, X., Kim, D.J., Kudo, E., Mao, T., Moriyama, M., Oh, J.E., Park, A., Silva, J., Song, E., Takehashi, T., Taura, M., Tokuyama, M., Venkataraman, A., Weizman, O.-E., Wong, P., Yang, Y., Cheemarla, N.R., White, E., Lapidus, S., Earnest, R., Geng, B., Vijayakumar, P., Odio, C., Fournier, J., Bermejo, S., Farhadian, S., Dela Cruz, C., Iwasaki, A., Ko, A.I., Landry, M.-L., Foxman, E.F., Grubaugh, N.D., 2020. Analytical sensitivity and efficiency comparisons of SARS-COV-2 qRT-PCR primer-probe sets. medRxiv. https://doi.org/10.1101/2020.03.30.20048108:PPR138009.

Wenjie, T.X.Z., Xuejun, M., Wenling, W., Peihua, N., Wenbo, X., George, F.G., Guizhen, W., 2020. Notes from the field: a novel coronavirus genome identified in a cluster of pneumonia cases — Wuhan, China 2019−2020. China CDC Weekly 2, 61–62.

Wu, X., Cai, Y., Huang, X., Yu, X., Zhao, L., Wang, F., Li, Q., Gu, S., Xu, T., Li, Y., Lu, B., Zhan, Q., 2020a. Co-infection with SARS-CoV-2 and influenza a virus in patient with pneumonia, China. Emerg. Infect. Dis. 26, 1324–1326.

Wu, C., Chen, X., Cai, Y., Xia, J., Zhou, X., Xu, S., Huang, H., Zhang, L., Zhou, X., Du, C., Zhang, Y., Song, J., Wang, S., Chao, Y., Yang, Z., Xu, J., Zhou, X., Chen, D., Xiong, W., Xu, L., Zhou, F., Jiang, J., Bai, C., Zheng, J., Song, Y., 2020b. Risk factors associated with acute respiratory distress syndrome and death in patients with coronavirus disease 2019 pneumonia in Wuhan, China. JAMA Intern. Med. 180, 934–943.

Yang, J., Zheng, Y., Gou, X., Pu, K., Chen, Z., Guo, Q., Ji, R., Wang, H., Wang, Y., Zhou, Y., 2020. Prevalence of comorbidities and its effects in patients infected with SARS-CoV-2: a systematic review and meta-analysis. Int. J. Infect. Dis. 94, 91–95.

Zhou, F., Yu, T., Du, R., Fan, G., Liu, Y., Liu, Z., Xiang, J., Wang, Y., Song, B., Gu, X., Guan, L., Wei, Y., Li, H., Wu, X., Xu, J., Tu, S., Zhang, Y., Chen, H., Cao, B., 2020. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. Lancet 395, 1054–1062.

Zhu, N., Zhang, D., Wang, W., Li, X., Yang, B., Song, J., Zhao, X., Huang, B., Shi, W., Lu, R., Niu, P., Zhan, F., Ma, X., Wang, D., Xu, W., Wu, G., Gao, G.F., Tan, W., 2020. A novel coronavirus from patients with pneumonia in China, 2019. N. Engl. J. Med. 382, 727–733.