ORIGINAL ARTICLE

Rapid Molecular Point of Care Testing for Detection of Influenza A, B Viruses and Respiratory Syncytial Virus Versus Multiplex PCR

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ABSTRACT

Introduction: Rapid detection of influenza viruses and respiratory syncytial virus (RSV) can be achieved by having rapid molecular point of care tests (POCTs). This expedites the diagnosis attributed by having similar clinical presentations leading to facilitation of precision medicine and reduction of antimicrobial resistance. The growing number of POCTs foster the need to ensure that these POCTs have satisfactory and reliable performance. With that the aim of this study is to evaluate the performance of rapid molecular POCT regarded as 'X' for the detection of Influenza viruses and RSV in comparison to multiplex PCR. **Methods:** A laboratory-based study was conducted from January to December 2020 which involved analysis of 116 nasopharyngeal swabs, tested using POCT X and multiplex PCR as a method of reference. The performance analysis incorporated the sensitivity, specificity, positive and negative predicted values determination. The cycle threshold values were reviewed for discordant results. **Results:** The POCT X demonstrated sensitivity of 88.57% with 100% specificity for Influenza A virus, and 85.71% of sensitivity with 100% specificity for influenza B virus detection. Meanwhile it revealed 100% sensitivity and specificity for RSV detection. There were ten specimens demonstrating discordant results whereby viruses were not detected by POCT X, however detected by multiplex PCR. The POCT X was not able to detect eight (12.9%) and two (16.7%) influenza A and B viruses respectively. **Conclusion:** The overall performance of POCT X was corresponded to multiplex PCR. This best served as a steadfast ancillary test for influenza and RSV infection.
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Keywords: Rapid molecular point-of-care test, Influenza A virus, Influenza B virus, Respiratory syncytial virus, Performance

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INTRODUCTION

Influenza and respiratory syncytial virus (RSV) are viruses known to be circulating worldwide and cause significant respiratory tract complications in certain populations associated to increased morbidity and mortality (1). Diagnosis of respiratory tract infection (RTI) caused by these viruses, include laboratory-based methods such as demonstration of cytopathic effect via viral isolation and serology, which remain essential. However, to date, these methods are mainly complemented and replaced with antigen detection via immunofluorescence (IF) technique and nucleic acid amplification tests (NAATs). This is mainly attributed to being laborious and costly, especially for maintaining cell lines and delayed turnaround time (TAT).

The rapid molecular POCTs in contrast to laboratory-based methods have the capacity to be accomplished at the site of specimen collection (e.g., emergency department, bedside, intensive care unit, clinics), offer results typically in less than two hours and require minimal training and handling of equipment (2,3). Furthermore, they require minimal hands-on time as most critical steps are automated in a single device (4).

Rapid and timely detection and identification of the aetiological agent may assist the healthcare personnel in the selection of the appropriate treatment, guide in decision making during outbreak situations, in relation to referral, hospital admission and quarantine (5).

The Infectious Diseases Society of America (IDSA) has recommended the use of rapid influenza molecular assays to detect influenza viruses in respiratory specimens over rapid influenza diagnostic tests (RIDTs). Besides that, the Centres for Disease Control and Prevention (CDC) advocated the use of reverse transcription PCR, as the method of reference for the diagnosis of influenza (6). Nevertheless, it is crucial to note that such advanced molecular technique is not widely available in most developing and less developed countries, owing to its high technicality and cost. In Malaysia, most laboratory tests that involves detection and identification of respiratory pathogens, which includes influenza virus and RSV by PCR were only offered to in-patients. Moreover, the molecular testing for respiratory pathogens is not routinely done but rather processed in batches. Thus, in reality, such investigation is not available in every centre nationwide, and even in the hospitals where it is offered; the timing from sampling to result may take up to a week.

Molecular technology had undergone a series of evolution and enabled multiple pathogen detection in a single test; also known as multiplex PCR. This technology has the ability in reducing the cost of performing multiple tests for one specimen, as well as assisting the detection of a wider range of pathogens. However, such multiplex PCR requires the initial process of decontamination and extraction. Risk of carry over contamination that could lead to false positive results is almost certain with well-trained personnel being needed to perform the related steps.

A more recent development of rapid molecular technique omits the specimen processing and reagent master mix mixing steps, where a sample can be tested directly by adding them to the appropriate pre-loaded cartridge, and the sampling process to result is shortened to less than an hour. This type of rapid molecular technique is appropriate as POCT, due to its simplicity, operator-friendly, fast TAT and yet uses advanced molecular methods which are generally more sensitive than antigen detection. Shedding light on the diagnostic accuracy or evaluation of POCT in detecting the abovementioned viruses may provide important information for policy purposes, including the provision of such kits in resource-poor-health facilities in rural areas, introduction of this simple and relatively affordable test kit (RM 150 for POCT versus RM 350 for multiplex PCR) in a suspected influenza outbreak for prompt prevention and control of the disease spread.

Management of influenza includes administration of antivirals that are best administered within 48 hours after the onset of symptoms. Meanwhile, management of RSV infection is principally supportive. As there is limited testing for influenza and RSV infection available at certain centres, patients may present to the emergency department at the later stage of respiratory infection, involving the lower respiratory tract and well past the 48 hours duration for antiviral treatment efficacy. Thus, there is a need for rapid molecular POCT to detect the pathogen at the first visit, long before the illness progress to the lower respiratory tract, causing severe symptoms. With that the study aims to evaluate the performance of rapid molecular POCT regarded as 'X' for the detection of Influenza A, B viruses and RSV in comparison to multiplex PCR, incorporating the determination of sensitivity and specificity of rapid molecular POCT X using multiplex PCR as a validation method.

MATERIALS AND METHODS

Study design and specimen recruitment

A multicentre laboratory-based study was conducted on the evaluation of a rapid molecular POCT which was regarded as POCT X throughout this study from January to December 2020. The inclusion criteria include nasopharyngeal swabs (NPS) that were tested positive for influenza viruses and RSV by multiplex PCR. Meanwhile the exclusion criteria were insufficient volume (less than 200µL) of viral transport media (VTM) that contained the NPS.

A total number of 120 specimens including NPS (n=119) and tracheal aspirate (TA, n=1) were recruited from Microbiology Laboratory, Department of Pathology, Hospital Sungai Buloh, and Quantum Diagnostic Gribbles Pathology Laboratory, Petaling Jaya Selangor, Malaysia. Of 120 specimens collected, 31 served as control and all these had none of the above three viruses detected. The controls were running in parallel to all tested specimens for both POCT X and multiplex PCR. The TA was excluded based on the criteria above, thus the total number of specimens tested for both platforms were 119. All tested specimens belonged to different patients.

Ethics approval

Ethical approval for this study was obtained from Research Ethics Committee, Universiti Teknologi MARA, REC/08/2020 (MR/200) and Medical Research & Ethics Committee (MREC), Ministry of Health, Malaysia, NMRR-19-1582-46748 (IIR).

Rapid molecular POCT X

This molecular device was designed for the detection of Influenza A and B viruses, as well as RSV. The POCT X operated based on an isothermal nucleic acid amplification technology using nicking enzyme amplification reaction (NEAR) to detect the PB2 gene in Influenza A virus, PA gene in Influenza B virus, and non-

structural gene NS2 and nucleocapsid gene N in RSV.

The POCT X provided step-by-step interactive instruction on the display screen and the test was initiated by placing the sample receiver into a blue panel on the machine. The initial process started with heating of the sample receiver containing the buffer for three minutes. Using a disposable pipette, 200µL of the specimen was withdrawn and mixed in the sample receiver for ten seconds. A transfer cartridge that also contained the reagent and master mix were used to draw and transfer the specimen from the sample receiver into a test base. The heating, mixing, PCR and detection process were embedded in the instrument. The process occurred for ten minutes, but could stop earlier if the above-mentioned genes were detected early in the cycle. The internal control is incorporated within the cartridge for each test, in which the control result will be obtained as 'Control Valid/Control Invalid' and the test result in the form of 'Detected / Not Detected'. In the event that the control was invalid, the entire test had to undergo re-run using a new cartridge.

Multiplex PCR

There were three multiplex PCR assays used as a method of reference in this study. The first was Fast Track Diagnostic™ Respiratory Pathogen 21 (FTD) that consists of probes to detect 21 different respiratory pathogens. Following extraction, the master mix was prepared based on the manufacturer's protocol. Apart from influenza A virus, a subtype of influenza A (H1N1) virus (swine-lineage), influenza B virus, and RSV A/B, FTD was also able to detect human coronaviruses NL63, 229E, OC43 and HKU1, human metapneumovirus A/B, human parainfluenza viruses 1,2,3 and 4, human bocavirus, enterovirus, human adenovirus, human parechovirus, human rhinovirus and *Mycoplasma pneumoniae*. The total time from specimen processing to result took four hours on average.

The second multiplex PCR assay used was Aries™ Flu A/B & RSV. This multiplex qualitative RT-PCR assay detected the matrix protein genes (M genes) of Influenza A and B virus, and the fusion gene of RSV. The VTM containing NPS were added through pipetting to the cassette, the cassette was then placed into ARIES magazine and then into ARIES instrument. The process from extraction, PCR amplification and result reading were fully automated in the machine. The total time from specimen to result took 120 minutes on average.

The third multiplex PCR assay used was Xpert™ Xpress Flu/RSV assay. This is also a multiplex qualitative RT-PCR assay detecting the matrix (M), basic polymerase (PB2) and acidic protein (PA) gene in influenza A virus, the matrix (M) and non-structural protein (NS) in influenza B virus, and the nucleocapsid of RSV A and B. The VTM containing NPS were added to the transfer chamber of the assay cartridge. The process from specimen to result was also fully automated as the Aries™ assay above. The total time from specimen to result took 30 minutes on average.

All the demographic data regarding age and gender of the patients were recorded and documented for analysis of both molecular testing methods.

Statistical analysis

The demographic data was analysed using descriptive analysis, while the performance of POCT X was evaluated by calculating the specificity and sensitivity, efficiency, positive predictive value (PPV) and negative predictive value (PPV). The cycle threshold (CT) value was obtained and recorded for discordant results between POCT X and multiplex PCR.

RESULTS

Demographic Characteristics

There were only 116 specimens out of 119 evaluated in this study as three specimens had contaminated VTM. The age of the patients for both POCT X and multiplex PCR methods ranged from five months to 82 years old, with the mean (standard deviation, SD) age being 33.2 (22.72) years old. There were 47 NPS from 116 specimens belonging to male while the remaining 69 were from female patients respectively, which made male to female ratio of 1:1.6.

In influenza A detected specimens by POCT X, the age ranged from one year to 81 years old, with the mean (SD) of 32.5 (24.02) years old. While in influenza B cases, the specimens were from patients aged four to 34 years old, with the mean (SD) of 17.1 (11.81) years old. The age ranged from six months old to 46 years old, with the mean (SD) of 14.3 (17.55) years old in RSV positive specimens.

The age for subjects evaluated using multiplex PCR, ranged from one year to 81 years old, with the mean (SD) of 32.5 (23.10) years old in influenza A positive cases. While in influenza B cases, the specimens were from patients aged four to 34 years old, with the mean (SD) of 18.9 (11.98) years old. The age ranged from six months old to 46 years old, with the mean (SD) of 14.3 (17.55) years old in RSV positive specimens.

Rapid Molecular POCT X

Of 116 specimens tested, influenza A virus was detected in 54 (46.6%) specimens, ten (8.6%) specimens had influenza B virus detected and RSV was detected in 11 (9.5%) specimens respectively. There were five specimens that had co-infection (detection of influenza A/B virus/RSV in the presence of other pathogens which were detected concurrently by multiplex PCR FTD. These were Influenza A virus with *Staphylococcus aureus* co-infection; Influenza A virus with Adenovirus co-infection; Influenza A virus, *Streptococcus pneumoniae*

and *Moraxella catarrhalis* co-infection; RSV and *Staphylococcus aureus* co-infection and Influenza A virus and Parechovirus co-infection.

Multiplex PCR

Of 116 NPS that were tested using multiplex PCR, Influenza A virus was detected in 62 (53.5%) specimens, Influenza B virus was detected in 12 (10.3%) specimens and RSV was detected in 11 (9.5%) specimens. Majority of the specimens had Influenza A virus detected, while both Influenza B virus and RSV had almost similar distribution as illustrated in Table I.

Table I: The distribution of POCT X result in comparison to multiplex PCR

Method	Control	FLU A*	FLU B*	RSV	Total
POCT X	31	54 (46.6%)	10 (8.6)	11(9.5)	116
Multiplex PCR	31	62 (53.5%)	12 (10.3%)	11(9.5)	116
Discordant results	-	8 (12.9%)	2 (16.7%)	0	10

FLU A, Influenza A virus; FLU B, Influenza B Virus; RSV, Respiratory syncytial virus

Rapid Molecular POCT X versus (vs) Multiplex PCR

There were ten specimens (n=10) demonstrating discordant results (viruses were not detected by POCT X, however detected by multiplex PCR). The POCT X was not able to detect eight (12.9%) and two (16.7%) influenza A and B viruses respectively. While for RSV, the POCT X managed to achieve 100% agreement to multiplex PCR. All specimens with discordant results were reviewed using CT value from the multiplex PCR as shown in Table II. The CT values varied from 17.8 to 42.69, however there were two specimens (Influenza B virus detected) where CT values were not available.

Table II: Discordant results by POCT X in corresponding to CT value of multiplex PCR

Sample No.	POCT X	Multiplex PCR	CT value
1	Influenza A not detected	Influenza A detected	17.80
2	Influenza A not detected	Influenza A detected	23.73
3	Influenza A not detected	Influenza A detected	37.80
4	Influenza A not detected	Influenza A detected	42.69
5	Influenza A not detected	Influenza A detected	27.3
6	Influenza A not detected	Influenza A detected	36.90
7	Influenza A not detected	Influenza A detected	40.91
8	Influenza A not detected	Influenza A detected	35.10
9	Influenza B not detected	Influenza B detected	40.1
10	Influenza B not detected	Influenza B detected	40.3

Based on Table II, it was evident that the viral load was appropriate for detection of Influenza A virus for multiplex PCR.

Performance of Rapid Molecular POCT X

The sensitivity, specificity, efficiency, NPV and PPV of POCT X were determined and illustrated in the Table III and IV. According to both Table III and IV, POCT X demonstrated 88.10% of overall sensitivity with 100% specificity for Influenza virus detection. This is inclusive of 100% PPV, 75.61% NPV with 91.3% efficiency. While the sensitivity of POCT X in detection of Influenza A virus was 88.57% with 100% specificity. The PPV and NPV for Influenza A was 100% and 79.48% respectively. Meanwhile, the efficiency for detecting Influenza A virus was 91.18%. For Influenza B virus, POCT X exhibited sensitivity of 85.71% with 100% specificity. The PPV, NPV and efficiency were 100%, 93.93%, and 95.55% respectively. The sensitivity and specificity of POCT X in detecting RSV were at 100%, and the PPV with NPV were equally 100%, as well as 100% efficiency. Table III outlines the sensitivity and specificity of POCT X.

Table III: Number of specimens tested with multiplex PCR and POCT X

	FLU A	FLU B	FLU A + FLU B	RSV
a	62	12	74	11
b	0	0	0	0
c	8	2	10	0
d	31	31	31	31

FLU A, Influenza A virus; FLU B, Influenza B Virus; RSV, Respiratory syncytial virus; a = Number of true positive; b = Number of false positive; c = Number of false negative; d = Number of true negative.

Table IV: The sensitivity, specificity, PPV and NPV of rapid molecular POCT X for detection of tested pathogens

	FLU A	FLU B	FLU A + FLU B	RSV
Sensitivity (%)	88.57	85.71	88.10	100
Specificity (%)	100	100	100	100
Efficiency (%)	91.18	95.55	91.3	100
PPV (%)	100	100	100	100
NPV (%)	79.48	93.93	75.61	100

FLU A, Influenza A virus; FLU B, Influenza B Virus; RSV, Respiratory syncytial virus.

DISCUSSION

The annual influenza and sporadic RSV outbreaks advocate the development of a rapid and reliable molecular diagnostic technique that are readily available and closer to healthcare worker, in order to expedite diagnosis and decision making. Such development is vital for a highly transmissible respiratory infection like influenza and RSV. An article on epidemic preparedness indicated that the development and employment of direct detection methods to detect the viraemia stage is crucial, so that cases can be appropriately managed while further transmission can be halted (8).

To date there are multiple molecular POCT assays available which indirectly display the great need to assess the performance of POCT assays, as such evaluation may assist healthcare providers to make a decision on which assay to purchase. In 2017, a systematic review and meta-analysis on diagnostic accuracy of rapid test for influenza compared with RT- PCR was conducted and yielded the pooled sensitivity of rapid molecular POCT X of 84.4% with 98.9% specificity for influenza A detection, while sensitivity and specificity for Influenza B were 87.3% and 98.7% respectively (9). Subsequently, in 2020 a study by Farfour E, et al. revealed an improved performance of rapid molecular POCT X with sensitivity of 92% for influenza A virus detection, and 96.2% for influenza B using RT-PCR assays (Allplex and Anyplex) as the method of reference. Here, the POCT X was found to be in between these two reported studies, whereby it has improved sensitivity and specificity in contrast to the earlier study conducted in 2017, while having slightly reduced sensitivity as compared to study by Farfour E,et al.

Meanwhile for RSV infection, the present study obtained 100% sensitivity and specificity using POCT X. In 2017, Schnee SV et al. reported that the overall sensitivity and specificity of the Alere i RSV test assay was 93% and 96% respectively in a paediatric point-of-care setting. In 2020, Verbakel JY et al., demonstrated that the cobas® Liat® POCT had a sensitivity and specificity of 100% and 99.4% for RSV respectively in a primary care setting. Hence our current evaluation is in concordance to other described studies and exhibited improved performance of POCT X especially for RSV infection.

On further perspective, it is evident in our study that the presence of other pathogen in the specimen detected by multiplex PCR did not affect the sensitivity and specificity of rapid molecular POCT X and this was contributed by the isothermal amplification methods that made this POCT X acquired a certificate of waiver from Clinical Laboratory Improvement Amendments (CLIA) (13, 14). The satisfactory performance of POCT X to detect influenza viruses and RSV in co-infections is important, as multiple infections may result in higher morbidity and mortality. Nevertheless, it is noteworthy that qualitative molecular methods such as POCT X is detecting the presence of RNA of these viruses in the specimen, without the ability to distinguish whether they are viable and causing disease, present as a colonizer or only remnants of RNA from a dead pathogen. Consequently, when more than one pathogen is detected, clinical consideration is crucial.

The CT value represents the number of cycles needed before the detection graph gives a sigmoid pattern and thus interpreted as positive / detected. In this study, the CT values were obtained from the multiplex PCR in order to rationale the false negative results of POCT X. There were previous studies (9, 15) conducting the analysis of CT values on the false negative results, where it was found that all false negative results had high CT value which correlated to low viral load that failed to be detected by POCT tested. This however was not the case for our study, as the CT value for discrepancy results ranged from 17.8 to 42.69 as illustrated in Table II. This may be attributed to two different nasopharyngeal swabs collected simultaneously for both tested methods which may have inconsistent viral load upon swabbing.

The present study has a few limitations including apparent reduction in the number of specimens collected after COVID-19 pandemic commenced, specifically after Malaysian government announced the Movement Control Order (MCO) in March 2020. The MCO involved closure of school, nurseries, and non-essential industries, as well as increasing the public awareness on wearing masks, social distancing and hand-washing. These contribute to reduced numbers of specimens for influenza and RSV infection. Moreover, as the national lock down is executed, patients may not seek medical attention for mild and moderate illness, which in turn has resulted in reduced hospital / clinic visits, thus the number of samples.

CONCLUSION

The POCT test is best suited as supplementary testing for influenza and RSV infection owing to its rapid detection, improved performance and reliability which is beneficial for proper patient's management.

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