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RESEARCH ARTICLE

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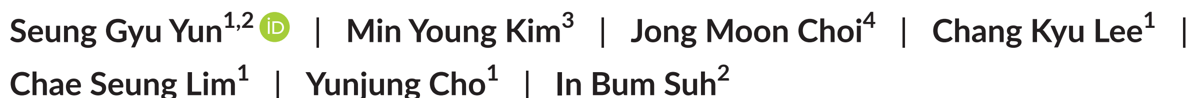
Comparison of three multiplex PCR assays for detection of respiratory viruses: Anyplex II RV16, AdvanSure RV, and Real-Q RV



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Background: Due to its great sensitivity, the nucleic acid amplification test (NAAT) is widely used for detection of respiratory viruses (RV). However, few reports have described a direct comparison between multiplex RT-PCR assays for RV. The objective of this study was to perform a direct comparison of three multiplex RT-PCR assays for the detection of respiratory viruses.
Methods: A total of 201 respiratory samples (161 nasopharyngeal swab samples and 40 sputum samples) were tested with three commercial RV assays: Seegene Anyplex II RV16 (AP), LG AdvanSure RV (AD), and Biosewoom Real-Q RV (RQ). The additional tests for the discrepant results were conducted by repeat RV assay or monoplex PCR coupled direct sequencing. Data analysis using percent agreement, kappa, and prevalence-adjusted and bias-adjusted kappa (PABAK) values was performed for comparisons among the three RV assays.
Results: Of the 201 samples, AP, AD, and RQ detected 105 (52.2%), 99 (49.3%), and 95 (47.3%) positive cases respectively. The overall agreement, kappa, and PABAK values for the three assays ranged between 97%-98%, 0.76-0.86, and 0.93-0.96 respectively. The performance of the three assays was very similar, with 94%-100% agreement for all comparisons, each virus types. The additional testing of samples showed discrepant results demonstrating that AD assay had the highest rate of concordance with original results.
Conclusions: We suggest that all multiplex assay would be suitable for the detection of for respiratory viruses in clinical setting.

KEYWORDS
agreement, nucleic acid amplification test, respiratory virus

1 | INTRODUCTION

Acute respiratory infections (ARI) are one of the major causes of mortality worldwide and approximately half of ARI are caused by respiratory viruses (RV).[1] There are several types of tests for detection of RV. Among them, the nucleic acid amplification test (NAAT) has widely been accepted in recent years.[2] NAAT has advantages in comparison to the classical methods of viral culture and direct fluorescent antibody tests. First, NAAT has superior sensitivity identifying RV cases not detected by classical methods. Second, RV testing results are

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available faster than those of viral cultures. Speed to result allows medical professionals to prescribe antiviral agents and perform appropriate infection control more rapidly. Lastly, NAAT does not need a strict transportation protocol for maintaining viability of RV.[3] In addition, the application of multiplexing technology in NAAT allows better detection than classical methods of a broad range of viruses. For all of these reasons NAAT has replaced classical methods.

There are a number of commercial multiplex kits that can detect between 12 and 23 virus types.[3,4] Performance evaluation studies for newly developed multiplex RV kits are weak, do not establish the reference standard method and therefore do not sufficiently calculate sensitivity and specificity of each test. Instead, some studies have suggested the reference test with in-house multiplex real-time PCR or commercial duplex PCR tests.[5-8] We performed a direct comparison of three commercial multiplex assays and produced the values of the agreement and kappa instead of sensitivity and specificity with the reference tests. The objective of this study was to perform a direct comparison of three multiplex RT-PCR assays for the detection of respiratory viruses.

2 | MATERIALS AND METHODS

2.1 | Clinical samples

Respiratory samples used in this study were collected between December 2015 and March 2016 at Armed Forces Medical Research Institute and Armed Forces Daejeon hospital (Daejeon, Republic of Korea) from those submitted for the detection of respiratory viruses. Clinical samples from 201 young male soldiers (age range 18-27 years, median 21 years) with acute respiratory illness were randomly selected for study without knowledge of previous results from Real-Q RV or Anyplex II RV16 tests. They consisted of nasopharyngeal (NP) swab samples (n=161) and sputum samples (n=40) and were stored at −70°C until this study was conducted.

Nasopharyngeal swab samples were obtained using flocked swabs and transported in 3 mL universal transport medium (COPAN Diagnostics, Murrieta, CA, USA). Sputum samples were received in sterile plastic containers and treated in order to homogenize samples using a 1:1 ratio dithiothreitol, which was diluted 1:100 with distilled water because of it viscosity. This study was approved by the Ethics Committee of Armed Forces Medical Command (AFMC-16-IRB-023).

2.2 | Nucleic acid extraction

The nucleic acid extraction system was used for each RV assay. For Anyplex II RV 16 (AP), nucleic acids were extracted from 500 μL of clinical samples using a MICROLAB Nimbus IVD workstation (Hamilton, Reno, NV, USA) with a STARMag 96 Virus Kit (Seegene, Seoul, Republic of Korea) and eluted into 80 μL of elution buffer. For AdvanSure RV real-time PCR (AD), nucleic acids were extracted from 200 μL of clinical samples using the TANBead Smart LabAssist-32 extraction system with a TANBead Viral Auto Plate kit (Taiwan Advanced Nanotech Inc., Taoyuan City, Taiwan) and eluted into 80 μL of elution buffer. For Real-Q RV Detection assay (RQ), nucleic acids were extracted from 200 μL of clinical samples using a Maxwell™ 16 device with the Maxwell 16 Viral Total Nucleic Acid Purification Kit (Promega, Madison, WI, USA) and eluted into 80 μL of elution buffer.

2.3 | Multiplex RT-PCR assays

Three commercial multiplex RV assays were performed based on manufacturers' protocols. Characteristics of the three multiplex assays are briefly reported in Table 1. The AP assay is composed of two-step RT-PCR. Before multiplex target PCR was performed, complementary DNA (cDNA) was synthesized from each sample's RNA through reverse transcriptase (RT) reaction with the cDNA Synthesis kit (Seegene). The mixture for CDNA synthesis is a 20 μL final volume, including 8 μL of the sample's nucleic acid, 8 μL of RT buffer, 2 μL of the RT enzyme Mix and 2 μL of random Hexamer with Veriti Thermal Cycler (Applied Biosystems, Foster City, CA, USA) under the following conditions: 25°C for 5 minutes, 37°C for 60 minutes and 95°C for 2 minutes. After cDNA synthesis, the 16 target viruses were multiplexed into two tubes according to target primer and probe. Multiplex real-time PCR was conducted in a 20 μL mixture containing 8 μL of cDNA, 5 μL of 4X TOCE Oligo Mix primer, 5 μL of 4X PCR master mix, and 2 μL of RNase-free water with the CFX96 Real-time PCR detection system (Bio-rad, Hercules, CA, USA). The thermal cycling conditions were: 50°C for 4 minutes; denaturation at 95°C for 15 minutes followed by 50 cycles of 95°C for 30 seconds, 60°C for 1 minutes and 72°C for 30 seconds. During the interval of the 50 cycles, melting temperature analysis was conducted by cooling the mixture to 55°C and maintaining for 15 seconds followed by heating the mixture from 55°C to 85°C (0.5°C/5 seconds) after 30, 40, and 50 cycles respectively. The fluorescence was continually detected during heating process and the data were converted to derive the melting temperature by plotting the negative derivative of fluorescence vs temperature ($-dF/dT$ against T). Seegene viewer software can automatically interpret melting peaks derived and present the results as '+, ++, and +++ positive' according to detection cycles of melting temperature analysis or 'negative.' Bacteriophage MS2 as an Internal Control (IC) was added to clinical specimens before nucleic acid extraction and was incorporated into the product as an exogenous whole process control in order to monitor during the period of time between nucleic acid isolation to result interpretation. The IC was co-amplified with the target nucleic acids within the clinical specimens.

The AD assay performs both reverse-transcription(RT) reaction with the extracted RNA from specimen and multiplex PCR reaction simultaneously in a single tube (1-step RT-PCR). It uses human RNase P gene, as an internal control, to give information for validity of the RNA extraction procedure and to prevent misjudgment from sampling error and RT-PCR reaction error. Briefly, the assay was multiplexed into five tubes targeting 14 RVs and an IC gene. This was conducted in a 20 μL mixture containing 5 μL of nucleic acid, 5 μL of primer/probe mixture and 10 μL of RT-PCR premix with the SLAN Real-time PCR detection system (Shanghai Hongshi Medical Technology Co., Shanghai, China). The thermal cycling conditions for the RT step were: 50°C for

TABLE 1 Summary of characteristics in multiplex assays for detection of respiratory viruses

Assay	AP	AD	RQ
Target virus	Influenza virus A	Influenza virus A	Influenza virus A
	Influenza virus B	Influenza virus B	Influenza virus B
	Respiratory syncytial virus A	Respiratory syncytial virus A	Respiratory syncytial virus A
	Respiratory syncytial virus B	Respiratory syncytial virus B	Respiratory syncytial virus B
	Parainfluenza virus 1	Parainfluenza virus 1	Parainfluenza virus 1
	Parainfluenza virus 2	Parainfluenza virus 2	Parainfluenza virus 2
	Parainfluenza virus 3	Parainfluenza virus 3	Parainfluenza virus 3
	Parainfluenza virus 4	—	—
	Coronavirus 229E	Corona Virus 229E	Corona Virus 229E
	Coronavirus OC43	Corona Virus OC43	Corona Virus OC43
	Coronavirus NL63	Corona Virus NL63	Corona Virus NL63/HKU1
	Bocavirus 1/2/3/4	Bocavirus	Bocavirus
	Adenovirus	Adenovirus	Adenovirus
	Rhinovirus A/B/C	Rhinovirus A/B/C	Rhinovirus
	Enterovirus	—	—
	Metapneumovirus	Metapneumovirus	Metapneumovirus
Technology	Melting curve analysis & Real-time RT PCR	Real-time RT PCR	Real-time RT PCR
	Tagging Oligonucleotide Cleavage and Extension (TOCE)	Taqman probe chemistry	Taqman probe chemistry
Samples per run	40	16	16
Automated result presentation	Yes	Yes	Yes
Method step	3	2	2
Turn-around time (h)[a]	4.5	2	2

AP, Anyplex II RV16; AD, AdvanSure RV; RQ: Real-Q RV.
[a]Turn-around time excludes nucleic acid extraction time.

10 minutes and 95°C for 30 seconds followed by 40 cycles of 95°C for 15 seconds; 53°C for 30 seconds and 60°C for 30 seconds.

The RQ assay is also a one-step RT- PCR method. Briefly, the assay was multiplexed into five tubes targeting 14 RVs and 1 IC gene, which was human RNase P, like in the AD assay. RQ was conducted in a 22 μL mixture containing 5 μL of nucleic acid, 12.5 μL of 2X PCR reaction mixture, 1 μL of RT-PCR enzyme, and 3.5 μL of water with CFX96 Real-time PCR detection system (Bio-rad). The RT-PCR reaction was performed with the following conditions for the RT step: 50°C for 30 minutes; 95°C for 15 minutes, followed by 45 cycles of 95°C for 15 seconds and 62°C for 45 seconds.

2.4 | Discrepant result analysis

A flow diagram of discrepant analysis is described in Figure 1. In summary, 167 samples (83.1%) with positive results from at least two RV assays or negative results from all three assays were not tested with the additional assay. However, the other 34 samples (16.9%), containing 36 discordant results showing a single positive result in any assay, were tested with the following additional assay. A single positive result from AP and RQ assay was retested individually with RV assay. Whereas a single positive result from AD assay was performed with monoplex PCR followed by direct sequencing. If the results between the original and additional tests were concordant, they were considered to be a consensus of positive results. Conversely, if the results between original and additional tests showed a discordance, they were considered to be a negative consensus. Because both AD and RQ assay cannot detect PIV4 and EV, the results about PIV4 and EV were excluded from the discrepant analysis.

2.5 | Statistical analysis

Statistical methods of inter-rater agreement, including percent agreement and kappa statistics, were calculated to compare the three RV assays. In addition, prevalence-adjusted and bias-adjusted kappa (PABAK) was calculated to compensate for underestimation of kappa values caused by low prevalence of each respiratory type.[9] The kappa value can be interpreted as follows: <0.20 as poor; 0.21-0.40 as fair; 0.41-0.6 as moderate; 0.61-0.8 as good and 0.81-1 as very good agreement.[10] Statistical analyses were performed using Microsoft

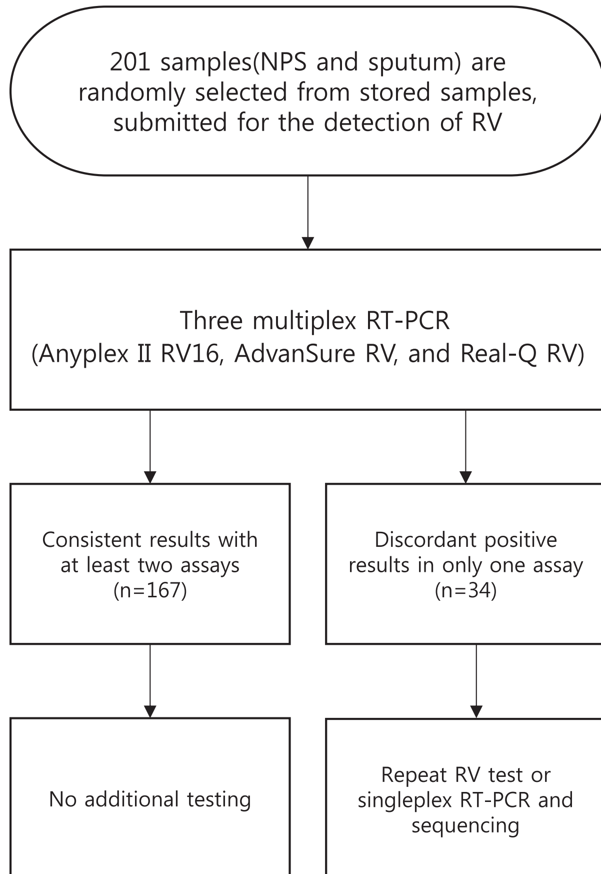

FIGURE 1 Flow diagram for evaluation of three commercial multiplex assays

Excel (Microsoft, Redmond, WA, USA) and MedCalc version 14.8.1 (MedCalc, Mariakerke, Belgium).

3 | RESULTS

Statistical analysis using percent agreement between RV assays in each virus type were high, ranging from 94% to 100%. Overall agreement between three RV assays ranged from 97% to 98%. The total kappa values were 0.76 between AP and AD, 0.82 between AP and RQ, and 0.86 between AD and RQ respectively. The kappa values ranged from 0.49 for PIV (AP vs AD and AD vs RQ) to 1.0 for PIV (AP vs RQ). In addition, PABAK values, which were adopted to compensate for the underestimation of kappa value when viruses show at a low prevalence rate, demonstrated very good agreement with ranges from 0.88 to 1.0 in comparison with kappa values.[11] Table 2 shows the above results.

A total of 167 samples (83.1%) showed concordant results. There was a total of 34 samples with a single positive result by one of the RV assays and two of these samples had discrepant results of two viruses. The 34 samples included 36 discordant results, 16, 18, and 2 viruses were positive only by AP, AD, and RQ respectively. Eighteen of the 36 discordant results from AP and RQ were resolved by repeat testing, with AD by monoplex PCR followed by direct sequencing. For the 36 discrepant virus results, additional testing revealed that 18.8% (3/16), 55.6% (10/18), and 50.0% (1/2) were consistent with the original virus result of AP, AD, and RQ assays respectively. These results were considered positive (Table 3).

Of the 201 samples, AP, AD, and RQ detected 103 (51.2%), 99 (49.3%), and 95 (47.3%) positive cases respectively (Table 4). Viral co-infection samples were identified in AP assay for 24 (11.9%) patients, in AD assay for 17 (8.5%) patients, and in RQ assay for 11 (5.5%) patients. In the co-infected samples: two viruses were detected in 24 patients by AP assay; two viruses were detected in 14 patients and three viruses in three patients by AD assay; two viruses were detected in 11 patients by RQ assay. INF A was the most commonly detected virus by co-infected samples, followed by ADV, RSVB, MPV and etc. Overall distribution of respiratory viruses from 3 RV assays is presented in Table 4. There are indications of the predominance of INFA, ADV, HRV, and MPV. Excluding these four major types, the rest of RV types accounted for only 0.8% to 8.9% of total viruses identified.

4 | DISCUSSION

The current study was performed for direct comparison between commercial multiplex RT-PCR for detection of respiratory viruses. A high degree agreement was found between AP, AD, and RQ assays. The agreement between 3 assays for INF A, INF B, PIV, CoV, HRV, MPV, RSV, and ADV ranged from 94% to 100% (Table 2). The agreement for PIV demonstrated the highest percent ranges, between 99% and 100%. While the kappa values varied according to virus types detected, PIV between AD vs AP and RQ was the lowest value (κ=0.49). This result was caused by low frequency of PIV in samples making them eligible for PABAK. The value of PABAK increased to 0.98. RSV between AP vs AD and RQ show a low kappa value; however, PABAK for RSV did not show a sharp increase as seen with PIV because of the number of discrepant results between three assays.

In real circumstances of a clinical laboratory, the introduction of multiplex RT-PCR assay for detection of RV was considered based on the assay's performance data and user friendliness.[2,3,12] The assay characteristics of 3 RV assays related to end-users was briefly summarized in Table 1. AP assay can detect 16 virus types and AD and RQ assays can detect 14 virus types. According to our study, the AP assay can test 40 samples in a single run whereas AD and RQ assay can simultaneously test 16 samples per run. However, the AP assay required the longest turn-around time compared with other assays: 4.5 hours for the AP assay vs 2 hours for both the AD and RQ assays. In addition, the AP assay consisted of two steps, reverse transcription for cDNA synthesis and real-time PCR coupled melting temperature analysis. As such it required end-users to spend more hands-on time to handle the reagents than other assays did. To overcome the time-consuming step in the AP assay, an automated liquid handling system of mixing and handling reagents for PCR mater mix setup could be automated using MICROLAB Nimbus IVD (Hamilton). The characteristics between AD and RQ assays were very similar. Both AD and RQ assays were used based on TaqMan probe and 1-step multiplex RT-PCR combined with

TABLE 2 Analysis of agreement among three multiplex assays

Virus	Assays	Agreement(95% CI)		Kappa value (PABAK)	
		AD	RQ	AD	RQ
INFA	AP	97 (93.9-99.1)	99 (96.8-100)	0.86 (0.93)	0.93 (0.97)
	AD		97 (94.7-99.4)		0.87 (0.94)
INFB	AP	98 (96.1-99.9)	99 (97.6-100)	0.70 (0.96)	0.85 (0.98)
	AD		99 (97.6-100)		0.85 (0.98)
PIV[a]	AP	99 (97.6-100)	100	0.49 (0.98)	1.00 (1.00)
	AD		99 (97.6-100)		0.49 (0.98)
CoV[b]	AP	96 (93.3-98.7)	98 (95.4-99.7)	0.79 (0.92)	0.84 (0.95)
	AD		95 (91.4-97.7)		0.69 (0.89)
HRV	AP	97 (94.0-99.1)	96 (93.3-98.7)	0.74 (0.93)	0.69 (0.92)
	AD		99.5 (98.5-100)		0.97 (0.99)
MPV	AP	98 (95.4-99.7)	99 (97.6-100)	0.77 (0.95)	0.90 (0.98)
	AD		99 (96.8-100)		0.86 (0.97)
RSV[c]	AP	94 (90.8-97.3)	95 (92.0-98.0)	0.57 (0.88)	0.64 (0.90)
	AD		99 (97.6-100)		0.90 (0.98)
ADV	AP	96 (93.3-98.7)	97 (94.0-99.1)	0.78 (0.92)	0.82 (0.93)
	AD		99 (96.8-100)		0.91 (0.97)
Total	AP	97 (95.8-97.6)	98 (97.0-98.4)	0.76 (0.93)	0.82 (0.95)
	AD		98 (97.5-98.8)		0.86 (0.96)

[a]PIV including type 1-4.
[b]Cov including type 229E, OC43 and NL63.
[c]RSV including type A and B.

TABLE 3 Discordant analysis of positive results by only one RV assay

Type of discrepant results	Result from AP	Result from AD	Result from RQ	Consensus	No. of samples
1	RSVB	Negative	Negative	Negative	9
2	RSVB	PIV2	Negative	Negative	1
3	RSVB	OC43	Negative	OC43	1
4	RSVA	Negative	Negative	Negative	1
5	Negative	RSVB	Negative	Negative	1
6	Negative	RSVB	Negative	RSVB	1
7	Negative	229E	Negative	Negative	4
8	Negative	229E	Negative	229E	1
9	Negative	Negative	NL63	Negative	1
10	Negative	MPV	Negative	MPV	1
11	Negative	NL63	Negative	NL63	1
12	Negative	Negative	ADV	ADV	1
13	Negative	OC43	Negative	OC43	1
14	Negative	INFA	Negative	Negative	1
15	Negative	INFA	Negative	INFA	3
16	Negative	MPV	Negative	MPV	1
17	Negative	MPV	Negative	Negative	1
18	MPV	Negative	Negative	MPV	1
19	INFA	Negative	Negative	INFA	1
20	ADV	Negative	Negative	Negative	1
21	ADV	Negative	Negative	ADV	1
Total no.					34

TABLE 4 Distribution of respiratory viruses detected by each assay

Target virus	AP (%)	AD (%)	RQ (%)	Consensus (%)
Influenza A	26 (20.2)	29 (24.4)	25 (23.6)	30 (24.4)
Influenza B	6 (4.7)	7 (5.9)	7 (6.4)	7 (5.7)
Parainfluenza 1	1 (0.8)	0 (0.0)	1 (0.9)	1 (0.8)
Parainfluenza 2	1 (0.8)	2 (1.7)	1 (0.9)	1 (0.8)
Parainfluenza 4	2 (1.6)	NT	NT	
Coronavirus 229E	7 (5.4)	12 (10.1)	7 (6.6)	8 (6.5)
Coronavirus OC43	6 (4.7)	9 (7.6)	5 (4.7)	9 (7.3)
Coronavirus NL63	3 (2.3)	4 (3.4)	3 (2.8)	4 (3.3)
Human rhinovirus	12 (9.3)	17 (14.3)	16 (15.1)	17 (13.8)
Metapnuemovirus	12 (9.3)	12 (10.1)	11 (10.4)	14 (11.4)
Respiratory syncytial virus A	2 (1.6)	1 (0.8)	1 (0.9)	1 (0.8)
Respiratory syncytial virus B	23 (17.8)	10 (8.4)	10 (9.4)	11 (8.9)
Adenovirus	21 (16.3)	16 (13.4)	19 (17.9)	20 (16.3)
Enterovirus	7 (5.4)	NT	NT	
Total viruses detected	129 (100)	119 (100)	106 (100)	123 (100)
No virus	96	102	106	98
1 virus	81	82	84	87
2 virus	24	14	11	16
3 virus	0	3	0	
Total samples	201 (100)	201 (100)	201 (100)	201 (100)

NT, Not tested for target virus.

reverse transcription followed by real-time PCR in a closed system. The test result of all assays was automatically presented by free analysis software offered from each company. The selection of multiplex RT-PCR assay for RV detection requires consideration of each laboratory's facility, human resources and the number of tests.

To the best of our knowledge there is no study that compares Anyplex II RV16, AdvanSure RV, and Real-Q RV in young adult patients with acute respiratory illness. Among the three assays, performance evaluation of AP assay was discussed in a number of articles. A first evaluation of AP assay against a combined standard of AP, xTAG Respiratory Viral Panel and Seeplex RV15 reported 95.2% sensitivity and ≥98.6% specificity rate.[13] About the same time, Cho et al.[14] reported that the performance of AP was superior in comparison with viral culture and Seeplex RV15. Huh et al.[15] analyzed agreement between AP and Seeplex RV12 without standard method and found that the AP assay produced an equivalent performance against Seeplex RV12. The result of present study also showed that AP assay produced good agreements in performance comparable with AD and RQ assay in performance. In a previous study, AD showed good agreement (98%) against conventional multiplex RT-PCR.[16] Similarly, the performance evaluation of AD vis-à-vis a composite standard method revealed the most sensitive performance compared with viral culture and Seeplex RV15.[17] The performance study of RQ assay was not found in the pubmed database, but evaluation of a previous version (1-step RV real-time PCR) against direct sequencing as a standard method reported 94.1% sensitivity and 96.6% specificity.[18] We demonstrated that the agreement between three assays was excellent. Moreover, the end-user in clinical laboratories selecting the multiplexed RT-PCR for RV will need to consider the benefits of each assay in terms of both performance and user-friendliness.

In the analysis of discrepant samples, 18.8% (3/16) from AP, 55.6% (10/18) from AD, and 50.0% (1/2) from RQ were consistent with the original virus results (Table 3). Of note, when repeat AP assay was done all RSVB samples (n=11) with an initial positive result by single AP identified as negative. Original test results of these samples were presented as + positive as detected by a melting temperature analysis after 50 cycles because they were supposed to contain low virus concentration. Unfortunately, the manufacturing company does not provide target range for virus detection and discloses that AP is a qualitative test for the detection of RV.

Likewise, samples of CoV 229E identified as positive by single AD assay identified as negative (80%, 4/5) from result of monoplex PCR followed by direct sequencing. Negative samples had a high C_t (threshold cycle) value (mean±SD, 25.17±1.19) close to 27.0 as a cut-off value, whereas one identified as positive using AD assay had a low C_t value (23.1). These findings suggest that the discordant results were frequently reported in samples containing low viral copy. Other studies also indicated that discrepant results between RV assay was quite associated with sample's viral load.[7,8,19-21] One samples by AP and three samples by AD identified as INF A positive by additional testing. This may be caused by the difference of target region in influenza genome in each assay. Other studies also indicated

that detection ability of the influenza virus between commercial RV assay was varied according to subtype.[22,23] Unfortunately, we did not perform the Influenza A subtyping on targeted discrepant samples so any assay in this study cannot differentiate between subtypes of INF A. Further evaluation of performance between assay with regard to influenza A subtype is needed.

There were some limitations to this study. First, this study used samples from only young adults for a winter season. Thus, we could not obtain adequate samples per target virus, such as PIV type 1-4, CoV 229E, OC43 and NL63, and RSV A. Those viruses could not be subjected to measure agreement values for each target due to low numbers. Second, another limitation was the use of stored frozen samples. This could skew results of RV detection based on storage conditions and the defrosting procedure. However, since all specimens were stored at the same storage condition and were processed by one skilled technician, we presume that comparison results between three assays was not significantly influenced by this characteristic of the sample.

In conclusion, the agreement of the three assays were very good, with 94%-100% agreement for all comparisons. We suggest that all multiplex assay would be suitable for the detection of for respiratory viruses in clinical setting.

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