

Clinical Performance of the Allplex™ Respiratory Panel 1 Test Compared to Simplexa™ Flu A/B and RSV for Detection of Influenza Virus and Respiratory Syncytial Virus Infection Including Their Subtyping

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Significance of the Study

- The performance of the Allplex™ Respiratory Panel was evaluated and compared to that of the Simplexa™ Flu A/B & RSV assay and other diagnostic tools for the detection of respiratory viruses. This panel can be used as a reliable and convenient assay for the detection of respiratory viruses because of its high sensitivity and specificity.

Keywords

Allplex™ Respiratory Panel 1 · Respiratory virus · Real-time polymerase chain reaction · Influenza · Respiratory syncytial virus

Abstract

Objective: The Allplex™ Respiratory Panel 1 (ARP) is a new assay based on a real-time polymerase chain reaction (RT-PCR) for the detection of influenza A (Flu A), influenza B virus (Flu B), and respiratory syncytial virus (RSV), including subtyping by multiple detection temperature (MuDT) technology. We evaluated the performance of the Allplex Respiratory Panel compared to the Simplexa™ Flu A/B & RSV assay (SP) and other diagnostic tools. **Materials and Methods:** A total of 372 samples were collected from patients at the Korea University Guro Hospital in Seoul, Korea. All samples were tested for influenza virus and RSV by ARP, SP, and an in-house RT-PCR. **Results:** The sensitivity of ARP was 95.56, 100, and 95.24% for Flu A, Flu B, and RSV, respectively. The

specificity of ARP was 100, 100, and 100% for Flu A, Flu B, and RSV, respectively. SP had sensitivities and specificities of 98.89 and 100% for Flu A, 100 and 100% for Flu B, and 100 and 100% for RSV. **Conclusion:** The Allplex panel showed high sensitivity, specificity, positive predictive, and negative predictive values for the detection of Flu A, Flu B, and RSV. This assay is fast and easy to perform because it takes only about 150 min and there is no need for post-PCR electrophoresis. The ARP can be used as a reliable and convenient assay in clinical laboratories.

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Introduction

Influenza virus is a major cause of morbidity and mortality in old and young patients and especially in immunocompromised individuals [1]. Influenza occurs globally with an estimated annual attack rate of 5–10% in adults

and 20–30% in children [2]. In 2009, pandemic influenza A (H1N1) caused 29,669 cases of infection, including 145 deaths, in 74 countries [3]. Respiratory syncytial virus (RSV) is the most common cause of bronchiolitis and pneumonia in infants and young children and it is the leading cause of respiratory infections that require hospitalization in infants [4]. Unfortunately, prior exposure to RSV does not protect against subsequent reinfection [5]. RSV reinfection also causes substantial morbidity in the elderly and in high-risk patients [6]. Although influenza virus and RSV are of considerable clinical importance, most research has focused on bacteria as the principal agent of community-acquired pneumonia in the past [7]. Influenza virus and RSV are important pathogens among children suffering from acute lower respiratory infections [8]. In particular, the pandemic H1N1 influenza A infection has steered public concern towards the causative virus in patients with respiratory symptoms, such as cough, rhinorrhea, sore throat, and fever.

To date, numerous diagnostic tools, such as viral culture, direct immunofluorescence assay, a rapid antigen test, and real-time polymerase chain reaction (RT-PCR) have been developed for the detection of respiratory viruses [9]. Viral culture has been the primary technique used to detect respiratory viruses, but it is time consuming and labor intensive [10]. Many rapid antigen tests still show an inferior sensitivity compared to RT-PCR, though they have several advantages, such as their rapid result times, ease of use and interpretation, and lower costs [11]. Various commercial multiplex RT-PCR assays have enabled clinical laboratories to detect the causative virus in patients with respiratory symptoms [1]. The Simplexa™ Flu A/B & RSV assay (SP) (Focus Diagnostics, Cypress, CA, USA) is an RT-PCR-based assay for in vitro detection of Flu A, Flu B, and RSV, but it cannot discriminate their subtyping.

The worldwide pandemic of influenza virus began with the Spanish influenza (type A/H1N1) in 1918, followed by the Asian flu (type A/H2N2) in 1957, the Hong Kong flu (type A/H3N2) in 1968, and the swine flu epidemic (type A/H1N1) in 2009 [12]. The subtyping of influenza viruses is important for predicting clinical characteristics to manage patients, and for estimating the prevalence rate epidemiologically. Therefore, it is important not only to detect whether an infection is caused by influenza virus but also to detect the subtype of influenza virus.

Allplex™ Respiratory Panel 1 (ARP) (Seegene, Seoul, Korea) is a new RT-PCR-based assay for detection of influenza virus and RSV infection including their subtyping. ARP employs multiple detection temperature (MuDT) technology, which provides multithreshold cy-

cle (Ct) values in a single fluorescence channel without requiring melt curve analysis as in RT-PCR instruments [13]. In ARP, 7 analytes can be detected in a single sample, including influenza A virus (Flu A), influenza B virus (Flu B), influenza A virus subtype H1 (Flu A-H1), influenza A virus subtype H3 (Flu A-H3), influenza A virus subtype H1N1 (Flu A-H1pdm09), RSV A, and RSV B. Nasopharyngeal swabs, nasopharyngeal aspirates, and bronchoalveolar lavage (BAL) can be used as specimens for ARP.

We evaluated the performance of the Allplex Respiratory Panel compared to SP and other diagnostic tools for the detection of respiratory viruses.

Materials and Methods

Subjects

This study was approved by the Institutional Research Ethics Board of the Korea University Guro Hospital (approval No. MD16056). Three hundred seventy-two samples were collected from patients at the Korea University Guro Hospital in Seoul, Korea, between January 2015 and February 2017. A total of 312 samples were collected from patients with respiratory symptoms, such as high fever, cough, and rhinorrhea; 60 samples were collected from healthy individuals as negative controls; 199, 153, 15, and 5 samples were from nasopharyngeal swabs, nasopharyngeal aspirates, BAL, and sputum, respectively.

Each sample was transported in a vial containing 3 mL of viral transport medium and immediately used for RT-PCR-based testing of influenza virus and RSV. The remaining specimens were then cryopreserved at -80°C until influenza rapid antigen testing was conducted. All specimens were subjected to a single freeze-thaw cycle. All samples were tested for influenza virus and RSV by ARP, SP, and an in-house RT-PCR. Influenza A samples were additionally tested for their subtyping using the Anyplex™ FluA 2 Real-Time Subtyping Kit (Seegene) (Fig. 1). ARP, SP, and Anyplex FluA 2 Real-Time Subtyping tests were carried out according to the manufacturer's instructions.

Nucleic Acid Extraction and Internal Control

Nucleic acid was extracted from patient specimens and assay controls using the NucliSENS® easyMAG™ extraction method (Biomérieux, Durham, NC, USA).

Internal control was added to each specimen to monitor the entire process from nucleic acid extraction to PCR, according to the manufacturer's instructions.

Allplex Respiratory Panel 1

The ARP assay can analyze samples to detect Flu A, Flu B, Flu A-H1, Flu A-H3, Flu A-H1pdm09, RSV A, and RSV B with each Ct value, and it was performed on a CFX96™ (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions.

The test results were interpreted automatically, and the analyzed data were presented using Seegene Viewer. If the Ct value was below 42 cycles, the sample was considered positive for presence of the pathogen. A negative specimen was defined by the absence of amplification for any pathogen with the presence of amplification of the internal control.

Table 1. In-house RT-PCR primer and probe sequences

Virus target	Primer or probe	Sequence
Influenza A virus	FluA-F	AGAT GAGTCTTCTAACC GAGGTC G
	FluA-R	TGACA GRAT YGGTCTT GTCTTTA GCCA YTCCA
	FluA-probe	[5FAM]TCA GGCCCCCTCAA GCC GAG[3BHQ1]
Influenza B virus	FluB-F	TACACA GCAAAAA GACCC
	FluB-R	TCCACTCCCTTTCTCCCC
	FluB-probe	[5HEX]ACACCCCCA GACCA GAT GA[3BHQ1]
Respiratory syncytial virus A	RSA-1	AGATCAACTTCTGTCATCCAGCAA
	RSA-2	ATTGATACTCCTAATTATGATGTGC
	RSA probe	CACCATCCAACGGAGCACAGGAGAT
Respiratory syncytial virus B	RSB-1	AAGATGCAAATCATAAATTCACAGGA
	RSB-2	CACTATAAAGATACTTAAAGATGCTGGATATCA
	RSB probe	AGGTATGTTATATGCTATGTCCAGGTTAGGAAGGGAA

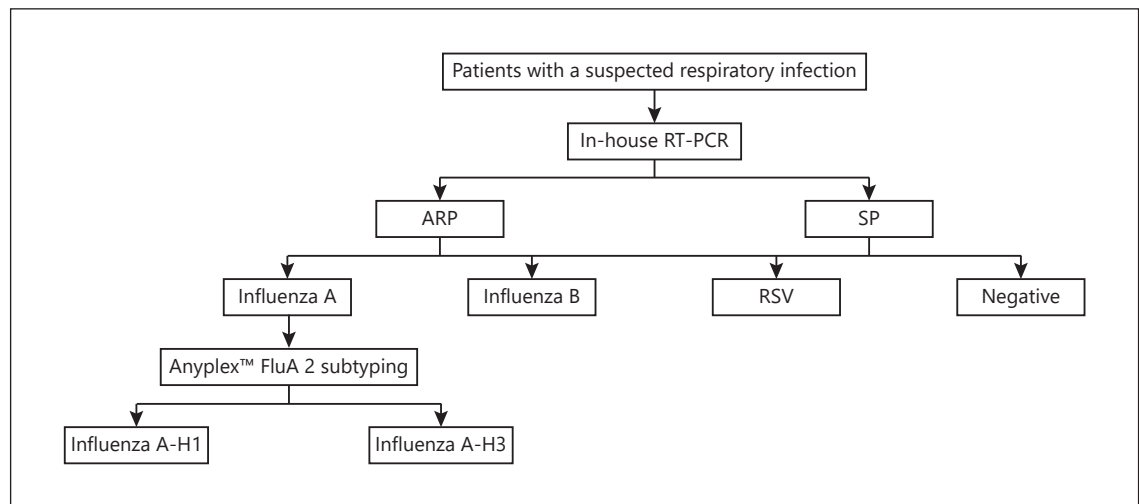


Fig. 1. Flow chart of tests for the detection of influenza virus and RSV.

Simplexa Flu A/B & RSV Assay

The SP assay can analyze samples to detect Flu A, Flu B, and RSV. SP for in vitro diagnostic use was approved by the US Food and Drug Administration (FDA) and Conformité Européenne (CE). This assay was performed on a 3M Integrated Cyler™ (Focus Diagnostics, CA, USA) according to the manufacturer's instructions.

A well-defined curve was considered a positive test result when it crossed the threshold cycle within 40 cycles of Flu A, Flu B, and RSV. In addition, multiple detections of 3 viruses were possible with this assay.

Anyplex FluA 2 Real-Time Subtyping

In parallel with the ARP assay, all the influenza A specimens were tested using an Anyplex FluA 2 Real-Time Subtyping Kit (Seegene), which is a commercial RT-, mPCR assay for the detec-

tion of influenza A and influenza B. This assay was performed on a CFX96™ according to the manufacturer's instructions. Viral RNA was extracted using the NucliSENS easyMAG apparatus, and the Anyplex FluA 2 Real-Time Subtyping Kit was used to detect influenza A and influenza B, according to the manufacturer's instructions. Results were visualized using Seegene Viewer.

In-House Real-Time PCR for the Detection of Respiratory Viruses

For the in-house method, viral RNA was extracted with a QIAamp® Viral RNA Mini Kit (Qiagen, Hilden, Germany) from 140 µL of the respiratory specimen by the in-house PCR protocol. A single-step real-time RT-PCR was performed as described previously with minor modifications [14]. The real-time PCR primers and probes are listed in Table 1. TaqMan amplification and detec-

Table 2. Comparison of ARP and SP

Virus	ARP		SP		Concordance, %	κ value	McNemar's p value
	positive	negative	positive	negative			
Influenza A	86	286	9	283	99.19	0.99	0.25
Influenza B	67	278	67	78	100.00	1.00	1.00
RSV	20	325	21	324	99.71	0.99	1.00

Table 3. Discrepancy between ARP and SP results

Case No.	ARP	Ct	ARP subtyping	Ct	SP	Ct
1	negative	none	influenza A-H3	37.09	influenza A	35.74
2	negative	none	influenza A-H3	34.32	influenza A	34.39
3	negative	none	influenza A-H3	39.56	influenza A	33.66
4	negative	none	negative	none	RSV	33.07

tion were performed on a real-time thermocycler CFX96 (Bio-Rad). Thermocycling conditions were as follows: reverse transcription at 50 °C for 20 min and initial denaturation at 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s and at 60 °C for 60 s. Each result was considered positive if the threshold cycle value (Ct) was less than 40.0. For quality control of in-house PCR, standard curves were produced with these 6 standard points of 10-fold dilution series of the virus culture stock samples (influenza A H1N1pdm/ H3N2 ATCC VR-544, influenza B ATCC VR-10-1, and RSV KBPV-VR-41, 42).

Data Analysis

Results of ARP and SP were analyzed and compared. Statistical analysis was performed with SPSS Statistics version 18 software (IBM Corporation, New York, NY, USA). The McNemar test for 2-by-2 contingency table analysis was performed between ARP and SP. Ct values of Flu A and Flu A subtyping, defined as an analysis of Flu A subtypes such as Flu A-H1, Flu A-H3, and Flu A-H1pdm09, were analyzed using a paired t test and the Pearson correlation coefficient. $p < 0.05$ was considered statistically significant.

Results

A total of 372 respiratory specimens were included in this study; 90 samples were positive for Flu A (including 24 Flu A-H1 and 66 Flu A-H3 samples), 67 samples were positive for Flu B, and 21 samples were positive for RSV. A total of 194 samples were negative for both influenza virus and RSV. Among the 372 samples, 173 (46.51%) were positive for 1 or more of the viruses tested by ARP while 177 (47.58%) samples were positive in the SP assay. Only 1 sample was positive for both Flu A and RSV. A total of 77 samples were taken from patients in the con-

valescent stage: 71 samples were negative and 4, 1, and 1 samples were positive for Flu A, Flu B, and RSV, respectively. In addition, 60 samples were taken from healthy individuals, and the results were all negative. There was no significant difference in the positive rate of the samples between 199 nasopharyngeal swabs, 153 nasopharyngeal aspirates, 15 BAL, and 5 sputum samples. All runs of ARP and SP were valid according to the specifications for internal control. The time required to perform the ARP assay was about 150 min, whereas the total assay time of SP was about 120 min.

A comparative analysis of viral detection by ARP and SP is shown in Table 2. In the detection of Flu A, the sensitivity and specificity of ARP were 95.56 and 100.00%, respectively, whereas these parameters in the case of Flu B were 100 and 100%, respectively. Similarly, the sensitivity and specificity of ARP in detecting RSV were 95.24 and 100.00%, respectively. The concordance rate between ARP and SP for detecting Flu A, Flu B, and RSV was 99.19, 100.00, and 99.71%, respectively. In the detection of Flu A, Flu B, and RSV, McNemar tests did not show any significant difference between ARP and SP ($p = 0.25, 1.00, \text{ and } 1.00$).

The influenza subtype of 90 samples, which were positive for influenza A, was tested using both the ARP and the Anyplex FluA 2 Real-Time Subtyping assay. A total of 24 samples were positive for Flu A-H1pdm09 by ARP and Anyplex FluA 2 Real-Time Subtyping assay and 66 samples were positive for Flu A-H3 by ARP and Anyplex FluA 2 Real-Time Subtyping assay. There were no discrepant results between ARP and Anyplex FluA 2 Real-Time Subtyping assay.

Table 4. Comparison of Ct between ARP and SP

Virus	ARP		SP	
	mean	SD	mean	SD
Influenza A	26.17	3.93	24.98	4.25
Influenza B	26.45	3.77	31.94	3.46
RSV	21.87	5.90	24.16	5.26

Table 5. Comparison of Ct between influenza A virus and influenza A subtyping by ARP

Influenza A		Influenza A subtyping ^a		Paired <i>t</i> test		Correlation coefficient ^b	
mean	SD	mean	SD	<i>t</i>	<i>p</i> value	<i>r</i>	<i>p</i> value
26.17	3.93	23.91	3.87	15.31	0.000	0.934	0.000

All Ct values used in this analysis were results of ARP test in samples of influenza A virus with all subtypes. ^a Tested using the ARP. ^b Pearson's correlation coefficient.

We observed discrepancies in 4 cases of viral detection by ARP and SP (Table 3). Three cases were positive for Flu A-H3 and negative for Flu A on investigation by ARP; however, they were positive for Flu A when tested by SP. Only a single case was negative for all of the respiratory viruses when tested by ARP, but RSV was found to be present in the sample when tested by SP.

All positive samples were analyzed for Ct values of ARP and SP (Table 4). The mean Ct values (\pm SD) of ARP and SP were 26.17 ± 3.93 and 24.98 ± 4.25 , respectively, in the detection of Flu A. In the case of Flu B, the mean Ct values (\pm SD) of ARP and SP were 26.45 ± 3.77 and 31.94 ± 3.46 , respectively. The Ct values determined by ARP and SP were 21.87 ± 5.90 and 24.16 ± 5.26 , respectively, for RSV detection.

Among 90 positive samples of Flu A, 86 positive samples were statistically analyzed for Ct values of Flu A and Flu A subtyping by correlation coefficients and paired *t* tests (Table 5). The other 4 positive samples were excluded because of discrepant results between Flu A and Flu A subtyping. The mean Ct values (\pm SD) of Flu A and Flu A subtyping were 26.17 ± 3.93 and 23.91 ± 3.87 for Flu A-positive samples, respectively. Ct values of Flu A were significantly different from those of Flu A subtyping as evaluated by a paired *t* test ($p = 0.000$). Between Flu A and Flu A subtyping, the correlation coefficient for the Ct values was 0.934 by Pearson's correlation analysis.

Discussion

Early diagnosis and treatment of the flu and RSV are very important because of the strong and rapid infectivity of the viruses. Mean serial intervals of Flu A-H3, Flu A-H1pdm09, and RSV and the duration between the onset of symptoms in a primary case and in its secondary cases are significantly shorter than in other contagious diseases, such as measles, varicella, smallpox, mumps, rubella, and pertussis [15]. Rapid diagnosis of a respiratory viral infection could reduce the rate of antibiotics use and unnecessary tests [16].

Because of the importance of early diagnosis for flu and RSV infections, numerous RT-PCR-based assays have been developed for the detection of respiratory pathogens. However, conventional RT-PCR assays have limitations, as they provide only a single Ct value in a single fluorescence channel. To overcome this limitation, conventional multiplex PCR assays were developed that have multiple fluorescence channels to acquire multiple Ct values for a faster analysis. FTD Respiratory pathogens 21 (Fast-track Diagnostics, Esch-sur-Alzette, Luxembourg) consists of 5 separate primer/probe mixes covering 16 human respiratory viruses, and each mix has 4 fluorescence channels [17]. The TaqMan array card (Life Technologies, Carlsbad, CA, USA) is comprised of 21 uniplex PCR assays that are conducted simultaneously in duplicate on a single 384-well microfluidic card with 8 ports [18]. Another alternative to overcome the limitations of conventional PCR assays is to use RT-PCR in combination with other technologies. The PLEX-ID Flu Detection™ Kit (Abbott Molecular, Des Plaines, IL, USA) detects influenza viruses using the PCR-coupled electrospray ionization mass spectrometry technology [19]. The Verigene Respiratory Virus Plus test (Nanosphere, Northbrook, IL, USA) is an automated, multiplex nucleic acid test based on nanoparticle technology that does not require preextraction of specimens [20].

On a single fluorescence channel, SP provides a single Ct value, while ARP provides multiple Ct values via MuDT technology. This technology shows that the intensity of the fluorescence signals of 2 discrete Tm targets is different at certain temperatures called detection temperatures. Consequently, a high-Tm target can be detected regardless of the presence of a low-Tm target. MuDT technology enables identification of the low-Tm target by utilizing a change in the fluorescence signals between 2 different detection temperatures [13]. MuDT also facilitates a shorter turnaround time because melt curve analysis after amplification is not necessary. It allows quantification of multiple targets in a single channel. In this con-

text, ARP provides multi-Ct values of 3 respiratory viruses with their subtyping by MuDT.

The ARP assay has 2 additional panels that allow the detection of 16 different types of respiratory viruses besides the influenza virus subtype and RSV [21, 22]. The ARP assay is highly useful as a routine examination in the hospital setting because the test time is the same even when other respiratory panels are added. In a recent study, ARP showed an overall sensitivity of 93.7% and a specificity of 100% for the detection of Flu A, Flu B, and RSV [23]. The ARP assay takes about 150 min, does not require electrophoresis after PCR, and can reduce the hands-on time by using automatic nucleic acid extraction equipment, such as MICROLAB STARlet IVD (Hamilton, Reno, NV, USA).

In this study, ARP demonstrated high sensitivity, specificity, positive predictive, and negative predictive values in patients with respiratory symptoms for causative viral detection. While SP was unable to identify the Flu A subtype, ARP could identify the Flu A subtype. The accuracy of the ARP assay for Flu A subtyping was 100%, compared to the Anyplex FluA 2 Real-Time Subtyping assay. In addition, the concordance rate between ARP and SP was very high. Ct values of positive samples where virus was detected were positively correlated between ARP and SP.

For the detection of Flu A, ARP showed no significant differences in sensitivity and specificity. Ct values of Flu A subtyping had a very strong correlation with Ct values of Flu A ($r = 0.934$). The mean Ct values for Flu A subtyping were significantly lower than those for Flu A. Flu A subtyping is more sensitive than detection of Flu A because of the strong correlation and significantly lower Ct values. For the detection of Flu B, ARP showed perfect agreement with SP assays. For detection of RSV, ARP showed no significant differences in sensitivity or specificity compared to SP.

Discrepancies between ARP and SP were observed in the detection of 3 cases of Flu A and in 1 case of RSV. In the detection of Flu A, 3 discrepant cases were detected only in Flu A subtyping but not in Flu A by ARP. We believe that Flu A subtyping is more sensitive than Flu A detection as the results of these samples would be interpreted as Flu A only by detection in Flu A subtyping. We suggest that if only the Flu A subtype is detected the sample indicates a very low viral load of influenza virus. However, if only Flu A is detected (and not the Flu A subtype), the subtype would be reported as unidentifiable and sequencing is recommended. For RSV detection, only 1 discrepant case was observed, having been detected by SP

but not by ARP. In-house RT-PCR was used to verify the 4 discrepant results between ARP and SP, and it was confirmed that ARP missed 4 positive samples. This can be explained by a possible lack of sensitivity using the ARP test, because ARP is a multiplexing assay for the detection of Flu A, Flu B, and RSV with their subtyping in a single tube. However, confirmative tests such as culture or sequencing were not performed for discrepant results between ARP and SP.

Among the 372 samples collected from the patients suspected of having respiratory infections, 24 samples were positive for Flu A-H1pdm09. We suggest the possibility of an epidemic Flu A-H1pdm09 because all of the Flu A-H1 positive samples were positive for Flu A-H1pdm09. This possibility may be verified in the future by epidemiologic surveys in multicenter studies.

Further studies are needed to compare the limits of detection by ARP, SP, and in-house PCR. However, confirmative tests such as culture or sequencing were not performed for discrepant results between ARP and SP. In addition, the relatively small numbers of positive RSV samples were a limitation in this study.

Conclusion

In this study, the Allplex Respiratory Panel Test showed high sensitivity, specificity, positive predictive, and negative predictive values for the detection of Flu A, Flu B, and RSV with multi-Ct values. The concordance rate between ARP and SP was also very high. ARP allows identification of the viral subtype in a single tube, unlike other RT-PCR methods, such as SP. The ARP assay is fast and easy to perform because it takes only about 150 min and there is no need for post-PCR electrophoresis. ARP can be used as a reliable and convenient assay in clinical laboratories.

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Disclosure Statement

The authors declare that they have no competing interests.

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