Comparison of two multiplex PCR assays for the detection of respiratory viral infections

Hanah Kim[1], Mina Hur[1], Hee-Won Moon[1], Yeo-Min Yun[1] and Hyun Chan Cho[2]

1 Department of Laboratory Medicine, Konkuk University School of Medicine, Seoul, Korea
2 Department of Laboratory Medicine, Hallym University College of Medicine, Seoul, Korea

Abstract

Introduction: Respiratory viruses are the main causes of upper and lower respiratory tract diseases. Rapid and accurate detection of respiratory viruses is crucial for appropriate patient treatment and prevention of endemic spread.

Objectives: We compared two multiplex polymerase chain reaction (PCR) assays for the detection of respiratory viral pathogens.

Methods: A total of 245 respiratory specimens (229 sputum samples, 14 bronchoalveolar lavage samples, 6 nasal swabs, 3 throat swabs, 7 unknown) were analyzed using two multiplex assays: One-step RV real-time PCR (BioSewoom, Seoul, Korea) and Seeplex RV 12 Detection kit (Seegene, Seoul, Korea). The results were further confirmed using sequencing as a reference.

Results: Among 245 samples (265 identifications including co-infections), the identification of respiratory viruses was 44.9% (119/265), 44.2% (117/265) and 45.3% (120/265) by One-step RV assay, Seeplex RV assay and sequencing, respectively. The concordance rate between One-step RV assay and sequencing was 95.5% (253/265), and that between Seeplex RV assay and sequencing was 89.8% (238/265) ($P = 0.0189$). The sensitivities of One-step RV and Seeplex RV assays were 94.1% [95% confidential interval (CI), 88.3%–97.6%] and 83.3% (95% CI, 75.4%–89.5%), respectively ($P = 0.0002$). The specificities of One-step RV and Seeplex RV assays were 96.6% (95% CI, 92.2%–98.9%) and 95.2% (95% CI, 90.3%–98.0%), respectively.

Conclusion: Although the performances of One-step RV and Seeplex RV assays were overall comparable, One-step RV assay showed better sensitivity and concordance with sequencing. One-step RV assay can be a useful option for respiratory virus testing in clinical laboratories.

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Conflict of interest
The authors have stated explicitly that there are no conflicts of interest in connection with this article.

Key words
multiplex PCR – One-step RV – performance – respiratory virus – Seeplex RV

Correspondence
Mina Hur, MD, PhD, Department of Laboratory Medicine, Konkuk University School of Medicine, Konkuk University Hospital, 120-1, Neungdong-ro, Hwayang-dong, Gwangjin-gu, 143-729 Seoul, Korea.
Tel: +82 2 2030 5581
Fax: +82 2 2636 6764
email: dearmina@hanmail.net

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H. Kim participated in the data analyses and wrote the draft manuscript. M. Hur participated in the design and running of the study, was responsible for all statistical analyses, and wrote the manuscript. H. W. Moon, Y. M. Yun, and H. C. Cho discussed findings and commented on the manuscript.

Ethics
This study was approved by the Institutional Review Board of the Konkuk University Medical Center. Informed consent from enrolled patients was exempted for this *in vitro* comparison study using routine, respiratory specimens.

Introduction

Respiratory viral infections are the major causes of upper and lower respiratory tract diseases. They are associated with significant morbidity and mortality in infants, children, the elderly and immunocompromised patients. The disease severity varies according to the host and viral predisposing factors, such as affecting age, immune status of patient, combined infection and infection mechanism of viral pathogen (1, 2). Respiratory infections caused by virus or bacteria are often indistinguishable, and rapid and accurate detection is crucial for appropriate patient treatment and prevention of endemic spread; therefore, selecting an appropriate diagnostic test is indispensable (3). The common respiratory viruses causing respiratory infections

include adenovirus (AdV), coronaviruses (CoV), human metapneumovirus (hMPV), influenza virus type A and B, parainfluenza virus type 1 (PIV 1), PIV 2, PIV 3, respiratory syncytial virus type A (RSV A), RSV B, rhinoviruses, enteroviruses and bocaviruses (4–6).

Although conventional techniques including viral isolation in cell culture and serology have been used to diagnose respiratory virus infections, their clinical value is limited because of weak sensitivity and slow turn-around time (6, 7). Recently, rapid antigen tests are used widely in clinical laboratories, but they are known to be less sensitive and less specific for the detection of respiratory viruses (8, 9). Molecular assay, especially polymerase chain reaction (PCR), is increasingly used for the diagnosis of viral infections. PCR is more sensitive and rapid, can be largely automated, and can detect and quantify viruses not amenable to routine cultures (9, 10). Moreover, a simultaneous detection of several viruses using multiplex PCR assay is advantageous to differentiate diagnosis of viral respiratory infections in one assay and facilitate cost-effectiveness in the clinical field (4, 6, 7, 11–13).

In this study, we compared two commercially available multiplex PCR assays: One-step RV real-time PCR (BioSewoom Inc., Seoul, Korea) and Seeplex RV 12 Detection kit (Seegene Inc., Seoul, Korea). Seeplex RV assay is one of the representative multiplex PCR platforms, and its performances have been evaluated in previous studies (14–19). We wanted to evaluate the diagnostic performance of a novel multiplex PCR assay, One-step RV assay, in comparison with that of Seeplex RV assay. Both assays were compared for the detection of the following pathogenic respiratory viruses: AdV, CoV, hMPV, human flu A (H1N1), human flu A (H3N2), influenza virus type A and B, PIV 1, PIV 2, PIV 3, rhinovirus A, RSV A and RSV B. All specimens positive for respiratory viruses by the two multiplex PCR assays were further sequenced to confirm the pathogens.

Materials and methods

Study population and sample collection

This study was approved by the Institutional Review Board of the Konkuk University Medical Center, a tertiary referral hospital with 900 beds. From July to September 2012, a total of 245 specimens (229 sputum samples, 14 bronchoalveolar lavage samples, 6 nasal swabs, 3 throat swabs, and 7 unknown) were consecutively collected from the 239 patients (134 males and 105 females) with respiratory symptoms, regardless of hospitalization. The patients consisted of 52 adults and 187 children, and their median age was 68 years and 3 years, respectively (range, 0–87 years). Bacteriological investigations (smear and culture) revealed the absence of bacterial co-infections. A total of 0.5–1 mL of specimen was added to viral transport medium [minimal essential medium with 2% fetal bovine serum, penicillin (100 U/mL), amphotericin B (20 ug/mL), neomycin (40 ug/mL), $NaHCO_3$ buffer], and an aliquot from the mixture of each sample was stored at –70°C for the analysis.

Nucleic acid extraction and multiplex PCR assays

Viral nucleic acid was extracted using the QiaAmp MinElute Virus spin kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. In brief, 25 uL of protease was added to 200 uL of specimen, and then a buffer AL solution was added to specimen/protease mixture. The mixture was incubated at 56°C for 15 min, and 250 uL of ethanol was added. The entire volume of sample was then transferred to a QiaAmp MinElute column and centrifuged for 1 min at 6000× *g*. A series of washes were performed.

Extracted nucleic acid was amplified by both One-step RV assay and Seeplex RV assay, according to the manufacturers' instructions. One-step RV real-time PCR was conducted in a final reaction volume of 35 uL, containing 5 uL of sample RNA, 5 uL of positive control, 5 uL of negative control, 3 uL of RV probe-primer mixture and 17 uL of 2× master mixture. PCR thermal cycling conditions were optimized to the following protocol: 30 min at 50°C, 15 min at 95°C and 45 cycles at 95°C for 15 s, followed by 62°C for 45 s. PCR was performed using Bio-Rad CFX96 real-time detection PCR system (Bio-Rad, Hercules, CA, USA).

Seeplex RV assay is an end-point reverse transcriptase PCR using dual specific oligonucleotide technology. After the cDNA synthesis using cDNA synthesis kit (Seegene Inc.), PCR was conducted in a final reaction volume of 20 uL containing 6 uL of cDNA, 4 uL of 5× RV primer and 10 uL of 2× master mix. PCR thermal cycling conditions were optimized to the following protocol: 15 min at 94°C for reverse transcription, 40 cycles at 94°C for 30 s, followed by 60°C for 1.5 min and 72°C for 1.5 min, and then final extension at 72°C for 10 min. PCR was performed using GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA). The amplified PCR products were separated on 2% agarose gels. Seeplex RV assay included a size marker that has the same lengths as those of amplicons for internal control and viruses. If a specimen showed an amplicon of the same size as one of a marker band, it was indicated as positive.

Table 1. Respiratory viruses identified by two multiplex PCR assays and sequencing*

	N (%) of positive results by each method			Concordance among the methods (*N*, %†)		
	One-step RV	Seeplex RV	Sequencing	One-step RV vs Seeplex RV	One-step RV vs sequencing	Seeplex RV vs sequencing
Negative	146 (55.1)	148 (55.8)	145 (54.7)	138/156 (88.5)	141/150 (94.0)	138/155 (89.0)
Positive	119 (44.9)	117 (44.2)	120 (45.3)	95/127 (74.8)	112/124 (90.3)	100/127 (78.7)
Adenovirus	9 (3.4)	9 (3.4)	8 (3.0)	7/11 (63.6)	8/9 (88.9)	7/10 (70.0)
Coronavirus OC 43	0	3 (1.1)	0	0/0 (100)	0/0 (100)	0/3 (0)
Human metapneumovirus	30 (11.3)	24 (9.1)	28 (10.6)	23/31 (74.2)	28/30 (93.3)	22/30 (73.3)
Influenza virus type A	0	1 (0.4)	0	0/0 (100)	0/0 (100)	0/1 (0)
Influenza virus type B	1 (0.4)	1 (0.4)	1 (0.4)	1/1 (100)	1/1 (100)	1/1 (100)
Parainfluenza virus type 1	8 (3.0)	5 (1.9)	8 (3.0)	5/8 (62.5)	8/8 (100)	5/8 (62.5)
Parainfluenza virus type 2	6 (2.3)	5 (1.9)	7 (2.6)	4/7 (57.1)	6/7 (85.7)	5/7 (71.4)
Parainfluenza virus type 3	25 (9.4)	26 (9.8)	25 (9.4)	22/29 (75.9)	23/27 (85.2)	22/29 (75.9)
RSV type A	5 (1.9)	5 (1.9)	4 (1.5)	4/6 (66.7)	4/5 (80.0)	4/5 (80.0)
RSV type B	8 (3.0)	6 (2.3)	7 (2.6)	5/9 (55.6)	7/8 (87.5)	5/8 (62.5)
Rhinovirus A	27 (10.2)	32 (12.1)	32 (12.1)	24/35 (68.6)	27/32 (84.4)	29/35 (82.9)
Total	265 (100)	265 (100)	265 (100)	233/265 (87.9)	253/265 (95.5)‡	238/265 (89.8)

*Coronavirus 229E is covered by both assays, and human flu A (H1N1) and human flu A (H3N2) are covered by One-step RV assay. All these three viruses were not detected in the enrolled specimens.

†Concordance rate (%) = (number of corresponding results by both methods/number of corresponding results by either method) × 100

‡$P = 0.0189$ vs concordance between Seeplex RV assay and sequencing.

PCR, polymerase chain reaction; *N*, number; RSV, respiratory syncytial virus.

The limits of detection for target viruses were 4–273 copies/uL for One-step RV assay and 100 copies/uL for Seeplex RV assay. Each assay included an internal control to rule out PCR inhibition.

Sequencing analysis

The positive results by multiplex PCR assays were further confirmed using sequencing. At the end of PCR reaction, 6 uL of an enzymatic purification mixture cocktail containing *Escherichia coli* exonuclease I and shrimp alkaline phosphatase was added to the PCR product. The mixture was incubated at 37°C for 30 min, followed by 85°C for 15 min, and then the mixture was kept at 4°C. The kept mixture was directly sequenced using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) in ABI PRISM 3730XL Autosequencer (Applied Biosystems). Sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) provided from The National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST).

Statistical analysis

The concordance rates between each multiplex PCR assay and sequencing were obtained. For each assay, sensitivity and specificity were calculated with their 95% confidence intervals (CI). The proportions between the two multiplex assays were compared using chi-squared test. Statistical analysis was performed using Analyse-it Software (Analyse-it Software, Ltd, Leeds, UK) and MedCalc Software (version 12.1.4, MedCalc Software, Mariakerke, Belgium). *P* values of equal to or less than 0.05 were considered statistically significant.

Results

A total of 265 results were obtained from 245 specimens. In 265 results, 120 results (45.3%) were positive by sequencing, 119 (44.9%) by One-step RV assay and 117 (44.2%) by Seeplex RV assay (Table 1). Coronavirus 229E, human flu A (H1N1) and human flu A (H3N2) were not detected in any specimens. Among the 11 types of viruses identified, rhinovirus A, hMPV and PIV 3 were the most frequently detected viruses. Overall concordance rates among the methods were: 87.9% (233/265) between One-step RV and Seeplex RV assays; 95.5% (253/265) between One-step RV assay and sequencing; and 89.8% (238/265) between Seeplex RV assay and sequencing. The concordance rate of each multiplex assay with sequencing showed a significant difference [5.7% (95% CI, 1.03%–10.5%), $P = 0.0189$].

Co-infections were detected in 16 out of 245 (6.5%) specimens (Table 2). The two multiplex assays showed discrepant results in 68.8% (11/16) of co-infected specimens, compared with 11.4% (28/245) of

Table 2. Clinical specimens with co-infections

Number	Specimen	One-step RV assay	Seeplex RV assay	Sequencing
1	Sputum	Adenovirus, Human metapneumovirus	Adenovirus, Human metapneumovirus	Adenovirus, Human metapneumovirus
2	Sputum	Adenovirus, PIV 3	Adenovirus, PIV 3	Adenovirus, PIV 3
3	Sputum	Human metapneumovirus, PIV 3	PIV 3	Human metapneumovirus
4	Sputum	Human metapneumovirus	Human metapneumovirus, PIV 3	PIV 3
5	Sputum	Human metapneumovirus, PIV 3	Human metapneumovirus, PIV 3	Human metapneumovirus, PIV 3
6	Sputum	Adenovirus, RSV B	Adenovirus, RSV A, RSV B	Adenovirus, RSV B
7	Sputum	RSV A	Rhinovirus A, RSV A	Rhinovirus A, RSV A
8	Sputum	Human metapneumovirus, PIV 3	PIV 3	Human metapneumovirus
9	BAL	Influenza virus type B, PIV 3	Influenza virus type B, PIV 3	Influenza virus type B, PIV 3
10	Sputum	PIV 2	PIV 2, PIV 3	PIV 2
11	Sputum	Adenovirus, Rhinovirus A	Adenovirus, Rhinovirus A	Adenovirus, Rhinovirus A
12	Sputum	RSV B	Adenovirus, RSV B	RSV B
13	Sputum	Rhinovirus A	Rhinovirus A, RSV B	Rhinovirus A
14	Sputum	RSV B	Rhinovirus A, RSV B	Rhinovirus A, RSV B
15	Sputum	Human metapneumovirus	Human metapneumovirus, Coronavirus OC 43	Human metapneumovirus
16	Sputum	Human metapneumovirus, PIV 1, PIV 3	Rhinovirus A, coronavirus OC 43	Human metapneumovirus, PIV 1, PIV 3

BAL, bronchoalveolar lavage; PIV, parainfluenza virus; RSV, respiratory syncytial virus.

discrepant results in total specimens. The proportion of discrepancy was significantly higher in co-infected specimens than in total specimens [difference of 57.4% (95% CI, 29.6%–78.0%), $P < 0.0001$].

The diagnostic performance of both multiplex assays was compared with sequencing (Table 3). The sensitivity and specificity of One-step RV assay were 94.1% (95% CI, 88.3%–97.6%) and 96.6% (95% CI, 92.2%–98.9%), respectively. In Seeplex RV assay, the sensitivity and specificity were 83.3% (95% CI, 75.4%–89.5%) and 95.2% (95% CI, 90.3%–98.0%), respectively. The sensitivity of One-step RV assay was significantly higher than that of Seeplex RV assay (94.1% vs 83.3%, $P = 0.0002$), although the specificities were comparable in both assays.

One-step RV assay showed excellent sensitivity (100%) for most of the viruses, but the sensitivity was lower for the detection of rhinovirus A, PIV 2 and PIV 3 (84.4%–92.0%). In Seeplex RV 12 assay, excellent sensitivity (100%) was observed only for influenza virus type B and RSV A. For the other viruses, the sensitivity varied widely, ranging from 62.5% to

Table 3. Diagnostic performance of two multiplex PCR assays for the detection and identification of respiratory viruses in comparison with sequencing

	One-step RV assay		Seeplex RV assay	
	Sensitivity (95% CI)	Specificity (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)
Adenovirus	100 (63.1–100)	99.6 (97.9–100)	87.5 (47.3–99.7)	99.2 (97.2–99.9)
Coronavirus OC 43	—	100 (98.6–100)	—	98.9 (96.7–99.8)
Human metapneumovirus	100 (87.7–100)	99.2 (97.0–99.9)	78.6 (59.0–91.7)	99.2 (97.0–99.9)
Influenza virus type A	—	100 (98.6–100)	—	99.6 (97.9–100)
Influenza virus type B	100 (2.5–100)	100 (98.6–100)	100 (2.5–100)	100 (98.6–100)
Parainfluenza virus type 1	100 (63.1–100)	100 (98.6–100)	62.5 (24.5–91.5)	100 (98.6–100)
Parainfluenza virus type 2	85.7 (42.1–99.6)	100 (98.6–100)	71.4 (29.0–96.3)	100 (98.6–100)
Parainfluenza virus type 3	92.0 (74.0–99.0)	99.2 (97.0–99.9)	88.0 (68.8–97.5)	98.3 (95.8–99.5)
RSV type A	100 (39.8–100)	99.6 (97.9–100)	100 (39.8–100)	99.6 (97.9–100)
RSV type B	100 (59.0–100)	99.6 (97.9–100)	71.4 (29.0–96.3)	99.6 (97.9–100)
Rhinovirus A	84.4 (67.2–94.7)	100 (98.4–100)	90.6 (95.1–99.2)	98.7 (96.3–99.7)
Total	94.1 (88.3–97.6)*	96.6 (92.2–98.9)	83.3 (75.4–89.5)	95.2 (90.3–98.0)

*$P = 0.0002$ vs Seeplex RV assay.

CI, confidence interval; RSV, respiratory syncytial virus.

90.6%. Regarding specificity, both multiplex assays showed very high performances, showing specificity of >98% for all targets.

Discussion

This study showed the diagnostic performances of two multiplex PCR assays for the detection of respiratory viruses: One-step RV and Seeplex RV assays. Compared with sequencing, the overall concordance rate of One-step RV assay was significantly better than that of Seeplex RV assay (95.5% vs 89.8%, $P = 0.0189$) (Table 1). For influenza virus type B and RSV A, both assays showed 100% of concordance with sequencing. For all the other viruses, except for these two viruses, the concordance rate in One-step RV assay was superior to that in Seeplex RV assay. The specificities of both assays were comparable and satisfactory, showing 96.6% in One-step RV assay and 95.2% in Seeplex RV assay (Table 3). Such a high performance was observed constantly for each virus, showing the lowest value of 98.3% for PIV 3 in Seeplex RV assay. However, the sensitivity of One-step RV assay was significantly better than that of Seeplex RV assay (94.1% vs 83.3%, $P = 0.0002$). Moreover, the sensitivity for each virus was more variable in Seeplex RV assay (62.5%–100%) than in One-step RV assay (84.4%–100%).

Accurate and rapid diagnosis of respiratory virus infection is essential for the initiation of early treatment and the prevention of viral spread (6, 14). An early start of proper anti-viral management can avoid unnecessary exposure to antibacterial agents, and some respiratory viral infections caused by influenza virus or AdV may benefit from specific anti-viral treatment. Knowing the subtype of influenza virus may be important predicting the efficacy of adamantanes and neuraminidase inhibitors as anti-viral agents (20–22). Viral culture is technically demanding and easily affected by specimen quality or virus type. Although shell vial culture and subsequent immunostaining has been widely used, it is not easy for routine testing in clinical laboratories (6, 7, 14, 23).

The nucleic acid amplification test including PCR is a powerful alternative for the respiratory virus detection. It is faster and more sensitive than virus culture, and some viruses that grow poorly in cell cultures can be also detected (24, 25). Multiplex PCR assay is advantageous for detecting common respiratory viruses simultaneously and is now widely used in clinical laboratories (7).

According to our data, the overall performance of One-step RV assay was superior to that of Seeplex RV assay. These two assays showed significant difference in terms of sensitivity and concordance rate with sequencing. Among the 11 respiratory virus targets, six viruses showed 100% of sensitivities in One-step RV assay, compared with variable performances (62.5%–100%) in Seeplex RV assay. Of note, the proportion of discrepancy between the two multiplex assays was significantly higher in co-infected specimens than in total specimens [68.8% vs 11.4%, difference of 57.4% (95% CI, 29.6%–78.0%), $P < 0.0001$] (Table 2). In the present study, the proportion of co-infections (6.5%) was relatively low compared with the previous data (8–11%) (6). We assume that the overall performance and concordance between the two assays would have shown a bigger difference, if we could enroll more co-infected specimens in this study. Further studies are awaited to clarify this assumption.

The hands-on time of Seeplex RV assay was at least 6 h: 2 h for RNA extraction and cDNA synthesis and additional 4 h for multiplex PCR and visualization by gel electrophoresis (6). On the contrary, the hands-on time of One-step real-time RV assay was reduced to approximately 3 h, without the steps of cDNA synthesis and gel electrophoresis. Although the cost per each case may be similar ($35–40) in both assays, the improved turnaround time of One-step RV assay would be beneficial for saving the overall hospital cost and for the timely reports.

Our study is limited in that the enrolled sample size was small: we analyzed only 265 results from 245 clinical specimens. Because these two assays are commercially available, we compared them only with clinical samples, without testing of known positive controls for each of the target viruses. Although 14 viruses can be identified using One-step RV assay, our analyses were confined to only 11 viruses because we could not enroll the clinical specimens with coronavirus 229E, human flu A (H1N1) or human flu A (H3N2). Moreover, the panel of both assays did not include some common respiratory viruses, such as enteroviruses and bocaviruses: the absence of these viruses might explain the relatively low number of positive samples in this set of clinical specimens. Another study would be needed to fully evaluate the diagnostic performances of One-step RV assay for such viruses. Another limitation is that the results of multiplex PCR assays were confirmed by sequencing only, without virus culture being performed. However, sequencing is generally used to confirm the culture negative but PCR positive results or to detect some poorly growing viruses in culture (14, 25).

In conclusion, a novel real-time multiplex PCR platform, One-step RV assay, showed satisfactory diagnostic performances in comparison with Seeplex RV assay.

With its excellent specificity and sensitivity, One-step RV assay can be one of the useful options for respiratory virus testing in clinical viral laboratories.

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