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Virology

Comparison of the Anyplex™ II RV16 and Seeplex® RV12 ACE assays for the detection of respiratory viruses

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1. Introduction

Respiratory viral infections are responsible for substantial morbidity in both pediatric and adult populations. Timely and accurate diagnosis of these infections is essential to patient care in settings as diverse as susceptible infants or children, older adults, patients with compromised immune systems, or individuals with underlying cardiopulmonary diseases (Glezen et al., 2000; Weinberg et al., 2004). Early diagnosis of respiratory viruses plays an important role in clinical management, reducing complications, antibiotic use, and unnecessary laboratory testing (Jernigan et al., 2011; Mahony et al., 2009; Renaud et al., 2012). The Seeplex RV12 ACE detection kit (RV12; Seegene) enables simultaneous detection of 12 respiratory viruses in 2 reactions per sample using dual priming oligonucleotides as PCR primers. The performance of RV12 has been demonstrated in previous studies, showing time and resource savings (Bibby et al., 2011; Drews et al., 2008; Weinberg et al., 2004; Yoo et al., 2007). Recently, Anyplex™ II RV16 detection (V1.1) kit (RV16; Seegene) with tagging oligonucleotide cleavage and extension (TOCE) technology has been developed. TOCE technology is a novel approach to real-time PCR, using the 2 components, the Pitcher and Catcher, to accomplish a unique signal generation. Through target bound Pitcher, TOCE assay moves the detection point from the target sequence to the Catcher. By designing unique Catchers, the resulting Duplex Catcher will have a predictable melting temperature profile (Cho et al., 2013; Chun, 2012; Lee, 2012). Both RV16 and RV12 achieved approval from CE, Health Canada, and the Ministry of Food and Drug Safety of Korea, being sold in more than 15 countries in Europe, Canada, and Asia. RV16 simultaneously detects 16 respiratory viruses: human bocavirus (HBoV); human enterovirus (HEV); influenza virus (INF) types A and B; parainfluenza virus (PIV) types 1, 2, 3, and 4; respiratory syncytial virus (RSV) types A and B; adenovirus (ADV); human metapneumovirus (HMPV); coronavirus (CoV) OC43, 229E, and NL63; and human rhinovirus (HRV). This profile is similar to the profile of RV12 but further includes HBoV, HEV, PIV 4, CoV 229E, and CoV NL63.

The aim of the present study was to compare the performance of RV16 and RV12. In addition, we evaluated the analytical performance of RV16. These studies were carried out in a routine diagnostic laboratory setting and used nonselective clinical specimens from Korean patients.

2. Materials and methods

2.1. Clinical specimens

This prospective study was approved by the Institutional Review Board of Samsung Medical Center. Three hundred sixty-five non-selective consecutive clinical respiratory specimens from 302 patients were obtained. The patients included 55 adults and 247 pediatric patients whose median age was 3 years and ranged from 1 day to 93 years. There were 320 nasopharyngeal (NPA) and 45 bronchoalveolar lavage (BAL) fluid samples submitted for RV12 testing from January to February 2013. These samples were simultaneously analyzed by RV16, and the aliquots of each specimen were immediately stored...
frozen at $-70^\circ$C. The volume of NPA and BAL fluid was approximately 10 mL.

2.2. Nucleic acid extraction

Nucleic acids were extracted from 100 μL of each specimen by MagNa Pure LC Total Nucleic Acid Isolation Kit (Roche, Mannheim, Germany) for RV12 and by MICROLAB STARlet (Hamilton, Reno, NV, USA) with STARMag 96 Virus Kit (Seegene) for RV16. The final elution volume of each sample was 50 μL in both kits. In RV16, bacteriophage MS2 was added as an internal control to each specimen, according to the manufacturer’s instructions.

2.3. RV12 testing

Random hexamer-primed complementary DNA (cDNA) synthesis products were generated using the Revertra First Strand cDNA synthesis kit (Fermentas, Ontario, Canada), according to the manufacturer’s instructions. Each cDNA preparation was subjected to the RV12 PCR procedure according to the manufacturer’s instructions (Seegene). Briefly, parallel 20 μL reactions were set up, each containing RV12 mastermix, 8-MOPS contamination control reagent, and 3 μL cDNA. One of each pair was supplemented with 4-μL primer mix A and the other, with 4-μL primer mix B. Thermal cycling conditions were as follows: 15 min at 95 °C, followed by 20 cycles of 95 °C for 30 s, 60 °C for 90 s, and 72 °C for 90 s, followed by a single incubation of 10 min at 72 °C. Amplification products were detected using capillary electrophoresis technology (Lab901 Screen Tape System; Lab901 Ltd, Loanhead, UK).

2.4. RV16 testing

cDNA synthesis was performed with cDNA Synthesis Premix (Seegene) from extracted RNAs. RV16 sets A and B were used, according to the manufacturer’s instructions. Briefly, the assay was conducted in a final volume of 20 μL containing 8 μL cDNA, 5 μL 4× RV primer, and 5 μL 4× master mix with the CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA) under the following conditions: 4 min at 50 °C and 15 min at 95 °C, followed by 50 cycles of 95 °C for 30 s, 60 °C for 1 min, and 72 °C for 30 s. After the reaction, Catcher Melting Temperature Analysis was performed by cooling the reaction mixture to 55 °C, maintaining the mixture at 55 °C for 30 s, 95 °C for 30 s, 60 °C for 90 s, and 72 °C for 90 s, followed by a single incubation of 10 min at 72 °C. Amplification conditions: 4 min at 50 °C and 15 min at 95 °C, followed by 30 cycles of 95 °C for 30 s, 60 °C for 90 s, and 72 °C for 90 s, followed by a single incubation of 10 min at 72 °C. Amplification products were detected using capillary electrophoresis technology (Lab901 Screen Tape System; Lab901 Ltd, Loanhead, UK).

2.5. Comparison of the RV12 and RV16 assays

Specimens that showed a discrepancy between RV12 and RV16 assay were further verified using monoplex PCR and sequencing in a blinded manner. The primers for monoplex PCR in the single or nested PCR format were identical to the primers of the RV12 or RV16 assay. The PCR products were purified with a power gel extraction kit (TaKaRa Bio Inc, Shiga, Japan). Purified templates were sequenced with a BigDye Terminator v3.1 cycle sequencing kit (Life Technologies, Foster City, CA, USA) and analyzed on an ABI 3730xl DNA analyzer (Life Technologies). Since RV12 cannot detect bocavirus (BoV), HEV, ADV F, PIV4, or HRV C, we excluded those results from comparison of the 2 assays.

2.6. Analytical sensitivity and specificity of the RV16 assay

Serially diluted plasmids containing the target gene were used for determination of analytical sensitivity. pUC19 vector was used for plasmid DNA preparation. Serial dilutions of the prepared plasmid DNA were made from $10^0$ to $10^5$ copies per reaction to determine the analytical sensitivity of the assay. MPV, BoV, and CoV-NL63 samples were isolated from patients, and their sequences were confirmed by direct sequencing. All other standard strains were obtained from American Type Culture Collection (ATCC). Ten replicates of each dilution step were performed. The lower detection limit was defined as the lowest concentration detected by 10 replicates of each assay.

The cross-reactivity of the RV16 assay was assessed using ten different bacteria: Streptococcus pneumoniae, Streptococcus pyogenes, Staphylococcus epidermidis, Moraxella catarrhalis, Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus, Neisseria meningitidis, and Haemophilus influenzae were obtained from the ATCC (Manassas, VA, USA). The DNA of supplied samples was extracted and assayed with the RV16 assay adhering to the same procedures used for sample processing.

2.7. Statistical analysis

Statistical analyses were performed using SPSS software, version 20.0 (SPSS Inc, Chicago, IL, USA) and the VassarStats website (http://vassarstats.net/). We used interrater agreement statistics (Kappa calculation) to compare the detection of respiratory viruses between the RV12 and RV16 assays. P-values less than 0.05 were considered statistically significant.

3. Results

3.1. Comparison of the RV16 assay with the RV12 assay

A total of 140 (38.4%) and 89 (24.4%) samples were RV16 and RV12 positive, respectively. Among viruses tested using both methods, the positive percent agreement between the RV16 and the RV12 assays was 95.5% (95% confidence interval [CI], 88.3–98.5), and the negative percent agreement was 80.0% (95% CI, 74.8–84.2). The kappa value for the 2 methods was 0.64 (95% CI, 0.55–0.71). Results by virus are presented in Table 1.

Sixty-five samples that were identified as positive samples by the RV16 assay were identified as negative by the RV12 assay: 8 samples had more than 1 discrepant viruses, and 71 discrepancies by each virus were detected. On the other hand, 4 clinical samples that were diagnosed negative resulted in 4 discrepancies by each virus.

| Virus | Agreement | Positive | % 95% CI | Negative | % 95% CI | Kappa value | Observed kappa | 95% CI |
|-------|-----------|----------|----------|-----------|----------|-------------|---------------|--------|
| Total | 95.5      | 88.3–98.5 | 80.0     | 74.8–84.2 | 0.64     | 0.55–0.71   | 0.32          | 0.08–0.56 |
| ADV   | 100       | 39.6–100 | 95.6     | 92.8–97.4 | 0.78     | 0.66–0.91   | 0             | 0.06–0.51 |
| INF A | 95.6      | 76.0–99.8 | 97.1     | 94.5–98.5 | 0.30     | 0.20–0.40   | 0             | 0.00–0.51 |
| INF B | 100       | NA       | 98.7–100 | 0         | 0        | 0           | 0             | 0.00–0.51 |
| PIV 1 | 0         | 0–94.5   | 100      | 98.7–100 | 1.00     | 1.00–1.00   | 0             | 0.00–0.51 |
| PIV 2 | 100       | 54.6–100 | 98.7–100 | 1.00      | 1.00–1.00 | 0           | 0             | 0.00–0.51 |
| PIV 3 | 100       | NA       | 98.7–100 | 1.00      | 1.00–1.00 | 0           | 0             | 0.00–0.51 |
| HRV   | 90.0      | 59.6–98.2 | 93.6     | 93.4–97.8 | 0.53     | 0.33–0.73   | 0             | 0.00–0.51 |
| RSV (A or B) | 97.1 | 83.4–99.8 | 98.2 | 95.9–99.3 | 0.90     | 0.82–0.97   | 0             | 0.00–0.51 |
| HMPV  | 100       | 46.3–100 | 98.3     | 96.2–99.3 | 0.62     | 0.34–0.90   | 0             | 0.00–0.51 |
| CoV   | 100       | 69.9–100 | 94.6     | 91.6–96.6 | 0.54     | 0.36–0.72   | 0             | 0.00–0.51 |

NA = not applicable.
sensitivities compared to the Seeplex® RV15 ACE detection kit (RV15; reported on the performance of RV16, which showed increased monoplex PCR and sequencing cannot be excluded. Recent studies RV16 may detect INF A with superior sensitivity compared to the gene sequencing. However, in the discrepant RV16-positive and the assay procedure used for the clinical samples for RV detection with the RV16 assay. All assay results were negative, and no nonspecific positive reaction was observed.

3.2. Analytical sensitivity and specificity of the RV16 assay

The detection limits of the RV16 detection kit for all 16 respiratory viruses were approximately 6 copies/μL (50 copies/reaction).

To evaluate the cross-reactivity and detection specificity, 10 different bacterial reference strains were tested using the same assay procedure used for the clinical samples for RV detection with the RV16 assay. All assay results were negative, and no nonspecific positive reaction was observed.

4. Discussion

We compared the performance of 2 multiplex PCR kits, RV12 and RV16, for detection of respiratory viruses using clinical respiratory samples. We performed monoplex PCR and direct sequencing in a blind manner for the samples that yielded discrepant results with the 2 assays.

In the present study, 38.4% and 24.4% of samples were RV16 and RV12 positive, respectively. The volume of sample added to the RV16 assay was almost 3 times more than the volume added to the RV12 assay, and this could have contributed to the higher number of positive samples by RV16 compared to RV12 assay.

Majority of the samples that were identified as positive from RV16 assays were confirmed as positive by monoplex real-time RT-PCR and gene sequencing. However, in the discrepant RV16-positive and the RV12-negative results for INF A, monoplex PCR and sequencing revealed that 50% were negative. This suggests the false positivity for INF A in RV16 assay by nucleic acid contamination or cross-reactivity from spurious primer interactions. However, the possibility that the RV16 may detect INF A with superior sensitivity compared to the monoplex PCR and sequencing cannot be excluded. Recent studies reported on the performance of RV16, which showed increased sensitivities compared to the Seeplex® RV15 ACE detection kit (RV15; Seegene), although we did not determine the diagnostic accuracy against a reference method (Cho et al., 2013; Kim et al., 2013).

The RV12 system had a limitation regarding its internal control and laboratory-developed real-time reverse transcription-polymerase chain reaction assays for respiratory virus detection. J Clin Microbiol 2009;47:2812–8.

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Yoo SJ, Kuak EY, Shin BM. Detection of 12 respiratory viruses with two-set multiplex reverse transcriptase-PCR assay using a dual priming oligonucleotide system. Korean J Lab Med 2007;27:420–7.

Table 2

| Virus    | RV16+/RV12+ | RV16+/RV12– | RV16–/RV12+ |
|----------|-------------|-------------|-------------|
|          | No. of samples | No. of positive results | No. of samples | No. of positive results |
| ADV      | 4           | 16          | 13          | 0         |
| INF A    | 22          | 10          | 5           | 1         |
| PIV 1    | 0           | 0           | 0           | 0         |
| PIV 2    | 9           | 14          | 13          | 1         |
| RSV (A or B) | 34        | 6           | 4           | 1         |
| HRV      | 5           | 6           | 6           | 0         |
| HMPV     | 12          | 19          | 17          | 0         |

ND = not performed due to lack of sample.

positivity only in the RV12 assay, none was concordant with the result by the RV12 assay. The overall results of monoplex PCR and sequencing revealed that majority of the samples that were identified as positive from RV16 assays also exhibited positive results, except INF A (Table 2).

3.2. Analytical sensitivity and specificity of the RV16 assay

The detection limits of the RV16 detection kit for all 16 respiratory viruses were approximately 6 copies/μL (50 copies/reaction).

To evaluate the cross-reactivity and detection specificity, 10 different bacterial reference strains were tested using the same assay procedure used for the clinical samples for RV detection with the RV16 assay. All assay results were negative, and no nonspecific positive reaction was observed.

4. Discussion

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In the present study, 38.4% and 24.4% of samples were RV16 and RV12 positive, respectively. The volume of sample added to the RV16 assay was almost 3 times more than the volume added to the RV12 assay, and this could have contributed to the higher number of positive samples by RV16 compared to RV12 assay.

Majority of the samples that were identified as positive from RV16 assays were confirmed as positive by monoplex real-time RT-PCR and gene sequencing. However, in the discrepant RV16-positive and the RV12-negative results for INF A, monoplex PCR and sequencing revealed that 50% were negative. This suggests the false positivity for INF A in RV16 assay by nucleic acid contamination or cross-reactivity from spurious primer interactions. However, the possibility that the RV16 may detect INF A with superior sensitivity compared to the monoplex PCR and sequencing cannot be excluded. Recent studies reported on the performance of RV16, which showed increased sensitivities compared to the Seeplex® RV15 ACE detection kit (RV15; Seegene), although we did not determine the diagnostic accuracy against a reference method (Cho et al., 2013; Kim et al., 2013).

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Table 2

Analysis for positive results in Anyplex™ II RV16 and Seeplex® RV16 ACE assays.

| Virus    | RV16+/RV12+ | RV16+/RV12– | RV16–/RV12+ |
|----------|-------------|-------------|-------------|
|          | No. of samples | No. of positive results | No. of samples | No. of positive results |
| ADV      | 4           | 16          | 13          | 0         |
| INF A    | 22          | 10          | 5           | 1         |
| PIV 1    | 0           | 0           | 0           | 0         |
| PIV 2    | 9           | 14          | 13          | 1         |
| RSV (A or B) | 34        | 6           | 4           | 1         |
| HRV      | 5           | 6           | 6           | 0         |
| HMPV     | 12          | 19          | 17          | 0         |

ND = not performed due to lack of sample.