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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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ABSTRACT

The Anyplex™ II RV16 detection kit (RV16; Seegene, Seoul, South Korea) is a multiplex real-time PCR assay based on tagging oligonucleotide cleavage extension. In this prospective study, we evaluated the RV16 assay by comparing with the Seeplex® RV12 ACE detection kit (RV12; Seegene), a multiplex end-point PCR kit. A total of 365 consecutive respiratory specimens were tested with both RV16 and RV12 assays in parallel and detected 140 (38.4%) and 89 (24.4%) positive cases, respectively. The positive percent agreement, negative percent agreement, and kappa values for the 2 assays were 95.6% (95% confidence interval [CI], 89.4–98.3%), 80.4% (95% CI, 75.3–84.6%), and 0.64 (95% CI, 0.56–0.72), respectively. The monoplex PCR and sequencing for the samples with discrepant results revealed that majority of the results were concordant with the results from RV16 assays. In conclusion, the RV16 assay produces results comparable to the RV12 assay.

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, Seoul, South Korea) 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 nonselective 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

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frozen at −70 °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 specimens 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 Revertaid 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-mL primer mix A, and the other, with 4-mL primer mix B. Thermal cycling conditions were as follows: 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).

2.4. 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, and heating the mixture from 55 °C to 85 °C. The fluorescence was measured continuously during the temperature increase. The melting peaks were derived from the initial fluorescence (F) versus temperature (T) curves.

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 blind 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 10^6 to 10^0 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 replicas 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 negative in the RV16 assay were identified as positive by the RV12 assay. For further analysis of the samples that yielded discrepant results with the RV16 assay and the RV12 assay, monoplex real-time reverse transcription-polymerase chain reaction (RT-PCR) and gene sequencing were performed. In the discrepant RV16-positive and the RV12-negative results, the results of monoplex PCR and sequencing revealed that 81.7% (58/71) were concordant with the result by the RV16 assay. On the other hand, in discrepant results that exhibited

Table 1
Detection of respiratory viruses by Anyplex™ II RV16 and Seeplex® RV12 ACE assays.

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

NA = not applicable.

Table 2
Analysis for positive results in Anyplex™ II RV16 and Seeplex® RV12 ACE assays.

Virus	RV16 +/ RV12 +	RV16+/RV12−		RV16−/RV12+	
		No. of samples	No. of positive results in	No. of samples	No. of positive results in monoplex PCR and sequencing
ADV	4	16	13	0	0
INF A	22	10	5	1	0
PIV 1	0	0	0	1	0
PIV 2	1	0	0	0	0
HRV	9	14	13	1	0
RSV (A or B)	34	6	4	1	ND
HMPV	5	6	6	0	0
CoV	12	19	17	0	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

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 facility. The artificial targets included in each PCR mastermix only allow validation of the PCR step, without control for the RNA extraction or reverse transcription steps (Bibby et al., 2011). As for RV16, the addition of bacteriophage MS2 to each extraction allows the RT-PCR system to monitor both the RNA extraction and the reverse transcription steps (Cho et al., 2013; Dreier et al., 2005). Nevertheless, the internal control in RV16 cannot give information for specimen quality or prevent misjudgment from sampling error, since it is an exogenous control.

RV16 has an advantage in workload compare to RV12. Given that the RV12 assay requires agarose gel detection after PCR, RV16 requires less time and labor than RV12 (less than 7 hours with reduced hands-on time) (Bibby et al., 2011; Kim et al., 2013). In addition, RV16 is a closed PCR system with a reduced chance of amplicon contamination.

The present study has some limitations. First, we could not compare a large number of some respiratory viruses between assays due to the low number of virus-positive specimens and respiratory virus seasonality, since this was consecutive prospective study performed over 2 months. Second, we did not compare RV16 and RV12 with viral cultures or direct fluorescent-antibody assays. Third, we used the same primers for RV12 and RV16 in the monoplex PCR and sequencing for discrepant analysis. However, we performed monoplex PCR and direct sequencing in a blind manner to overcome the limitation of the study design. Fourth, since the analytical sensitivities were determined using plasmid DNA, the reverse transcription step could not be included in the estimate.

In conclusion, the RV16 assay produces results comparable to the RV12 assay. Further analysis of the samples that yielded discrepant results demonstrated that the RV16 assay exhibited a higher concordance with the results of monoplex PCR and sequencing, compared to the RV12 assay.

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