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Comparison of Anyplex II RV16 with the xTAG Respiratory Viral Panel and Seeplex RV15 for Detection of Respiratory Viruses

Hyun-Ki Kim, Sung-Hee Oh, Kyung Ah Yun, Heungsup Sung, Mi-Na Kim

Department of Laboratory Medicine, Asan Medical Center and University of Ulsan College of Medicine, Seoul, South Korea

A novel multiplex real-time PCR approach (Anyplex II RV16 [RV16]; Seegene, South Korea) was compared with a multiplex endpoint PCR kit (Seeplex RV15 ACE detection kit [RV15]; Seegene) and a liquid bead-based assay (xTAG respiratory viral panel [xTAG]; Abbott, United States). Of nasopharyngeal swabs or aspirates and bronchoalveolar lavage fluid samples submitted for RV15 testing, 199 retrospectively collected positive specimens and 283 prospectively collected specimens were further tested with RV16 and xTAG. A true-positive result was defined as a positive result from all three methods or RV16 and xTAG or RV15 and xTAG. For specimens with discrepant results, monoplex PCR and sequencing of the target viruses were performed. In total, 300 virus-positive specimens yielded 386 viruses. When the bocavirus results were excluded, the overall sensitivities of RV16, RV15, and xTAG were 95.2%, 93.3%, and 87.2%, respectively (95% confidence intervals, 93.0 to 97.4%, 90.8 to 95.8%, and 83.8 to 90.6%, respectively). RV16 was more sensitive than xTAG for coronavirus OC43/HKU1 (100% versus 26.1%; $P < 0.0001$) and adenovirus (100% versus 79.5%; $P < 0.01$) but was less sensitive than xTAG for rhinovirus/enterovirus (89.4% versus 97.9%; $P < 0.05$). RV16 demonstrated higher sensitivity than RV15 for the detection of adenovirus (100% versus 82.1%; $P < 0.05$). The specificities of all three methods ranged from 98.6% to 100%. Sequencing analysis of 64 rhinovirus-positive samples revealed that RV16 accurately differentiated between rhinovirus and enterovirus. RV16 most frequently missed rhinovirus C. In conclusion, the overall sensitivity of RV16 was better than that of xTAG. However, improvement of the sensitivity for rhinovirus is required.

Respiratory viral infections are a major cause of morbidity and mortality worldwide (1–3). The rapid and accurate diagnosis of respiratory viral pathogens aids in antiviral therapy. Early diagnosis has the potential to reduce complications, antibiotic use, and unnecessary laboratory testing (4–6). Clinical laboratories traditionally use methods such as direct fluorescent-antibody assay (DFA) and viral culture to detect respiratory viruses. Compared with such traditional methods, nucleic acid amplification tests show superiority in terms of sensitivity for the detection and spectrum of target viruses (7, 8). In addition, the use of multiplex assays significantly reduces the hands-on time and cost compared to those for DFA and culture (5, 9, 10).

The Luminex xTAG respiratory viral panel (xTAG; Abbott, United States) is a multiplex PCR assay that is approved by the U.S. Food and Drug Administration (FDA). The xTAG assay is capable of detecting 20 viruses and subtypes simultaneously in a single patient sample (5). The Seeplex RV15 ACE detection kit (RV15; Seegene, South Korea) is able to detect 15 viruses in three reactions per sample (11). The recently developed Anyplex II RV16 detection kit (RV16; Seegene) is a novel multiplex real-time PCR based on tagging oligonucleotide cleavage extension (TOCE) (12, 13). TOCE introduces two novel components, the "pitcher" and "catcher," to accomplish a unique signal generation in real time. In a TOCE reaction, the 5′ nuclease activity specifically cleaves a target-bound pitcher in such a manner that a designed tagging portion is released. The released tagging portion hybridizes to the capturing portion of the catcher. The formation of the duplex catcher through tagging portion extension and physical distancing of the quencher from the fluorescent moiety results in the fluorescent signal. What becomes apparent is the control over the melting-temperature properties of the catcher: by designing unique catchers, the resulting duplex catcher will have a predictable and unique melting-temperature profile. As a result, multiple catchers with unique melting-temperature profiles can be detected by catcher melting-temperature analysis (CMTA), in the same reaction and in the same color channel (12, 13). Therefore, multiple target analytes can be detected simultaneously in a single fluorescence channel.

In this study, we evaluated the diagnostic performance of RV16 compared to those of xTAG and RV15. In addition, we analyzed rhinovirus (RhV)- and enterovirus (EV)-positive samples by sequencing to determine whether RV16 could differentiate between RhV and EV accurately.

MATERIALS AND METHODS

Specimens. From the nasopharyngeal swabs (NPSs) or aspirates (NPAs) and the bronchoalveolar lavage (BAL) fluid samples submitted for RV15 testing from November 2011 to June 2012, 199 positive specimens (102 NPSs, 92 NPAs, and 5 BAL fluid samples) were retrospectively collected by considering the viral variety. These samples were further tested by RV16 and xTAG. In addition, 283 specimens (196 NPSs, 64 NPAs, and 23 BAL fluid samples) were consecutively collected on certain dates of each month and tested by RV15, RV16, and xTAG. NPSs were obtained using flocked swabs (Copan Diagnostics, Italy) and were transported in 3.0 ml of universal transport medium (UTM; Copan Diagnostics). The volume of NPA and BAL fluid was approximately 10 ml. Aliquots of retrospectively collected samples were stored at −70°C before testing. Prospectively collected samples were tested simultaneously upon receipt on all three assays. As a result, a total of 482 specimens were tested by RV15, RV16, and xTAG. The patients included 287 males and 195 females whose median

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Address correspondence to Heungsup Sung, sung@amc.seoul.kr.
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TABLE 1 Melting-temperature ranges of targets

Channel	A set		B set	
	Target	T_m (°C)[a]	Target	T_m (°C)
FAM	PIV4	64–68.5	hMPV	64–68.5
	ADV	77–81.5	BoV	77–81.5
HEX	PIV1	62.5–68	CoV 229E	62.5–67.5
	PIV2	70–74	CoV NL63	70–74
	PIV3	77.5–81.5	CoV OC43	76.5–81.5
R610	INF A	61.5–66.5	RSV A	62.5–67.5
	INF B	69–73	RSV B	69.5–73.5
	RhV	75.5–80.5	EV	77–81
Q670	IC[b]	63.5–68.5	IC	63.5–68.5

[a] T_m, melting temperature.
[b] IC, internal control.

age was 51 years and ranged from 14 days to 93 years. To evaluate the ability of RV16 to differentiate between RhV and EV, 64 samples that were positive for RhV and/or EV from the above-mentioned multiplex assays were further tested by sequencing.

Nucleic acid extraction and internal control. Nucleic acids were extracted from 500 μl of specimens by easyMAG (bioMérieux, France) for RV15 and by STARlet (Hamilton Robotics, NV) for RV16 and xTAG. The final elution volume of each sample was 50 μl in both kits. In RV16 and xTAG testing, an internal control was added to each specimen in order to check the entire process from nucleic acid extraction to PCR, according to the manufacturer's instructions. In RV15 testing, an internal control and its specific primer set were added to the multiplex primer mix so as to check the PCR process, according to the manufacturer's instructions.

RV16 testing. The cDNAs were synthesized from extracted RNAs with the cDNA Synthesis Premix (Seegene). Respiratory virus detection kits A and B were used to detect 14 types of RNA viruses and two types of DNA viruses, according to the manufacturer's instructions. Briefly, the assay was conducted in a final volume of 20 μl containing 8 μl of cDNA, 4 μl of 5× RV primer, 4 μl of 8-methoxypsoralen (8-MOP) solution, and 4 μl of 5× master mix with the CFX96 real-time PCR detection system (Bio-Rad, CA) under the following conditions: denaturation at 95°C for 15 min and 50 cycles at 94°C for 30 s, 60°C for 1 min, and 72°C for 30 s. CMTA 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 rise. The melting peaks were derived from the initial fluorescence (F) versus temperature (T) curves by plotting the negative derivative of fluorescence over temperature versus temperature (−dF/dT versus T). Table 1 shows the melting temperature for each target. The melting-temperature analysis was done by Seegene viewer software, which interpreted the results as "+" or "−." The user also could check the "−dF/dT versus T curve" on this program.

According to the data provided by the manufacturer, the analytical sensitivity, i.e., limit of detection, of RV16 was 50 copies per reaction for each type of virus. RV16 could detect 16 viruses covering many serotypes of each virus, including influenza A virus (INF A) (subtypes H1, H2, H3, H5, H6, H7, H9, H10, and H11), influenza B virus (INF B), respiratory syncytial viruses A and B (RSV A and RSV B), adenovirus (ADV) (serotypes A to F), human metapneumovirus (hMPV), coronavirus (CoV) 229E, CoV NL63, CoV OC43/HKU1, parainfluenza viruses (PIV) 1 to 4, RhVs A to C, EV, and bocaviruses (BoV) 1 to 4. This profile is similar to the profile of RV15, except for CoV 229E and CoV NL63, which only RV16 can differentiate.

xTAG testing. For xTAG, the cDNA and amplification steps were performed in a single-tube format. Amplification and detection were performed according to the manufacturer's instructions. The xTAG assay required two PCRs. For multiplex amplification PCR, a GeneAmp PCR System 9700 (Life Technologies, Carlsbad, CA) thermocycler was used under the following conditions: denaturation at 95°C for 15 min and 35 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. For multiplex target-specific primer extension PCR, the thermocycler (Life Technologies) was used under the following conditions: denaturation at 96°C for 2 min and 35 cycles at 95°C for 30 s, 54°C for 30 s, and 72°C for 30 s. Virus detection was performed by xMAP technology with a Luminex 100 system (Austin, TX). The cutoff values for xTAG were predetermined by the manufacturer.

RV15 testing. RV15 required a separate cDNA synthesis step before the multiplex PCR step could be performed. The cDNA synthesis was performed with a RevertAid First Strand cDNA synthesis kit (Fermentas, Canada) according to the manufacturer's instructions. The samples were tested for 13 types of RNA viruses and two types of DNA viruses with respiratory virus detection kits A, B, and C according to the manufacturer's instructions. PCR was conducted in a final volume of 20 μl containing 3 μl of cDNA, 4 μl of 5× RV15 ACE primer (A, B, or C), 3 μl of 8-MOP solution, and 10 μl of 2× master mix using the thermocycler (Life Technologies) under the following conditions: denaturation at 94°C for 15 min and 40 cycles at 94°C for 30 s, 60°C for 1.5 min, and 72°C for 1.5 min. The amplified PCR products were analyzed by agarose gel electrophoresis.

Sequencing. Specimens with discordant results were tested by monoplex PCR and sequencing. The primers for monoplex PCR in the single or nested PCR format were identical with the primers of the RV15 or RV16 assay. The PCR products were purified with a power gel extraction kit (TaKaRa Bio Inc., Shiga, Japan). Purified templates were sequenced with an ABI Prism BigDye Terminator v3.1 cycle sequencing kit (Life Technologies) and analyzed on an ABI 3730xl DNA analyzer (Life Technologies).

Definitions. A positive result from all three methods or from RV16 and xTAG or from RV15 and xTAG was considered to be a true positive without sequencing. In the case of a positive result of a single target from one method or RV16 and RV15, additional monoplex PCR and sequencing with specific primers were performed. A positive result from one method or RV16 and RV15 with a negative result from monoplex PCR and sequencing was considered to be a false positive. Because xTAG cannot detect BoV, we excluded BoV results from the sensitivity analysis.

Statistical analysis. Confidence intervals for a mean and comparison of proportions were analyzed. All statistical analyses were performed using SPSS (version 18.0; SPSS, Chicago, IL).

RESULTS

Distribution of respiratory viruses. Of the 199 retrospectively collected specimens, 198 gave true-positive results. Of the 283 prospectively collected specimens, 102 (38.0%) gave true-positive results. Table 2 shows the distributions of the viruses as single infections and coinfections. Overall, 300 positive specimens yielded 386 viruses, including BoV results. A single virus was detected in 228 specimens, two viruses in 62 specimens, three in 7 specimens, four in 2 specimens, and five in 1 specimen. When the BoV data were excluded, 296 true-positive specimens yielded 374 viruses.

Comparison of assays. The sensitivity and specificity were calculated for each target and assay according to our definition of a true or false positive (Table 3). The overall sensitivities of RV16, RV15, and xTAG were 95.2% (356/374), 93.3% (349/374), and 87.2% (326/374), respectively (95% confidence intervals, 93.0 to 97.4%, 90.8 to 95.8%, and 83.8 to 90.6%, respectively). RV16 was more sensitive than xTAG for CoV OC43/HKU1 (100% [23/23] versus 26.1% [6/23]; $P < 0.0001$) and ADV (100% [39/39] versus 79.5% [31/39]; $P < 0.01$) but was less sensitive than xTAG for RhV and EV (89.4% [84/94] versus 97.9% [92/94]; $P < 0.05$). RV16 demonstrated higher sensitivity in the detection of ADV (100%

TABLE 2 Distribution of respiratory viruses in samples

Virus	Subtype	Infection no.		Total no. (%)
		Single	Multiple	
RhV/EV		42	52	94 (24.4)
RSV	A	21	16	37 (9.6)
	B		1	1 (0.3)
INF A	H1	1		1 (0.3)
	H3	21	4	25 (6.5)
	Unidentified	3		3 (0.8)
INF B		31	3	34 (8.8)
PIV	1	10	4	14 (3.6)
	2	2	1	3 (0.8)
	3	19	10	29 (7.5)
	4	1		1 (0.3)
hMPV		37	13	50 (13.0)
CoV	OC43/HKU1	11	12	23 (6.0)
	229E/NL63	15	5	20 (5.2)
BoV		4	8	12 (3.1)
ADV		10	29	39 (10.1)
Total (%)		228 (59.1)	158 (40.9)	386 (100)

[39/39] versus 82.1% [32/39]; $P < 0.05$) than did RV15. The specificities of the three methods were generally very high, ranging from 98.6% to 100%.

Identification of multiple viruses. Multiple viruses were detected in 67 specimens (22.6% of positive specimens, excluding BoV results). RV16, RV15, and xTAG detected all of the contained viruses in 83.6% (56/67), 73.1% (49/67), and 70.1% (47/67) of the samples containing multiple viruses, respectively. Among the samples containing multiple viruses, 49 (73.1%), 28 (41.8%), and 18 (26.9%) specimens contained RhV, ADV, and both RhV and ADV, respectively.

RhV and EV. Because xTAG has combined RhV and EV targets, these viruses were lumped together as RhV/EV to perform a sensitivity and specificity analysis. The cases detected by xTAG alone as RhV/EV were tested by monoplex PCR and sequencing. Therefore, all of the cases of RhV were differentiated from EV in the RhV/EV-positive cases; 81 were positive for RhV only, 5 for EV only, and 8 for both RhV and EV. RV16 detected 79 RhV cases (88.8%) and 13 EV cases (100%), whereas RV15 detected 80 RhV cases (89.9%) and 12 EV cases (92.3%). xTAG detected 87 RhV-positive specimens (97.6%) and 13 EV-positive specimens (100%).

Monoplex PCR and sequencing were used to evaluate 64 samples that were positive for RhV and/or EV. Among the 64 samples, 58 were positive for RhV only, 2 for EV only, and 4 for both RhV and EV. The result of sequencing corresponded with that of the multiplex PCR assays. RV16 and RV15 differentiated RhV and EV accurately. Coinfections of RhV and EV in four samples were also confirmed by sequencing. No cross-reactivity between RhV and EV was found for either RV16 or RV15. The sequencing analysis identified RhV as RhV A ($n = 34$), RhV B ($n = 4$), or RhV C ($n = 24$). RV16 missed four cases of RhV C. RV15 missed one case of RhV A and one case of RhV C. xTAG missed one case of RhV A.

DISCUSSION

This study compared the performance of RV16 with those of RV15 and xTAG. All three methods showed good sensitivities for the detection of INF A and INF B. xTAG had an advantage for the detection of INF A because it further detected INF A virus subtypes H1, H3, and H5. RV16 had improved sensitivities for ADV. RV15 and RV16 showed good sensitivities for CoV. The xTAG assay showed decreased sensitivity for this virus, especially for CoV OC43/HKU1, as has also been noted in previous studies (7). The low sensitivity for CoV could be a noticeable disadvantage of xTAG because CoV has been suggested as a causative agent of croup (14, 15).

RV16 and RV15 had lower sensitivities than xTAG for RhV. The use of specific targets for each genus outside the 5′ noncoding region may compromise the sensitivity of detection, especially of RhV (7). For this reason, the xTAG assay combined the RhV and EV targets. However, RV16 and RV15 differentiated between RhV and EV, which may account for their reduced sensitivities. Our analysis of the abilities of RV16 and RV15 to differentiate between RhV and EV showed that both methods differentiated these viruses accurately, with no cross-reactivity between RhV and EV. The clinical significance of distinguishing RhV and EV is not well defined. However, the specific diagnosis of respiratory viruses is of considerable im-

TABLE 3 Percent sensitivity and specificity of three multiplex assays for detection and identification of respiratory viruses

Target	RV16		RV15		xTAG	
	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
RhV/EV	89.4	99.5	89.4	100	97.9	100
RSV A/B	100	99.8	89.5	99.5	84.2	100
INF A	100	100	100	100	96.6	100
INF B	97.1	100	97.1	100	94.1	100
PIV 1–4	97.9	98.6	100	99.3	83.0	99.8
PIV 1	100	98.9	100	100	78.6	100
PIV 2	100	100	100	100	100	99.8
PIV 3	96.6	99.8	100	99.6	82.8	100
PIV 4	100	100	100	99.8	100	100
hMPV	88.0	100	96.0	100	96.0	100
CoV OC43/HKU1	100	100	95.7	100	26.1	100
CoV 229E/NL63	100	99.8	100	100	90.0	100
ADV	100	100	82.1	100	79.5	100

portance in order to understand the epidemiology and pathogenesis of viral respiratory tract infection. In addition, while the importance of RhV for the induction of lower respiratory infections has become increasingly valued during the past several years, many questions regarding the epidemiology and pathogenicity of these viruses still need to be answered. The assays differentiating RhV from other picornaviruses could be useful in this respect (16). RV16 had particularly low sensitivity for RhV C. RhV C could be associated with more severe clinical illnesses, including lower respiratory tract infections and asthmatic exacerbations, than are RhV A and RhV B (17, 18). Considering the importance of RhV, the sensitivity of RV15 and RV16 assays for RhV should be improved. Otherwise, the advantage of differentiating RhV and EV would significantly diminish with lower sensitivity.

In addition to sensitivity and specificity, ease of use and required time are important factors to consider when choosing a multiplex PCR assay. Among the assays considered in this study, xTAG was the most time- and labor-intensive, requiring 8 to 9 h, five distinctive reagent preparation steps, two different thermocycler programs, and complex enzymes and reagents. In contrast, RV16 and RV15 required less time (6 to 7 h: 3 h for RNA and DNA extraction and reverse transcription and 3 to 4 h for PCR) and labor (5, 11, 19). Given that the RV15 assay requires agarose gel detection after PCR, RV16 seems to require the least labor and time. In contrast to RV15 and xTAG, which are open PCR systems and have a potential for amplicon contamination, RV16 is a closed PCR system, which offers some decreased contamination risk. With regard to data interpretation, RV15 has a disadvantage because it requires the visual inspection of products. RV16 and xTAG provide results as numerical data. Therefore, RV16 seems to be the most user-friendly of the three assays.

In our study, multiple viruses were detected in 67 specimens (22.6% of true-positive specimens). RhV and ADV were the viruses that were commonly associated with infection by multiple agents. However, we included retrospectively selected samples; thus, our data may not represent the actual prevalence of multiple-agent infection. Interestingly, xTAG and RV15 detected 100% and 90% of ADV in specimens harboring ADV as a single agent but only 71.4% and 79.6% of ADV in specimens harboring multiple agents. This result could have been affected by the reduced copies of ADV in samples from multiple-virus illnesses compared with single-virus illnesses (20, 21). The impact of multiple viruses on the severity of clinical illness is still unclear (21–23).

A limitation of our study may be that we did not compare RV16 with viral culture or DFA. However, Seeplex RV detection kits and xTAG have shown superior or comparable sensitivities compared with culture and DFA; therefore, this disadvantage should not be a major pitfall of the study (6–8, 19, 24–26). The use of the same primers as RV15 and RV16 in the monoplex PCR for discrepant analysis is a major limitation of our study design. The positive results seen only in xTAG were in one case of PIV2, one case of hMPV, and five cases of RhV. Six cases (1 hMPV and 5 RhVs) were confirmed with true-positive results, although one case (PIV2) was confirmed as a false-positive result. This would yield a bias in favor of RV16 and RV15.

In conclusion, the overall sensitivity of RV16 was better than that of xTAG for relevant respiratory viruses. However, improvement of the sensitivity for RhV is required.

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We declare that we have no financial conflicts of interest.

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