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Evaluation of a novel real-time RT-PCR using TOCE technology compared with culture and Seeplex RV15 for simultaneous detection of respiratory viruses

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ABSTRACT

Background: Various kinds of commercial molecular systems have been developed for fast and more accurate detection of respiratory viruses. Anyplex™ II RV16 [RV16] was designed for simultaneous detection of 16 respiratory viruses using multiplex PCR coupled with TOCE™ technology.
Objectives: To compare the performance of RV16 with those of culture and Seeplex® RV15 ACE [RV15] by determining their sensitivity and specificity.
Study design: Seven hundred and thirty respiratory samples were tested by modified shell vial culture method, RV16, and RV15. For molecular tests, automated nucleic acid extraction and liquid handling system using MICROLAB Nimbus IVD (Hamilton, USA) was adopted to maximize the workflow and accuracy. Performance of each assay was determined against a composite reference standard.
Results: Two hundred and one samples (28%) out of 730 samples were positive by culture, while additional 281 (39%) were positive by RV16 or RV15. Sensitivities of RV16, RV15, and culture for virus tested were as follows: 100/93/63% for influenza A, 90/80/69% for influenza B, 98/94/63% for RSV, 98/52/23% for adenovirus, and 100/75/46% for PIV. For viruses not covered by culture, sensitivities of RV16 and RV15 were as follows: 99/81% for rhinovirus, 92/100% for coronavirus OC43, 100/56% for coronavirus 229E/NL63, 92/88% for metapneumovirus, 100/62% for bocavirus, and 91/91% for enterovirus. Overall, the specificities of culture, RV16, and RV15 (Seegene) were 100/99.9/99.9%.
Conclusions: RV16 assay was superior to culture method and RV15 and will be a promising tool for patient management and public health epidemiology.

1. Background

Respiratory viral infections are one of the leading causes of morbidity and mortality. The rapid and accurate identification of viral etiology is critical in ruling out non-viral infections, initiating timely therapeutic interventions and limiting the spread of infections.[1–3]

Diagnoses of viral respiratory tract infections have been made generally by culture-based methods using cell culture and/or detection of antigens. Although these methods are effective and often complementary, they do have some disadvantages.[4] Cell culture, which has been considered the "gold standard", takes time, and detection of antigens often lacks sensitivity or specificity.[5] Even when these methods are applied simultaneously, some samples are still found negative despite clinical evidence of a viral respiratory infection.[4]

Molecular techniques offer quicker turn-around times, greater analytical sensitivity, and allow identification of strains that are difficult to culture using standard methods in clinical laboratories.[6] Especially, multiplexed RT-PCR enabled laboratorians to detect a panel of viruses simultaneously while maintaining excellent sensitivity and specificity.[3,7,8] Several studies have demonstrated the advantages of multiplex PCR including Seeplex® RV15 ACE detection [RV15] (Seegene, Seoul, Korea), which achieved approval from CE (2009), Health Canada (2011), and KFDA (2013), being sold in more than 20 countries in Europe, Canada and Asia.[6,9–12]

Recently, Anyplex™ II RV16 [RV16] with Tagging Oligonucleotide Cleavage and Extension (TOCE) technology (Seegene,

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Seoul, Korea) has been developed. TOCE assay is a novel approach to real-time PCR, using the two components, 'pitcher' and 'catcher'.[13] Through target bound pitcher, TOCE assay moves the detection point from the target sequence to the catcher, enabling predictable analysis of melting temperature for catcher duplex and offering multiplex real-time PCR capability to RV16.[13] RV16 achieved approval from CE (2012), Health Canada (2012), and KFDA (2013), being sold in more than 15 countries in Europe, Canada and Asia. RV16 uses 16 primer sets for simultaneous detection of 16 respiratory viruses: human bocavirus, human enterovirus, influenza virus type A and B, parainfluenza virus type 1, 2, 3, 4, RSV type A and B, adenovirus, human metapneumovirus, coronavirus OC43, 229E, NL63, and rhinovirus.[3–15]

2. Objectives

We compared the new multiplex RT-PCR, RV16 with well-known RV15 and culture by determining their sensitivity and specificity.

3. Study design

3.1. Subjects

From October 2011 to May 2012, 730 nasopharyngeal aspirate (NPA) samples (each NPA from each patient) were consecutively collected from 730 patients with acute respiratory infection at the Korea University Anam Hospital, Seoul, Korea. All of our samples (flocked nasopharyngeal swabs) had been transported in 3 mL of UTM (COPAN, Murrieta, CA, USA) and were freshly used for virus culture, Seeplex® RV15 assay and Anyplex™ II RV16 assay (Seegene, Korea). This study was approved by the Research ethics board of the Korea University Anam Hospital for patients.

3.2. Cell culture

FrozenFreshCell R-Mix Too cells (Diagnostic HYBRIDS, Athens, Ohio) were provided as cryovials shipped on dry ice. According to the manufacturer's instructions, the thawed cells were diluted in planting medium and delivered into 24 well plates. Then, two hundred microliters of specimen were then inoculated and the plates were centrifuged. After overnight incubation, cell monolayers were washed with phosphate-buffered saline, fixed with acetone, and stained with a respiratory virus fluorescent antibody pool, D3 DFA (Diagnostic HYBRIDS, Athens, Ohio). When virus-specific fluorescence was noted, virus identification was performed by using Diagnostics HYBRIDS' individual monoclonal antibodies. The presence of three or more cells per well with specific apple-green fluorescence was considered positive identification. When the initial screen was negative, the second vial was examined at day 3.

3.3. RV15 testing

Nucleic acids were extracted from 500 μl of NPA samples using a MICROLAB Nimbus IVD (Hamilton, Reno, NV, USA) with STARMag 96 Virus Kit (Seegene, Korea) and eluted in 100 μl of elution buffer. cDNA synthesis was carried out with RevertAid™ Firtst Strand cDNA sysnthesis kit (Fermentas, Burlington, ON, Canada) according to the manufacturer's suggested methods. The samples are tested with Respiratory Virus Detection Kit-A, B, and C (Seeplex® RV15 ACE detection kit; Seegene, Korea) according to the manufacturer's instructions. To detect amplification products was coupled with capillary electrophoresis technology (Lab901Screen Tape system; Lab901 Ltd, Loanhead, UK).

3.4. RV16 testing

RNAs were extracted from 500 μl of NPA samples with addition of 10 μl of bacteriophage MS2 as an internal control (Anyplex™II RV16 detection, Seegene) using a MICROLAB Nimbus IVD (Hamilton, USA) with STARMag 96 Virus Kit (Seegene, Seoul, Korea). Automated protocol for extraction, RT-PCR and PCR setup was implemented using Nimbus automated liquid handling workstation to maximize the workflow and accuracy.

The internal control added to each specimen works as an exogenous control to check the whole process from nucleic acid extraction to RT-PCR. cDNA synthesis was performed with cDNA synthesis Auto mix (Seegene, Korea) from extracted RNAs. Respiratory Virus Detection Kit-A and B (Anyplex™II RV16 detection; Seegene, Korea) were used according to the manufacturer's instructions. Briefly, TOCE assay[13] was conducted in the final volume of 20 μl using a real-time thermocycler CFX96 (Bio-Rad, Hercules, CA, USA). After reaction, Catcher Melting Temperature Analysis (CMTA)[15] was performed by cooling the reaction mixture to 55 °C, holding at 55 °C for 30 s, heating from 55 °C to 85 °C. The fluorescence was measured continuously during the temperature rise, while the melting peaks were derived from the initial fluorescence (F) versus temperature (T) curves.

3.5. Analytical methods

Performance of each assay was determined against a composite reference standard.[12] As for uncultivable viruses, we compared RV16 and RV15. If the results between RV16 and RV15 were concordant, they were considered to be a true positive without sequencing. However, if the results between RV16 and RV15 were discrepant, singleplex PCR and sequencing with specific primers was performed as a confirmatory test. The main outcomes of this study were the sensitivity and specificity of culture and RV16 and RV15 assay. All analyses were performed using SPSS (version 20.0; SPSS, Chicago, IL, USA).

4. Results

4.1. Distribution of respiratory viruses

A total of 472 (64.7%) and 437 (59.9%) samples were RV16 and RV15 positive, while 201 (27.5%) samples were culture-positive. All 201 culture-positive samples were RV16 positive, except 4 samples (3 influenza B, 1 RSV). Of 730 samples tested, respiratory viral pathogens were detected from 482 samples (66.0%). A single virus was detected in 356 samples (48.6%, 356/730). One hundred and twenty-six samples (17.2%) had viruses more than one. Two, three and four viruses-infected specimens were detected in 14.2 (104/730), 2.7 (20/730) and 0.3% (2/730) of total specimens, respectively (Table 1). In our study, RSV A and rhinoviruses were the viruses most commonly associated with multiple agent infection, followed by adenovirus, metapneumovirus, and coronavirus (Table 1). In our study, two (100%) of two quadruple viruses infected specimens were detected by RV16 assay only, while 9 (45%) of 20 triple viruses infected specimens were detected by RV16 alone. The results remained the same at repeated tests, singleplex PCR, and confirmatory sequencing analysis.

Table 1 shows the overall distribution of respiratory viral pathogens, indicating the predominance of influenza viruses, RSV and HRV. It also shows that coronavirus, metapneumovirus and adenovirus accounted for about 13.1% (63/482), 10.4% (50/482) and 12.7% (61/482) respectively, followed by enterovirus and bocavirus at around 2.3% (11/482) and 4.4% (21/482).

Table 1
Distribution of respiratory viruses in samples by RV15, RV16 and culture.

Target virus	Virus subtype	Total positive specimen no. by two PCR (%)[a]	Single virus (%)	Two viruses (%)	Three viruses (%)	Four viruses (%)	Total positive specimen no. by culture (%)
RSV	A	160 (33.2)	109 (22.6)	40 (8.3)	10 (2.1)	1 (0.2)	101 (16.0)[b]
	B	1 (0.2)		1 (0.2)			
HRV		126 (26.1)	63 (13.1)	50 (10.4)	11 (2.3)	2 (0.4)	NA
INF	A	76 (15.8)	70 (14.5)	4 (0.8)	2 (0.4)		48 (7.6)
	B	39 (8.1)	31 (6.4)	7 (1.5)	1 (0.2)		27 (4.3)
ADV		61 (12.7)	23 (4.8)	26 (5.4)	10 (2.1)	2 (0.4)	14 (2.2)
MPV		50 (10.4)	33 (6.8)	12 (2.5)	5 (1.0)		NA
Coronavirus	OC43	24 (5.0)	9 (1.9)	13 (2.7)	2 (0.4)		NA
	NL63	20 (4.1)	2 (0.4)	14 (2.9)	4 (0.8)		NA
	229E	19 (3.9)	2 (0.4)	15 (3.1)	2 (0.4)		NA
Parainfluenza	1	2 (0.4)	1 (0.2)	1 (0.2)			1 (0.16)
	2	3 (0.6)	1 (0.2)	2 (0.4)			–
	3	16 (3.3)	7 (1.5)	7 (1.5)	2 (0.4)		10 (1.6)
	4	3 (0.6)	1 (0.2)	1 (0.2)	1 (0.2)		NA
HboV	–	21 (4.4)	3 (0.6)	9 (1.9)	7 (1.5)	2 (0.4)	NA
HEV	–	11 (2.3)	1 (0.2)	6 (1.2)	3 (0.6)	1 (0.2)	NA
Total	–	482 (100.0)	356 (73.9)	104 (21.6)	20 (4.1)	2 (0.4)	201 (31.8)

[a] % means the percentage against the number of total virus-infected specimens, 482.
[b] Viral culture does not differentiate RSV A from RSV B. The number, 101, means the counting of RSV irrespective of any subtype.

4.2. Sensitivity according to each target virus (Table 2)

Of the 76 influenza A positive samples, 76 (100%), 71 (93.4%), 48 (63.2%) samples were positive by RV16, RV15 and viral culture, respectively. Of 39 influenza B positive samples, 35 (89.7%), 31(79.5%), 27 (69.2%) samples were positive by RV16, RV15 and viral culture, respectively.

RV16, RV15, and culture detected 24 (100%), 18 (75%), and 11(45.8%) PIV-positive samples among 24 PIV-positive samples, respectively. The predominant PIV subtype was type 3. Ten PIV3 samples and one PIV1 were positive by viral culture. Sixteen PIV3, 3 PIV2, and 2 PIV1 samples were positive by RV16, while thirteen PIV3, 2 PIV1, and 2 PIV2 samples were positive by RV15.

Among 161 RSV positive samples, 158 (98.1%), 151 (93.8%), and 101 (62.7%) samples were positive by RV16, RV15 and culture, respectively.

Among 61 ADV positive samples, 60 (98.4%), 32 (52.4%), and 14 (23%) samples were detected as ADV-positive by RV16, RV15 and culture, respectively.

HRV, coronavirus, bocavirus, enterovirus and metapneumovirus were not included in our viral culture panel. HRV was detected in 125 (100%) samples by RV16 and in 101 (80.8%) samples by RV15. Of the 24 coronavirus OC43 positive samples, 22 (91.7%) and 24 (100%) samples were positive by RV16 and RV15, respectively. As for coronavirus 229E/NL63, 27 (100%) and 15 (55.6%) samples were positive by RV16 and RV15, respectively. Bocavirus was detected in 21 (100%) samples by RV16 and in 13 (61.9%) samples by RV15. Metapneumovirus was detected in 46 (92.0%) samples by RV16 and in 44 (88.0%) samples by RV15. Also enterovirus was detected in 10 (90.9%) samples by RV16 and in 10 (90.9%) samples by RV15.

4.3. Comparison of the assay performance

Table 2 shows that RV16 was more sensitive than RV15 for specific viruses tested in this study. RV16 demonstrated sensitivity of ≥98% for all viruses except coronavirus OC43 (91.7%), metapneumovirus (92.0%), enterovirus (90.9%), and influenza B (89.7%). RV15 had good sensitivity for coronavirus OC43 (100%), influenza A (93.4%), and RSV (93.8%), but lower sensitivity for PIV, influenza B and rhinovirus (75–80.8%) and lowest sensitivity for coronavirus 229E and bocavirus (55.6–61.9%). Culture was less sensitive than two multiplex assays for all viruses tested. Two multiplex assays had similarly good sensitivity for the detection of RSV, and coronavirus OC43 (91.6–100%). The sensitivity for detecting PIV (100%), adenovirus (98.4%), rhinovirus (100%), coronavirus 229E/NL63 (100%), and bocavirus (100%) was better for RV16 than that for RV15 (75%, 52.4%, 80.8%, 55.6%, and 61.9%, respectively). RV16 assay showed higher sensitivity for influenza A (100%) than RV15 assay (93.4%). Compared with RV15 and culture, 15.5% (35/730) of samples demonstrated additional viral positivity by RV16. Overall, the specificities of culture, RV16, and RV15 were 100/99.9/99.9%.

5. Discussion

In this study, we evaluated the performance of the novel RV16 assay with those of RV15 assay and viral culture. RV16 and RV15 assays offered good sensitivities (79.5–100%) for the detection of common respiratory viral agents (influenza A or B, PIV 1–3, RSVA or B), while viral culture showed low sensitivities (45.8–69.2%). As for adenovirus, the RV16 assay showed superior sensitivity to RV15 (98.4% vs. 52.4), while viral culture demonstrated low sensitivity (23.0%). In our study, twenty-eight ADV-positive specimens were detected by RV16 alone. A confirmatory sequencing demonstrated that nineteen, six, two and one specimens were adenovirus C, B, F (41) and D type, respectively. In a previous study, through evaluating the four commercial multiplex PCR assays, the performance for adenovirus was not as good as the other viruses, assuming due to various serologic types of adenovirus.[12] In addition, the molecular assays detected viral pathogens from culture negative 282 (39%) samples. The overall detection rate in our study was similar to those in the previous reports. As for RV15, a Chinese study based on 164 nasopharyngeal swab presented the detection rate of 78.7% for all target respiratory viruses,[9] while a Canadian study based on 750 nasopharyngeal swabs showed the detection rate of 66.9%.[12] As for Seegene RV12, a Korean study performed on 101 respiratory specimen from 92 patients showed the detection rate of 75.2% for all target respiratory viruses,[3] while other Canadian study using 231 nasopharyngeal specimen demonstrated 69.8%.[6]

Viral culture has limited ability to detect some viruses.[16] Rhinovirus is difficult to detect by viral culture due to its diversity of serotypes. In our study, only RV16 and RV15 detected 125 rhinoviruses (24%) from 529 culture negative samples. Although MPV causes a lower respiratory disease similar to that caused by RSV,[17] it is difficult to detect MPV by cell culture due to its selective,

Table 2
Sensitivities of two multiplex assays and culture for detection of respiratory viruses.

Target virus	Culture positive no. (%)	RV16 assay positive no. (%)	RV15 assay positive no. (%)	Statistical difference[a]
INF A	48 (63.2%)	76 (100%)	71 (93.4%)	NS
INF B	27 (69.2%)	35 (89.7%)	31 (79.5%)	NS
RSV	101 (62.7%)	158 (98.1%)	151 (93.4%)	NS
ADV	14 (23.0%)	60 (98.4%)	32 (52.4%)	<0.05
PIV	11 (45.8%)	24 (100%)	18 (75%)	<0.05
HRV	–	125 (100%)	101 (80.8%)	<0.05
OC43	–	22 (91.7%)	24 (100%)	NS
229E/NL	–	27 (100%)	15 (55.6%)	<0.05
HBoV	–	21 (100%)	13 (61.9%)	<0.05
MPV	–	46 (92.0%)	44 (88.0%)	NS
HEV	–	10 (90.9%)	10 (90.9%)	NS
Total positive	201 (31.8%)	604 (97.4%)	510 (82.3%)	NS

[a] Between RV16 and RV15 by McNemar's test.

slow growth and mild cytopathicity. In this study, RV16 and RV15 detected 46 MPV (9%) from 529 culture negative samples.

RV16 assay was more sensitive for detecting all targets except for coronavirus OC43 than RV15 (Table 2). Since the clinical impact of influenza A, adenovirus, PIV, coronavirus, and bocavirus is becoming increasingly important, RV16 may have a strong point in the management of patients with respiratory tract infections.[6,10–12] Given that the coronavirus and human bocavirus are recently discovered, and detected worldwide and sometimes associated with severe respiratory illness, RV16 will be helpful to take care of the patients who have previously had the undefined or fatal respiratory illness.[16,18–20]

The advantage of the PCR-based assay was detecting co-infections which are usually missed by viral culture.[6] In the literature, the detection rate of multiple infections by multiplex PCR assay ranged from 5% to 40%.[3,6,9,12,16] Whereas, similar to our study results, Gharabaghi et al. using multiplex PCR reported two, three and four viruses-infected specimens as 15.3, 0.8 and 0.2% of total respiratory specimens, respectively. Although the role of multiple viral agents in affecting the clinical course of disease is at present unknown, multiple agents may be clinically significant, especially in immune compromised individuals.[12] RV16 assay would provide clinicians with better infectious status of patients than RV15 or culture, resulting in the more effective prevention of such multiple infections.

Recently, Kim et al. compared the performance of RV16, RV15, and Xtag,[21] which showed similar sensitivities for RV16 and RV15 to ours for almost all target viruses. But they reported about 10% lower sensitivity of RV16 and RV15 for rhinovirus compared to ours. This might be caused by use of xTAG with good performance for rhinovirus as an arbitrator. As for influenza B, RV16 and RV15 sensitivity (97.1% and 97.1%, respectively) by Kim et al. were higher than those in our study (89.7% and 79.5%, respectively).[21] In our study, three cases of influenza B, which were culture positive and the molecular assays negative, were detected. These seem to make such difference in sensitivities of influenza B. A case of 2009 H1N1 influenza positive by culture and negative by the ProFlu+ assay (Gen-Probe Prodesse, Inc., Waukesha, WI) was reported.[22] Two site mismatches were found later between the probe used and target sequence. Such kind of mismatch might have happened in our influenza B cases.

While the market price of RV16 is as same as that of RV15 in this country, RV16 compared with RV15, marked technical advancements was made with introduction of TOCE and CMTA.[13–15] TOCE bypassed the need to directly measure the target, employing an artificial template of defined and predictable T_m, the Catcher.[13] Because the sequence of the Catcher is defined during assay design, the resulting Duplex Catcher will yield a distinct and predictable melting profile in melting temperature analysis, referred as CMTA. Thereby, TOCE and CMTA system can stably detect multiple targets in a single channel and furthermore increase the number of targets up to 8 in a single tube.

A major challenge of several multiplex RT-PCR systems is the quantification of multiple viral targets in a reaction tube.[15] Very recently repeated melting temperature analysis, named cyclic-CMTA was introduced.[15] The upgraded version of RV16 with cyclic-CMTA comes to quantify multiple viral targets by repeating melting curve analysis at three preselected cycles.[15]

A limitation to RV15 system is its internal control facility. The artificial targets included in each PCR mastermix allow validation only of the PCR step.[10] 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 step.

In conclusion, RV15 and the novel RV16 assay increased the sensitivity of detection of respiratory viruses by 38.1% over viral culture, while maintaining excellent specificity. The new multiplex PCR assay was superior to RV15 for detection of most of 16 viral targets, including multiple infections. In this era of emerging viral infections, RV16 assay will be beneficial in determining patient management and public health epidemiology.

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Competing interests

None.

Ethical approval

This study was approved by the Human Use Ethical Committee at Korea University Anam Hospital.

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