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## Evaluation of the AdvanSure™ real-time RT-PCR compared with culture and Seeplex RV15 for simultaneous detection of respiratory viruses<sup>☆,☆☆,★</sup>

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### ABSTRACT

Recently, AdvanSure™ kit based on multiplex real-time PCR was developed for simultaneous detection of 14 respiratory viruses (RVs). We compared the performance of AdvanSure with those of Seeplex® RV 15 ACE and culture by determining their sensitivities and specificities against a composite reference standard. Four hundred thirty-seven respiratory samples were tested by modified shell vial culture method, RV 15 ACE, and AdvanSure. One hundred fourteen samples (26.2%) out of 437 samples were positive by culture, while additional 91 (20.8%) were positive by AdvanSure or RV15. One hundred twelve of 114 culture-positive samples were positive by AdvanSure except 2 samples (1 adenovirus, 1 respiratory syncytial virus [RSV]). Overall, the sensitivities of culture, RV15, and AdvanSure were 74.5%, 89.8%, and 95.1%, respectively. Sensitivities of culture, RV15, and AdvanSure for each virus tested were as follows: 91/100/96% for influenza A, 60/0/100% for influenza B, 63/95/97% for RSV, 69/81/89% for adenovirus, and 87/93/93% for parainfluenza virus. For viruses not covered by culture, sensitivities of RV15 and AdvanSure were as follows: 77/88% for rhinovirus, 100/100% for coronavirus OC43, 40/100% for coronavirus 229E/NL63, 13/100% for metapneumovirus, and 44/100% for bocavirus. The overall specificities of culture, RV15, and AdvanSure were 100/98.9/99.5%, respectively. Of 45 coinfecting specimens, AdvanSure detected 41 specimens (91.1%) as coinfecting, while RV15 detected 27 specimens (60.0%) as coinfecting. AdvanSure assay demonstrated exquisite performance for the detection of RVs and will be a valuable tool for the management of RV infection.

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### 1. Introduction

Human viral respiratory infections are often associated with significant morbidity and mortality (Thompson et al., 2003). The rapid and accurate diagnostic methods are important in identifying the causative pathogens at early stage of the illness, initiating timely therapeutic interventions and limiting the misuse of drugs (Coiras et al., 2003; Zhang et al., 2012).

Diagnoses of viral respiratory tract infections have been made generally by non-molecular approaches such as direct immunofluorescence and viral culture. Although these methods are effective and often complementary, they are time-consuming, labor-intensive, and often lack sensitivity or specificity (Bellau-Pujol et al., 2005; Gooskens

et al., 2008; Vallieres and Renaud, 2013; Zhang et al., 2012). However, various nucleic acid amplification tests have been developed and employed for a number of years, their utility having been demonstrated largely by comparison with these classical diagnostic methods (Bibby et al., 2011; Gharabaghi et al., 2011).

As molecular assays offer reduced turnaround times and considerably improved sensitivity (Choudhary et al., 2013; Drews et al., 2008; Vallieres and Renaud, 2013), they have been progressively multiplexed to simultaneously detect several different viruses in a single assay while maintaining excellent sensitivity and specificity (Choudhary et al., 2013; Vallieres and Renaud, 2013). Several studies have demonstrated the advantages of multiplex PCR assays such as xTAG RVP, RVP fast (Luminex Molecular Diagnostics, Toronto, ON, Canada), Resplex II (Qiagen, Mississauga, ON, Canada), FilmArray® Respiratory panel (Idaho Technology Inc., Salt Lake City, UT, USA), and Seeplex RV assays (Seegene, Seoul, Korea), which are used routinely for the detection of respiratory viral infection (Bibby et al., 2011; Couturier et al., 2013; Gharabaghi et al., 2011; Kim et al., 2013; Zhang et al., 2012).

Recently, AdvanSure™ respiratory virus (RV) has been introduced as multiplex real-time reverse transcription-polymerase chain reaction (real-time RT-PCR) (LG Life Science, Seoul, Korea), using 14 primer sets for simultaneous detection of 14 RVs with 5 tubes: influenza virus type A and B; parainfluenza virus (PIV) type 1, 2, 3;

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respiratory syncytial virus (RSV) type A and B; adenovirus, human bocavirus, human metapneumovirus (MPV), coronavirus OC43, 229E, NL63; and rhinovirus. The aim of this study was to compare the AdvanSure™ real-time RT-PCR with a multiplex conventional PCR, Seeplex® RV 15 ACE (Seegene), and culture by determining their sensitivities and specificities.

## 2. Materials and methods

### 2.1. Subjects

From August 2012 to February 2013, 437 nasopharyngeal samples (each from each patient) were consecutively collected from 437 patients with acute respiratory infection at the Korea University Anam Hospital, Seoul, Korea. All of our samples (nasopharyngeal aspirates or flopped nasopharyngeal swabs) had been transported in 3 mL of UTM (COPAN, Murrieta, CA, USA) and were freshly used for virus culture, Seeplex® RV 15 assay, and AdvanSure™ real-time RT-PCR (LG Life Science). This study was approved by the Research Ethics Board of the Korea University Anam Hospital for patients.

### 2.2. Cell culture

FrozenFreshCell R-Mix Too cells (Diagnostic HYBRIDS, Athens, OH, USA) 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. Once the cells had formed a confluent monolayer, 200 µL of each specimen was inoculated into each of 4 wells of 24-well plates, and the plates were centrifuged. The first well was used for screening at day 1. When the initial screen was negative, the second well was examined at day 3. If the second well tested positive at day 3, the third well was used to confirm the result. The fourth well was used as viral stock. For viral detection, RV fluorescent antibody pool, D3 DFA (Diagnostic HYBRIDS), and Diagnostics HYBRIDS' individual monoclonal antibodies were used.

### 2.3. Seeplex® RV 15 ACE detection kit

Nucleic acids were extracted from 500 µL of nasopharyngeal samples using a NucliSENS® easyMAG® (BioMérieux, Marcy l'Etoile, France) as per the manufacturer's protocol. Complementary DNA (cDNA) synthesis was carried out with RevertAid™ First Strand cDNA synthesis kit (Fermentas, Burlington, ON, Canada) according to the manufacturer's suggested methods. The samples were tested with Respiratory Virus Detection Kit-A, B, and C (Seeplex® RV 15 ACE detection kit) according to the manufacturer's instructions. Amplification products were detected using capillary electrophoresis technology (Lab901Screen Tape system; Lab901 Ltd, Loanhead, UK).

### 2.4. AdvanSure™ RV real-time RT-PCR

Nucleic acids (RNAs and DNAs) were extracted from 200 µL of nasopharyngeal samples using a TANBead fully automated magnetic bead operating platform, Smart LabAssist-32 with TANBead Viral Auto Plate (96) (Taiwan Advanced Nanotech Inc., Taoyuan City, Taiwan). AdvanSure™ RV real-time kits (LG Life Science) were used to detect 12 types of RNA viruses and 2 types of DNA viruses according to the manufacturer's instructions. Briefly, 5 µL of extracted nucleic acids was added in an AdvanSure RV real-time PCR reaction tube containing 5 µL of primer probe mixture and 10 µL of 1-step premix operating cDNA synthesis and real-time PCR. For the reverse transcription step, this mixture was incubated at 50 °C for 10 min. Denaturation followed at 95 °C for 30 s, then 10 cycles of PCR (15 s at 95 °C, 30 s at 53 °C, and 30 s at 60 °C), and 30 additional cycles of PCR for the detection of fluorescence signals (15 s at 95 °C, 30 s at 53 °C, 30 s at 60 °C). AdvanSure™ RV real-time RT-PCR performed both reverse-transcription

reaction with the extracted RNA and multiplex PCR reaction simultaneously in a single tube and used endogenous RNase P, as an internal control, to give information for validity of RNA extraction procedure and to prevent misjudgment from sampling error and RT-PCR reaction error.

### 2.5. RV 16 testing

RNAs were extracted from 500 µL of nasopharyngeal samples with addition of 10 µL of bacteriophage MS2 as an internal control (Anyplex™II RV 16 detection; Seegene) using a MICROLAB Nimbus IVD (Hamilton, Reno, NV, USA) with STARMag 96 Virus Kit (Seegene). 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) from extracted RNAs. Respiratory Virus Detection Kit-A and B (Anyplex™II RV 16 detection; Seegene) were used according to the manufacturer's instructions. Briefly, the assay 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) was performed by cooling the reaction mixture to 55 °C, holding at 55 °C for 30 s, heating from 55 °C to 85 °C (Hwang, 2012).

### 2.6. Singleplex PCR and sequencing

For singleplex PCR and sequencing, the primers for singleplex PCR in the single or nested PCR format were identical with the primers of previous studies (Arden et al., 2005; Kapoor et al., 2010; Lam et al., 2007). The PCR products were purified with a QIA quick PCR Purification Kit (QIAGEN, Hilden, Germany). Purified templates were sequenced with an ABI Prism 3730xl DNA sequencer (Life Technologies, Grand Island, NY, USA).

### 2.7. Analytical methods

Performance of each assay was determined against a composite reference standard (Gharabaghi et al., 2011). A positive result from all 3 methods or from culture or from both RV15 and AdvanSure™ was considered to be a true positive. A negative result from all 3 methods was considered to be a true negative. In the case of a positive result from only 1 of 2 PCR assays, the confirmatory test was as follows: A positive result from only RV15 was confirmed with RV16 (Seegene). A positive result from only RV15 with a negative result from RV16 was considered to be a false positive. A positive result from AdvanSure only was confirmed with RV16. In case of a positive result from AdvanSure only with a negative result from RV16, another confirmatory test, singleplex PCR and sequencing, was performed. The main outcomes of this study were sensitivities and specificities of culture, AdvanSure™, and RV 15 assay. Because culture cannot detect human rhinovirus (HRV), MPV, coronavirus, and bocavirus, we excluded HRV, MPV, coronavirus, and bocavirus results from the sensitivity analysis of culture. Specificity was calculated as the percentage of the number of target virus-negative specimens by each assay against the number of target virus-negative specimens by composite reference standard. All statistical analyses were performed using SPSS (version 20.0; SPSS, Chicago, IL, USA).

## 3. Results

### 3.1. Distribution of RVs

Four hundred thirty-seven respiratory samples were tested by culture, RV15, and AdvanSure. Of 437 samples tested, respiratory viral

pathogens were detected from 205 samples (46.9%, 205/437). The distributions of the viruses as single infections and coinfections were shown in Table 1. Forty-five samples (10.3%) had viruses more than 1. Of 45 coinfecting specimens, AdvanSure detected 41 specimens (91.1%) as coinfecting, while RV15 detected 27 specimens (60.0%) as coinfecting. Thirty-seven samples (8.5%) had 2 viruses in their samples, while 8 (1.8%) had 3 viruses. In our study, RSV and rhinoviruses were the viruses most commonly associated with multiple agent infection, followed by adenoviruses and bocaviruses.

A total of 195 (44.6%, 195/437) and 184 (42.1%, 184/437) samples were AdvanSure and RV15 positive, while 114 (26.1%, 114/437) samples were culture positive. One hundred twelve of 114 culture-positive samples were AdvanSure positive, except 2 samples (1 adenovirus, 1 RSV) (Table 2).

### 3.2. Comparison of the assay performance (Table 3)

The sensitivity for each target and assay was calculated with the number of true confirmed cases (Table 3). Overall, the sensitivities of culture, RV15, and AdvanSure were 74.5, 89.8, and 95.1%, respectively. Eight target viruses (influenza A, influenza B, RSV, PIV, coronavirus OC43, coronavirus 229E/NL63, bocavirus, MPV) demonstrated sensitivities of more than 93% in AdvanSure (Table 3). Four target viruses (influenza A, RSV, PIV, coronavirus OC43) had sensitivities of more than 93% in RV15. Influenza B, coronavirus 229E/NL, bocavirus, and MPV showed poor sensitivities in RV15. However, there was no statistically significant difference between the 2 PCR assays for all viruses except MPV. Culture showed fairly good sensitivities in influenza A (90.9%) and PIV (86.7%). However, culture showed lower sensitivities in RSV and adenovirus (63.2 and 69.2%, respectively).

The overall specificity of culture, RV15, and AdvanSure were 100/98.9/99.5%, respectively. As for each target virus, the specificities of RV15 and AdvanSure were as follows: 100/99.8% for influenza A, 100/100% for influenza B, 100/100% for RSV, 99.8/100% for adenovirus, 100/99.8% for PIV, 99.5/100% for rhinovirus, 99.8/100% for coronavirus OC43, 100/100% for coronavirus 229E/NL63, 99.8/99.8% for MPV, and 100/100% for bocavirus.

### 3.3. Discrepant results

Thirty samples were only positive by AdvanSure, of which 25 (83.3%) were positive by RV16. As for 5 discrepant samples, an

**Table 2**

Distribution of positive samples according to culture, RV15, and AdvanSure.

Assay	Culture (%)	RV15 (%)	AdvanSure (%)
Culture (114)	114 (100%)	108 (94.7%)	112 (98.2%)
RV15 (184)	104 (56.5%)	184 (100%)	172 (93.5%)
AdvanSure (195)	110 (56.4%)	174 (89.2%)	195 (100%)

additional confirmatory test, singleplex PCR and sequencing, was performed. Taken together, 29 (96.7%) were confirmed as true positive by composite reference standard. Confirmed true-positive viruses were 11 rhinoviruses, 6 MPVs, 4 bocaviruses, 3 RSVs, 2 influenza B viruses, 2 adenoviruses, and 2 coronavirus NL63. One MPV was false positive.

Sixteen samples were only positive by RV15, of which 11 (68.8%) were confirmed as true positive by RV16. They were 6 rhinoviruses, 2 RSVs, 1 influenza A virus, and 2 adenoviruses. Two rhinoviruses, 1 coronavirus OC43, 1 adenovirus, and 1 MPV were false positive.

## 4. Discussion

In this study, we evaluated the performance of the AdvanSure assay with those of RV15 assay and viral culture. The agreement between AdvanSure and RV15 assays was 92.7% (405/437). For PIV and coronavirus OC43, 2 PCR assays showed the same sensitivities of 93.3% and 100%, respectively. The sensitivity for AdvanSure was slightly higher than that for RV15 (95.1% versus 89.8%); there was no statistically significant difference between 2 PCR assays. For detection of influenza B, coronavirus 229E/NL63, bocavirus, and MPV, there might be an apparent difference between AdvanSure and RV15. Nevertheless, the difference was not statistically significant, which is likely due to the low number of target virus-positive specimens (Table 3).

The good performance of AdvanSure was found in multiple viruses infected specimens. Of 45 coinfecting specimens, AdvanSure detected 41 specimens (91.1%) as coinfecting, while RV15 detected 27 specimens (60.0%) as coinfecting. Although the contradictory conclusions on the clinical impact of viral coinfections have been reported (Vallieres and Renaud, 2013), several studies have pointed out that multiple virus infections are associated with a more severe clinical course (Bruijnesteijn van Coppenraet et al., 2010; Martin et al., 2013). As simultaneous control of all coinfecting viruses becomes more important goal of medical treatment, AdvanSure assay would provide

**Table 3**

Sensitivities of 2 multiplex assays and culture for detection of RVs.

Target virus	Total true-positive specimen, no.	Culture positive, no. (%)	RV15 assay positive, no. (%)	AdvanSure positive, no. (%)	Statistical difference <sup>a</sup>
INF A	22	20 (90.9%)	22 (100%)	21 (95.5%)	NS
INF B	5	3 (60.0%)	0 (0%)	5 (100.0%)	NS
RSV	95	60 (63.2%)	90 (94.7%)	92 (96.8%)	NS
ADV	26	18 (69.2%)	21 (80.8%)	23 (88.5%)	NS
PIV	15	13 (86.7%)	14 (93.3%)	14 (93.3%)	NS
HRV	68	-	52 (76.5%)	60 (88.2%)	NS
OC43	5	-	5 (100%)	5 (100%)	NS
229E/NL	5	-	2 (40.0%)	5 (100%)	NS
HboV	9	-	4 (44.4%)	9 (100%)	NS
MPV	8	-	1 (12.5%)	8 (100%)	<0.05
Total positive	205	114 (74.5% <sup>b</sup> )	184 (89.8% <sup>c</sup> )	195 (95.1% <sup>c</sup> )	<0.05

HEV = human enterovirus.

<sup>a</sup> Between RV15 and AdvanSure™ by McNemar's test.

<sup>b</sup> % means the percentage against the number of total cultivable virus-infected specimens, 153.

<sup>c</sup> % means the percentage against the number of total virus-infected specimens, 205.

**Table 1**

Distribution of RVs in samples by RV15, AdvanSure, and culture.

Target virus	Virus subtype	Total true-positive specimen, no. (%) <sup>a</sup>	Single virus (%) <sup>b</sup>	Two viruses (%) <sup>b</sup>	Three viruses (%) <sup>b</sup>
RSV		95 (45.6)	72 (75.8)	19 (20.0)	4 (4.2)
HRV		68 (32.7)	43 (63.2)	21 (30.9)	4 (5.9)
INF	A	22 (10.6)	19 (86.4)	2 (9.1)	1 (4.5)
	B	5 (2.4)	3 (60.0)	2 (40.0)	0 (0)
ADV		26 (12.5)	9 (34.6)	12 (46.2)	5 (19.2)
MPV		8 (3.8)	4 (50.0)	3 (37.5)	1 (12.5)
Coronavirus	OC43	5 (2.4)	2 (40.0)	2 (40.0)	1 (20.0)
	NL63/	5 (2.4)	1 (20.0)	3 (60.0)	1 (20.0)
	229E				
PIV		15 (7.2)	7 (46.7)	6 (40.0)	2 (13.3)
HboV		9 (4.3)	0 (0)	4 (44.4)	5 (55.6)
Total		205 (100.0)	160 (78.0)	37 (18.0)	8 (3.9)

ADV = adenovirus; HboV = human bocavirus; INF = influenza virus; NA = not applicable.

<sup>a</sup> % means the percentage against the number of total virus-infected specimens, 205.

<sup>b</sup> % means the percentage against total true-positive specimen number of target virus.

clinicians with better infectious status of patients than RV15 (Zhang et al., 2012).

As expected, AdvanSure and RV15 showed sensitivities superior to culture. For influenza A, viral culture showed good sensitivity similar to both of them, but lower sensitivities for RSV and adenovirus, as in other studies (Bruijnesteijn van Coppenraet et al., 2010; Sanghavi et al., 2012). Among 5 influenza B positives, 3 were detected by culture, and 5, by AdvanSure, while none of them were detected by RV15. In the previous study evaluating Seeplex RV12 detection kit (Seegene, Rockville, MD, USA), viral culture, RV12, and real-time PCR detected 8, 6, and 11 of 11 influenza B–positive specimens, respectively (Bruijnesteijn van Coppenraet et al., 2010). In that study, RV12 based on a dual priming oligonucleotide system (DPO) demonstrated less clinical sensitivity for detection of influenza B than viral culture, while real-time PCR showed the greatest analytical and clinical sensitivity. Similarly, our study demonstrated that RV15 with DPO technology was less sensitive for influenza B than viral culture, while AdvanSure was the most sensitive for influenza B virus.

Viral culture is not a sensitive method for detection of rhinovirus and other viruses such as MPV and coronavirus (Lee et al., 2007). Viral culture has limited ability to detect many serotypes of rhinovirus (Greenberg, 2011). In our study, only AdvanSure and RV15 detected 48 rhinoviruses (14.9%) from 323 culture-negative samples. Although MPV causes acute respiratory tract infections similar to that caused by RSV in children, the elderly, and immune-compromised patients, it is difficult to detect MPV by cell culture due to its fastidious growth requirements and mild cytopathicity (Feuillet et al., 2012; Lee et al., 2007). In this study, AdvanSure and RV15 detected 6 MPVs (1.9%) from 323 culture-negative samples.

In spite of the relatively low sensitivity of culture, a positive result from culture only was considered to be a true positive because it has been regarded as traditional gold standard. As shown in a case of 2009 H1N1 influenza positive by culture and negative by the ProFlu+ assay (Gen-Probe Prodesse, Inc., Waukesha, WI, USA), mutations of target site could raise the false negativity by PCR method (Zheng et al., 2010). There was 1 case of adenovirus positive by culture and negative by both PCR assays in this study, which may be caused by mismatch between primer and/or probe and its binding region.

Among 29 specimens, which were positive by only AdvanSure and confirmed as true positive by composite reference standard, 21 specimens (72%) had high cycle threshold values ( $\geq 20$ , cut-off = 27) by AdvanSure, indicating low amounts of target virus in the specimens. As expected, the real-time PCR system of AdvanSure was more sensitive than RV15 based on capillary electrophoresis.

Five false-positive cases in RV15 in comparison with 1 case in AdvanSure might be caused by carry over or contamination during the process of 2-step RT-PCR and/or gel electrophoresis after PCR, while the AdvanSure system, being a 1-step real-time PCR with automated nucleic acid extraction system, seems to be able to reduce such chance (Caliendo, 2011; Kehl and Kumar, 2009; Olofsson et al., 2011). Besides, AdvanSure as real-time PCR technology has the advantages such as decreasing the turnaround time, evaluating PCR products in real time during each PCR cycle, and aiding as a semi-quantitative method in distinguishing a true etiological agent from an innocent bystander (Kehl and Kumar, 2009; Olofsson et al., 2011).

With the introduction of simultaneous molecular diagnostics, current estimates of the prevalence of viral coinfections were documented to be 5%–50% (Martin et al., 2013; Vallieres and Renaud, 2013). Similar to the previous study evaluating multiplex real-time PCR, 2 and 3 viruses-infected specimens in this study were 8.5% and 1.8% of total respiratory specimens, respectively (Gharabaghi et al., 2011; Wang et al., 2009).

RSV and rhinovirus were the most common viruses detected with other viruses. However, coinfection rates were highest in bocavirus and coronavirus. Bocavirus was always detected with other viruses (9/9, 100%). Seven coronavirus (70%) was co-detected with other

viruses among 10 coronavirus-positive cases. In the previous study analyzing multiple viral respiratory infections among 225 childcare attendees, viruses frequently coinfecting with bocavirus were adenovirus, coronavirus, and HRV (Martin et al., 2013). Similarly, in this study, bocaviruses presented most frequently with adenoviruses (77.8%, 7/9). Our result that all of bocavirus-infected samples were coinfecting supports the claim that the development of human bocavirus pathogenicity may require its reciprocal interaction with other coinfecting viruses (Zhang et al., 2012).

Two different extraction methods with different input volumes (NucliSENS® easyMAG® versus Smart LabAssist-32) in this study were used according to manufacturer's instruction. Different extraction method could partly affect the difference observed. However, further study is needed to evaluate accurately the impact of different extraction systems on the performance of AdvanSure or RV15.

The main limitation of this study is the small number of specimens with specific virus such as influenza B virus, coronavirus OC43, coronavirus 229E/NL63, bocavirus, and MPV, requiring future study with more specimens as for those viruses. Nevertheless, it should be noted that AdvanSure showed improved overall sensitivity over RV15. Also, we compared not only AdvanSure and multiplex conventional PCR but also culture, complementing the shortcoming in the previous study evaluating AdvanSure (Rheem et al., 2012).

In conclusion, AdvanSure assay demonstrated exquisite performance, compared with RV15 as well as culture method. In addition, the AdvanSure showed improved sensitivity over RV15 for detection of coinfection, while maintaining excellent specificity. AdvanSure will be a promising alternative for molecular multiplexing testing.

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