

Comparison of the Seeplex Reverse Transcription PCR Assay with the R-mix Viral Culture and Immunofluorescence Techniques for Detection of Eight Respiratory Viruses

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Abstract. This study evaluated the clinical usefulness of a newly introduced multiplex reverse transcription PCR assay (Seeplex RV; Seegene, Inc., Seoul, Korea) in patients with respiratory symptoms. Fifty clinical respiratory specimens (45 from children, 5 from adults) were tested for 8 viruses (influenza virus type A and B, parainfluenza virus type 1, 2, 3, respiratory syncytial virus type A and B, and adenovirus) by Seeplex RV (S-RV) and R-mix viral culture with immunofluorescence (VC-IF). Forty (80%) of the 50 samples showed concordant results between S-RV and VC-IF; 24 of these showed the same positive and 16 showed the same negative results. Among the 10 discrepant samples, 9 were S-RV-positive and VC-IF-negative. Six were obtained in patients with lower respiratory tract infection. Only 1 sample was VC-IF-positive and S-RV-negative. This patient had pneumonia. In 3 cases, more than 1 virus was identified by S-RV. The total running time of S-RV was 6 hr, which shortens the detection time for the viral presence by 2 workdays compared to VC-IF. In conclusion, S-RV is reliable, rapid, relatively easy to perform, and able to detect more than 1 virus simultaneously. Therefore, implementation of the S-RV assay in clinical laboratories will aid rapid diagnosis and treatment of major viral infections of the respiratory tract.

Keywords: respiratory viruses, reverse transcription PCR, virus culture, Seeplex RV assay

Introduction

An acute respiratory tract infection (ARTI) is one of the common causes for both outpatient clinic visits and hospital admissions of children [1,2]. Although the bacterial infection rate has declined with the use of antimicrobial agents, viral infection rate has been increasing. Influenza virus type A and B, parainfluenza virus type 1, 2, 3 (PIV I, PIV II, PIV III), respiratory syncytial virus (RSV) type A and B, and adenovirus are major causes of lower respiratory tract infections in infants and young children under 5 yr old. Human metapneumovirus,

also identified in children with respiratory infection, rhinovirus, and coronavirus are known as causative agents of the common cold [3-8]. These respiratory viruses are responsible for pneumonia, bronchiolitis, and croup. Early detection methods for these viruses are important in planning for the appropriate treatment in the hospital. R-mix viral culture with immunofluorescence (VC-IF) has been widely performed for the diagnosis of lower respiratory viral infections, but VC-IF has a long turn around time (TAT); more than 3 workdays are generally required. Several types of molecular biological methods, including reverse transcription PCR (RT-PCR), PCR-hybridization, and real time PCR, have been introduced as more rapid detection methods for respiratory infections [9-13]. However, the limited number of detectable viruses and the

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labor intensity of multiple PCR reactions have hampered routine clinical use of the molecular methods. Recently, the Seeplex RV assay (S-RV), a multiplex RT-PCR method, has been introduced and been shown to run 12 primer sets simultaneously for detection of 12 respiratory viruses: influenza virus type A and B, parainfluenza virus type 1, 2, 3, RSV type A and B, adenovirus, human metapneumovirus, coronavirus OC43 and 229E, and rhinovirus. This study evaluates the performance of the S-RV assay in patients with respiratory symptoms.

Materials and Methods

Subjects. Fifty clinical specimens were obtained from patients with suspected ARTI who were seen at the Korea University Medical Center (KUMC), Anam and Guro Hospitals in Seoul, and Ansan Hospital in Gyeonggi, Korea. The subjects ranged in age from three mo to 72 yr, with children 6 yr of age or younger comprising 84% of the study population. A lower respiratory tract infection was defined as a patient with one or more of the following clinical features: bronchiolitis, pneumonia, wheezing, or laryngotracheobronchitis, or a patient who required ventilation without an underlying physiological disease. The medical record of each patient was reviewed using an electronic medical record system. Patient age, gender, type of clinical sample, bacterial culture results, and clinical diagnosis were collected.

Specimens. The specimens were predominantly nasopharyngeal aspirates (NPAs, 92%) collected either in the outpatient clinic or after hospitalization. NPAs were collected from the nasopharynx via a catheter into a collecting tube that contained virus transport media (VTM). The NPAs were stored in a refrigerator at 4°C for 1-2 days until inoculation for viral culture. The specimens were vigorously vortexed for 10 sec, followed by centrifugation at 4,200 × g for 5 min to pellet the epithelial cells and bacteria. The supernatant was used for virus culture. For comparisons with S-RV, the specimens were stored at -70°C until tests were performed.

R-mix viral culture. A cryopreserved R-mix cell monolayer on a glass coverslip in shell vials on dry ice was obtained. Two shell vials were thawed for 4 min, and the medium was removed and new medium was added. Then, 200 µl of the patient specimen supernatant was inoculated and the vials were centrifuged at 700 × g for 60 min at room temperature. After overnight incubation at 36°C in a CO₂ incubator, the coverslip containing the cells was fixed with acetone and stained with a respiratory virus fluorescent antibody pool (Respiratory Panel 1 Viral Screening & Identification Kit, Chemicon, Temecula, CA, USA). Positive screens were further identified with a second R-Mix shell vial on day 2. Coverslips were scraped and spotted to 5-well slides. Dried and fixed slides were then stained with virus-specific

monoclonal antibodies. If the initial R-Mix screening was negative, a second shell vial was examined on day 7 [14].

DNA and RNA extraction. Viral DNA and RNA were extracted from 300 µl of each respiratory specimen. Briefly, frozen aliquots of clinical specimens were thawed and viral DNA/RNA were extracted using a Gene-spin™ kit (iNtRON Biotechnology, Seoul, Korea) according to the manufacturer's protocol. Extracted RNA, random hexamers, and murine leukemia virus reverse transcriptase (RevertAid M-MuLV RT, Fermentas, Burlington, Canada) were used to synthesize the cDNA.

First-strand cDNA synthesis. Total RNAs extracted from clinical samples were used for the synthesis of first-strand cDNAs. Reverse transcription was performed for 1.5 hr at 37°C in a final reaction volume of 20 µl with 1 µl of random hexamers (0.2 µg/µl), 7.5 µl of total RNA (100 ng), 1 µl of murine leukemia virus reverse transcriptase (200 u/µl), 1 µl of RNase inhibitor (20 u/µl), 2 µl of dNTP (10 mM) 5X RT buffer (a final concentration of 4 mM MgCl₂), and 3.5 µl of water. The product was stored at -20°C until use.

Polymerase chain reaction. PCR amplification was performed using 10 µl of Seeplex RV master mix (Seegene, Inc., Seoul, Korea), 4 µl of 5X multiplex primer sets, 3 µl of 8-MOP solution, and 3 µl of newly synthesized first-strand cDNA (1 µg/3 µl). The Seeplex RV contained A and B sets of primers designed from highly conserved regions of genetic sequences for the 12 respiratory viruses. These primers were specifically designed by use of DSO technology of Seegene, Inc. and the specific primer sequences of the A set were released [15]. An initial pre-PCR step of 94°C for 15 min was performed in a thermocycler 9600 (Perkin-Elmer, Waltham, MA, USA), followed by a total of 35 PCR cycles under the following conditions: 94°C for 30 sec, 60°C for 1.5 min, and 72°C for 1.5 min. The final cycle was followed by an extension step at 72°C for 10 min to complete any partial polymerizations. The amplified PCR products were separated on 2% agarose gel and stained with ethidium bromide. The type of respiratory virus was identified by comparison with the reference band size provided by the manufacturer (Fig. 1).

As an internal control, plasmids containing amplicons (719 bp) were used during viral RNA/DNA extraction. This internal control could detect any problems that occurred during the viral RNA/DNA preparation or PCR reaction. As a negative control, sterile deionized water was used in place of cDNA as the PCR template. To check the integrities of primers used in the RT-PCR assay, positive RNA controls from the manufacturer and from clinical specimens that were positive by viral culture methods were assayed in the presence of all primer pairs. No cross-reactivity was detected.

Results

Fifty clinical specimens were tested. Table 1 summarizes the patient demographics. Twenty-six of the 50 samples were from male patients and 24

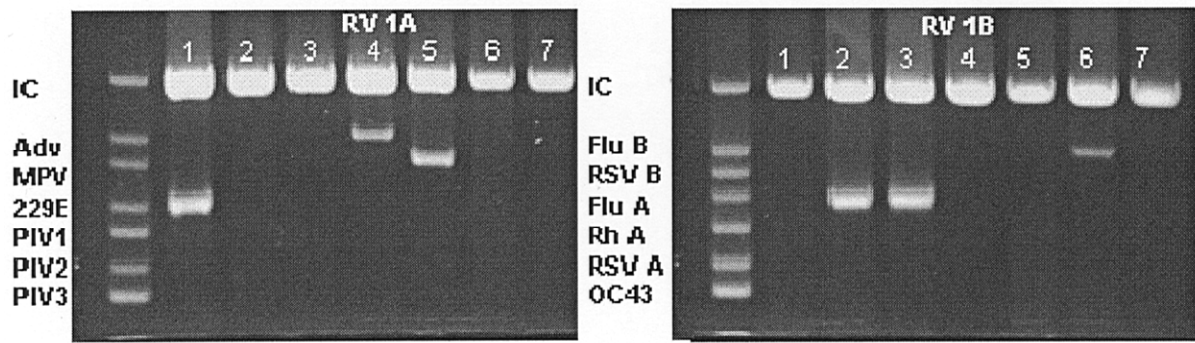


Fig. 1. Seeplex RV analysis for respiratory viruses. Samples were electrophoresed and were visualized by use of a UV illuminator. A 2% agarose gel was used (100V, 30 min electrophoresis). In the RV 1A set, internal control (IC), adenovirus (Adv), metapneumovirus (MPV), coronavirus 229E (229E), parainfluenza virus 1 (PIV1), parainfluenza virus 2 (PIV2), and parainfluenza virus 3 (PIV3) were used as controls. In the RV 1B set, internal control (IC), influenza A (Flu A), influenza B (Flu B), RSV B, rhinovirus A (Rh A), RSV A, and coronavirus OC43 (OC43) were used. For each set: lane 1 = coronavirus 229E; lanes 2 and 3 = RSV B; lane 4 = adenovirus; lane 5 = MPV; lane 6 = Flu A; lane 7 = negative control.

Table 1. Patient characteristics.

Characteristics	Number of patients
Gender	
Male	26
Female	24
Age	
< 6 yr	42
6 - 18 yr	3
> 18 yr	5
Type of Specimen	
Nasopharyngeal aspirate	46
Nasopharyngeal swab	1
Throat swab	1
Tracheal aspirate	2

Table 2. Comparison of viral culture with IF and Seeplex RV (S-RV) assays for detection of 8 major respiratory viruses.^a

Seeplex RV (S=RV)	Viral culture with IF		
	Positive	Negative	Total
Positive	24 ^b	9 ^c	33
Negative	1 ^d	16	17
Total	25	25	50

^a The 8 major respiratory viruses were influenza virus type A and B, parainfluenza virus type 1, 2, 3, respiratory syncytial virus (RSV) type A and B, and adenovirus.

^b More than 1 virus detected with S-RV in 2 specimens: influenza B + parainfluenza 1; RSV type B + adenovirus.

^c More than 1 virus detected with S-RV in 1 specimen: influenza B + adenovirus.

^d Parainfluenza virus detected only by viral culture and IF.

Table 3. Comparison of the viral culture and Seeplex RV assays with clinical data for the 10 patients with discordant results.

Patient	Gender/Age	Specimen	Viral culture	Seeplex RV assay	Clinical diagnosis
1	M/28 mo	NPA	Neg	RSV B	Pneumonia
2	F/5 yr	NPA	Neg	PIV3	Acute lymphadenitis
3	M/9 mo	NPA	Neg	PIV3	Acute bronchiolitis
4	F/25 mo	NPA	Neg	PIV3	Pneumonia
5	M/17 mo	NPA	Neg	Adenovirus, influenza B	Acute pharyngitis
6	M/3 mo	NPA	Neg	PIV3	Acute bronchiolitis
7	F/4 yr	NPA	Neg	Influenza B	Acute pharyngitis
8	F/3 yr	NPA	Neg	PIV3	Pneumonia
9	F/33 mo	NPA	Neg	Influenza A	Pneumonia
10	M/3 mo	NPA	PIV	Neg	Pneumonia

NPA, nasopharyngeal aspirate; Neg, negative; PIV3, parainfluenza type3.

from female patients. Forty-two specimens were from patients under the age of 6 yr; 3 samples were from patients 6 to 18 yr old; 6 samples were from patients above 18 years old. Most specimens (46 cases) were from nasopharyngeal aspirates, and the rest were from a nasopharyngeal swab (1), a throat swab (1), or tracheal aspirates (2).

Seeplex RV vs viral culture assays for 8 major respiratory viruses. The same 50 clinical specimens were tested for respiratory viruses by the S-RV and VC-IF assays. A concomitant comparison between VC-IF and S-RV was performed only for 8 major respiratory viruses (influenza virus type A and B, parainfluenza virus type 1, 2, 3, respiratory syncytial virus type A and B, and adenovirus). VC-IF assay results revealed 25 positive samples. Among these positive samples, only 1 sample was S-RV-negative, and the virus identified by VC-IF was parainfluenza (Table 2). Twenty-five samples were VC-IF-negative as measured for 7 days. The clinical diagnosis of the VC-IF-positive, S-RV-negative case was pneumonia. S-RV also detected double infection in 2 VC-IF-positive clinical specimens: influenza B and parainfluenza 1 for influenza B VC-IF-positive, and RSV type B and adenovirus for adenovirus VC-IF-positive.

In 25 VC-IF-negative samples, S-RV detected 10 respiratory viruses from nine clinical specimens: five parainfluenza virus 3, two influenza B, one influenza A, one RSV, and one adenovirus. From one sample two different viruses (adenovirus and influenza B) were identified (Table 2). Six of the nine S-RV-positive and VC-IF-negative cases were diagnosed as acute bronchiolitis or pneumonia (Table 3).

Seeplex RV assays for 4 additional respiratory viruses. The S-RV assay includes additional viruses (rhinovirus, coronavirus 229E, coronavirus OC43, and metapneumovirus), so we identified 8 more viruses (3 rhinovirus, 3 coronavirus, and 2 metapneumovirus) from 8 specimens. Four of these 8 cases had a double infection: one coronavirus 229E + parainfluenza 3, one coronavirus OC43 + adenovirus, one rhinovirus + RSV A, and one rhinovirus + adenovirus. Two metapneumovirus, one rhinovirus, and one coronavirus were suspected

as single infections. The clinical diagnoses of the metapneumovirus- and rhinovirus-positive cases were pneumonia. The diagnosis of the coronavirus-positive case was maxillary sinusitis.

Discussion

Influenza virus type A and B, parainfluenza virus type 1, 2, 3 (PIV I, PIV II, PIV III), respiratory syncytial virus (RSV) type A and B, and adenovirus are frequently isolated in acute respiratory infection patients in Korea [6]. Laboratory diagnoses of these infections have been made by virus culture, direct fluorescent-antibody (DFA), immunochromatographic (ICG) assay, multiplex RT-PCR, or real time PCR [9,10,13,16-18].

Rapid detection of respiratory viruses is important with the advent of antiviral drug therapy. The availability of a rapid viral diagnostic assay will enable physicians to make more accurate treatment decisions, reduce unnecessary antimicrobial agent use, and shorten hospital stays for patients. A conventional virus culture requires up to 7 days observation time for cytopathic effect. Immunoperoxidase staining of the cultures with monoclonal antibodies speeds viral identification, with results usually available within 48 hr [14]. Other rapid tests, such as the DFA test and the ICG assay, have been shown to provide very rapid results, but have low sensitivity; 87.6% for the DFA assay and 43.8–64.9% for the ICG assay [16-18].

PCR methods have been introduced with debatable sensitivity. Liolios et al [9] reported that a reverse transcription-PCR-enzyme hybridization assay method was 100%-sensitive and 93%-specific. The recently developed reverse transcriptase multiplex PCR system, Seeplex RV, needs only 6 hr of assay time: 1 hr for RNA/DNA extraction, 2 hr for reverse transcription, and 3 hr for PCR and agarosegel detection. In comparison to a conventional virus culture, the results can be obtained within 1 workday and 2 workdays can be saved. Moreover, the ability to detect coronavirus and rhinovirus, major causative organisms of upper respiratory tract infections, is helpful for differential diagnosis of lower respiratory tract infections.

In the present study, the detection rate of respiratory viruses by S-RV was better than that by

the R-mix viral culture method. These results are consistent with those of other studies that used RT-PCR assays for respiratory viral infections [9,10]. In our study, S-RV detected 96% of viral culture-positive clinical samples and 9 additional positive samples, which were missed by the R-mix virus culture method. S-RV can be especially useful when the viral load is low or when the virus is not replication-competent.

Human metapneumovirus has been identified as a causative agent of lower respiratory tract infections in children [19,20]. Detection of metapneumovirus is difficult and the virus culture is not performed routinely in clinical laboratories. Chung et al [21] reported that 12.1% of children with acute respiratory tract disease were infected with human metapneumovirus in Korea [21]. In the present study, for 2 metapneumovirus-positive samples, the clinical diagnoses were pneumonia. In both of these cases, metapneumovirus was a possible causative viral agent. The medical histories for a rhinovirus-positive and a coronavirus-positive patient were reviewed, but the patients were not found to have respiratory disease. For these cases, upper respiratory tract colonization might influence the results obtained with S-RV. Seven cases of double infection were detected only by use of S-RV, suggesting a more widespread clinical applicability of this system.

An intrinsic limitation of the PCR method is that it is unable to discriminate between a dead or a live virus and this limitation needs to be considered in the interpretation of the method's results. In our study, in 3 of the 9 cases that were positive only by the S-RV assay, the clinical diagnosis was an upper respiratory infection.

For 1 VC-IF-positive, S-RV-negative case, re-testing of S-RV was impossible due to lack of the specimen. Specimens for S-RV were stored for up to 4 mo, whereas relatively fresh specimens were used for VC-IF. Specimen storage conditions might cause false negative results in S-RV.

In conclusion, the S-RV assay is rapid, highly sensitive, and able to detect a double infection. Therefore, the clinical use of S-RV will be helpful in diagnosis and early treatment of viral respiratory infections in children, especially when the clinical symptoms are taken into consideration.

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