

Identification of Adenovirus, Influenza Virus, Parainfluenza Virus, and Respiratory Syncytial Virus by Two Kinds of Multiplex Polymerase Chain Reaction (PCR) and a Shell Vial Culture in Pediatric Patients with Viral Pneumonia

Jong-Han Lee,¹ Jin-Kyong Chun,² Dong Soo Kim,² Yongjung Park,¹
Jong Rak Choi,¹ and Hyon-Suk Kim¹

Departments of ¹Laboratory Medicine and ²Pediatrics, Yonsei University College of Medicine, Seoul, Korea.

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Corresponding author: Dr. Hyon-Suk Kim,

Department of Laboratory Medicine,
Yonsei University College of Medicine,
250 Seongsan-ro, Seodaemun-gu,
Seoul 120-752, Korea.

Tel: 82-2-2228-2443, Fax: 82-2-364-1583

E-mail: kimhs54@yuhs.ac

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Purpose: Early identification of causative agents in lower respiratory infection of pediatric patients can reduce morbidity and prevent an overuse of antimicrobials. Two kinds of multiplex polymerase chain reaction (PCR) and a commercial shell vial viral culture were performed to identify causative agents in pediatric patients. **Materials and Methods:** Nasopharyngeal aspirates of 220 children diagnosed with viral pneumonia were obtained. Two kinds of multiplex PCR (Seeplex™ RV detection kit, and Labopass™ RV detection kit), and a shell vial culture by R-Mix were performed. **Results:** Positive samples from 220 total samples by two multiplex PCRs were 52.7% and 46.4%, respectively. We also cultured 103 samples that showed positive results of the adenovirus, influenza virus, parainfluenza virus, and respiratory syncytial virus (RSV) by two multiplex PCR. The RSV was most frequently detected in 53.0% (Seeplex) and 51.7% (Labopass) of patients. The detection rate of adenovirus (AdV) was 10.3% and 12.1%, influenza virus (IFV) A and B was 12.5% and 3.4%, and parainfluenza virus (PIFV) 1, 2, and 3 were 2.9% and 2.6%. Shell vial cultures showed concordant results with each multiplex PCR by 96.1% and 77.7%, respectively. Sequencing results were 90% consistent with multiplex PCR. **Conclusion:** Multiplex PCR showed more positivity than the shell vial culture and it can be an effective primary test. Other complementary efforts such as viral cultures and sequencing analysis could be considered, according to clinical and laboratory conditions.

Key Words: Multiplex PCR, respiratory virus, virus culture, viral pneumonia

INTRODUCTION

Acute respiratory viral infections are a major cause of morbidity in pediatric patients.¹ Although they are most often self-limited and confined to the upper respiratory tract, they lead to a substantial number of upper or lower respiratory tract complications. Therefore, accurate and rapid diagnosis of respiratory virus infection is essential for the initiation of early treatment and the prevention of viral spread.

Several studies evaluating polymerase chain reaction (PCR)-based methods for the detection and typing of respiratory viruses were reported.^{2,3} The adenovirus

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(AdV), influenza virus (IFV), parainfluenza virus (PIFV), and respiratory syncytial virus (RSV) have been identified as significant pathogens in community-acquired and nosocomial respiratory infections.^{4,5} Recently, some respiratory viral infections caused by IFV, enterovirus (EnV), or AdV may now benefit from specific antiviral treatment.⁶⁻⁸

The aim of this study is to evaluate and suggest clinical usefulness of two kinds of multiplex PCR for identifying causative viruses in pediatric viral pneumonia, which is the most severe lower respiratory infection in children.

MATERIALS AND METHODS

Sample collection

Nasopharyngeal aspirates from 220 pediatric patients during a period of 6 months were obtained by a mucus extractor (Sewoon Inno-Vision Medical, Seoul, Korea) and transferred to each vial of the universal transport medium (Diagnostic Hybrids, Inc., Athens, OH, USA). All 220 pediatric patients were admitted with symptoms of severe lower respiratory infection. For definitions of cases, moderate to severe lower respiratory infections were determined according to the World Health Organization (WHO) recommended surveillance standards, 2nd edition (1999). Lower respiratory viral infections were diagnosed by an expert pediatrician through physical examinations, chest X-rays, blood tests, erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP), excluding bacterial origin.

The 220 aspiration samples were immediately made aliquot and stored at -75°C until nucleic acid extraction, viral culture, or sequencing analysis proceeded. All patients' samples were collected according to the protocols of the Institutional Review Board of Yonsei University Health System.

Nucleic acid extraction

QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) with automated QIAcube[®] (QIAGEN) was used to extract nucleic acid. The 220 nucleic acid extracts were kept in a deep freezer at -75°C until analysis.

Multiplex PCR for detection of respiratory virus

Seeplex[™] RV detection kit

(<http://www.seegene.co.kr/en/index.php>)

Nucleic acids extracted from nasopharyngeal aspirates were used for the synthesis of first-strand cDNAs by Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). The Seeplex[™] RV detection kit (Cosmo Genetech, Seoul, Korea) contained A and B sets of primers designed by highly conserved regions of genetic sequences for the 12 respiratory viruses. The Seeplex[™] kit

is designed to identify AdV, human metapneumovirus (hMPV), Human coronavirus (HCoV) 229E/NL63, parainfluenza virus (PIFV) 1, PIFV 2, and PIFV 3, and the Seeplex[™] RV detection kit B is designed to detect IFV A, IFV B, RSV A, RSV B, rhinovirus (RhV), and HCoV OC43/HKU1. Each PCR was conducted in a final reaction volume of 20 μL containing 3 μL of cDNA, 3 μL of 8-methoxypsoralen (MOP) solution, 4 μL of $5 \times \text{RV}$ Primer, and 10 μL of $2 \times$ master mix. The PCR protocol was 94°C for 30 sec, followed by 35 cycles of 60°C for 1.5 min, and 72°C for 1.5 min, followed by a 10 min final extension at 72°C . The amplified products were separated on a 2% agarose gel stained with ethidium bromide. Each run included a molecular size marker and internal control. Also, American Type Culture Collection (ATCC) standard viruses were used for positive control and 10 μL distilled water as negative control.

Labopass[™] RV detection kit

(http://www.cosmo4.com/index_eng.html)

Nucleic acids from nasopharyngeal aspirates were also used for the Labopass[™] kit. This kit was also designed to detect 12 types of viruses. The final reaction volume was 50 μL composed of 40 μL premixture and 10 μL nucleic acid. Two kinds of PCR protocols were used. AdV and human bocavirus (HboV) operated at 95°C for 3 min, then 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by a 5 min final extension at 72°C and preserved at 4°C . HCoV, EnV, PIFV, RhV, and RSV all operated at 42°C for 60 min, 95°C for 3 min, then 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by a 5 min final extension at 72°C and preserved at 4°C . The PCR products were analyzed by identifying bands with a 2% agarose gel stained with ethidium bromide.

Virus culture by R-Mix ReadyCells with antigen staining

Samples showing any positive results for AdV, IFV, PIFV, and RSV from the two multiplex PCR kits were performed with shell vial cultures by R-Mix ReadyCells (Diagnostic HYBRIDS, Inc., Athens, OH, USA) according to manufacturer's procedures. Firstly, the cryopreserved R-Mix cell reagents were heated in a 37°C heat block for 4 min, and rinsed with a rinse buffer and then remained for 4 min at room temperature. In the meantime, the re-feed medium and 1.0 mL R-Mix ReadyCells were mixed. Then, 200 μL specimens were inoculated into R-Mix ReadyCells and centrifuged at $700 \times g$ for 60 min at room temperature. After overnight incubation at 35°C in the incubator, cell monolayers of the shell vial were washed and fixed with acetone, and stained with respiratory virus fluorescent antibodies by D3 DFA (Diagnostic Hybrids, Inc., Athens, OH, USA). If virus-specific fluorescence was noted by

screening, virus identification was performed using individual monoclonal antibodies staining (IFV A and B, AdV, PIFV 1,2,3 and RSV). When the initial screening was negative, the vial was re-examined on day 3 and day 5.

Sequencing analysis

Ten samples, which were culture negative but multiplex PCR positive, were proceeded with sequencing analysis to identify these equivocal results. All sequencing analyses were proceeded after repeating PCR then proper primers were prepared for specific viruses identification. An automated sequencing analyzer (Applied Biosystems, Foster City, CA, USA) accompanied with its recommended reagent was used according to the manufacturer’s protocol. Finally, results of all sequences were analyzed by matching those of GenBank data using Basic Local Alignment Search Tool (BLAST) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

RESULTS

Nucleic acid amplification results by 2 kinds of multiplex PCR

The basic characteristics and major results of 220 pediatric patients are summarized in Table 1. The positive rate of the Seeplex™ RV detection kit was 52.7% (116 positive samples/total 220 samples). Seeplex™ identified 136 viruses which were the sum of AdV (n = 14, 10.3%), HCoV 229E/NL63 (n = 5, 3.7%), HCoV OC43/HKU1 (n = 4, 2.9%), IFV A (n = 8, 5.9%), IFV B (n = 9, 6.6%), HMPV (n = 2, 1.5%), PIFV 1 (n = 0, 0%), PIFV 2 (n = 0, 0%), PIFV 3 (n = 4, 2.9%), RhV A/B (n = 18, 13.2%), RSV A (n = 39, 28.7%), and RSV B (n = 33, 24.3%).

The Labopass™ RV detection kit showed 46.4% positivity (102 positive samples/total 220 samples). The Labopass™ RV detection kit identified 116 viruses which were the sum of AdV (n = 14, 12.1%), HBoV (n = 4, 3.4%), HCoV NL63 (n = 1, 0.9%), HCoV OC43 (n = 2, 1.7%), EV (n = 0, 0%), IFV A (n = 2, 1.7%), IFV B (n = 2, 1.7%), HMPV (n = 22, 19.0%), PIFV 1 (n = 2, 1.7%), PIFV 2 (n = 0, 0%), PIFV 3 (n = 1, 0.9%), RhV A/B (n = 6, 5.2%), and RSV (n = 60, 51.7%). The coinfection (more than 2 virus species identified) rate of the Seeplex™ RV kit was 6.8%

(15/220) and of the Labopass™ RV kit was 5.9% (13/220).

Shell vial cultures with direct immunostaining for identifying AdV, IFV, PIFV, and RSV

If one or more of the viruses were identified from a multiplex PCR, R-Mix ReadyCell cultures were proceeded with direct immunofluorescence staining. There were 103 samples that showed a positive multiplex PCR of AdV, IFV, PIFV, and RSV. The shell vial culture assay showed 93 positive samples of the total 103 samples (positive rate 90.3%). There were two samples which showed 2 kinds of viruses. A total of 95 positive numbers of the R-Mix culture was noted from 15 AdV, 6 IFV A, 9 IFV B, 2 PIFV, and 63 RSV. Positive viral culture rate from the Seeplex™ RV detection kit was 96.1% (99 samples of the total 103 samples) and that of the Labopass™ RV detection kit was 77.7% (80 samples of the total 103 samples).

Fifty samples of the 103 total samples showed the same results by the two kinds of multiplex PCR and viral culture. However, there were 53 samples which showed discrepant results by each test. The 53 results were divided into 5 groups according to discrepant characters (Table 2). The R-Mix culture which we used in this study targeted only four viruses (AdV, IFV, PIFV, and RSV), and so other viruses could not be isolated. The positive results of multiplex PCR and R-Mix culture for detecting AdV, IFV, PIFV, and RSV are compared in Fig. 1.

Sequencing results of multiplex PCR positive but viral culture negative samples

Ten samples of culture negative but any multiplex PCR positive were further analyzed by sequencing. The results are showed in Table 3. The corresponding viruses were identified with a homology of 91-100%. Of the ten sequencing results, nine were consistent with multiplex PCR results. There was one completely discrepant result between multiplex PCR and sequencing analysis (Case No. 10: IFV A versus RhV, Table 3).

DISCUSSION

Rapid and accurate identification of causative agents in viral

Table 1. Basic Characteristics of 220 Pediatric Patients

Patients’ characteristics	Value
Age [mean ± SD; (range)]	2.5 yrs ± 1.4; (0 - 6)
Sex ratio (M : F)	1.3 (124 : 96)
Final diagnosis	Viral pneumonia
No. of positive mutiplex PCR patients (% , Seeplex vs. Labopass)	116 vs. 102 (52.7, 46.4)
No. of positive shell vial culture patients (% , Seeplex vs. Labopass)*	99 vs. 80 (96.1, 77.7)

*Total 103 samples were proceeded shell vial cultures.

Table 2. Comparison Results of Multiplex PCR Positive Cases

	Multiplex PCR (Seeplex™)	Multiplex PCR (Labopass™)	R-Mix culture	Total no. (103 cases)	
Consistent (50)	AdV	AdV	AdV	10	
	IFV B	IFV B	IFV B	2	
	RSV A	RSV	RSV	38	
Inconsistent (36)	AdV	AdV + IFV A + RhV	AdV + IFV A	1	
	AdV	hMPV + PIFV 3	PIFV 3	1	
	AdV	Negative	AdV	1	
	IFV A	Negative	IFV A	4	
	IFV A	Negative	IFV B	1	
	IFV A	Negative	No growth	1	
	IFV A	Negative	No growth	1	
	IFV A	Negative	No growth	1	
	IFV B	Negative	IFV B	5	
	IFV B + RSV A	Negative	RSV + IFV B	1	
	IFV B + RSV A	RSV	RSV	1	
	hMPV + RSV B	hMPV	No growth	1	
	Negative	IFV A	IFV A	1	
	Negative	MPV + PIFV 1	No growth	1	
	Negative	RSV	RSV A + RSV B	2	
	PIFV 3	AdV	AdV	1	
	PIFV 3 + RhV A/B	Negative	No growth	1	
	PIFV 3 + RhV A/B	PIFV 1	PIFV 3	1	
	PIFV 3 + RhV A/B + RSV B	AdV	AdV	1	
	RSV A	Negative	No growth	2	
	RSV A	Negative	RSV	4	
	RSV A	RSV	No growth	1	
	RSV A + RSV B	Negative	RSV	1	
	RSV B	RSV	No growth	1	
	Indeterminate (17)	AdV + HCoV OC43	AdV + HCoV OC43	AdV	1
		HCoV 229E/NL63 + RSV B	RSV	RSV	3
		HCoV OC43 + RSV A	RSV	RSV	1
		HCoV OC43 + RSV B	RSV	RSV	1
HCoV 229E/NL63 + RSV A		RSV	RSV	1	
RhV A/B + RSV A		RSV	RSV	2	
RSV A		hMPV + RSV	RSV	3	
RSV A + RSV B		RSV + HBoV	RSV	1	
RSV B		hMPV + RSV	RSV	2	
RSV B	RSV + hMPV	RSV	2		

PCR, polymerase chain reaction; AdV, adenovirus; HCoV, human coronavirus; IFV, influenza virus; PIFV, parainfluenza virus; hMPV, human metapneumovirus; RhV, rhinovirus; RSV, respiratory syncytial virus; HBoV, human bocavirus.

*R-Mix culture could only detect AdV, IFV, PIFV and RSV in this study.

pneumonia of pediatric patients is essential to prevent disease propagation. It is also helpful for early initiation of proper management. An early start of proper anti-viral management can reduce unnecessary overuse of antibacterial agents. In addition, some respiratory viruses, including IFV, EnV, and AdV, may now benefit from specific anti-

ral treatment.⁶⁻⁸ So, we designed and performed this study to evaluate two kinds of multiplex PCR kits and a commercial shell vial culture method for their clinical efficacy with prospectively collected samples of pediatric patients.

Until now, virus isolation by a cell culture and a direct immunofluorescent antigen staining assay has been the

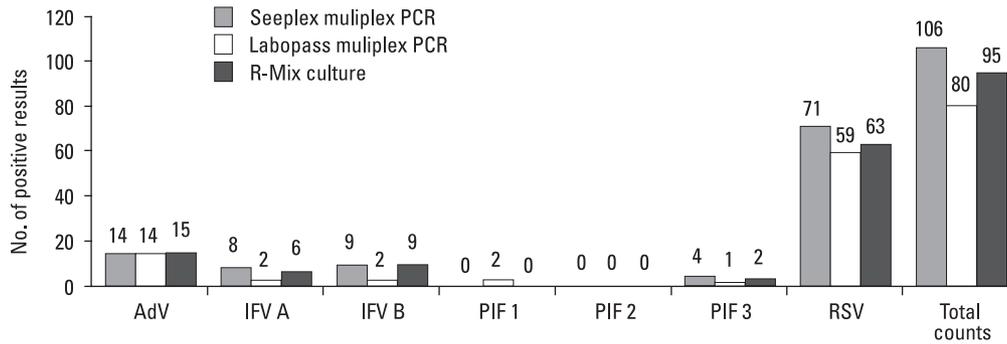


Fig. 1. Comparison of multiplex PCRs and R-Mix culture for detecting adenovirus (AdV), influenza virus (IFV), parainfluenza virus (PIF) and respiratory syncytial virus (RSV) in pediatric patients with viral pneumonia.

Table 3. Sequencing Confirmations of 10 Samples that Showed Multiplex PCR Positive and Culture Negative Results

Case no.	Gender (M : 1, F : 2)	Age (mo)	Respiratory disease	Multiplex PCR (Seeplex™)	Multiplex PCR (Labopass™)	R-Mix culture	Sequencing results	% Homology based on maximum identity (%)*
1	1	56	Viral Pneumonia	IFV A	Negative	No growth	RSV A + RSV B + IFV A	100 + 98 + 97
2	2	22	Viral Pneumonia	IFV A	Negative	No growth	IFV A	99
3	1	28	Viral Pneumonia	RSV A	Negative	No growth	RSV A	98
4	2	16	Viral Pneumonia	RSV A	Negative	No growth	RSV A	97
5	1	51	Viral Pneumonia	RSV A	RSV	No growth	RSV A	97
6	2	19	Viral Pneumonia	RSV B	RSV	No growth	RSV B	98
7	2	38	Viral Pneumonia	hMPV + RSV B	hMPV	No growth	hMPV	98
8	2	20	Viral Pneumonia	PIFV 3 + RhV A/B	Negative	No growth	RhV A	91
9	2	50	Viral Pneumonia	Negative	hMPV + PIFV	No growth	hMPV	98
10	1	46	Viral Pneumonia	IFV A	Negative	No growth	RhV A	97

PCR, polymerase chain reaction; IFV, influenza virus; PIFV, parainfluenza virus; hMPV, human metapneumovirus; RhV, rhinovirus; RSV, respiratory syncytial virus. *Respiratory viruses were confirmed by the Basic Local Alignment Search Tool (BLAST) program.

most commonly used method for identifying respiratory viruses.⁹ An enzyme immunoassay may be quicker but it is less sensitive. These conventional methods may be affected by specimen quality, virus type, and technical skill.¹⁰⁻¹² The virus culture is still considered the gold standard for respiratory virus detection, but it has limitations in turnaround time, specimen transport, and storage conditions in maintaining the infectivity of the virus.^{11,13} To compare the test methods, Choi, et al.¹⁴ reported that the positive rate of the direct antigen test, viral culture method, and multiplex PCR for detecting AdV, IFV, PIFV, and RSV was 28.4%, 36.2%, and 44.8%, respectively.

Recently, virus identification by immunostaining after shell vial culture is the most widely accepted laboratory standard method of virus testing. But, this method is still not easy for routine testing in clinical laboratories. The nucleic acid amplification method including multiplex PCR is a powerful alternative, but it has some limitations of false positivity and false negativity. The nucleic acid amplification test is faster than the culture method and has

been reported to be more sensitive^{9,15-17} and can be automated these days. In addition, there are advantages in detecting some viruses which grow poorly in cell cultures such as HMPV.¹⁸

A Korean national survey¹⁹ of respiratory virus testing was performed and thirty-one clinical laboratories responded that they provided respiratory virus testing for clinical diagnosis in Korea. Among the responders, PCR and/or culture were most widely adopted, in 42% of the institutes, rapid immunochromatographic method 29%, immunofluorescent antigen assay 23%, and enzyme immunoassay 7%.

We focused on the four major respiratory viruses of AdV, IFV, PIFV, and RSV. The exact concordance rate between the two kinds of multiplex PCR and R-Mix viral culture was 48.5% (50/103). Our results of the two multiplex PCR showed an equivalent or higher positive rate than in other studies.^{9,10,14} Two kinds of multiplex PCR reagents showed different positivity according to the virus type. The positive rate of the Seeplex™ RV detection kit was higher than the Labopass™ RV kit for IFV and RhV. However, the

Labopass™ RV kit detected more HMPV than the Seeplex™ RV kit.

Discrepant results between these two types of multiplex PCR and R-Mix culture comprised 51.5% (53/103) of the total. Weinberg, et al.¹⁷ reported that the PCR method could detect viruses two times more, compared with virus culture. Our study showed that the concordance rate of Seeplex PCR to the virus culture was 83% (90/103), and that of Labopass PCR, 80.6% (83/103). These results were similar to previous reports, of 83.2% (556/668)¹⁷ and 80% (40/50).²⁰

The discrepancy might be caused from multiplex PCR limitations. One of the major limitations of PCR detection is false-negative results as a consequence of PCR inhibitors present in clinical samples that are not removed by the extraction process.⁹ Another limitation is the principle of PCRs, which could produce false results if a primer region has nucleotide variation and is unable to detect new types or strains of a virus. This is the reason that direct antigen tests or virus cultures cannot be completely substituted by solitary multiplex PCR tests until now.²¹

We noticed a 6.8% (15/220) coinfection rate (more than 2 virus types identified) by the Seeplex™ RV kit and 5.9% (13/220) by the Labopass™ RV kit. Previous studies have suggested that double-virus infections are associated with greater severity of respiratory tract infection.²² In our study, we could not find out any remarkable parameters or difference of clinical severity in cases of coinfection.

Ten cases of culture negative but multiplex PCR positive were further evaluated by direct sequencing in our study. Sequencing results confirmed almost all the original results of multiplex PCR.

In conclusion, identification of respiratory viruses by multiplex PCR can be more rapid, an easier method, and show more positive results than the viral culture method. Hence, multiplex PCR can be the first choice for detection of respiratory viruses in a clinical laboratory. Other complementary efforts such as viral cultures and sequencing methods could be selectively proceeded in selected cases according to each laboratory's environment.

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