

sensitive and specific detection of infectious agents, including fastidious organisms or low-copy-number agents. Molecular testing for the diagnosis of ARTIs is especially useful for human metapneumovirus (hMPV), rhinovirus, and coronaviruses[1, 2].

Multiplex PCR, which detects various agents using multiple primer sets, offers significant benefits in regard to costs, labor, and accurate diagnosis. Furthermore, it has advantages of simultaneous testing for multiple agents in any clinical syndrome where a variety of agents may be involved. Respiratory viruses cause a wide spectrum of respiratory illness in children ranging from pharyngitis, otitis media, laryngitis, and bronchitis to bronchiolitis and pneumonia. It is difficult to distinguish clinically the causative agents involved in infection[3]. The diversity of pathogens in ARTIs requires a sensitive and specific multiplex PCR assay. However, limited sensitivities and specificities hampered the routine use of multiplex PCR in clinical laboratories.

We intended to evaluate the usefulness of a recently developed multiplex reverse transcriptase-polymerase chain reaction (mRT-PCR) assay kit as a routine method for the detection of a large panel of respiratory viruses in the clinical laboratory. A two-set commercial mRT-PCR was designed to detect one DNA virus (adenovirus) and 11 RNA viruses commonly associated with pediatric ARTIs (coronavirus OC43, coronavirus 229E/NL63, hMPV, influenza A virus, influenza B virus, RSV A, RSV B, PIV 1, PIV 2, PIV 3, and rhinovirus). Each set contains six pairs of primers adopting a dual priming oligonucleotide (DPO) system[4]. Each primer consists of two separate priming regions and a polydeoxyinosine linker. A shorter 3'-segment of primer is designed to block non-specific annealing and the other 5'-segment initiates stable annealing. The linker contributes to lowering melting temperature in spite of the length of primer with more than 35 bases. We performed a hospital-based epidemiological screening over a one-year period with DPO-based mRT-PCR assays.

MATERIALS AND METHODS

1. Materials

From January through December 2004, NPAs were collected from 709 children ≤ 5 yr old. Inclusion criteria

were: any child admitted to our institution, a 700-bed tertiary care hospital in Seoul, Korea, with an episode of ARTI. Fresh respiratory samples were sent to the clinical laboratory within 1 hr of collection and then stored in 4°C until FA assays. Aliquots of NPAs were also stored at -70°C. We selected 30% (N=212) of children from 709 nonconsecutive pediatric patients by means of a systematic random sampling process, using a random number table. Twelve cases had small quantities of specimen not enough for RNA extractions, thus the final sample size was 200 individuals.

1) Case definition

The medical records were reviewed retrospectively and the final diagnosis was obtained from the discharge letter. The diagnosis was made on the basis of chest radiographs and clinical findings. Bronchiolitis was defined as an acute respiratory illness characterized by rhinorrhea, cough, and diffuse wheezes, with peribronchial thickening or hyperaeration on the chest radiograph. Pneumonia was diagnosed if a patient had dyspnea with focal rales or decreased breath sounds, and the presence of a lobar or interstitial infiltrate on the chest radiograph. Laryngotracheobronchitis (croup) was an acute lower respiratory tract infection characterized by hoarseness, cough, and stridor. Exacerbation of asthma was characterized by aggravation of wheezing or dyspnea without other symptoms or signs in children with underlying asthma. The co-infection was the cases with simultaneous detections of two or more respiratory viruses in the same specimen. Co-infected cases were discarded in the review of disease entity or seasonal distribution to avoid bias of epidemiologic data.

2. Methods

1) Respiratory virus antigen assays with direct fluorescent antibody method

The refrigerated NPA specimens were processed and incubated with fluorescence-labeled antibodies for adenovirus, influenza A/B virus, RSV, and PIV according to the manufacturer's instructions (Imagen, Dako, Glostrup, Denmark). The slides were read under a fluorescence microscope. Slides were scored as positive if they had at least two fluorescing respiratory cells.

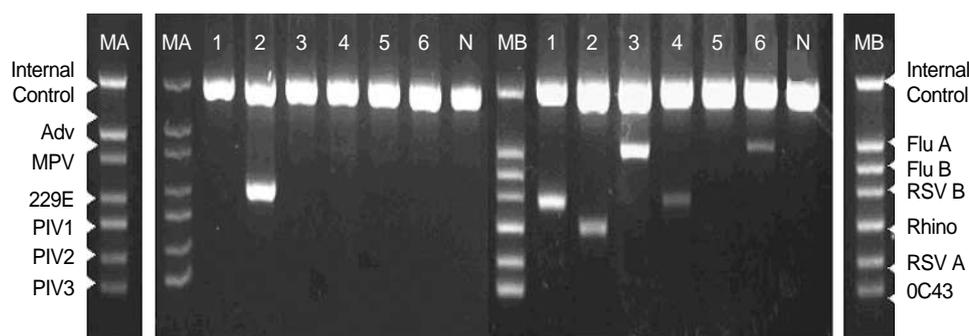


Fig. 1. Two sets (A set in the left and B set in the right) of mRT-PCR using dual priming oligonucleotide systems in 6 clinical specimens. Lane MA, Size marker of A set; MB, Size marker of B; N, negative control; 1-6, Patients 1-6; Patient 1, Positive for RSV B; Patient 2, coronavirus 229E/NL63 and rhinovirus; Patient 3, influenza A; Patient 4, RSV B, Patient 5, negative; Patient 6, influenza A. Each lane presents internal control band (719 bp). Adv, Adenovirus (534 bp); MPV, Human metapneumovirus (469 bp); 229E, Coronavirus 229E/NL63 (375 bp); PIV1, Parainfluenza virus 1 (324 bp); PIV2, Parainfluenza virus 2 (264 bp); PIV3, Parainfluenza virus 3 (219 bp); Flu A, Influenza A virus (516 bp); Flu B, Influenza B virus (455 bp); RSV B, Respiratory syncytial virus B (391 bp); Rhino, Human rhinovirus A (340 bp); RSV A, Respiratory syncytial virus A (273 bp); OC43, Coronavirus OC43 (231 bp).
Abbreviation: mRT-PCR, multiple reverse transcriptase PCR.

2) Multiplex RT-PCR for respiratory virus detection

RNAs were extracted from 530 μL of frozen NPA samples, using a MagMaxTM Viral RNA Isolation Kit (Ambion, Austin, TX, USA). In this process, we added 5 μL of an internal control (SeeGene, Seoul, Korea), which was DNA extracted from plants to assess any problems that may have occurred during the viral RNA preparation or the PCR reaction. RNAs purified from NPAs were used for the synthesis of first-strand cDNAs by Moloney murine leukemia virus reverse transcriptase (200 U/ μL ; Promega, Madison, WI, USA).

Respiratory Virus Detection Kit-A and B (Seeplex RV detection kit, SeeGene) were used to detect 11 types of RNA and one type of DNA virus according to the manufacturer's instructions. Briefly, PCR was conducted in a final reaction volume of 20 μL containing 6 μL of cDNA, 4 μL of 5xRV Primer, and 10 μL of 2xMaster Mix. The PCR protocol was 35 cycles at 94°C for 30 sec, followed by 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 PCR products were separated on 2% agarose gels stained with ethidium bromide. Each kit includes a size marker, which has the same lengths as those of amplicons for internal control and six viruses (Fig. 1). If a specimen showed an amplicon of the same size as one of a marker band, it was scored as positive.

3) Sequence analysis for coronavirus 229E/NL63

We performed sequence analysis to discriminate coronavirus 229E and NL63. mRT-PCR positive band for coro-

navirus 229E/NL63 from patient's samples were extracted from the gel by using the GENCLEAN II Kit (Q-BIO gene, Carlsbad, CA, USA), and directly sequenced with ABI PRISM 3100-*Avant* Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using target PCR primer.

3. Statistical analysis

Proportions between groups were compared by the Pearson's chi-square test or Fisher's exact test. The mean age was compared using the Mann-Whitney U test. *P* values less than or equal to 0.05 were considered statistically significant.

RESULTS

1. Demographics of patients

The 200 patients tested were not different in age, sex, clinical diagnosis, and monthly distribution compared to the archived 709 patients. The median age of the 200 children tested was 16 months. The age distribution of patients was 12.5% for infants 0-3 months old, 30% for infants 4-12 months old, 27% for children 13-24 months old, 18% for children 25-36 months old, and 12.5% for children older than 36 months of age. One hundred and nineteen children (59.5%) were male. One hundred-nine children (54.5%) were diagnosed as pneumonia, 49 (24.5%) as bronchiolitis, 17 (8.5%) as croup, nine (4.5%) as bronchitis,

and 16 (8.0%) as exacerbation of asthma. The FA assays were positive in 66 patients (33.0%) (Table 1, 2).

2. mRT-PCR results

Overall positive rate was 56.0% (112/200) with mRT-PCR. From the 112 PCR-positive patients, a total of 129 viruses were identified (Table 1). Sixteen cases (8.0% of

total, 14.3% of mRT-PCR-positive cases) showed co-infections: 15 specimens were positive for two viruses and one specimen was positive for three viruses (RSV A, PIV 1, and influenza B virus). Thirteen of the 16 (81.3%) co-infections involved RSV A or B and ten (62.5%) involved one of PIVs.

Table 1. Viruses identified in 200 nasopharyngeal aspirates obtained from children with acute respiratory tract infections by mRT-PCR and fluorescent antibody assays

Virus identified	N (%) of positive specimens, by the indicated method of detection (N=200)		Concordance of mRT-PCR and fluorescent Ab assay			
	mRT-PCR	Fluorescent Ab assay	Both (+)	PCR (+) Ab (-)	PCR (-) Ab (+)	Concordance (%)*
RSV A	49 (24.5)	45 (22.5) [†]	43	6	2	84.3
RSV B	10 (5.0)	2 (1.0) [†]	2	8	0	20.0
Influenza virus A	13 (6.5)	6 (3.0)	5	8	1	35.7
Influenza virus B	7 (3.5)	2 (1.0)	0	7	2	0
Parainfluenza virus 1	9 (4.5)	2 (1.0) [†]	2	7	0	22.2
Parainfluenza virus 2	4 (2.0)	0 [†]	0	4	0	0
Parainfluenza virus 3	10 (5.0)	7 (3.5) [†]	7	3	0	70.0
Adenovirus	6 (3.0)	2 (1.0)	2	4	0	33.3
Coronavirus 229E/NL63	8 (4.0)	Not tested				
Coronavirus OC43	2 (1.0)	Not tested				
Human metapneumovirus	6 (3.0)	Not tested				
Rhinovirus	5 (2.5)	Not tested				
Total	129 (56.0) [‡]	66 (33.0)	61	47	5	54.0

*Concordance rate (%)=(N of positive results by both of the two methods*100)/(N of positive results by both of the two methods + N of positive results in multiplex RT-PCR only+N of positive results in fluorescent Ab assay only); [†]The direct fluorescent antibody assay was designed not to discriminate respiratory syncytial virus A and B or parainfluenza virus 1, 2, and 3. The results were classified according to the results of RT-PCR; [‡]A total of 129 viruses were identified from 112 specimens (56.0%) by multiplex RT-PCR. Abbreviations: mRT-PCR, multiple reverse transcriptase PCR; RSV, Respiratory syncytial virus.

Table 2. Disease entities in 200 children with acute respiratory tract infections and their causative agents detected by mRT-PCR*

Respiratory virus	N (%) isolated virus in each disease entities				
	Bronchitis	Croup	Bronchiolitis	Pneumonia	Asthma exacerbation
RSV A	1 (11.1)	0	13 (26.5)	20 (18.3)	4 (25.0)
RSV B	0	0	1 (2.0)	7 (6.4)	0
Influenza A	3 (33.3)	3 (17.6)	0	6 (5.5)	0
Influenza B	1 (11.1)	1 (5.9)	0	1 (0.9)	2 (12.5)
Parainfluenza virus 1	1 (11.1)	1 (5.9)	0	1 (0.9)	0
Parainfluenza virus 2	0	0	0	2 (1.8)	0
Parainfluenza virus 3	0	2 (11.8)	2 (4.1)	3 (2.8)	1 (6.3)
Adenovirus	0	1 (5.9)	1 (2.0)	2 (1.8)	0
Coronavirus 229E/NL63	0	5 (29.4)	2 (4.1)	0	0
Coronavirus OC43	0	0	2 (4.1)	0	0
Metapneumovirus	1 (11.1)	0	0	2 (1.8)	0
Rhinovirus	0	0	2 (4.1)	2 (1.8)	0
Co-infection	2 (22.2)	0	5 (10.2)	9 (8.3)	0
Negative	0	4 (23.5)	21 (42.9)	54 (49.5)	9 (56.3)
Total	9	17	49	109	16

*Co-infected cases were excluded in each causative virus count. Abbreviations: See Table 1.

3. Seasonal and epidemiologic distributions of viruses

For 96 children with single positive mRT-PCR results, the monthly distributions of detected viruses are shown in Fig. 2. mRT-PCR positive rates were from 10% in August to 100% in February and March. Most of the isolated RSV were RSV B for the first three months and RSV A for the last four months in 2004. Influenza A showed a peak in the spring and influenza B in the fall followed by influenza A in December. PIV 1 and PIV 2 were detected in the winter-spring season; however, PIV 3 was most prevalent in June and July. A small number of hMPV were observed in spring. Coronavirus 229E/NL63 was identified in the spring and fall.

mRT-PCR showed that about a half of the bronchiolitis (28/49, 57.1%) or pneumonia (55/109, 50.5%) cases were caused by one of the 12 tested viruses, and RSV was their most frequent causative agent (Table 2). Chil-

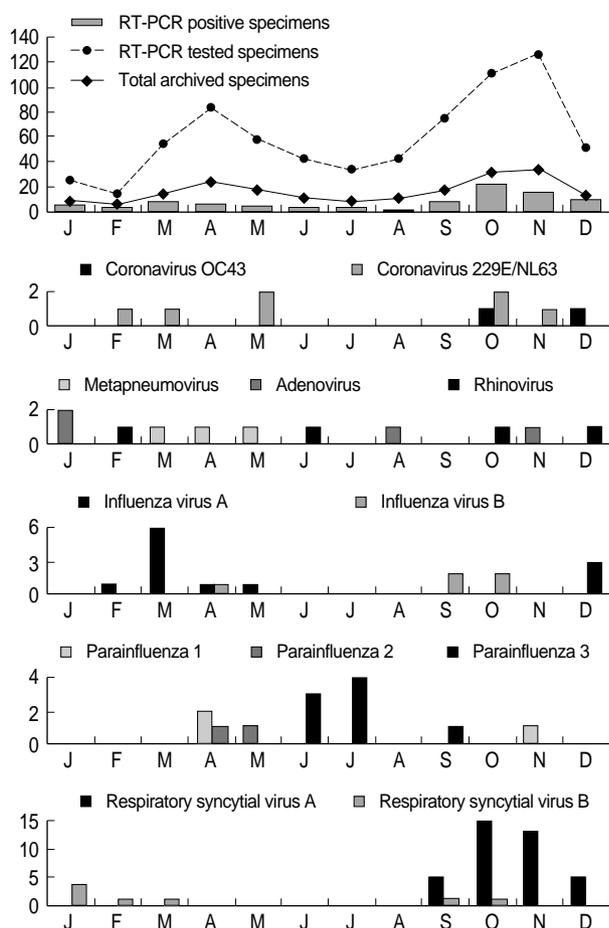


Fig. 2. Monthly distribution of 12 respiratory viruses isolated from children with acute respiratory tract infections in 2004 (Co-infected cases were excluded).

dren infected with RSV A or B predominantly presented with bronchiolitis or pneumonia (41/46, 89.1%) compared with the other viruses (28/50, 56.0%; $P < 0.001$). Thirteen of the 16 coinfecting cases involved RSV and most of them were also manifested as bronchiolitis or pneumonia (12/13, 92.3%). hMPV was identified in six children (3.0%), including three children co-infected with RSV. All of them were younger than 36 months (9 to 34 months).

Causative agents were found in 13 of 17 croup cases and coronavirus 229E/NL63 was the most frequent agent (5/17, 29.4%). Sequence analysis of eight coronavirus 229E/NL63 amplicons found six coronavirus NL63 cases and two coronavirus 229E cases. Six coronavirus NL63 infected children were under 24 months old (5-15 months) and five of them caused croup in the spring or fall seasons.

The detection rates of mRT-PCR were not different among age groups; it ranged from 50.0% to 64.0%. In children >36 months, influenza viruses were detected more frequently than in younger patients (24.0% vs 8.0%, $P = 0.033$).

4. Reproducibility of mRT-PCR assay

We confirmed the reproducibility of the assay kits by blind tests. Three technicians performed duplicate tests with 23 specimens. All 23 specimens showed the same results in each of six runs.

DISCUSSION

For the five kinds of viruses tested by conventional FA assays (adenovirus, influenza A/B, RSV, and PIV), the mRT-PCR method detected 47 additional cases of respiratory viral infections, including 31 co-infecting viruses (Table 1). This meant that in an additional 13% of patients (26/200) their presumptive causative agents were identified by mRT-PCR, suggesting a higher sensitivity of mRT-PCR compared to FA assays. FA assays for respiratory viruses require refrigerated fresh respiratory epithelial cells, and the analytic yields are frequently hampered by the poor quality of nasopharyngeal aspirates. In our hospital, many FA specimens did not have an enough number of respiratory epithelial cells for reliable antigen

assays. However, PCRs made it possible to detect viruses in spite of a small number of respiratory cells. Moreover, the additional items tested by mRT-PCR (coronavirus, hMPV, rhinovirus) yielded more positive results in 10% of patients (20/200). In our study, DPO-based mRT-PCR provided sensitive results for 12 kinds of viruses within a day at a reasonable cost compared to FA assays for 5 kinds of viruses.

Negative PCR results were observed in the five specimens that had positive results in FA assays (Table 1). RNA degradation during freezing, thawing, and storage was considered as a possible cause of PCR negativity. The stained materials used for FA assays had limitations for the review of findings or additional testing. In our retrospective study, we could not review FA assay slides and FA false positive reaction could not be ruled out. Prospective comparative studies with other standardized methods such as culture or real-time PCR method, and evaluation of analytical sensitivity are required to validate the performances of this new mRT-PCR assay.

Conventional multiplex PCRs occasionally make false positive bands due to primer dimers, primer competitions, or their different melting temperatures[5]. This false positivity necessitates further validation techniques such as nested PCR or probe hybridization. Another step for specific detection increases a cost for tests and needs a longer time. DPO-based multiplex PCR showed no non-specific band on gels. Individual PCR with the same DPO primer showed the same results with multiplex PCR for a few co-infected PCR (data not shown); however, further validation of the bands with different primers should be performed. Clinical significance of the sensitive PCR results need more evaluations because they do not always mean active infections, especially for those of co-infected cases. In our series, many co-infected cases showed one strong band and another faint band on electrophoresis gels. Thirteen of the 16 (81.3%) co-infections involved RSV A or B and showed similar clinical findings with RSV A or B; All of them were manifested as bronchiolitis or pneumonia in winter-spring season.

In this study, we evaluated the overall prevalence of 12 respiratory viruses with mRT-PCR in childhood ARTIs. In addition, we compared the relative contributions among different age groups, disease entities, and seasonal occurrences, and most findings were not different from previous studies. The positive rates for adenovirus, rhinovirus,

and hMPV in our study population were lower than those in previous reports[6-11]. Their annual or local epidemic shown in previous studies was considered as a cause of the differences. Additional investigations are planned with mRT-PCR assays for extended periods based on the current study results. One year is a short period of time for an epidemiologic study; however, our preliminary data provide useful findings for further investigations in Korea, where only a few studies have been published on the epidemiology of hMPV, coronaviruses or antigenic groups of RSV[6, 7, 10, 12].

Recently, coronavirus has also been considered as a causative agent of croup[7, 12]. The mRT-PCR technique used in this study detected three genotypes of coronavirus, OC43, 229E, and NL63, however, it did not discriminate between 229E and NL63. In our cases, sequence analysis found six NL63 infections and five of the NL63 caused croup in the spring or fall seasons in children under 24 months of age. Choi et al[7], indicated that NL63 was an important etiologic agent of croup during the spring in Korea. However, they did not find coronavirus during the autumn-winter season. In our group, three patients infected with 229E/NL63 were hospitalized with croup in October and November suggesting a role for NL63 in croup during the autumn-winter season. Han et al[12], detected NL63 from 1.7% (14 or 872) of NPA of children with ARTIs with a pattern of periodic epidemics. They were manifested as croup (64.2%) or bronchiolitis (21.4%) with the peak prevalence in November (28.5%). These findings confirmed the role of NL63 for croup in fall-winter-spring seasons. An additional study with DPO-based multiplex PCR would give a more informative data on the epidemiologic role of coronaviruses without increasing a laboratory workload.

There are two major antigenic groups of RSV, A and B, and additional antigenic variability occurs within these groups[13, 14]. The antigenic diversity of this virus may contribute to the ability of RSV to establish reinfections throughout life. During individual epidemic periods, both antigenic groups co-circulate or one group may predominate[15, 16]. Intragroup differences have also been observed in individual epidemics over consecutive years[17]. Our patients demonstrated individual epidemics of RSV A and B in the winter-spring season and the fall-winter season, respectively. Further epidemiologic investigations, to discern intergroup and intragroup differences, would improve

our understanding of the molecular evolution of RSV.

In this university-hospital based one-year epidemiologic study, the results were suggestive of a usefulness of the DPO technique-based multiplex PCR method as a diagnostic tool in the clinical laboratory.

요약

배경 : 저자들은 dual priming oligonucleotide system (DPO) 기법을 도입하여 개발된 다중 역전사중합효소연쇄반응(mRT-PCR) 키트의 소아 호흡기바이러스감염에서의 진단적 유용성을 평가하고자 하였다.

방법 : 2004년 급성호흡기감염으로 입원한 5세 이하 소아의 비인두흡인검체 중 200개를 대상으로 하였다. 신선한 검체로 호흡기바이러스 항원검사를 실시하였고, 냉동 보관한 검체를 이용하여 12종의 호흡기바이러스를 검출하기 위한 mRT-PCR 검사(Seeplex RV detection kit, SeeGene, 서울, 한국)를 실시하였다.

결과 : 66명(33.0%)의 환자에서 5가지 호흡기바이러스에 대한 항원검사 양성결과를 얻었다. mRT-PCR 검사에서는 112명(56.0%)의 환자에서 12가지 호흡기 중 하나 이상이 검출되었으며 이중 16명(8.0%)은 두 가지 이상의 바이러스가 동시감염되어 있었다. 112명으로부터 129개의 바이러스가 검출되었으며 바이러스별 양성률은 respiratory syncytial virus A/B (38.0%/7.8%), influenza virus A/B (10.1%/5.4%), parainfluenza virus 1/2/3 (7.0%/3.1%/7.8%), coronavirus 229E or NL63 (6.2%), human metapneumovirus (4.7%), adenovirus (4.7%), rhinovirus (3.9%), and coronavirus OC43 (1.6%) 순이었다.

결론 : DPO 기법을 이용한 mRT-PCR 키트를 이용해 소아 호흡기감염의 원인 바이러스를 민감하게 검출할 수 있었으며, 양성 결과의 임상적 해석에 대해서는 추가적인 연구가 요구된다.

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