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Epidemiology of Respiratory Viral Infection Using Multiplex RT-PCR in Cheonan, Korea (2006–2010)

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Multiplex RT-PCR was used to detect respiratory viruses in 5,318 clinical samples referred to the laboratory of a tertiary teaching hospital from December 2006 to November 2010. The acquired data were analyzed with respect to types, ratio, and co-infection trends of infected respiratory viruses. Trends in respiratory viral infection according to sex, age, and period of infection were also analyzed. Of the 5,318 submitted clinical samples, 3,350 (63.0%) specimens were positive for at least one respiratory virus. The infection rates were 15.8% for human rhinovirus, 14.4% for human respiratory syncytial virus A, 9.7% for human respiratory syncytial virus B, 10.1% for human adenovirus, 5.4% for influenza A virus, 1.7% for influenza B virus, 4.7% for human metapneumovirus, 2.3% for human coronavirus OC43, 1.9% for human coronavirus 229E/NL63, 3.7% for human parainfluenza virus (HPIV)-1, 1.1% for HPIV-2, and 5.3% for HPIV-3. The co-infection analysis showed 17.1% of double infections and 1.8% of triple infections. The median age of virus-positive patients was 1.3 years, and 91.5% of virus-positive patients were under 10 years old. Human respiratory syncytial virus was the most common virus in children under 5 years of age, and the influenza A virus was the most prevalent virus in children over 5 years of age. These results help in elucidating the tendency of respiratory viral infections.

Key words: Respiratory virus, epidemiology, multiplex RT-PCR

Acute respiratory tract infections (ARTIs) are some of the most common diseases that occur in both children and adults, with over 80% of identified infections attributed to respiratory viruses [8]. ARTIs constitute 30–50% of outpatients and 20–40% of pediatric hospital admissions [14]. It has been estimated that 2.5 million cases occur annually in Europe [28]. The most frequently implicated viruses among hospitalized children are human adenovirus (HAdV), influenza A virus (FLUAV), influenza B virus (FLUBV), human parainfluenza virus (HPIV), human respiratory syncytial virus (HRSV), human rhinovirus (HRV), human coronavirus (HCoV), and human metapneumovirus (HMPV) [10, 27, 28]. The epidemiology of respiratory viruses shows various patterns. Although all age groups are susceptible to infection, there is a higher rate of infection among children than adults. Respiratory viruses are highly contagious in humans and infect many people in a short period of time [21].

The viruses that infect the respiratory tract show various patterns according to the seasons. HRSV infections are prevalent around the world throughout the year. In temperate regions, infections begin in winter, last 4–5 months, peaking as early as from December to March and sometimes persisting until June. Influenza virus infections are prevalent in the cold season in temperate regions. These infections can be fatal in the elderly or patients who already have cardiopulmonary diseases. Children without influenza antibodies show high infection rates [23]. It is difficult to identify respiratory viral pathogens, because viruses that infect the respiratory tract can infect other organs, and the initial symptoms of respiratory viruses are similar to each other [14].

In Korea, a 2004 study on viral respiratory infections reported the use of the indirect immunofluorescence assay [25], and a 2007 study in children with acute viral respiratory infection was conducted using multiplex RT-PCR [5]. However, little is known of viral infections in the Choongchung Province. In this study, we screened 5,318 samples referred to Dankook University Hospital for the presence of respiratory viruses using multiplex RT-PCR. Pathogens that caused infections from December 2006 to

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November 2010 were classified into types, and determinations were made of the ratio of pathogens, patterns of contagion, and trends of co-infection. These results highlight the tendency of viral infection according to sex, age, or seasons and can be helpful in developing effective and rapid diagnostics and treatments for respiratory viral infections.

Materials and Methods

Materials

Between December 2006 and November 2010, 5,318 specimens were collected from patient samples submitted to Dankook University Hospital for respiratory virus screening. Multiplex RT-PCR was performed.

Methods

Specimen collection. Nasopharyngeal aspirations were collected from patients with respiratory illnesses using a mucus trap with a sterile suction catheter. Samples were kept for immediate testing or refrigerated at 4°C for up to 48 h.

Screening target virus. HAdV, FLUAV, FLUBV, HRV, HCoV-229E/NL63, HCoV-OC43, HMPV, HPIV-1, HPIV-2, HPIV-3, HRSV-A, and HRSV-B were analyzed. Classification and abbreviation of viruses followed the International Committee on Taxonomy of Viruses (2008).

Multiplex RT-PCR analysis using nucleic acids.

RNA extraction. A Viral Minelute RNA Spin kit (Qiagen, Germany) was used for the extraction of nucleic acids from all samples. As the starting material, 200 μl of each respiratory sample was used for the nucleic acid isolation.

cDNA synthesis. Total RNAs extracted from the clinical samples were used for the synthesis of the first-strand cDNAs (RevertAid First Strand cDNA Synthesis Kit; Fermentas, Hamilton, ON, Canada). Reverse transcription was performed for 1.5 h at 37°C in a final reaction volume of 20 μl with 1 μl of random hexamer, 8 μl of total RNA, 1 μl of reverse transcriptase, 1 μl of RNase inhibitor, 2 μl of dNTP, 4 μl of 5× RT buffer, and 3 μl of water. The products were stored at −20°C until used.

RT-PCR. PCR amplification was performed using the Seeplex RV12 Detection Kit (Seegene, Seoul, Korea), according to the manufacturer's instructions, with a PTC200 PCR system (MJ Research, Alameda, CA, USA). Briefly, PCRs cycled the following sequence 40 times: 94°C for 30 s, 60°C for 90 s, and 72°C for 90 s. The final cycle was followed by an extension step at 72°C for 10 min to complete any partial polymerizations. Positive and negative RT-PCR controls containing standardized viral RNA extracts and nuclease-free water, respectively, were included in each run.

Electrophoresis. PCR products were separated on 2% agarose gels containing 0.5 g of ethidium bromide per milliliter in Tris-borate-EDTA (TBE) buffer and were visualized under ultraviolet light.

Results

Virus Detection in Patients

The total number of specimens from December 2006 to November 2010 was 5,318. Of these, 3,350 specimens were positive (63.0%); in these, the number of detected virus was 4,045. The most prevalent virus was HRV, comprising 20.8% (841/4,045) of the identified viruses, followed by HRSV-A viruses (18.9%; 766/4,045). HPIV-2 was the least detected, with a prevalence of 1.4% (57/4,045). The sex ratio of requested specimens was 59.9% in men (3,188/5,318) and 40.1% in women (2,130/5,318). The virus isolation rate was 63.0% in men and 63.1% in women. Depending on the type of virus, HPV-2 showed

Table 1. Distribution of virus isolation and the infection rate according to gender.

Virus[a]	No. (%)[b] of isolated viruses	Infection rate			Gender ratio of infection rate
		All	Male	Female	
HCoV-229E	102 (2.5%)	1.9%	2.0%	1.8%	1.11
HAdV	535 (13.2%)	10.1%	10.0%	10.1%	0.99
FLUAV	288 (7.1%)	5.4%	5.5%	5.4%	1.02
FLUBV	89 (2.2%)	1.7%	1.6%	1.8%	0.89
HMPV	248 (6.1%)	4.7%	4.6%	4.8%	0.96
HCoV-OC43	123 (3.0%)	2.3%	2.0%	2.8%	0.71
HPIV-1	199 (4.9%)	3.7%	4.0%	3.3%	1.21
HPIV-2	57 (1.4%)	1.1%	1.3%	0.7%	1.86
HPIV-3	281 (6.9%)	5.3%	4.8%	6.0%	0.80
HRSV-A	766 (18.9%)	14.4%	14.2%	14.7%	0.97
HRSV-B	516 (12.8%)	9.7%	9.8%	9.5%	1.03
HRV	841 (20.8%)	15.8%	16.1%	15.4%	1.05
Total	4,045 (100%)	63.0%	63.0%	63.1%	1.00

[a]Abbreviations: HCoV-229E, human coronavirus 229E/NL63; HAdV, human adenovirus; FLUVA, influenza A virus; FLUBV, influenza B virus; HMPV, human metapneumovirus; HCoV-OC43, human coronavirus OC43; HPIV-1, 2, 3, human parainfluenza virus 1, 2, 3; HRSV-A, human respiratory syncytial virus A; HRSV-B; human respiratory syncytial virus B; HRV, human rhinovirus.

[b]Percentage of virus isolates: Number of the isolates of each virus group/Number of total virus isolates.

Table 2. Age distribution of the specimen requests and the virus detection.

Age group	No. (%) of total specimens	No. (%) of virus-isolated specimens
0–9 years	4,212 (79.2%)	3,065 (91.5%)
10–19 years	188 (3.5%)	77 (2.3%)
20–39 years	131 (2.5%)	28 (0.84%)
40–59 years	218 (4.1%)	45 (1.34%)
60–79 years	466 (8.8%)	112 (3.3%)
80–99 years	103 (1.9%)	23 (0.7%)
Total	5,318 (100%)	3,350 (100%)

the greatest difference, with an isolation ratio of 1.86 between males and females (Table 1).

Distribution of Patient Age

The mean and median ages of all patients were 12.7 and 1.9 years (range, 3 days to 93 years), respectively. The mean and median ages of virus-positive patients were 5.9 and 1.3 years (range, 9 days to 93 years), respectively, and 91.5% of patients who were positive for respiratory virus were <10 years old (Table 2). HRSV-A was the most common virus detected in children ≤2 years of age, whereas FLUAV was the most prevalent virus among children >5 years of age. HPIV-2 displayed the lowest infection rate at every age (Table 3).

Annual and Seasonal Distributions

Determination of the seasonal average positive rate revealed autumn to have the highest (29.7%; 1,202/4,045) rate, followed by spring (27.8%; 1,125/4,045) (Table 4). HRSV infection showed seasonal variation with peaks during the cold season. The average isolation rate of HRSV was 25.6% (119/464) in October, 50.9% (350/688) in November, 57.9% (341/589) in December, and 38.2% (165/432) in January. HPIV infection was also apparent during the spring and summer seasons. The average isolation rate of HPIV was 15.6% (88/561) in May, 27.0% (113/419) in June, 11.1% (32/287) in July, and 3.0% (8/265) in August.

Viral Co-Infection

The rates for single, dual, and triple infections were 81.1% (2,717/3,350), 17.1% (572 cases), and 1.8% (60 cases), respectively (Table 5). The ratio of co-infection was lowest in FLUBV (9.0%) and the highest was HAdV (48.0%). Co-infections with HRV occurred most frequently, in 6.6% of samples (Table 6). Concerning double infection, co-infection with HAdV with HRV was most frequent (14.0%, 80/572), followed by HRSV-A with HRV (9.8%, 56/572), and HRSV-B with HRSV (7.7%, 44/572). Viral triple infection was detected in six samples with the HAdV, FLUAV, and HRV combination and five samples with the HCoV-OC43, HRSV-A, and HRV combination. There was one case of quadruple infection, in which HAdV, HMPV, HPIV-3, and HRV were isolated in the same specimen.

DISCUSSION

In this study, we report viral agents of ARTIs in 5,318 patients in Dankook University Hospital enrolled over 4 years from December 2006 to November 2010. Nasopharyngeal aspirates from 3,350 of the 5,318 (63.0%) patients tested

Table 3. Age distribution of the number of virus isolates.

Virus[a] \ Age group	No. (%) of isolates			
	≤2 Years	3–5 Years	6–9 Years	≥10 Years
HCoV-229E	65 (2.5%)	18 (2.1%)	8 (2.7%)	11 (3.5%)
HAdV	258 (10.0%)	199 (22.9%)	51 (17.5%)	27 (8.7%)
FLUAV	109 (4.2%)	60 (6.9%)	76 (26.1%)	43 (13.9%)
FLUBV	19 (0.7%)	28 (3.2%)	24 (8.2%)	18 (5.8%)
HMPV	148 (5.7%)	70 (8.0%)	11 (3.8%)	19 (6.1%)
HCoV-OC43	74 (2.9%)	24 (2.8%)	5 (1.7%)	20 (6.5%)
HPIV-1	138 (5.4%)	46 (5.3%)	5 (1.7%)	10 (3.2%)
HPIV-2	33 (1.3%)	16 (1.8%)	6 (2.1%)	2 (0.6%)
HPIV-3	209 (8.1%)	40 (4.6%)	8 (2.7%)	24 (7.7%)
HRSV-A	619 (24.0%)	99 (11.4%)	16 (5.5%)	32 (10.3%)
HRSV-B	393 (15.3%)	73 (8.4%)	12 (4.1%)	38 (12.3%)
HRV	509 (19.8%)	197 (22.6%)	69 (23.7%)	66 (21.3%)
Total 4,045 isolates	2,574 (100%)	870 (100%)	291 (100%)	310 (100%)

[a]Abbreviations: HCoV-229E, human coronavirus 229E/NL63; HAdV, human adenovirus; FLUVA, influenza A virus; FLUBV, influenza B virus; HMPV, human metapneumovirus; HCoV-OC43, human coronavirus OC43; HPIV-1, 2, 3, human parainfluenza virus 1, 2, 3; HRSV-A, human respiratory syncytial virus A; HRSV-B; human respiratory syncytial virus B; HRV, human rhinovirus.

Table 4. Distribution of virus isolates by month and season.

Virus[a]	Spring			Summer			Autumn			Winter			Total
	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sep.	Oct.	Nov.	Dec.	Jan.	Feb.	
HCoV-229E	6	4	8	2	0	1	4	14	8	23	18	14	102
	5.9%	3.9%	7.8%	2.0%	0%	1.0%	3.9%	13.7%	7.8%	22.6%	17.7%	13.7%	
HAdV	28	45	58	48	34	52	66	66	66	34	27	11	535
	5.2%	8.4%	10.8%	9.0%	6.4%	9.7%	12.3%	12.3%	12.3%	6.4%	5.1%	2.1%	
FLUAV	30	55	27	5	0	0	7	18	25	39	69	13	288
	10.4%	19.1%	9.4%	1.7%	0%	0%	2.4%	6.3%	8.7%	13.5%	24.0%	4.5%	
FLUBV	24	20	9	1	0	0	0	0	0	0	4	31	89
	27.0%	22.5%	10.1%	1.1%	0%	0%	0%	0%	0%	0%	4.5%	34.8%	
HMPV	28	92	75	27	10	2	0	1	1	3	3	6	248
	11.3%	37.1%	30.2%	10.9%	4.0%	0.8%	0%	0.4%	0.4%	1.2%	1.2%	2.4%	
HCoV-OC43	14	10	16	15	2	3	1	2	5	21	11	23	123
	11.4%	8.1%	13.0%	12.2%	1.6%	2.4%	0.8%	1.6%	4.1%	17.1%	8.9%	18.7%	
HPIV-1	10	9	15	19	22	24	36	22	23	8	5	6	199
	5.0%	4.5%	7.5%	9.6%	11.1%	12.1%	18.1%	11.1%	11.6%	4.0%	2.5%	3.0%	
HPIV-2	1	1	7	4	0	6	7	17	8	2	2	2	57
	1.8%	1.8%	12.3%	7.0%	0%	10.5%	12.3%	29.8%	14.0%	3.5%	3.5%	3.5%	
HPIV-3	0	17	88	113	32	8	5	9	5	2	2	0	281
	0%	6.1%	31.3%	40.2%	11.4%	2.9%	1.8%	3.2%	1.8%	0.7%	0.7%	0%	
HRSV-A	48	39	23	11	0	5	11	65	181	233	107	43	766
	6.3%	5.1%	3.0%	1.4%	0%	0.7%	1.4%	8.5%	23.6%	30.4%	14.0%	5.6%	
HRSV-B	23	30	8	5	1	1	12	54	172	115	61	34	516
	4.5%	5.8%	1.6%	1.0%	0.2%	0.2%	2.3%	10.5%	33.3%	22.3%	11.8%	6.6%	
HRV	71	83	103	86	64	61	93	114	84	40	24	18	841
	8.4%	9.9%	12.3%	10.2%	7.6%	7.3%	11.1%	13.6%	10.0%	4.8%	2.9%	2.1%	
Monthly no. of virus isolates.	283	405	437	336	165	163	242	382	578	520	333	201	4,045
Monthly no. of infected specimens	239	327	347	258	148	138	186	308	497	438	286	178	3,350
Monthly rate of infection	59.2%	58.2%	61.9%	61.6%	51.6%	52.1%	54.4%	66.4%	72.2%	74.4%	66.2%	58.4%	63.0%
Monthly percentage of virus isolates	7.0%	10.0%	10.8%	8.3%	4.1%	4.0%	6.0%	9.4%	14.3%	12.9%	8.2%	5.0%	100%
Seasonal percentage of virus isolates		27.8%			16.4%			29.7%			26.1%		100%

[a]Abbreviations: See Table 1.

Table 5. Distribution of infection types.

Infection type	No. (%) of infected specimens
Single infection	2,717 (81.1%)
Double infection	572 (17.1%)
Triple infection	60 (1.8%)
Quadruple infection	1 (0.03%)
Total	3,250 (100%)

positive for at least one viral pathogen. HRSV was detected in 1,282 (31.7%) patients showing the highest frequency throughout the study period, followed by HRV (20.8%), HAdV (13.2%), FLUAV (7.1%), HPIV-3 (7.0%), and HMPV (6.1%) (Table 1). In this study, HRSV was the most commonly detected and showed similar results to culture-based studies [5, 14]. In these prior studies, HAdV and HPIV were reported as the second most common viral pathogens. However, presently, HRV was the second most prevalent. There are several explanations for these differences. Detection methods and PCR primers can differ from one study to another, making direct comparison of data difficult [2, 7]. Further studies can clarify this issue by evaluating the sensitivity and specificity of the different detection methods used. Second, the infection rates may be specific to geographical locations and the particular study period [2, 16].

The median age of positive patients in this study was 1.3 years; RSVs were the most commonly isolated. Similar results have also been reported in other studies [3, 12]. Albuquerque *et al.* [1] reported that the median age for recently discovered respiratory viruses was 2.7 years; HMPV and human bocavirus were the most commonly isolated. In the present study, HMPV was the significant cause of viral infections in patients aged under 5 years (Table 3). The percentage of viruses isolated from patients aged under 5 years was 85.1% (3,444/4,045). Meanwhile, 10.7% (569/5,318) of specimen requests in patients aged over 60 years accounted for about half of 20.8% (1,106/5,318) of the subtotal of specimens from patients aged more than 10 years (Table 2). This may be due to the August 2009 (H1N1) flu epidemic outbreak, which increased awareness in young adults and the elderly [9, 11, 24] and prompted more medical examinations for respiratory viruses. In children under 2 years of age, the prevalence of infection for HRSV-A and HRV was 24.0% and 19.8%, respectively (Table 3). On the other hand, children 3–5 years of age were infected mainly by HRV (22.6%) and HAdV (22.9%), whereas FLUAV (26.1%) and HRV (23.7%) were the main viruses infecting those >5 years of age. HPIV-2 showed a lower positivity in most age groups. The analysis of the age distribution of viral infections revealed that younger and elderly adults were more frequently infected. These data coincide with previous reports on respiratory infections, in which a similar age distribution was reported [8, 14, 19]. Additional studies are required to determine its asymptomatic prevalence in the population, pathogenicity, and viral shedding characteristics.

Respiratory viruses show various patterns depending on the geography and season [17, 18]. The highest infection frequency was evident in the winter season. In our study, the highest monthly percentage of virus isolates was found in November (14.3%), followed by December (12.8%). The monthly infection rate was the highest in December (74.4%), followed by November (72.2%), October (66.4%), and January (66.2%), in which the rates were more than the average infection rate of 63.0% (Table 4).

Among the positive cases, multiple (≥2) respiratory viruses were obtained in 633 (18.9%) patients. The number

Table 6. Rate and ratio of infections and co-infections by each virus group.

Virus[a]	No. of infected specimens	No. of co-infected specimens	Ratio of co-infection[b]	Rate of co-infection[c]	Rate of infection[d]
HCoV-229E	102	39	38.2%	0.7%	1.9%
HAdV	535	257	48.0%	4.8%	10.1%
FLUAV	288	60	20.8%	1.1%	5.4%
FLUBV	89	8	9.0%	0.1%	1.7%
HMPV	248	79	31.9%	1.5%	4.7%
HCoV-OC43	123	59	48.0%	1.1%	2.3%
HPIV-1	199	66	33.2%	1.2%	3.7%
HPIV-2	57	21	36.8%	0.4%	1.1%
HPIV-3	281	105	37.4%	2.0%	5.3%
HRSV-A	766	165	21.6%	3.1%	14.4%
HRSV-B	516	119	23.1%	2.2%	10.0%
HRV	841	350	41.6%	6.6%	15.8%

[a]Abbreviations: See Table 1.

[b]Ratio of co-infection: co-infected specimens/infected specimens.

[c]Rate of co-infection: co-infected specimens/submitted specimens.

[d]Rate of infection: infected specimens/submitted specimens.

of patients who were double infected was 572 (17.1%), 60 patients (1.8%) were triple infected, and one patient (0.03%) was quadruple infected (Table 5). Co-infections were significantly more frequent in younger and older groups. Co-infection ratios were higher for HAdV, HCoV-OC43, and HRV (48.0%, 48.0%, and 41.6%, respectively) and lower for FLUBV (9.0%). HRV had the highest co-infection rate at 6.6%, which was thought to be due to the highest prevalence of 15.8% (Table 6). According to the researches of the past, HRSV, FLUAV, and HPIV were reported as viral agents for the most common co-infections [26, 29, 30]. On the other hand, in a recent study by the multiplex RT-PCR method, HPIV, HRV, and HAdV were reported as viruses that causes co-infections frequently [4]. These differences are attributed to the seasonal incidence of viral agents and the difference of the virus detection methods [2, 31]. Recently, the incidence of pneumonia due to two or more viruses was reported to be higher. Co-infection was detected at 30% in one study [13], whereas other studies [15, 22] reported 3–30%. The immature immune system of the infants and the lack of previous exposure to respiratory viruses could increase their susceptibility to a simultaneous infection by two or more respiratory viruses [6]. Another possible explanation for the increased rate of dual respiratory virus infection among children may be that a unique characteristic of respiratory syncytial virus facilitates infection with a second respiratory virus. It is also plausible that prolonged shedding of respiratory viruses occurs more commonly in children [6].

We have described the epidemiology of acute respiratory virus infections, including co-infections over four years in Cheonan by multiplex RT-PCR, which is a rapid and sensitive method for the detection of viral agents. Recently, the identification of respiratory viruses was improved in sensitivity by the application of multiplex PCR and real-time PCR. The time efficiency of real-time PCR is more than twice that of conventional PCR [19, 20]. Early detection of a viral respiratory infection is crucial for the implementation of timely and appropriate treatments. A future study may include quantification of the viral load to elucidate the role of each virus involved in the infection.

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