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Comprehensive detection of causative pathogens using real-time PCR to diagnose pediatric community-acquired pneumonia

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Abstract We have developed a real-time reverse transcription-PCR (RT-PCR) method to detect 13 respiratory viruses: influenza virus A and B; respiratory syncytial virus (RSV) subgroup A and B; parainfluenza virus (PIV) 1, 2, and 3; adenovirus; rhinovirus (RV); enterovirus; coronavirus (OC43); human metapneumovirus (hMPV); and human bocavirus (HBoV). The new method for detection of these viruses was applied simultaneously with real-time PCR for the detection of six bacterial pathogens in clinical samples from 1700 pediatric patients with community-acquired pneumonia (CAP). Of all the patients, 32.5% were suspected to have single bacterial infections; 1.9%, multiple bacterial infections; 15.2%, coinfections of bacteria and viruses; 25.8%, single viral infections; and 2.1%, multiple viral infections. In the remaining 22.6%, the etiology was unknown. The breakdown of suspected causative pathogens was as follows: 24.4% were *Streptococcus pneumoniae*, 14.8% were *Mycoplasma pneumoniae*, 11.3% were *Haemophilus influenzae*, and 1.4% were *Chlamydophila pneumoniae*. The breakdown of viruses was as follows: 14.5% were RV, 9.4% were RSV, 7.4% were hMPV, 7.2% were PIV, and 2.9% were HBoV. The new method will contribute to advances in the accuracy of diagnosis and should also result in the appropriate use of antimicrobials.

Key words Real-time RT-PCR · Respiratory virus · Community-acquired pneumonia · Child

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Introduction

Community-acquired pneumonia (CAP) is the most common infectious disease occurring in children. The etiological agents are varied, including a number of viruses, and bacteria such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Mycoplasma pneumoniae*, and *Chlamydophila pneumoniae*.^{1–4} To select an appropriate antimicrobial for a patient with bacterial pneumonia, identification of the causative pathogen quickly on the first day of hospitalization would be extremely useful. If at all possible, this would also decrease hospital costs and length of stay.

We constructed a simultaneous identification system, using a real-time PCR assay in our laboratory, for the six main bacterial pathogens from clinical samples collected from pediatric patients with CAP.⁵ Subsequently, the method was improved by adding a multiplex PCR (MPCR) kit for seven viruses. The total time required for obtaining the results for viruses was about 5.0 h. Using this improved method, we⁶ detected etiological agents in 117 patients who were hospitalized with CAP. In that study, antibody titers for some pathogens were also measured for paired sera from the patients to verify the PCR results. Based on the identification of etiological agents, our cases in that study were categorized as viral infection (23.1%); viral and bacterial coinfection (38.5%); bacterial infection (21.4%); *M. pneumoniae* infection, including coinfection with another pathogen (16.2%); and *C. pneumoniae* infection (0.9%). Our data were very similar to those previously reported by Michelow et al.⁷ and Jüven et al.⁸

It is well known that the causative viruses of respiratory tract infections (RTIs) are quite varied, such as influenza viruses A, B (Flu A, Flu B); respiratory syncytial virus (RSV) subgroup A and B; parainfluenza virus 1–3 (PIV1, PIV2, PIV3); adenovirus (AdV); rhinovirus (RV); enterovirus (EV); and coronavirus (CoV).^{9–13} Human metapneumovirus (hMPV)^{14–18} and human bocavirus (HBoV)^{19–22} have also been reported recently as etiological agents of CAP.

Recently, real-time reverse-transcription-PCR (RT-PCR) methods have been applied for the identification of

^a See Acknowledgment

respiratory viruses.^{23–28} The PCR methods appear to be more sensitive than culture and are less affected by specimen quality and transport, and provide an objective interpretation of results. The United States Food and Drug Administration cleared for marketing a test called the xTAG Respiratory Viral Panel (Luminex Molecular Diagnostics, Toronto, Canada) that simultaneously detects and identifies 12 respiratory viruses.^{29,30} This method can detect these viruses within 5 h.

For the use of real-time RT-PCR as a routine method in diagnosis, we focused on developing methods to cover the detection of all of the above 13 respiratory viruses within 3 h, combining the methods with our previously described method for detecting the six main bacterial pathogens involved in CAP.⁵

In this article, we describe the results obtained when a comprehensive identification system with real-time PCR was applied for clinical samples collected from pediatric patients with CAP.

Patients, materials, and methods

Patients and clinical samples

A total of 1700 nasopharyngeal swab samples were sent to our laboratory from pediatricians at ten medical institutions that participated in the “Acute Respiratory Diseases (ARD) Study Group” between January 2005 and December 2006. These samples were collected from pediatric patients with CAP who were aged from 0 to 19 years. The CAP was diagnosed from the presence of pulmonary infiltrates on chest X-ray. After informed consent was obtained from the patients’ parents/guardians, blood samples for the determination of WBC, C-reactive protein (CRP), and serum antibody titers for several pathogens, and nasopharyngeal swab samples to determine the causative pathogen by real-time PCR were collected. An application form (in which patient names and doctors’ names were withheld), written by the doctor in charge, was sent to our laboratory with the clinical samples.

Patients with duration of symptoms of 6 days or more before visiting the hospitals and patients already administered with intravenous antibiotics at another hospital or clinic were excluded from the study, counting without reservation in consideration of the percentage of virus positive apparently dropped to a lower value.

DNA/RNA extraction

The nasopharyngeal samples were suspended in 1.5 ml of pleuropneumonia-like organisms (PPLO) broth (Difco, Detroit MI, USA) immediately after they were received. PPLO broth was selected for cultivating *M. pneumoniae*. A 1.0-ml aliquot of the PPLO broth was transferred to an Eppendorf tube and centrifuged at 5000 rpm for 5 min at 4°C. Nine hundred microliters of the supernatant was discarded, and the remaining solution including the pellet was

used as the sample. DNA/RNA was extracted using Extra-gen II (TOSOH, Tokyo, Japan) according to the manufacturer’s protocol, as follows: first, a 100-μl aliquot of the suspension was transferred to an Eppendorf tube, which contained 8 μl of detergent for DNA/RNA coprecipitation. Next, the Eppendorf tube was vortexed for 10 s. To this mixture, 500 μl of 60% (vol/vol) isopropanol-containing protein-denaturing detergent was added, and the resulting mixture was centrifuged at 12 000 rpm for 3 min at 4°C after vortexing for 10 s. The supernatant was discarded, and the residue was treated with 200 μl of 40% (vol/vol) isopropanol again, as described above. Finally, the harvested DNA/RNA pellet was resuspended in DNase- and RNase-free H₂O to provide 40 μl of DNA/RNA sample. The extraction process was finished within 10 min.

cDNA synthesis

Reverse transcription (RT) was performed in an Eppendorf tube containing 25.5 μl reaction mixture after the addition of a 10-μl aliquot of the DNA/RNA sample as described above. The reaction mixture consisted of: (i) 100 U Rever Tra Ace (TOYOBO, Osaka, Japan), (ii) 8.5 μl of 2 mM dNTPs, (iii) 1 μl of 25 pmol/μl random primer, (iv) 40 U RNase inhibitor, and (v) 4 μl of 5 × RT buffer. The RT reaction was carried out at 30°C for 10 min, 42°C for 50 min, and terminated by incubation at 99°C for 10 min, using a thermal cycler (Gene Amp PCR System 9600-R; Perkin-Elmer Cetus, Norwalk, CT, USA). After the RT reaction, 25 μl of DW was added.

Real-time PCR for viruses

Table 1 shows four sets of virus-specific primers and molecular beacon (MB) probes (RSV subgroup B, AdV, CoV, and HBoV) that were newly constructed for this study. The primers and MB adenovirus probes were modified from those of He and Jiang³¹ in order to amplify serotypes 7 and 14. The HBoV and CoV (OC43) primers were designed based on alignment from the The National Center for Biotechnology Information (NCBI) database. The reference sequences were as follows: HBoV, accession no. DQ296618; CoV (OC43), accession no. NC_005147. The other probes were slightly modified for MB probes as previously described in each of the following references: RSV subgroup A;³² Flu A, Flu B, and PIV1–3;²⁶ RV;³³ EV;³⁴ and hMPV.³⁵ The MB probes and primers were prepared by Sigma-Aldrich Japan (Tokyo, Japan) and Operon Biotechnologies (Tokyo, Japan), respectively. All of the MB probes were labeled with a fluorescent reporter, 6-carboxyfluorescein (FAM) or carboxy-X-rhodamine (ROX) at the 5′ end and labeled with black hole quencher 1 (BHQ-1) at the 3′ end.

The reaction mixture consisted of 25 μl of 2 × real-time PCR Master Mix (TOYOBO), 0.3 μM of each primer, and 0.3 μM of MB probe; the final volume of the mixture was adjusted to 50 μl with the addition of DNase- and RNase-free H₂O. The multiplex reaction for RSV subgroup A and

Table 1. Primers and probes newly constructed for this study

Viruses, primer, and probe	Primer or probe ^a sequence
Respiratory syncytial virus subgroup B	
Sense primer	5'-AACAGACATAAGCAGCTCAGTAATT-3'
Reverse primer	5'-CGATTTTGTGGATGCAGTGCATTT-3'
Probe	5'-GCGAGCAGGAGCTATAGTGTCTATGGTAGCTCGC-3'
Adenovirus	
Sense primer	5'-CCCTGGTAKCCRATRTTGTA-3'
Reverse primer	5'-AATGACAGGCTGYTGAGYC-3'
Probe	5'- <u>GCTGCCA</u> ACCAGTCYTTGGTCATGTTRCATTGGGCAGC-3'
Coronavirus (OC43)	
Sense primer	5'-TGTTTCTCAACAGCTTAGTG-3'
Reverse primer	5'-TCCTAGATGATTGGCTTTTG-3'
Probe	5'-GCTGCCGTGCAGCACAAGCTATGGAGAAGGCAGC-3'
Human bocavirus	
Sense primer	5'-GAAAGACAAGCATCGCTCC-3'
Reverse primer	5'-TGGGTGTTCTCTGATGATATG-3'
Probe	5'- <u>CGCGCT</u> GGAGCAGGAGCCGCAGCCGATAGCGCG-3'

^a Stem oligonucleotides are underlined

Table 2. Specificity after 40 cycles of real-time PCR amplification for 13 viruses

Virus	RSV-A	RSV-B	Flu A	Flu B	PIV1	PIV2	PIV3	AdV	RV	EV	CoV	hMPV	HBoV
RSV subgroup A	+	-	-	-	-	-	-	-	-	-	-	-	-
RSV subgroup B	-	+	-	-	-	-	-	-	-	-	-	-	-
Influenza virus A	-	-	+	-	-	-	-	-	-	-	-	-	-
Influenza virus B	-	-	-	+	-	-	-	-	-	-	-	-	-
Parainfluenza virus 1	-	-	-	-	+	-	-	-	-	-	-	-	-
Parainfluenza virus 2	-	-	-	-	-	+	-	-	-	-	-	-	-
Parainfluenza virus 3	-	-	-	-	-	-	+	-	-	-	-	-	-
Adenovirus	-	-	-	-	-	-	-	+	-	-	-	-	-
Rhinovirus	-	-	-	-	-	-	-	-	+	(±)	-	-	-
Enterovirus	-	-	-	-	-	-	-	-	-	+	-	-	-
Coronavirus (OC43)	-	-	-	-	-	-	-	-	-	-	+	-	-
Human metapneumovirus	-	-	-	-	-	-	-	-	-	-	-	+	-
Human bocavirus	-	-	-	-	-	-	-	-	-	-	-	-	+

subgroup B was performed by mixing each set of primers and MB probes.

Real-time RT-PCR for 12 reaction mixtures (one well contained the reaction mixture of RSV subgroup A and subgroup B), corresponding to the 13 viruses, was performed in a 96-well PCR plate (Bio Medical Equipment, Tokyo, Japan) for eight samples, in one assay in which 12 wells were arranged in one strip for one sample, and the plates were stored at -30°C until use.

Prior to amplification, 1.5 µl of cDNA was added to each of the 12 wells on ice, and amplification was immediately started at 95°C for 30 s as the first step, followed by 40 cycles of PCR: 95°C for 15 s, 55°C for 30 s, and 75°C for 15 s, using Mx3000P (Stratagene, La Jolla, CA, USA) or Thermal Cycler Dice TP800 (Takara Bio, Kyoto, Japan). The real-time PCR was finished within 1.5 h. The total time from sample disposition to finish was 3.0 h or less.

Sensitivity and specificity of real-time RT-PCR

The sensitivity of real-time RT-PCR was determined by testing tenfold serial dilutions of a 50% tissue culture infectious dose (TCID₅₀) titrated stock solution of each of the

following viruses: RSV subgroup A, RSV subgroup B, Flu A, PIV2, PIV3, AdV, and CoV. The threshold cycle (Ct) values of every dilution for each virus were determined by duplicate PCR tests.

The limits of detection with Ct values for each virus were as follows: 10¹ TCID₅₀/ml with 38.0 cycles for RSV subgroup A, 10¹ TCID₅₀/ml with 36.1 cycles for RSV subgroup B, 10¹ TCID₅₀/ml with 37.2 cycles for Flu A, 10¹ TCID₅₀/ml with 39.2 cycles for PIV2, 10² TCID₅₀/ml with 36.6 cycles for PIV3, 10¹ TCID₅₀/ml with 39.4 cycles for AdV, and 10¹ TCID₅₀/ml with 38.2 cycles for CoV.

From these results, the limitation of Ct values estimated to be positive for the corresponding virus was defined as 40 or fewer amplification cycles. The specificities of the MB probe and primer sets for the 13 viruses after 40 cycles of amplification are shown in Table 2. No nonspecific positive results were obtained from those viruses selected at random from laboratory stock cDNA, and the specificity of primers and probes was high.

The sensitivity and specificity of real-time RT-PCR were also compared with the results obtained with an "MPCR kit for respiratory infection associated viruses set-3" (Maximbio, San Francisco, CA, USA) that is designed to identify seven viruses; namely, RSV, Flu A, Flu B, PIV1,

PIV2, PIV3, and AdV in one tube. The reactions were performed according to the manufacturer's protocol, using 1.5 µl of cDNA. The sensitivity and specificity of real-time RT-PCR relative to those of conventional PCR for the seven viruses were 96.3%–100% and 84.1%–98.9, respectively (data not shown).

Real-time PCR for bacteria

Six pathogens: *S. pneumoniae*, *H. influenzae*, *M. pneumoniae*, *C. pneumoniae*, *S. pyogenes*, and *Legionella pneumophila* were identified using the real-time PCR with a Respiratory Tract Infection (RTI) kit (Code: CY214; Takara Bio, Kyoto, Japan) as described previously.⁵ The total time from DNA extraction to the finish was 1.5 h.

Bacterial cultures

Bacterial culture and species identification were performed according to the *Manual of clinical microbiology*.³⁶

Results

Etiological agents

Table 3 shows the etiological agents found in 1700 CAP patients who had visited the hospital within 5 days of symptom onset (mean, 3.8 days). These etiological classifications were made according to the tentative criteria described by Nakayama et al.⁶

Single bacterial infection was identified in 32.5% ($n = 553$) of the 1700 patients, multiple bacterial infection was identified in 1.9% ($n = 32$), mixed infection of bacteria and viruses was identified in 15.2% ($n = 258$), single viral infection was identified in 25.8% ($n = 438$), and multiple viral infection was identified in 2.1% ($n = 35$). Infections for which the etiological agent was unknown occurred in 22.6% ($n = 384$) of all the patients examined here.

Table 4 shows the bacteria suspected to be the causative pathogens and the differentiation whether they were single infections or coinfections. Bacterial infection accounted for 49.6% ($n = 843$) of all cases, among which 24.4% ($n = 415$) were caused by *S. pneumoniae*, 14.8% ($n = 251$) were caused by *M. pneumoniae*, 11.3% ($n = 192$) were caused by *H. influenzae*, 1.4% ($n = 24$) were caused by *C. pneumoniae*, and 0.1% ($n = 1$) were caused by *S. pyogenes*. Of the cases in which causative pathogens were suspected to be *S. pneumoniae*, *H. influenzae*, or *C. pneumoniae*, half were single infections and the others mostly showed coinfection with viruses. In contrast, *M. pneumoniae* infection cases were mostly single infections.

Table 3. Etiology in 1700 children with CAP

Pathogenic agents	<i>n</i> (%)
Bacterial (single)	553 (32.5)
Bacterial (multiple)	32 (1.9)
Bacterial and viral	258 (15.2)
Viral (single)	438 (25.8)
Viral (multiple)	35 (2.1)
Unknown	384 (22.6)
Total	1700 (100.0)

Table 4. bacterial pathogens suspected as etiological agents with high probability in pediatric patients with CAP

Pathogen	Total (%) ^a	Single infection (<i>n</i> = 553)	Coinfection with:		
			Bacteria (<i>n</i> = 32)	Virus (<i>n</i> = 252)	Bacteria and virus (<i>n</i> = 6)
<i>S. pneumoniae</i>	415 (24.4)	236 (56.9) ^b	30 (7.2)	144 (34.7)	5 (1.2)
<i>M. pneumoniae</i>	251 (14.8)	206 (82.1)	9 (3.6)	34 (13.5)	2 (0.8)
<i>H. influenzae</i>	192 (11.3)	95 (49.5)	24 (12.5)	68 (35.4)	5 (2.6)
<i>C. pneumoniae</i>	24 (1.4)	16 (66.7)	3 (12.5)	5 (20.8)	0
<i>S. pyogenes</i>	1 (0.1)	0	0	1	0

^aPercentage in 1700 patients

^bPercentage of each pathogen

Table 5. Viral pathogens identified by real-time RT-PCR in clinical samples from pediatric patients with CAP

Pathogen	Total (%) ^a	Single infection (<i>n</i> = 438)	Coinfection with:		
			Bacteria (<i>n</i> = 253)	Virus (<i>n</i> = 35)	Bacteria and virus (<i>n</i> = 5)
Rhinovirus	247 (14.5)	122 (49.4) ^b	101 (40.9)	22 (8.9)	2 (0.8)
Respiratory syncytial virus	159 (9.4)	107 (67.3)	44 (27.7)	7 (4.4)	1 (0.6)
Human metapneumovirus	125 (7.4)	84 (67.2)	30 (24.0)	9 (7.2)	2 (1.6)
Parainfluenza virus 1, 2, 3	122 (7.2)	74 (60.7)	37 (30.3)	10 (8.2)	1 (0.8)
Human bocavirus	50 (2.9)	17 (34.0)	18 (36.0)	13 (26.0)	2 (4.0)
Adenovirus	30 (1.8)	11 (36.7)	11 (36.7)	7 (23.3)	1 (3.3)
Others ^c	38 (2.2)	23 (60.5)	12 (31.6)	2 (5.3)	1 (2.6)

^aPercentage in 1700 patients

^bPercentage for each virus

^c“Others” denotes Flu A, Flu B, CoV, and EV

Table 6. Pathogenic agents identified in 1700 pediatric patients with CAP: distribution by age

Pathogens	Total no. of positive cases	Age ^a	Number of isolates (%)						
			<1 year	1 year	2 years	3 years	4 years	5 years	≥6 years
Bacteria									
<i>S. pneumoniae</i>	415	2.5 ± 2.3	77 (18.6)	120 (28.9)	60 (14.5)	63 (15.2)	40 (9.6)	18 (4.3)	37 (8.9)
<i>H. influenzae</i>	192	2.4 ± 1.9	35 (18.2)	53 (27.6)	30 (15.6)	23 (12.0)	26 (13.5)	10 (5.2)	15 (7.8)
<i>M. pneumoniae</i>	251	6.1 ± 3.7	7 (2.8)	24 (9.6)	22 (8.8)	23 (9.2)	22 (8.8)	17 (6.8)	136 (54.2)
<i>C. pneumoniae</i>	24	5.4 ± 3.8	3 (12.5)	3 (12.5)	2 (8.3)	0 (0.0)	2 (8.3)	2 (8.3)	12 (50.0)
Virus									
Rhinovirus	247	2.9 ± 2.7	42 (17.1)	61 (24.8)	36 (14.6)	30 (12.2)	28 (11.4)	13 (5.3)	37 (15.0)
Respiratory syncytial virus	159	1.6 ± 1.4	52 (32.7)	47 (29.6)	28 (17.6)	20 (12.6)	8 (5.0)	1 (0.6)	3 (1.9)
Human metapneumovirus	125	2.4 ± 1.9	15 (12.0)	35 (28.0)	21 (16.8)	24 (19.2)	20 (16.0)	6 (4.8)	4 (3.2)
Parainfluenzavirus 1, 2, 3	122	2.3 ± 2.6	28 (23.0)	45 (36.9)	9 (7.4)	16 (13.1)	11 (9.0)	3 (2.5)	10 (8.2)
Human bocavirus	50	1.3 ± 0.9	14 (28.0)	27 (54.0)	5 (10.0)	2 (4.0)	1 (2.0)	1 (2.0)	0
Adenovirus	30	1.5 ± 2.7	9 (29.6)	6 (50.1)	1 (11.5)	6 (4.6)	1 (2.4)	3 (2.4)	4 (0.1)
Others ^b	38	2.7 ± 2.2	6 (15.8)	12 (31.6)	3 (7.9)	3 (7.9)	4 (10.5)	2 (5.3)	8 (21.1)

^aMedian ± SD^b“Others” denotes Flu A, Flu B, EV and CoV**Table 7.** Diagnostic value of WBC count and CRP according to etiological agent

Pathogens	<i>n</i>	WBC (×10 ³ /mm ³)		CRP (mg/dl)	
		Median	Range	Median	Range
Bacterial					
<i>S. pneumoniae</i>	235	17.1	5.6–39.9	5.4	0.1–26.2
<i>H. influenzae</i>	94	13.2	5.6–29.1	3.2	0.1–17.5
<i>M. pneumoniae</i>	189	6.6	2.3–19.8	1.8	0.1–13.4
<i>C. pneumoniae</i>	16	8.8	4.2–12.8	0.2	0.0–0.6
Bacterial + viral	216	14.5	6.2–35.6	3.7	0.1–19.3
Viral					
Rhinovirus	116	11.0	4.0–21.4	0.6	0.0–5.9
Respiratory syncytial virus	101	8.8	3.4–22.8	0.8	0.0–9.5
Human metapneumovirus	82	6.9	2.7–17.5	0.8	0.0–10.0
Parainfluenza virus 1, 2, 3	70	7.9	2.6–19.1	0.7	0.0–8.1
Human bocavirus	17	10.5	5.6–17.6	0.5	0.0–1.9
Adenovirus	11	12.1	7.4–26.5	4.0	0.7–8.4
Influenza virus	10	8.1	5.3–11.3	1.1	0.4–3.8

Patients infected with *S. pyogenes*, EV, and CoV were few, so they are not included

Table 5 shows the identified viral pathogens. Viral infection accounted for 43.0% ($n = 731$) of all cases. The cumulative positive cases determined by real-time RT-PCR showed that RV accounted for 14.5% ($n = 247$) of the viral infections; RSV, for 9.4 % ($n = 159$); hMPV for 7.4 % ($n = 125$); PIV for 7.2% ($n = 122$); HBoV for 2.9% ($n = 50$); and AdV for 1.8% ($n = 30$; the remaining viruses [Flu A, Flu B, CoV, and EV] are combined as “others” in Table 5).

The percentages of single viral infections were relatively higher for RSV (67.3%), hMPV (67.2%), and PIV (60.7%) than for RV (49.4%) and HBoV (34.0%).

Distribution of pathogenic agents according to age

Table 6 shows the distribution of each pathogenic agent identified according to patient age. The median ages of patients who were infected with *S. pneumoniae* and *H. influenzae* were 2.5 and 2.4 years, respectively, and the median ages of those infected with *M. pneumoniae* and *C. pneumoniae* were 6.1 and 5.4 years, respectively. The median ages of patients positive for infection with RSV, HBoV, and AdV

ranged from 1.3 to 1.6 years, while the median ages of those positive for infection with RV, hMPV, and PIV were slightly higher.

WBC count and CRP according to etiological agents

Both the WBC count and CRP were determined in 1157 of the 1700 patients on the same day as the sample collection for PCR.

Table 7 shows the median values and ranges for the WBC count and CRP in the patients with bacterial infections, those with bacterial and viral coinfections, and those with viral infections.

In the patients with *S. pneumoniae* or *H. influenzae* infection, and bacterial and viral coinfection, the median values were comparatively high, at $13.2\text{--}17.1 \times 10^3/\text{mm}^3$ for WBC and 3.2–5.4 mg/dl for CRP. In contrast, these values in the patients with *M. pneumoniae* and *C. pneumoniae* infections were apparently low, at $6.6 \times 10^3/\text{mm}^3$ and $8.8 \times 10^3/\text{mm}^3$, respectively, for WBC, and 1.8 mg/dl and 0.2 mg/dl, respectively, for CRP.

The median values for the WBC count and CRP in the patients who were positive for viruses were all $11.0 \times 10^3/\text{mm}^3$ or less and 1.1 mg/dl or less, respectively, except for AdV.

Seasonal epidemiology of viruses

Figure 1 shows the seasonal epidemiology for RSV, Flu, PIV, RV, hMPV, and HBoV viruses by month over the 2 years from January 2005 to December 2006. The percentage for each virus is given as the ratio per month in the CAP cases. The HBoV shown in this Fig. was examined for only 1 year, beginning in January 2006.

RSV was detected mainly from August to January, corresponding to the late summer to winter season. Although the numbers were small, Flu was detected from February to March in the winter season. PIV3 accounted for 86.1% of all the PIV cases, and they predominated from May to July during the spring to summer season. RV was detected in all months of the year, but the peak incidence was observed from August to September. hMPV peaked predominantly from February to July in early spring to summer, while HBoV was detected from May to June.

Discussion

Antimicrobial resistance in *S. pneumoniae*,³⁷ *H. influenzae*,^{38,39} and *M. pneumoniae*⁴⁰ in CAP poses major clinical problems in the treatment of patients. Therapeutic antibiotics administered to patients upon hospitalization are usually selected empirically, based on the likely etiological agent after viewing of the chest X-ray, hematological studies, consideration of the history, respiratory symptoms, and age of the patient. To avoid inappropriate antimicrobial therapy and to select the most appropriate agent, guidelines for CAP in pediatric and adult patients have been proposed in Japan,^{41,42} as well as in other countries.⁴³

However, we often encounter a patient in whom it is difficult to predict the causative pathogen from the clinical findings described above. It is preferable that the etiological agent be identified on the day of hospitalization, so that the diagnosis of CAP can be made without waiting for bacterial cultures.

Recently, real-time PCR for *M. pneumoniae*, *C. pneumoniae*, and *L. pneumophila* causing pneumonia,^{5,44} and real-time PCR for respiratory viruses,^{24,26} have been receiving attention as effective methods. To detect or to identify etiological agents accurately in children with CAP, it is necessary to improve the comprehensive PCR detection of both bacteria and viruses from one sample in order to lighten the load of the patient and improve cost-effectiveness.

As described in the "Methods" section, an important characteristic of our examination protocol included a labor-saving procedure for the extraction of DNA and RNA from the clinical samples, using an Extragen II kit (TOSOH), that took less than 10 min. The extracted

samples were divided and used for real-time PCR for bacterial detection and real-time RT-PCR for viral detection. The real-time PCR results for these etiological agents can be obtained within 3 h from the time of the receipt of clinical samples.

Recently, we further improved the methods of cDNA synthesis using a new enzyme, PrimeScript RTase (TakaraBio, Tokyo, Japan), so that the synthesis can be finished in 15 min. Accordingly, the results of bacterial and viral analysis by real-time PCR can be obtained within 2.0 h at present. If these improved methods are applied to laboratory examination, it is expected that they could contribute greatly to the use of appropriate antimicrobial chemotherapy.

We note that, because sputum collection is rather difficult in children, compared with adults, nasopharyngeal samples are commonly used for real-time PCR in children. Although nasopharyngeal samples are appropriate for identifying viruses and *M. pneumoniae* and *C. pneumoniae*, such specimens are not always appropriate for the detection of *S. pneumoniae* and *H. influenzae*. This is because these bacteria are often isolated from nasopharyngeal sites in healthy controls as well. Positive results for bacteria obtained by real-time PCR should be carefully evaluated as to whether they are infection cases or whether the results reflect the presence of indigenous bacteria. Although WBC and CRP values may provide useful information to determine the etiological agents of CAP, further studies will be necessary to construct an appropriate indicator.

As previously reported by Nakayama et al.,⁶ 90% or higher percentages of patients who were PCR-positive for *M. pneumoniae*, *C. pneumoniae*, and viruses showed significantly high antibody titers to the corresponding pathogens. In other words, if the PCR result turns out to be positive, you may determine that the target is the causative agent.

In the present study, the percentage of cases in which *H. influenzae* was suspected to be the causative pathogen was apparently higher than in other studies.⁸ We assume that this reflects the situation in which *H. influenzae* type b (Hib) vaccination was not available during the period of this study. Hib vaccine was, however, approved in 2007 by the Japanese Ministry of Health Labor, and Welfare, and will be distributed in Japan in 2008.

hMPV¹⁴ and HBoV,¹⁹ which have been newly identified as etiological agents causing CAP, were included in our real-time PCR detection system for 13 viruses. Real-time PCR using pathogen-specific-MB probes that annealed to the inner portion of the amplified DNA products apparently improved the specificity of the assays compared to conventional PCR (data not shown).

Although it is well known that RSV and PIV are the main causative viruses in CAP patients, the comparatively high rates of 7.4% for hMPV and 2.9% for HBoV, shown in the "Results" section, were notable in this study. Epidemics due to hMPV may have occurred during our study period. The age peak for RSV or HBoV infection was at 1 year old or less, but that for hMPV was slightly higher. In addition, the prevailing viruses differ depending on the season, which may serve as a good reference to determine CAP causative viruses.

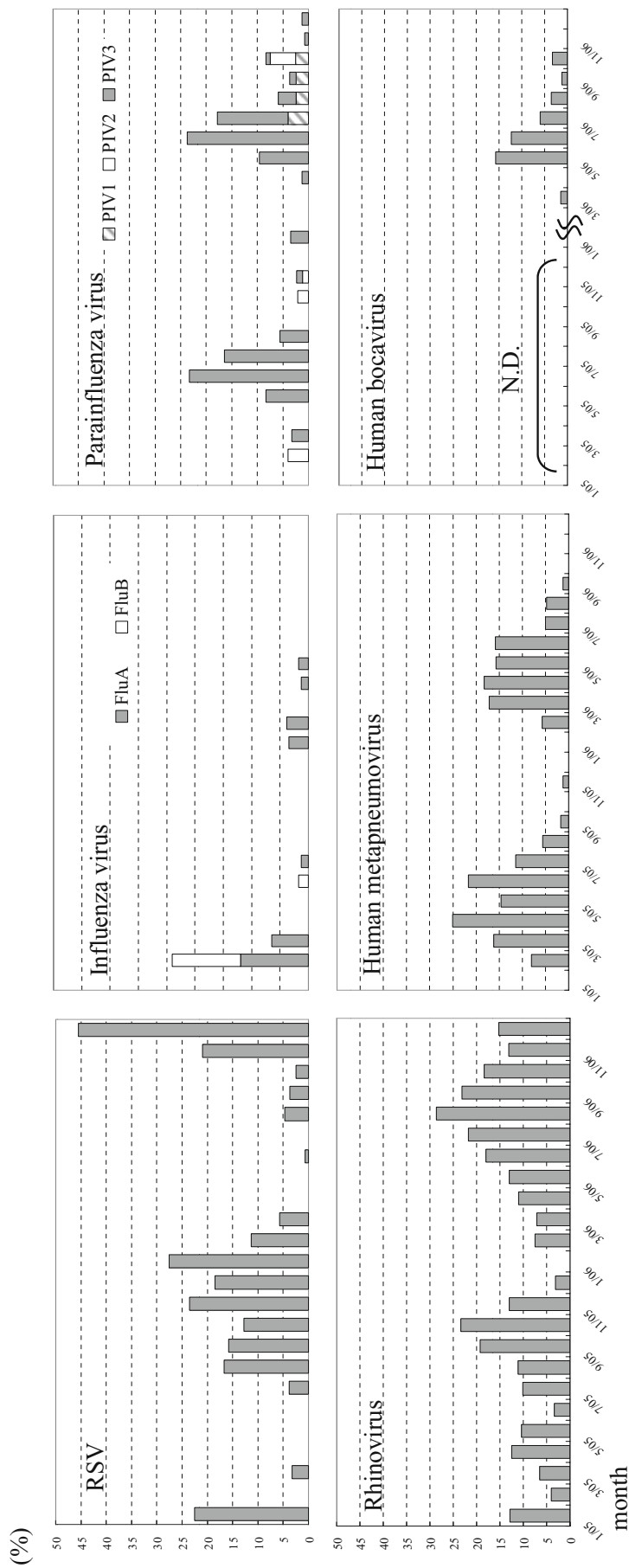


Fig. 1. Monthly prevalence of respiratory syncytial virus (RSV), influenza virus (Flu), parainfluenza virus (PIV), rhinovirus, human metapneumovirus, and human bocavirus from January 2005 through December 2006. N. D., no data

Finally, we anticipate that real-time RT-PCR can be developed to be a multiplex and one-step method, in order to establish a rapid and comprehensive system for the identification of viruses and bacteria.

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