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ORIGINAL ARTICLE

A practical approach estimating etiologic agents using real-time PCR in pediatric inpatients with community-acquired pneumonia

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Abstract To evaluate pathogens in pediatric inpatients with community-acquired pneumonia (CAP), an Acute Respiratory Diseases Study Group organized by ten Japanese medical institutions devised a rapid, reliable process based on real-time PCR results in nasopharyngeal swab samples plus admission blood test results. From April 2008 to April 2009, we enrolled 903 children with CAP based on chest radiographs and clinical findings who were hospitalized within 5 days of onset. Comprehensive real-time PCR was used to detect 6 bacteria and 11 respiratory viruses. The swab specimens also were used for bacterial cultures. After initial determination of presence or absence of viral and mycoplasmal infections, significant bacterial contributions were defined by bacterial identification, clinical efficacy of antimicrobial agent, and reference to blood test results. Children were stratified by age: below 1 year, 1 year, 2–5 years, or at least 6 years old. Among patients studied, 34.4 % were diagnosed with viral infection; 21.8 %, bacterial infection; 17.5 %, viral/bacterial

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co-infection; 5.9 %, mycoplasmal infection; 0.3 %, mycoplasmal/bacterial co-infection; and 1.7 %, viral/mycoplasmal co-infection. The remaining 18.4 % had unknown pathogens. Purely viral infection was suggested mainly in infants younger than 1 year; mycoplasmal infection typically occurred in children at least 6 years old. Our results suggest usefulness of real-time PCR for nasopharyngeal samples together with blood tests in estimating etiologic agents in clinical settings.

Keywords Real-time PCR · Community-acquired pneumonia · Pediatric inpatient · Comprehensive detection · Etiologic agents

Introduction

Pediatricians commonly encounter children with community-acquired pneumonia (CAP) in outpatient settings. Etiologic agents for CAP are various; several viruses can cause primary respiratory infections, whereas bacteria produce mainly secondary infections. Respiratory viruses most responsible for CAP vary according to patient age, season, and epidemic environment.

Lower respiratory tract specimens that are informative regarding pathogens are difficult to obtain consistently from children with CAP. Nasopharyngeal swab (NPS) samples represent a practical alternative but are limited by the potential presence of *Streptococcus pneumoniae* and *Haemophilus influenzae* among the normal flora of the upper respiratory tract [1].

Recently, methods combining multiplex real-time polymerase chain reaction (PCR) and a fluid microbead-based assay have identified 20 respiratory viruses [2]. We previously reported a new protocol to comprehensively identify microorganisms within 2.5 h, including real-time PCR for 6 bacteria [3] and reverse transcription (RT) real-time PCR for 12 viruses [4] using specific molecular beacon probes.

In this study, we set out to incorporate real-time PCR in a practical method for estimating etiologic agents of CAP in children using noninvasively obtained NPS specimens. Interpretation of results consisted of two steps: the first was to determine whether a virus, an atypical bacterial organism such as *Mycoplasma pneumoniae*, or both contributed to CAP onset; the next step was to estimate the significance of any typical bacterial contribution. Determination whether bacterial contribution was present was based on both organism identification and blood test results, according to Japanese guidelines published in 2007 [5].

Subjects eligible for our investigation were pediatric patients with pulmonary infiltrates demonstrated by chest radiography who were hospitalized within 5 days after symptom onset. Combining results of real-time PCR in NPS specimens with admission blood tests, we estimated etiologic agents with high accuracy in CAP patients.

Subjects and methods

Patients and clinical samples

Thirteen pediatricians affiliated with ten medical institutions participated actively in an Acute Respiratory Diseases (ARD) study from April 2008 to April 2009. All Japanese pediatric patients with CAP were enrolled in this study if they were 1 month to 15 years old, diagnosed based on pulmonary infiltrates in chest radiographs, hospitalized within 5 days from onset of fever, and had attenuated or abnormal breath sounds consistent with the 2007 Japanese guidelines for management of respiratory infectious diseases in children [5].

Our research protocol was approved by the review board of the National Hospital Organization Tokyo Medical Center. After informed consent was obtained from the parents, NPS samples were collected from all subjects by the pediatricians, and sent immediately to our laboratory at Kitasato Institute for Life Sciences together with a study entry form that was completed by the pediatrician but maintained patient anonymity. Real-time PCR and bacterial cultures for every sample were performed at Kitasato Institute just after their receipt according to a previously described protocol [3, 4]. Hematological and serological tests including white blood cell (WBC) count, neutrophil count, lymphocyte count, C-reactive protein (CRP), and acute-phase serum antibody titers for several pathogens were determined routinely for all patients at the time of admission to each hospital. After about 2 weeks, serum was resampled for convalescent-phase titers.

Real-time PCR to detect viruses and bacteria

Clinical samples suspended in 0.5 ml pleuropneumonia-like organism (PPLO) broth (Difco, Detroit, MI, USA) were used as a starting sample for extraction of DNA and RNA and for bacterial culture. Steps from DNA/RNA extraction to real-time PCR are shown in Fig. 1.

A CycleavePCR kit (Takara Bio, Shiga, Japan; catalog number CY216) containing reverse transcription (RT) reagent to produce cDNA from RNA was used for viral identification. Cycleave probes were labeled with a fluorescent reporter, 6-carboxyfluorescein (FAM) or carboxy-X-rhodamine (ROX), at the 5′-end, and with eclipse dark quencher at the 3′-end, to search for 11 respiratory viruses (1 or 2 per well) as follows: respiratory syncytial virus (RSV) subgroup A (RSV-A) and RSV subgroup B (RSV-B); parainfluenza virus (PIV) type 1 (PIV1) and PIV type 2 (PIV2); PIV type 3 (PIV3) and human metapneumovirus (hMPV); influenza virus (FLU)

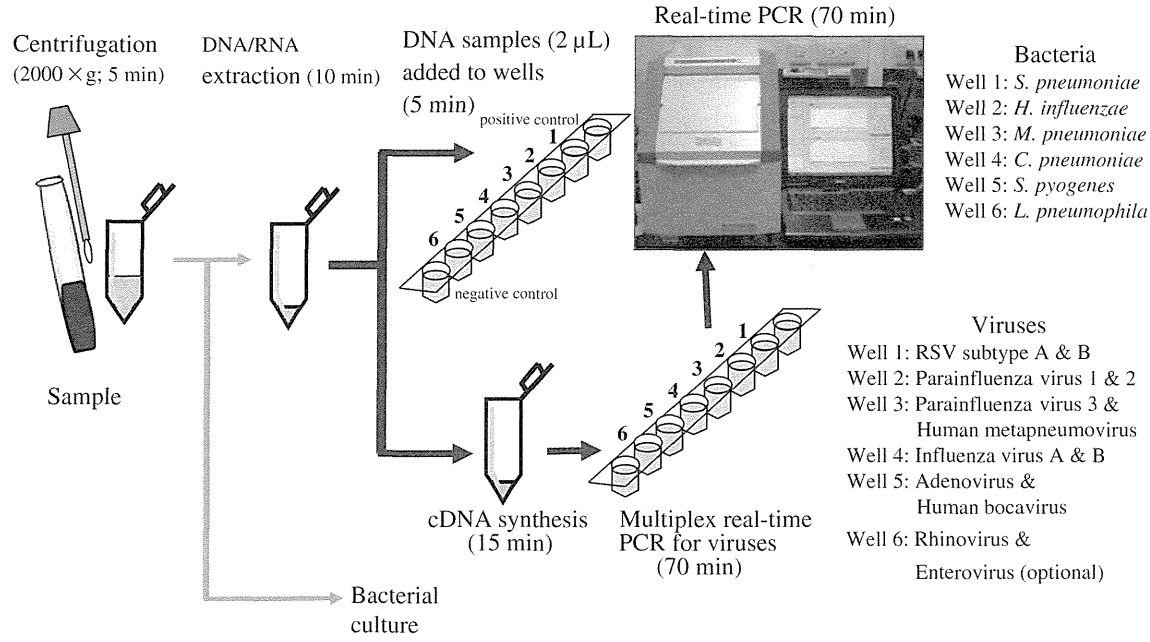

Fig. 1 Protocol for comprehensive viral and bacterial detection in clinical nasopharyngeal swab samples by real-time polymerase chain reaction (PCR). DNA/RNA samples were extracted using Extragen II (Tosoh, Tokyo, Japan). Two CycleavePCR kits, CY216 for viruses and CY214 for bacteria (Takara Bio, Shiga, Japan), were adapted. The swab samples also were used for bacterial cultures. Total PCR time from initial sample handling to reporting to each pediatrician was kept within 2 h

type A (FLU A) and FLU type B (FLU B); adenovirus (ADV) and human bocavirus (HBoV); and rhinovirus (RV), with an option to include enterovirus (EV) [4].

PCR sensitivity represented about 10 plaque-forming units (pfu) per well for RSV, PIV, and FLU [4]. The RT reaction was carried out at 37 °C for 15 min using a thermal cycler (Gene Amp PCR System 9600-R; Perkin-Elmer Cetus, Norwalk, CT, USA). Viral cDNA samples were brought to a final volume of 40 μl by adding 25 μl distilled water (DW) to the RT reaction tube.

Another CycleavePCR kit (catalog number CY214) was used to identify six bacterial pathogens: *S. pneumoniae*, *H. influenzae*, *M. pneumoniae*, *Chlamydophila pneumoniae*, *Streptococcus pyogenes*, and *Legionella pneumophila*. The sensitivity of this kit similarly has been described to be about 10 colony-forming units (cfu) per well [3].

Before amplification, 2.0 μl cDNA or DNA sample was added to wells containing the reaction mixture and kept at 4 °C until amplification was begun at 95 °C for 30 s, followed by 45 cycles of PCR: 95 °C for 15 s, 55 °C for 30 s, 75 °C for 15 s, using an Mx3000P (Stratagene, La Jolla, CA, USA) or a Thermal Cycler Dice TP800 (Takara Bio, Shiga, Japan). Total time from DNA/RNA extraction to reporting was 2.0 h.

Bacterial cultures

Bacterial culture including *M. pneumoniae* and culture-based species identification were carried out according to the *Manual of Clinical Microbiology* [6].

Categorization to assess etiologic agents

Our process for estimating agents causing pediatric CAP and number of cases classified into each category is shown in Fig. 2.

First, we assessed the categories of viral and mycoplasmal infection based on detection of the agents using both RT real-time PCR and real-time PCR, as appropriate. In this step, etiologic agents initially were classified as viral, mycoplasmal, mixed viral and mycoplasmal, or other.

Second, we evaluated the significance of bacterial contributions by semiquantitative bacterial load in NPS using a real-time PCR and bacterial cultures, defervescence within 24 h after administration of antimicrobial agent, and reference to blood test results for WBC, neutrophil counts, and CRP [5, 7]. Positive bacterial blood cultures were considered to indicate a significant bacterial contribution.

Categories were finally classified into (1) viral infection, (2) viral and bacterial co-infection, (3) mycoplasmal infection, (4) mycoplasmal and viral co-infection, (5) mycoplasmal and bacterial co-infection, (6) bacterial infection, and (7) unknown. The category of "unknown" was included when blood test data did not fulfill the foregoing criteria, even though bacteria were detected in NPS samples.

Statistical analyses

We used Microsoft Excel 2010 for Statistics (SSRI, Tokyo, Japan) and Prism Version 5.0 (GraphPad Software, La Jolla, CA, USA) for data analyses. WBC and neutrophil

counts were analyzed using box-and-whisker plots, as was CRP. Categorical variables were compared using chi-square tests, with a P value below 0.05 indicating a significant difference between groups.

Results

Etiologic category by age group in enrolled subjects

A total of 903 inpatients enrolled in our investigation included 219 (24.3 %) <1 year old; 283 (31.3 %) 1 year old; 283 (31.3 %) from 2 to 5 years old; and 118 (13.1 %) at least 6 years old.

Pathogens estimated to be etiologic agents are categorized by age group are in Table 1. These agents included viral infection (34.4 %), viral and bacterial co-infection (17.5 %), bacterial infection (21.8 %), mycoplasmal infection (5.9 %), mycoplasmal and viral co-infection (1.7 %), and mycoplasmal and bacterial co-infection (0.3 %). In the remaining 18.4 % of patients, the causative pathogen was considered unknown.

Apparently pure viral infection was most common among patients ≤1 year old, and least common in children at ≥6 years old. Consistently, the rate of viral and bacterial co-infection decreased at school age. On the other hand, percentages for bacterial infection exceeded 20 % irrespective of age group. Single or mixed mycoplasmal infections were rare among patients ≤1 year old, whereas the rate of single mycoplasmal infection was 32.2 % in children at ≥6 years old.

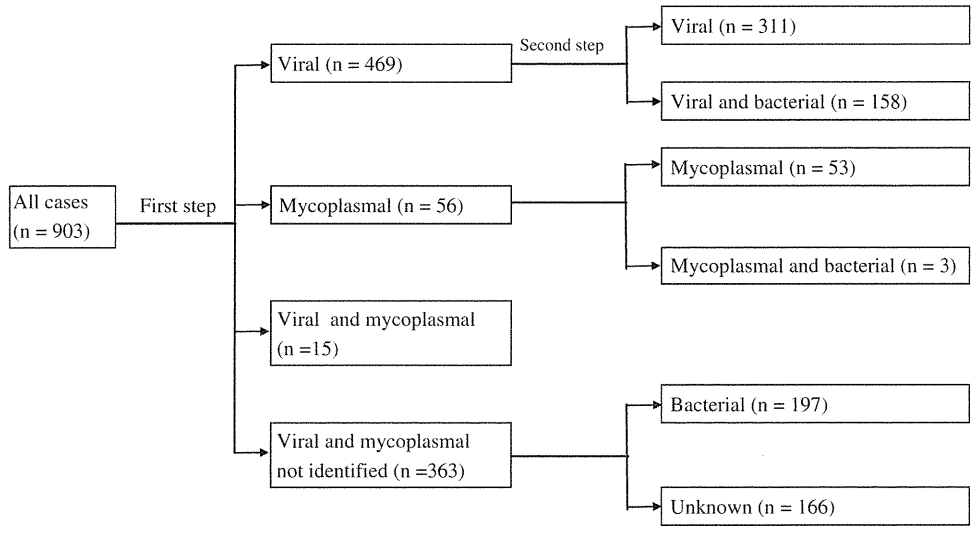

Fig. 2 Categorization of etiologic agents in pediatric inpatients with community-acquired pneumonia (CAP). Patients were classified into (1) viral infection, (2) viral and bacterial co-infection, (3) mycoplasmal infection, (4) mycoplasmal and viral co-infection, (5) mycoplasmal and bacterial co-infection, (6) bacterial infection, and (7) unknown. Viral and mycoplasmal identification in first step was assessed by RT real-time PCR or real-time PCR for nasopharyngeal swab (NPS) samples. Bacterial contribution was assessed totally from clinical effect of antibiotics, and blood test results, in addition to semiquantitative real-time PCR for NPS sample

Table 1 Pathogens identified as etiologic agents in 903 pediatric patients with community-acquired pneumonia (CAP), according to age

Etiologic agent	Classification by age: number of cases (%)				Total
	<1 year	1 year	2–5 years	≥6 years	
Viral	95 (43.4)	113 (40.0)	89 (31.4)	14 (11.9)	311 (34.4)
Viral and bacterial	34 (15.5)	66 (23.3)	53 (18.7)	5 (4.2)	158 (17.5)
Bacterial	47 (21.5)	63 (22.3)	69 (24.4)	18 (15.3)	197 (21.8)
Mycoplasmal	0	2 (0.7)	13 (4.6)	38 (32.2)	53 (5.9)
Mycoplasmal and viral	1 (0.5)	0	8 (2.8)	6 (5.1)	15 (1.7)
Mycoplasmal and bacterial	0	2 (0.7)	1 (0.4)	0	3 (0.3)
Unknown	42 (19.2)	37 (13.1)	50 (17.7)	37 (31.3)	166 (18.4)
Total	219	283	283	118	903

CAP community-acquired pneumonia

Viral contribution by age group

Table 2 shows viral pathogens according to age group. Based on results of RT real-time PCR, 484 patients (53.6 %) were determined to have single ($n = 449$) or mixed viral infections ($n = 35$). In these patients, viral loads determined semiquantitatively by RT real-time PCR assay 70.1 % were found to be 3(+), corresponding to at least 10^4 particles per well. Common viral contributors were RSV A (30.8 %), followed by RV (28.9 %) and PIV 1–3 (12.9 %); others were RSV B (9.1 %), HBoV (8.3 %), EV (3.5 %), FLU A/B (3.5 %), and ADV (2.7 %). Together, RSV A and B accounted for 39.9 %, RSV or PIV represented mainly single viral infection, and RV or HBoV usually was involved in viral/bacterial co-infection (Supplement 1).

Rates for RV and PIV involvement varied little by age. In contrast, viral contributions by RSV A or B were common among children 5 years old or younger, but much less so in patients more than 5 years old. The rate of HBoV involvement was highest in 1-year-old patients. Rates of FLU A and B contributions increased at school age.

Bacterial contribution by age group

Bacterial contributions are classified by age group in Table 3. Based on categorization to assess etiologic agents described materials and methods, 423 patients (46.8 %) had single or mixed bacterial infections. Common bacterial contributions included *S. pneumoniae* (46.6 %), followed by *H. influenzae* (17.0 %), *S. pneumoniae* and *H. influenzae* (17.5 %), and *M. pneumoniae* (16.1 %). *C. pneumoniae*, *S. pyogenes*, and *S. aureus* involvement was very infrequent for each species.

In children of ages ≤5 years old, the most common contributors were *S. pneumoniae* and *H. influenzae*; such involvement was far less frequent among patients ≥6 years old. Single infection by *M. pneumoniae* (65.7 %) was most common at school age, with only very rare involvement of patients ≤1 year old.

Characteristics of blood test results according to etiologic category and age group

Distributions of WBC and CRP values together with the median, 25th percentile, and 75th percentile values as

Table 2 Viral pathogens identified by reverse transcription (RT) real-time polymerase chain reaction (PCR) in clinical samples from pediatric inpatients with CAP, by age

Viral pathogen[a]	Classification by age: number (%)				Total
	<1 year	1 year	2–5 years	≥6 years	
Rhinovirus	50 (35.5)	38 (19.9)	50 (31.3)	12 (44.4)	150 (28.9)
Respiratory syncytial virus A	45 (31.9)	66 (34.6)	48 (30.0)	1 (3.7)	160 (30.8)
Respiratory syncytial virus B	14 (9.9)	19 (9.9)	13 (8.1)	1 (3.7)	47 (9.1)
Influenza virus A and B	1 (0.7)	0	9 (5.6)	8 (29.6)	18 (3.5)
Parainfluenza virus 1, 2, and 3	15 (10.6)	29 (15.2)	21 (13.1)	2 (7.4)	67 (12.9)
Human bocavirus	9 (6.4)	27 (14.1)	7 (4.4)	0	43 (8.3)
Human metapneumovirus	1 (0.7)	0	1 (0.6)	0	2 (0.4)
Enterovirus	4 (2.8)	10 (5.2)	4 (2.5)	0	18 (3.5)
Adenovirus	2 (1.4)	2 (1.0)	7 (4.4)	3 (11.1)	14 (2.7)
Total	141	191	160	27	519

CAP community-acquired pneumonia

[a] Include 35 cases identified to be positive to two viruses

Table 3 Bacterial pathogens highly considered to be etiologic agents in pediatric patients with CAP

Pathogen	Classification by age: number (%)				Total
	<1 year	1 year	2–5 years	≥6 years	
S. pneumoniae	45 (55.6)	77 (57.9)	68 (47.9)	7 (10.4)	197 (46.6)
H. influenzae	17 (21.0)	27 (20.3)	21 (14.8)	7 (10.4)	72 (17.0)
Spn and *Hflu*	18 (22.2)	24 (18.0)	28 (19.7)	4 (6.0)	74 (17.5)
M. pneumoniae	1 (1.2)	2 (1.5)	21 (14.8)	44 (65.7)	68 (16.1)
Mpn and *Spn*	0	0	0	0	0
Mpn and *Hflu*	0	2 (1.5)	0	0	2 (0.5)
Mpn, *Spn*, and *Hflu*	0	0	1 (0.7)	0	1 (0.2)
Other[a]	0	1 (0.8)	3 (2.1)	5 (7.5)	9 (2.1)
Total	81	133	142	67	423

Spn Streptococcus pneumoniae, *Hflu Haemophilus influenzae*, *Mpn Mycoplasma pneumoniae*, *CAP* community-acquired pneumonia

[a] Other includes *Chlamydophila pneumoniae* and *Streptococcus pyogenes*

demonstrated by box-and-whisker plots are shown in Figs. 3 and 4, respectively, according to age group and the four main categories of agent identified by RT real-time PCR and real-time PCR for NPS samples; viral infection, bacterial infection (*S. pneumoniae* or *H. influenzae*), viral and bacterial co-infection, and single mycoplasmal infection. Similar analytical data for neutrophils in each age group are shown in Supplement 2.

Differences were significant for WBC, neutrophils, and CRP between viral infection and bacterial contribution in all age groups. In particular, 25th percentiles of WBC and neutrophil in both bacterial infection and viral and bacterial co-infection appeared greater than the 75th percentiles for viral infection. Based on these results, the cutoff value for presence of an important bacterial contribution was estimated at about to 13.0×10^3 cells/μl for WBC and 7.0×10^3 cells/μl for neutrophils. Although significant differences were recognized for CRP between viral infection and bacterial infection, no clear cutoff value was apparent. However, it was quite reasonable that the cutoff value for CRP was set at 3.0 mg/dl based on both 75th percentile value in viral infection (2.3 mg/dl) and 25th percentile value in bacterial infection (3.1 mg/dl).

If these cutoff values applied to classification of bacterial infection, bacterial contributions including either bacterial infections or viral and bacterial co-infection categories accounted for 34.4 % according to WBC plus neutrophils and CRP, 53.2 % according to WBC plus neutrophil counts, and 12.4 % according to CRP alone.

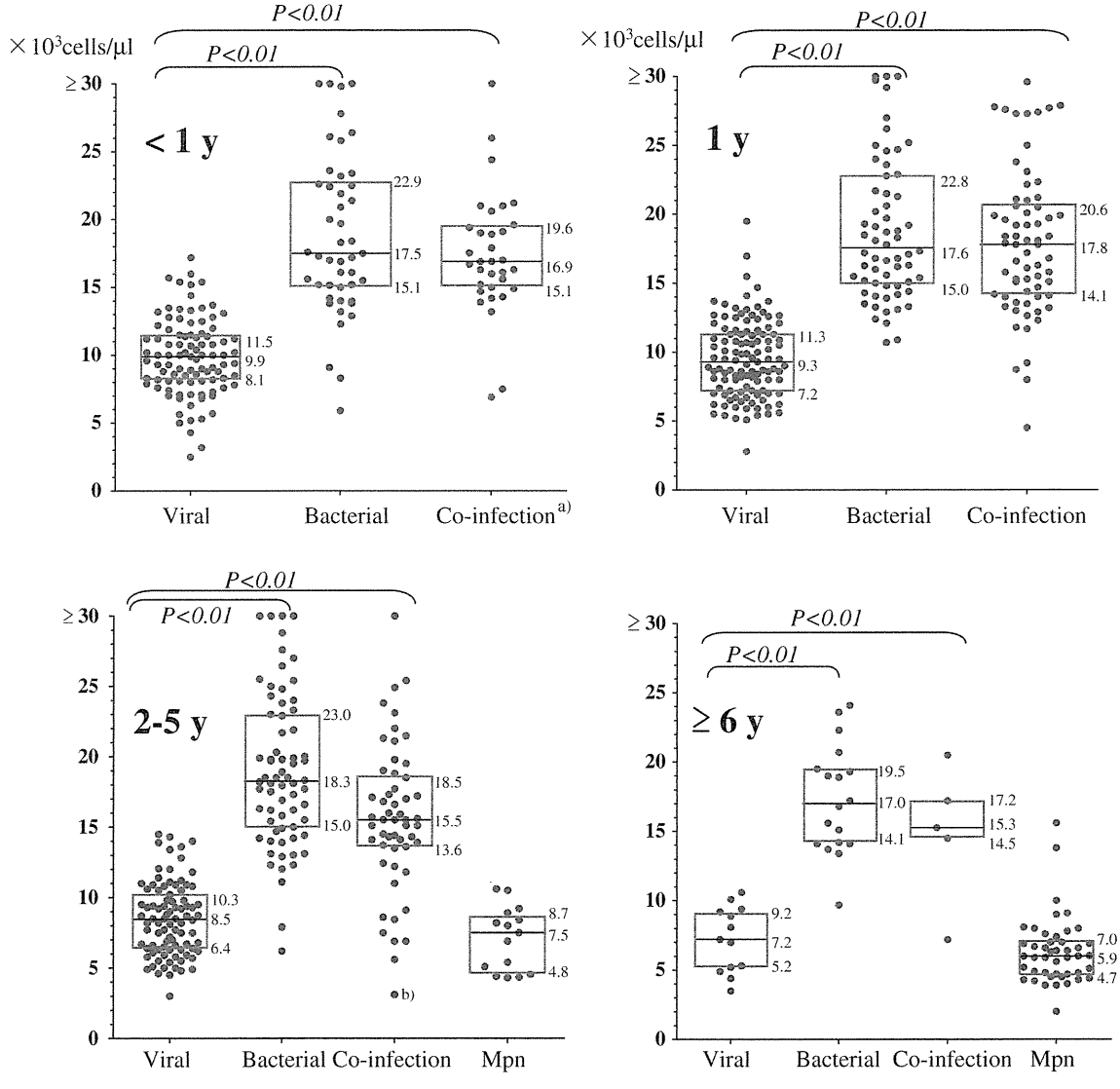

Fig. 3 Plots of white blood cell (WBC) count obtained on hospitalization are shown by age group and by etiologic category. In these *box-and-whisker plots*, 50 % of data are included in each box and the median is represented by a *horizontal line*. Categorical variables were compared using chi-squared tests: a *P* value below 0.05 indicated a significant difference between groups. [a]Co-infection, viral and bacterial co-infection; [b]a patient having disseminated intravascular coagulation (DIC) following pneumonia; *Mpn Mycoplasma pneumoniae*

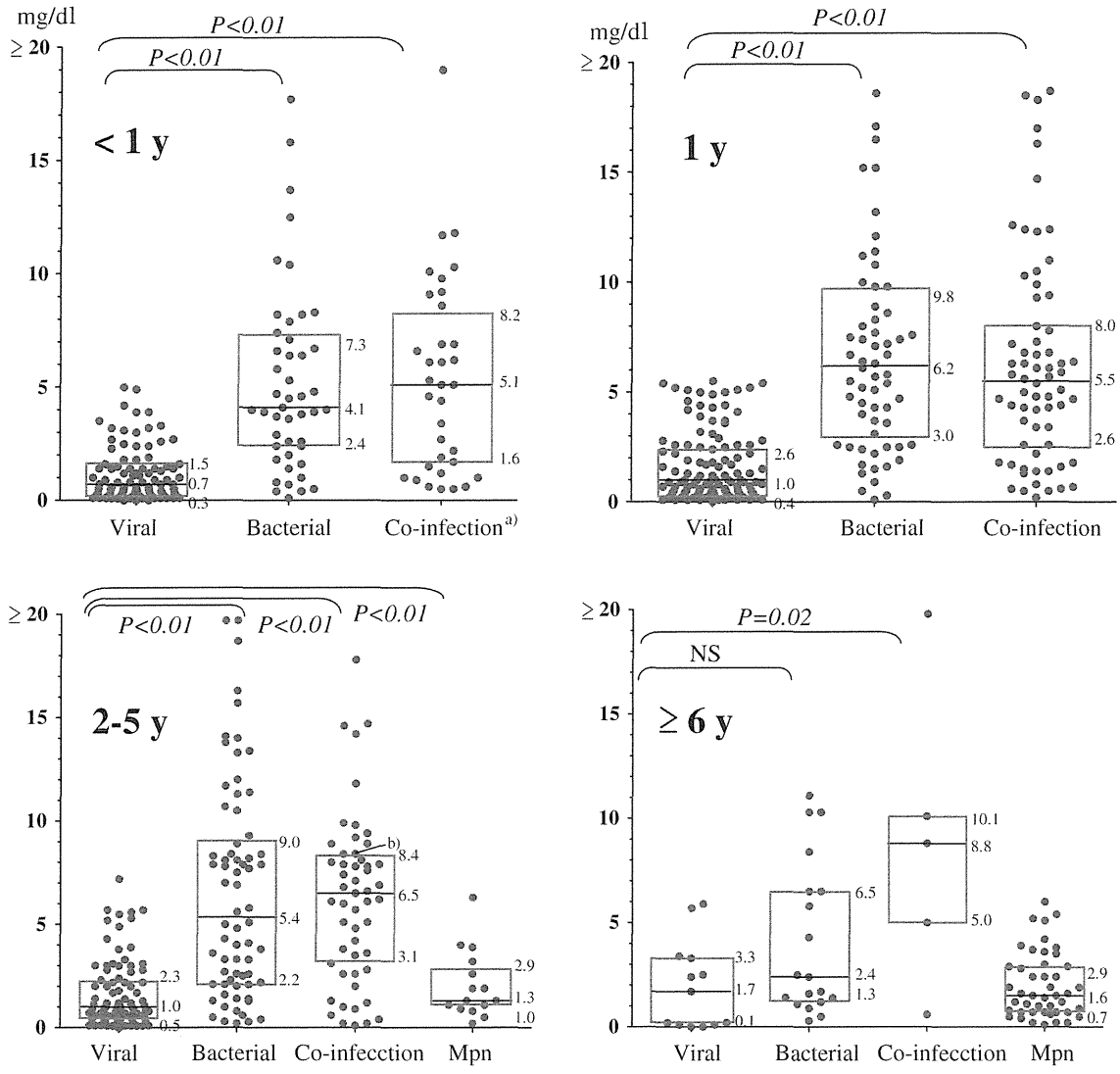

Fig. 4 Plots of C-reactive protein (CRP) concentration at time of hospitalization are shown by age group and by etiologic category. [a]Co-infection, viral and bacterial co-infection; [b]a patient having DIC following pneumonia; *Mpn Mycoplasma pneumoniae*

Discussion

Ideally, estimation of etiologic agents including discrimination among viral, mycoplasmal, or bacterial contributions should be completed within hours to guide antimicrobial treatment for pediatric inpatients with CAP [4, 7]. Such decision making in clinical settings is not easy, given the variety of causative microorganisms present in the respiratory tract [8], difficulties in obtaining good-quality specimens from children [9], and the very low rate of bacterial detection using blood cultures [10, 11].

Several recent studies concerning comprehensive viral detection with RT-PCR methods have been reported [12–14], but assessment of bacterial contributions except for *M. pneumoniae* [15] has shown little progress. For instance, a significant rise in antibodies between serum samples has been required to define the causative agent as *S. pneumoniae*, a frequent contributor to CAP in children [16–22]. Even such confirmation can be unavailable at many medical facilities.

A number of pediatricians in Japan currently determine CAP agents and select antimicrobial agents based on findings from chest radiographs, blood tests, and bacterial isolation using NPS. However, mere isolation of *S. pneumoniae* or *H. influenzae* from NPS cannot identify either as a causative agent, because both may colonize in the nasopharynx. In addition, empiric therapy for CAP threatens to create serious problems concerning the emergence of *S. pneumoniae*, *H. influenzae*, or *M. pneumoniae* resistant to antimicrobials [23–25].

To guide treatment of pediatric inpatients with CAP, we presently set out to establish a diagnostic flowchart for

rapid estimation of responsible agents based on comprehensive detection of both viruses and bacteria using real-time PCR of NPS samples. First, viral and mycoplasmal infections were evaluated, because detection of these organisms in NPS can result directly in etiologic determination. As a next step, significance of involvement by *S. pneumoniae* or *H. influenzae* needs to be assessed when either organism is detected in NPS. Several clinical trials reported distinguishing bacterial from nonbacterial infections based on CRP, but reliability was insufficient for determination in individual patients [26, 27]. Previously, combined increases in WBC, CRP, procalcitonin, and erythrocyte sedimentation rate have been reported as methods to estimate bacterial involvement [28, 29].

Our particular approach to assessment of bacterial contributions combined semiquantitative evidence of a heavy bacterial load using PCR and/or bacterial cultures with laboratory blood data at the time of admission. As described in the results, the 25th percentiles for WBC (13.6–15.1 $\times$ 10^3 cells/μl) and neutrophils (6.5–12.0 $\times$ 10^3 cells/μl) in cases with bacterial contribution apparent to exceed the 75th percentiles for WBC (9.2–11.5 $\times$ 10^3 cells/μl) and neutrophils (4.7–6.3 $\times$ 10^3 cells/μl) in the cases with viral infection; this permitted determination of cutoff values for these biomarkers indicating bacterial involvement. No clear cutoff value was found for CRP; the rise in CRP is known to occur 1 day after the rise in WBC [30] for WBC and for neutrophils.

Data not shown here, antibody titers against RSV, PIV, FLU, and ADV, were determined in paired sera from the acute and convalescent phase using the complement fixation (CF) test. However, the rise of antibody titers was not recognized in about 60 % of cases that were verified to have viruses by RT real-time PCR. Regarding *M. pneumoniae*, antibody titers were determined using the passive agglutination (PA) test; sensitivity and specificity of real-time PCR for antibody titers were 83 % and 95 %, respectively.

We were able to generally estimate etiologic agents in 81.6 % of 903 CAP inpatients at a high rate. Of those patients, 34.4 % had viral infection; 21.8 %, bacterial infection; 17.5 %, viral and bacterial co-infections; 5.9 %, mycoplasmal infection; and 2.0 %, mycoplasmal/viral or mycoplasmal/bacterial infections. These results suggest the usefulness of comprehensive RT real-time PCR and real-time PCR for NPS samples together with blood tests in estimating etiologic agents in clinical settings, especially for virus, *M. pneumoniae*, and *C. pneumoniae* identifications.

Bonzel and colleagues [12] reported dominant contributions of viruses (64.6 %), especially RSV infection (44.1 %), using comprehensive PCR-based detection in children with respiratory infections, including upper respiratory illnesses and bronchitis. Moreover, approximately 70 % viral detection, with the main agent being RSV, has been described recently in lower respiratory illnesses [13, 14]. In our study, the rate of viral involvement was slightly low, with the differences in detection rates probably reflecting differing site of the main respiratory illness: upper airway, bronchial, or alveolar.

Japanese guidelines for management of respiratory infectious diseases in children, including CAP, were published in 2007 [5]. Severe versus non-severe illnesses were distinguished based on patient age, respiratory condition, oxygen saturation, presence of labored breathing or cyanosis, and general condition as well as blood test results. The guidelines recommended antimicrobial therapy according to severity. Although evaluating severity is important, timely selection of antimicrobials based on determination of etiologic agents is essential.

In conclusion, we propose a practical combinated approach using RT real-time PCR for viruses and real-time PCR for bacteria as well as physical and blood test findings on admission as a way to improve management of pediatric CAP.

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Application of the Real-Time PCR Method for Genotypic Identification of β-Lactam Resistance in Isolates from Invasive Pneumococcal Diseases

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We sought to identify genotypic resistance classes by real-time PCR in 300 *Streptococcus pneumoniae* isolates from invasive pneumococcal diseases. Primers and molecular beacon probes were designed for the *lytA* gene, 3 *pbp* genes, and the *mef*A/*erm*B genes. Targeted sequences of *pbp1a*, *pbp2x*, and *pbp2b* genes in susceptible strain R6 corresponded to those of penicillin G-nonsusceptible strains, including sites within or adjacent to conserved amino acid motifs. If amplification did not occur, the corresponding penicillin-binding protein (PBP) was considered to possess amino acid substitution(s) affecting minimal inhibitory concentrations (MICs) of β-lactam antibiotics. Real-time PCR required 90 min or less. Strains were assigned to six genotypic classes: Genotypic penicillin-susceptible *S. pneumoniae* (gPSSP) with 3 normal genes (22.3%); genotypic penicillin-intermediate *S. pneumoniae* (gPISP) (*pbp2x*) with an abnormal *pbp2x* gene (25.3%); gPISP (*pbp2b*) with an abnormal *pbp2b* gene (7.3%); gPISP (*pbp1a+2x*) with abnormal *pbp1a+2x* genes (11.3%); gPISP (*pbp2x+2b*) with abnormal *pbp2x+2b* genes (4.7%); or genotypic penicillin-resistant *S. pneumoniae* (gPRSP) with 3 abnormal PBP genes (29.0%). Sensitivity and specificity of real-time PCR compared with those of conventional PCR were high, 73.7–100% and 97.7–100%, respectively. As for relationships between genotype and β-lactam MICs, 90% of MICs for every resistance class were distributed within three serial dilutions for almost all antibiotics. MICs of each β-lactam antibiotic were estimated with high probability from genotypic patterns. In conclusion, determination of genotypic classes of *S. pneumoniae* using rapid real-time PCR is useful in selecting effective therapeutic agents for patients with pneumococcal infection.

Introduction

Streptococcus pneumoniae is a leading etiologic agent in children and adults with severe invasive infections that contribute importantly to morbidity and mortality.[20,32] Strains resistant to penicillin G (PEN) have emerged and spread rapidly worldwide.[1,15]

In Japan, clinical isolates of PEN-resistant *S. pneumoniae* (PRSP) and PEN-intermediate *S. pneumoniae* (PISP) have increased rapidly since the late 1990s among school and preschool children as well as patients aged 65 years or older with either respiratory tract infections (RTI) or invasive pneumococcal diseases (IPD).[28,30] The mortality rate reportedly is higher in elderly IPD patients than in pediatric patients.[8]

Characteristically, PRSP and PISP strains show simultaneous resistance to cephalosporin antibiotics used in ambulatory practice.[30] The resistance mechanism for β-lactam antibiotics in PRSP and PISP is a decrease in affinities of three PEN-binding proteins (PBP) involved in peptidoglycan synthesis. These three enzymes, PBP1A, PBP2X, and PBP2B, are encoded by the *pbp1a*, *pbp2x*, and *pbp2b* genes, respectively. Among PEN-nonsusceptible strains (PRSP and PISP), abnormal genetic mosaic patterns of *pbp1a*, *pbp2x*, and/or *pbp2b* were found to differ from those of PEN-susceptible *S. pneumoniae* (PSSP).[9,14] Although a variety of mosaic regions have been detected in each gene, the main contributors to β-lactam resistance are amino acid substitutions identified within or adjacent to conserved amino acid motifs such as Ser-Thr-Met-Lys (STMK), Ser-Ser-Asn (SSN), and Lys-Ser-Gly (KSG).[2,3,13,23,24]

Therefore, we established a conventional PCR method to determine whether or not a pneumococcal isolate is PEN-susceptible according to molecular evidence.[31] This PCR was completed within 2.5 hr from selection of a colony for testing by amplification and gel electrophoresis. The resistance pattern based on the results of conventional PCR

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was defined as genotypic (g) resistance and represented by designations such as gPRSP (*pbp1a+pbp2x+pbp2b*), gPISP(*pbp1 a+pbp2x*), gPISP(*pbp2x*), and gPSSP. Currently, the prevalence of gPRSP possessing three abnormal *pbp* genes exceeds 46% among pediatric patients and 17% among adults in Japan.[8]

Given this situation, therapeutic choices for Japanese IPD patients have gradually eroded, with empirical first-line therapy shifting from penicillins or third-generation cephalosporins to carbapenem antibiotics. At the same time, numbers of adults and elderly persons with various underlying diseases posing high risk of IPD have increased rapidly.

In the present study, we aimed to construct a novel assay using real-time PCR that eliminates the need for gel electrophoresis, allowing completion of all procedures within 90 min. We describe sensitivity and specificity of our real-time PCR compared with conventional PCR and efforts to estimate MICs of therapeutic agents against various strains belonging to different PBP genotypic classes.

Materials and Methods

Strains and serotyping

Clinical isolates of *S. pneumoniae* obtained from IPD patients were collected from 186 clinical laboratories at medical institutions participating in our program of active nationwide surveillance for emerging and re-emerging of infectious diseases. We randomly selected 300 strains as follows: Blood ($n=218$), cerebrospinal fluid ($n=56$), pleural fluid ($n=14$), joint fluid ($n=6$), and others ($n=6$). These strains were sent to our laboratory from August, 2006, to July, 2007, accompanied by application form with a similar format as the Active Bacterial Core Surveillance (ABCs) case report.

The serotypes of all strains were determined in real time by the Quellung reaction using antiserum purchased from the Statens Serum Institute (Copenhagen, Denmark). The serotypes of these strains were mainly 6B ($n=47$), 12F ($n=28$), 14 ($n=27$), 3 ($n=26$), 4 ($n=22$), 9 ($n=20$), 19F ($n=19$), 23F ($n=18$), 6A/6C ($n=16$), 19A ($n=14$), 15 ($n=12$), and others ($n=51$).

Real-time PCR primers and molecular beacon probes

Sequences of six sets of primers and molecular beacon (MB) probes and amplicon sizes (bp) applied for our real-time PCR are shown in Table 1. Target genes and the DNA amplification positions were the *lytA* gene encoding the autolysin enzyme specific to *S. pneumoniae*[12]; the *pbp1a* gene detected in susceptible strains,[18] located in the region including a conserved amino acid motif, STMK, corresponding to that of resistant strains; the *pbp2x* gene detected in susceptible strains,[16] located in the region surrounding the STMK motif corresponding to divergent sequences of resistant strains; the *pbp2b* gene detected in susceptible strain,[10] located in the region adjacent the SSN motif; the *mef*A gene

TABLE 1. PRIMERS AND MOLECULAR BEACON PROBES FOR REAL-TIME PCR

Target gene	*Sequence (5′ to 3′)*	*Position*	*Amplicon size (bp)*	*Target amino acid substitution*
Autolysin (*lytA*)				
Sense primer	CAGAATTAGGTTTTTTCTCGC	723–743	188	—
Reverse primer	TAAGAGTTCGATATAAAGGCG	890–910		
Probe	FAM-CGCGATCAGGTCTCAGCA TTCCAACCGCCGATCGCG-BHQ1	809–830		
PBP 1A (*pbp1a*)				
Sense primer	AAACCGCGACTGGGGATCAAC	2037–2057	239	S(T)MK
Reverse primer	GGTTGAGTCCGACCTTGTTT	2275–2256		↓
Probe	FAM-CGCGATCACTGGGATAGGGG CTACTTTGGCGATCGCG-BHQ1	2174–2196		A or S
PBP 2X (*pbp2x*)				
Sense primer	CCAGGTTCCACTATGAAAGTG	1255–1275	197	S(T)(M)K
Reverse primer	ATCCCAACGTTACTTGAGTGT	1451–1431		↓ ↓
Probe	FAM-CGCGATCAGATGCCACGATTC GAGATTGGGGATCGCG-BHQ1	1353–1375		A F
PBP 2B (*pbp2b*)				
Sense primer	CCTATATGGTCCAAACAGCCT	1566–1586	147	SSN(T)
Reverse primer	GGTCAATTCCTGTCGCAGTA	1712–1693		↓
Probe	FAM-CGCGATCTCGGCACCAGCAAT CTAGAGTCTGATCGCG-BHQ1	1626–1648		A or S
Macrolide efflux (*mef*A)				
Sense primer	GGGACCTGCCATTGGTGTGC	180–199	402	—
Reverse primer	CCCAGCTTAGGTATACGTAC	581–562		
Probe	FAM-CGCGATCCCCCAGCACTCAAT GCGGTTACACGATCGCG-BHQ1	359–382		
Adenine methylase (*erm*B)				
Sense primer	CGTACCTTGGATATTCACCG	721–740	224	—
Reverse primer	GTAAACAGTTGACGATATTCTCG	944–922		
Probe	FAM-CGCGATCCCGCCATACCACAG ATGTTCCGATCGCG-BHQ1	852–872		

encoding the efflux protein for 14-membered macrolide (ML) antibiotics[25]; and the *ermB* gene encoding adenine methylase for 14- and 16-membered ML antibiotics.[27]

Primers and MB probes corresponding to *pbp1a*, *pbp2x*, and *pbp2b* genes were designed to amplify the DNA only in susceptible strains. All MB probes were labeled with a fluorescent reporter of 6-carboxyfluorescein (FAM) at the 5′ end and also with a black hole quencher 1 (BHQ-1) at the 3′ end. Reporters and quenchers were connected to stem oligonucleotides.

Real-time PCR conditions

The real-time PCR reaction mixture consisted of 15 μl of 2× real-time PCR Master Mix (Toyobo, Tokyo, Japan), each primer at 0.2 μM, and each MB probe at 0.3 μM. The final volume of the mixture was adjusted to 30 μl by addition of DNase- and RNase-free H_2O. After each reaction mixture was pipetted into a 96-well plate, plates were stored at −30°C until use.

One colony grown on a sheep blood agar plate was picked up and suspended in 30 μl of lysis solution.[29] The tube then was placed in a thermal cycler (Gene Amp PCR System 9600R; Perkin-Elmer Cetus, Norwalk, CT) and heat-treated for 5 min at 60°C and for 5 min at 94°C to obtain template DNA. Next, after wells of the frozen real-time PCR reagent were thawed on ice, 2 μl of each template DNA was added to each well. Real-time PCR was performed immediately with a Stratagene Mx3000P (Stratagene, La Jolla, CA). The PCR conditions included an initial DNA denaturation step of 95°C for 30 sec, followed by 40 cycles of 95°C for 15 sec, 50°C for 20 sec, and 75°C for 15 sec. The time required from the lyses reaction to completion of real-time PCR was 90 min.

Conventional PCR

Conventional PCR was performed as a control assay for the real-time PCR in the same strains, using a commercially available kit (Wakunaga Pharmaceuticals, Hiroshima, Japan). The right of commercial production for this kit had been transferred to the company from Ubukata et al.[19,30]

Sequencing of pbp *genes with discrepancies between the two PCR methods*

Both the *pbp1a* and *pbp2x* genes in *S. pneumoniae* strains for which a discrepancy in the PCR data was recognized between the conventional and real-time methods were sequenced to identify the amino acid substitution. PCR primers used for analysis were a sense primer for *pbp1a*, 5′-TGGGA TGGATGTTTACACAAATG-3′; a reverse primer for *pbp1a*, 5′-TGTGCTGGTTGAGGATTCTG-3′; a sense primer for *pbp2x*, 5′-TATGAAAAGGATCGTCTGGG-3′; and a reverse primer for *pbp2x*, 5′-AGAGAGTCTTTCATAGCTGAAGC-3′, as described previously.[2,3]

Amplified DNA fragments were purified using a QIAquick PCR purification kit (Qiagen, Tokyo, Japan) and used as templates. Sequencing reactions were carried out using a BigDye® Terminator Cycle Sequencing kit, version 3.1 (Applied Biosystems, Foster City, CA). DNA sequencing was performed with an ABI 3130/3130xl genetic analyzer (Applied Biosystems).

Susceptibility testing

MICs of the five β-lactam antibiotics PEN, ampicillin (AMP), cefotaxime (CTX), meropenem (MEM), and panipenem (PAM) were determined by an agar dilution method using Mueller–Hinton II agar (MH, Becton Dickinson, Franklin Lakes, NJ) supplemented with 5% defibrinized sheep blood. Bacterial inoculum size and culture conditions were in accordance with a previously described method.[28]

S. pneumoniae ATCC49619 and R6 reference strains were used as quality controls.

Multilocus sequence typing and eBURST analysis

Multilocus sequence typing (MLST) performed for *S. pneumoniae* strains recognized discrepancy in the data of the two PCR methods. MLST and eBURST analysis was performed according to the MLST site (http://spneumoniae.mlst.net/).

Results

Resistant genotypes determined by real-time PCR

Figure 1 shows four patterns from a computer display connected to the real-time PCR instrument shown just after the PCR reaction was completed. Each tested strain was identified as follows: A, as gPSSP by DNA amplification corresponding to *lytA* (a), *pbp1a* (b), *pbp2x* (c), and *pbp2b* (d) genes; B, as gPISP (*pbp2x*), with only the *pbp2x* gene not amplified; C, as gPISP (*pbp1a*+*pbp2x*), with *pbp1a* and *pbp2x* genes not amplified; and D, as gPRSP (*pbp1a*, *pbp2x*, and *pbp2b*), with all 3 *pbp* genes not amplified. With regard to ML resistance, a strain showing DNA amplification for *mef*A and/or *erm*B genes was identified as ML resistant.

Genotypic classification of β-lactam and macrolide resistance

All strains tested were classified into six genotypic categories by real-time PCR for 3 *pbp* genes: gPSSP with three normal genes ($n=67$, 22.3%); gPISP (*pbp2x*) with an abnormal *pbp2x* gene ($n=76$, 25.3%); gPISP (*pbp2b*) with an abnormal *pbp2b* gene ($n=22$, 7.3%); gPISP (*pbp1a*+*2x*) with abnormal *pbp1a*+*2x* genes ($n=34$, 11.3%); gPISP (*pbp2x*+*2b*) with abnormal *pbp2x*+*2b* genes ($n=14$, 4.7%); and gPRSP with three abnormal PBP genes ($n=87$, 29.0%). Strains examined included 106 from pediatric patients (35.3%) and 194 from adult patients (64.7%). Percentages of the strains representing gPRSP accounted for 50.5% of isolates from children and 17.5% of those from adults.

Although detailed data are not shown, ML resistance in these strains was classified into four genotypic categories based on presence or absence of resistance genes: ML-susceptible strains ($n=58$, 19.3%); 14-membered ML-resistant strains possessing an *mef*A gene ($n=79$, 26.3%); 14- and 16-membered ML-resistant strains possessing an *erm*B gene ($n=150$, 50.0%); or an ML-resistant strain possessing *mef*A and *erm*B genes ($n=13$, 4.3%). Proportions of strains showing ML resistance were 85.9% of isolates from children and 77.9% of the isolates from adults.

Sensitivity and specificity of real-time PCR

As shown in Table 2, sensitivity and specificity for all strains were compared between conventional and real-time

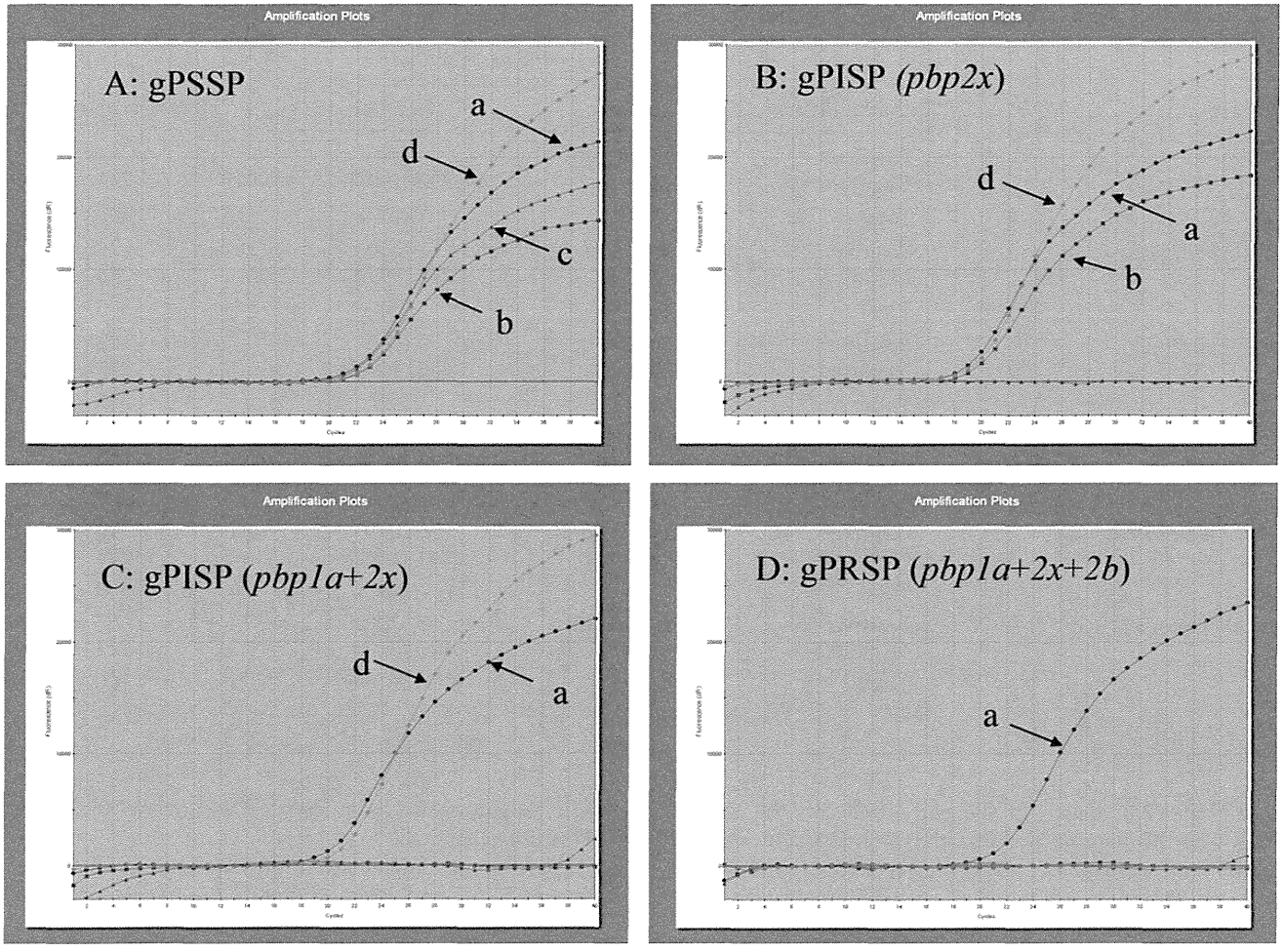

FIG. 1. Four genotypic resistance patterns from a computer display connected to the real-time PCR instrument, seen just after the PCR reaction was completed. (**A**) Genotypic penicillin-susceptible *Streptococcus pneumoniae* (gPSSP) by DNA amplification corresponding to *lytA* (a), *pbp1a* (b), *pbp2x* (c), and *pbp2b* (d) genes. (**B**) Genotypic penicillin-intermediate *S. pneumoniae* (gPISP) (*pbp2x*), with only the *pbp2x* gene not amplified. (**C**) gPISP (*pbp1a*+*pbp2x*), with *pbp1a* and *pbp2x* genes not amplified. (**D**) Genotypic penicillin-resistant *S. pneumoniae* (gPRSP) (*pbp1a*, *pbp2x*, and *pbp2b*), with 3 *pbp* genes not amplified.

PCR. The sensitivity and specificity for the *mefA* gene and the *ermB* gene were calculated to be 100%.

Table 3 shows detailed information for the nine strains (3.0%) showing a discrepancy between real-time PCR and conventional PCR. In these strains, DNA amplification for the *pbp1a* or *pbp2x* gene corresponding to the susceptible strain occurred weakly in conventional PCR but not at all in real-time PCR. According to susceptibility testing for AMP, and CTX, results of real-time PCR proved more accurate than those of conventional PCR. Overall, our new real-time PCR method showed to have excellent sensitivities and specificities compared with those of conventional PCR.

Relationships between PBP gene alterations and MIC of β-lactam agents

Figure 2 shows relationships between MICs of five β-lactam agents and results of real-time PCR for *pbp1a*, *pbp2x*, and *pbp2b* genes in the tested strains. MICs of PEN, MEM, and PAM were affected by *pbp2b* alterations rather than those in *pbp2x*. On the other hand, the MIC of CTX was 4–8 times lower than that of PEN due to *pbp2x* alterations. Notably, 90% of MICs in each genotype resistance class were distributed essentially within three serial dilution concentrations (for instance, gPRSP in PEN, from 0.5 to 2 mg/L) for almost all antibiotics. However, eight gPSSP strains with a CTX MIC ranging from 0.125 to 0.25 mg/L possessed substitutions of Thr550Ala adjacent to a KSG motif in PBP2X that could not be detect with the real-time PCR constructed in this study.

Estimated MIC_{50} values and corresponding ranges for 90% of β-lactam antibiotics among six PBP genotypic categories are listed in Table 4. On the basis of these data, MIC estimation for parenteral β-lactam antibiotics associated with clinical efficacy could be made with high probability.

Discussion

The ultimate global public health goal in the 21st century is to develop and disseminate vaccination to prevent infectious diseases caused by various viruses and bacteria more effectively. For immunity against pneumococcal infections, development of 23-valent pneumococcal polysaccharide

TABLE 2. SENSITIVITES AND SPECIFICITIES OF REAL-TIME PCR COMPARED WITH THOSE CONVENTIONAL PCR

Genotype	Real-time PCR	Conventional PCR (%)		Total no. of samples
		Positive	Negative	
gPSSP	Positive	67 (98.5)[a]	0 (0.0)	67
	Negative	1 (1.5)	232 (100.0)[b]	233
	Total	68	232	300
gPISP (*pbp2x*)	Positive	75 (96.2)	1 (0.5)	76
	Negative	3 (3.8)	221 (99.5)	224
	Total	78	222	300
gPISP (*pbp2b*)	Positive	22 (100.0)	0 (0.0)	22
	Negative	0 (0.0)	278 (100.0)	278
	Total	22	278	300
gPISP (*pbp1a+2x*)	Positive	31 (100.0)	3 (1.1)	34
	Negative	0 (0.0)	266 (98.9)	266
	Total	31	269	300
gPISP (*pbp2x+2b*)	Positive	14 (73.7)	0 (0.0)	14
	Negative	5 (26.3)	281 (100.0)	286
	Total	19	281	300
gPRSP (*pbp1a+2x+2b*)	Positive	82 (100.0)	5 (2.3)	87
	Negative	0 (0.0)	213 (97.7)	213
	Total	82	218	300

[a]Sensitivity.
[b]Specificity.

vaccine (PPV23)[5] began in the early 1980s in the United States, and this vaccine was introduced in Japan in 1988. In Japan, 7-valent pneumococcal conjugate vaccine (PCV7) has just been approved on a voluntary basis to prevent IPD among children with immunologic immaturity.

In countries where PCV7 has been introduced into the vaccine schedule, incidence of pediatric IPD caused by vaccine-type strains has decreased significantly,[4,6,22] while a related decrease of IPD among adults also has been reported.[17] However, prevalence of IPD caused by serotypes 19A and 6A (nonvaccine types) has increased, accompanied by a shift from PEN-susceptible to PEN-resistant strains.[7,11,21] Some investigators also have reported that overall incidence of IPD is little changed.[26]

In Japan, great clinical attention has been paid to the increase of PRSP and PISP among *S. pneumoniae* isolates from IPD,[8] which strongly reflects the difference in use of oral antibiotics between pediatricians and internists. Specifically, in pediatric practice, oral cephalosporins are favored over penicillins for outpatients, although a recent shift back toward amoxicillin and AMP has been noted. On the other hand, in internal medicine, ML and fluoroquinolone agents rather than β-lactam antibiotics are preferred. This might contribute significantly to rates of

TABLE 3. DETAILS OF 9 STRAINS SHOWING A DISCREPANCY IN RESULTS IN BETWEEN REAL-TIME PCR AND CONVENTIONAL PCR

No of strain	Genotype		MIC (mg/L)					Serotype	ST	CC
	Conventional PCR	Real-time PCR	PEN	AMP	CTX	MEM	PAM			
Ref R6	gPSSP	gPSSP	0.016	0.016	0.016	0.008	0.004	—	—	—
RS-009	gPISP(*pbp2x*)	gPISP(*pbp1a+2x*)	0.125	0.5	1	0.031	0.008	14	13	15
RS-027	gPISP(*pbp2x*)	gPISP(*pbp1a+2x*)	0.125	0.5	1	0.031	0.008	6B	385	156
RS-083	gPISP(*pbp2x*)	gPISP(*pbp1a+2x*)	0.125	0.5	1	0.031	0.004	6B	2983	156
RS-046	gPISP(*pbp2x+2b*)	gPRSP	0.5	1	2	0.063	0.016	14	343	554
RS-101	gPISP(*pbp2x+2b*)	gPRSP	0.5	2	2	0.063	0.016	14	343	554
RS-193	gPISP(*pbp2x+2b*)	gPRSP	0.5	1	0.5	0.063	0.016	14	343	554
RS-311	gPISP(*pbp2x+2b*)	gPRSP	0.5	1	2	0.125	0.016	14	343	554
RS-065	gPISP(*pbp2x+2b*)	gPRSP	1	4	1	0.25	0.031	6B	6939	None[a]
RS-208	gPSSP	gPISP(*pbp2x*)	0.063	0.125	0.125	0.016	0.004	6A	4542	156

When DNA amplification occurred, the corresponding *pbp* gene showed the same sequences as the susceptible strain; for example, a strain showing amplification of *pbp1a* and *pbp2b* genes was designation gPISP(pbp2x).
[a]ST6939 is not present in any group of clonal complexes.
MIC, minimum inhibitory concentration; PEN, penicillin; AMP, ampicillin; CTX, cefetaxime; MEM, meropenem; PAM, panipenem; ST, sequence type; CC, clonal complex; gPSSP, genotypic penicillin-susceptible *Streptococcus pneumoniae;* gPISP, genotypic penicillin-intermediate *S. pneumoniae*; gPRSP, genotypic penicillin-resistant *S. pneumoniae.*

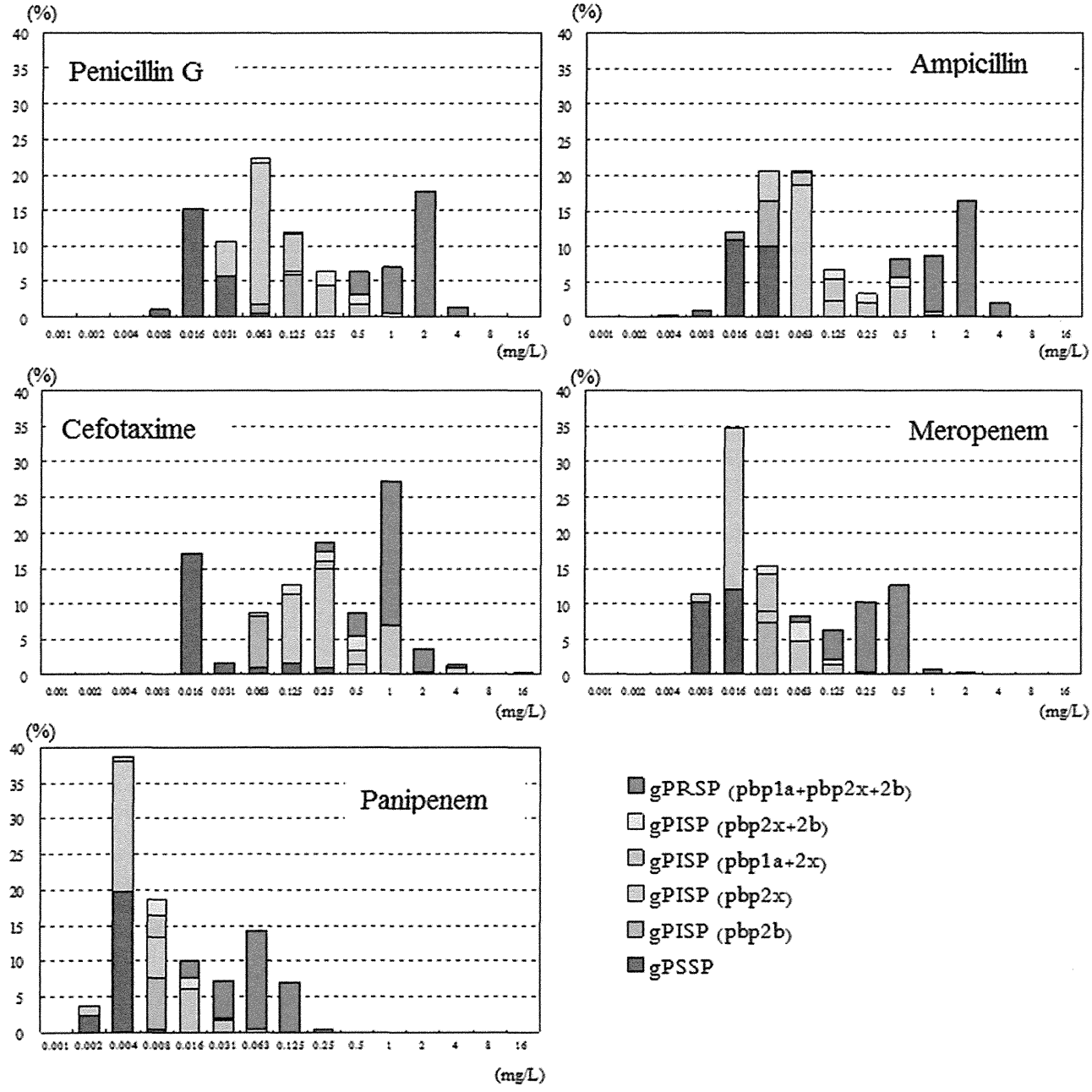

FIG. 2. Correlation between minimal inhibitory concentrations (MICs) of five β-lactam antibiotics and penicillin-binding protein (PBP) gene alterations for 300 *Streptococcus pneumoniae* isolates from invasive infections.

gPRSP isolated from pediatric patients and gPISP isolated from adult patients.

These situations concerning antibiotic resistance, in addition to the present state of pneumococcal vaccination, show that a need for rapid and accurate determination of resistance in clinical isolates is necessary for appropriate selection of chemotherapeutic agents in pneumococcal infections.

We initially identified species and antibiotic resistance using colony samples likely to be *S. pneumoniae* from blood agar plate using a conventional PCR method completed within 2.5 hr using gel electrophoresis.[30] Intrinsically, three primer sets designed on *pbp1a*, *pbp2x*, and *pbp2b* genes detect the most important amino acid substitutions affecting β-lactam susceptibilities, all positioned within or adjacent to conserved amino acid motifs in each PBP—substitutions from STMK to SAMK or SSMK in PBP1A, substitutions from STMK to SAMK or SAFK and from (L)KSG to (V)KSG in PBP2X, and substitution from SSN(T) to SSN(A) or SSN(S) in PBP2B. The genotypic resistance pattern based on the *pbp* gene analysis was divided into six categories: gPSSP, gPISP(*pbp2x*), gPISP(*pbp2b*), gPISP(*pbp1a+pbp2x*), gPISP (*pbp2x+pbp2b*), and gPRSP (*pbp1a+pbp2x+pbp2b*).

This was not shown in the results, but each class of resistance genes was not of a single clone. For example, gPRSP was divided into 11 serotypes with various clonal complexes (CCs). The major serotypes and CCs were serotype 6B with

TABLE 4. ESTIMATED MIC_{50s} AND FITTING RANGES OF 90% OF β-LACTAM ANTIBIOTICS FOR 6 PBP GENOTYPE CLASSES

Genotype	n	Estimated MIC (mg/L)				
		PEN	AMP	CTX	MEM	PAM
gPSSP	67	0.016 (0.016–0.031)	0.016 (0.016–0.031)	0.016 (0.016–0.125)	0.016 (0.008–0.016)	0.004 (0.002–0.004)
gPISP (*pbp2b*)	22	0.125 (0.063–0.125)	0.031 (0.016–0.031)	0.063 (0.063)	0.031 (0.031)	0.008 (0.008)
gPISP (*pbp2x*)	76	0.063 (0.031–0.063)	0.063 (0.031–0.063)	0.25 (0.125–0.25)	0.016 (0.016–0.031)	0.004 (0.002–0.008)
gPISP (*pbp1a+2x*)	34	0.25 (0.125–0.5)	0.25 (0.063–0.5)	1 (0.25–2)	0.063 (0.031–0.125)	0.016 (0.008–0.031)
gPISP (*pbp2x+2b*)	14	0.25 (0.063–0.5)	0.25 (0.063–0.5)	0.25 (0.125–0.5)	0.063 (0.031–0.125)	0.016 (0.008–0.031)
gPRSP (*pbp1a+pbp2x+2b*)	87	2 (0.5–2)	2 (0.5–2)	1 (0.5–2)	0.5 (0.125–0.5)	0.063 (0.031–0.125)

MIC, minimum inhibitory concentration; PEN, penicillin; AMP, ampicillin; CTX, cefetaxime; MEM, meropenem; PAM, panipenem; gPSSP, genotypic penicillin-susceptible *Streptococcus pneumoniae*; gPISP, genotypic penicillin-intermediate *S. pneumoniae*; gPRSP, genotypic penicillin-resistant *S. pneumoniae*.

CC156 and CC490, serotype 19F with CC320, serotype 23F with CC156, CC242 and CC1437, serotype 6A with CC3115, CC3787 and CC81, and serotype 14 with CC320 and CC554.

As stated in the Results section, real-time PCR yielded satisfactory sensitivity and specificity compared with conventional PCR. Accurate estimation of MICs of each β-lactam antibiotic on the basis of genotypic patterns is highly important. Our novel real-time PCR assay also can be completed within 90 min after selection of colony samples, with elimination of gel electrophoresis, saving both time and labor.

Another merit of this assay is possible direct testing of usually sterile specimens (such as cerebrospinal fluid, joint fluid, and pleural fluid) from IPD patients, because primers and MB probes for amplification of the *lytA* gene are included in the real-time PCR. In the future, simultaneous performance of speciation and identification of resistance gene(s) by real-time PCR should optimize cost and benefit in clinical settings.

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β-ラクタム系薬耐性肺炎球菌およびインフルエンザ菌に対する経口抗菌薬作用後の形態変化

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ペニシリン耐性肺炎球菌（penicillin-resistant *Streptococcus pneumoniae*: PRSP）とβ-ラクタマーゼ非産生アンピシリン耐性インフルエンザ菌（β-lactamase-nonproducing, ampicillin-resistant *Haemophilus influenzae*: BLNAR）に対する経口抗菌薬作用後の経時的形態変化について位相差顕微鏡下で観察した。また，BLNARに関しては，その形態変化を電子顕微鏡下でも観察した。使用菌株は，肺炎球菌がME19株（genotype: gPRSP，莢膜19F型），インフルエンザ菌がJPH002株（gBLNAR，莢膜b型）である。薬剤はamoxicillin（AMPC），cefditoren（CDTR），tebipenem（TBPM），tosufloxacin（TFLX）を対象とした。作用濃度はそれぞれの薬剤を通常投与量で小児に投与した際に得られるCmax到達から1時間後の血中濃度とした。gPRSPでは，TBPMを作用させた場合のみ20分後から溶菌像が認められ，2時間後には90%の細胞が溶菌した。溶菌までのスピードやその割合の高さから，本薬の殺菌性の強さが裏づけられた。AMPCとTBPMを作用させたgBLNARは，スフェロプラスト化（膨化）した菌からの溶菌細胞と，細胞内に空胞を形成した細胞が観察された。CDTRでは，著しくフィラメント化した細胞からの溶菌像，TFLXではややフィラメント化したまま死滅したと推定される細胞が観察された。それぞれの薬剤の殺菌性やすでに報告されているPBPに対する親和性の違いを反映する経時的形態変化は，抗菌薬選択の上で有益な情報と結論された。

肺炎球菌やインフルエンザ菌は，小児の呼吸器系感染症において最も重要な起炎菌であり，時に致命的な敗血症や化膿性髄膜炎等を惹起する。これらの菌の病原性発揮には，菌が産生する多様な病原因子とともに，宿主側の免疫学的未熟性も関わっていることが知られている[1,2]。

本邦においては，1990年代初めからペニシリン耐性肺炎球菌（penicillin-resistant *Streptococcus pneumoniae*: PRSP），1990年代後半からβ-ラクタマーゼ非産生アンピシリン耐性インフルエンザ菌（β-lactamase-nonproducing, ampicillin-resistant *Haemophilus influenzae*: BLNAR）が急速に増加し，治療上の問題となってきた[3]。以前は，経口抗菌薬の投与によって治療可能であった気管支炎等の呼吸器疾患の重症化や，急性中耳炎の難治化が挙げられる[4,5]。

肺炎球菌やインフルエンザ菌は，それらに対する抗体産生能が低い年齢層の乳幼児では上咽頭などに定着し，常在化しやすい[6]。このため，小児における両菌は，成人よりも抗菌薬に曝される機会が多いと推定され，β-ラクタム系薬耐性化が問題化したのも小児からである。耐性化の特徴として，肺炎球菌のβ-ラクタム系薬に対する耐性化は，PBP2Xをコードする*pbp2x*遺伝子の変異による抗菌力のわずかな低下から始まっている。これら変異株に対する経口セフェム系薬のMIC値はそれぞれの薬剤の上咽頭や中耳病巣内濃度をわずかに上回る程度で，菌を完全に死滅させることはできない。そのような菌がさらに同系統の薬剤に触れると，PBP1Aや2Bをコードする*pbp1a*あるいは*pbp2b*遺伝子に変異が生じ，遺伝子変異が積み重なることになる。すなわち，遺伝子学的にはgenotypic (g) なgPRSPと表される耐性菌である[7]。

インフルエンザ菌のβ-ラクタム系薬耐性化は，PBP3をコードする*ftsI*遺伝子の変異によるアミノ酸置換であるが，薬剤のPBP3への結合親和性に影響するアミノ酸置換が重なると感受性は低下，すなわち耐性化する[8]。これらも遺伝子学的にはgBLNARとして生物学的手法による耐性菌とは区別する。このような呼吸器系感染症由来のgPRSPやgBLNARの耐性レベルは，経口抗菌薬で得られる血中濃度あるいは病巣濃度よりもやや上まわった程度であることが特徴である。

これら耐性菌による小児の治療用抗菌薬として，2009年6月に承認され同年8月に発売されたのがTebipenem pivoxil（TBPM-PI）である[9]。また，2009年12月にはtosufloxacin（TFLX）[10]が承認され，翌年発売に至っている。前者は小児を対象とした世界初の経口カルバペネム系抗菌薬であり，その特徴は，耐性菌に対しペニシリン系薬やセフェム系薬よりも優れた抗菌活性と殺菌性を示す[11,12]こと，比較的高い血中濃度が得られる点にある。TFLXもTBPMと同様に小児のPRSPおよびBLNARを含む感染症に限定されているが，インフルエンザ菌に対する抗菌活性に優れる[10]。

本論文においては，amoxicillin (AMPC)，cefditoren (CDTR)，TBPMおよびTFLXの経時的殺菌効果の特徴を，主に薬剤作用後の形態変化の上から比較した成績について述べる。

材料と方法

1. 被験菌株

被験菌株とした肺炎球菌は，TBPM-PIの小児における治験時に急性中耳炎例より分離されたME19株である。本菌は莢膜型19F，耐性遺伝子型はgPRSP（*pbp1a*+*2x*+*2b*）である。寒天平板希釈法により測定した肺炎球菌の感受性測定の基準薬であるpenicillin G（PCG）に対する感受性は2μg/mL，また，主たる経口抗菌薬に対する感受性は，ampicillin (ABPC): 2μg/mL, AMPC: 1μg/mL, CDTR: 1μg/mL, cefdinir (CFDN): 8μg/mL, TBPM: 0.063μg/mL, TFLX：0.5μg/mL, erythromycin (EM): 2μg/mLである[13]。

インフルエンザ菌は，化膿性髄膜炎由来の莢膜b型株で*ftsI*遺伝子のPCR解析によってgBLNARと判定されたJPH002株を用いた。寒天平板希釈法により測定した本菌の主たる経口抗菌薬に対する感受性は，ABPC: 2μg/mL，AMPC: 4μg/mL，CDTR: 0.25μg/mL，CFDN: 2μg/mL，TBPM: 0.5μg/mL，およびTFLX: 0.008μg/mLである。

2. 位相差顕微鏡観察

i) 肺炎球菌

gPRSPのME19株は，Todd Hewitt（TH）Broth に接種し，37℃で60分間の静置培養後，film agar に接種した。薬剤フリーで30分間培養後，抗菌薬を灌流法で作用させた。薬剤の作用濃度は，小児の体内動態により近づけて評価したいと考え，小児に対してそれぞれの薬剤を投与した後のCmax

から約1時間後に得られる血中濃度に設定した。この濃度設定は，被験菌がそれぞれの薬剤の2×MIC以上に曝される時間が2.5時間以上保たれることを勘案，その時間の最も短いCDTRに合わせ一律に設定したものである。

すなわち，AMPCは45mg/kg投与で得られる12.7μg/mL（Cmax 15.7μg/mL）[14]，CDTRは6mg/kgで得られる2.1μg/mL（Cmax 2.9μg/mL）[15]，TBPMは4mg/kg投与で得られる1.7μg/mL（Cmax 3.5μg/mL）（未発表データ）とした。

肺炎球菌細胞の形態学的変化の観察は，抗菌薬を作用させる30分前から開始し，薬剤作用2.5時間後まで10秒間隔で撮影した。使用した位相差顕微鏡は，DIAPHOT（Nikon）である。

ii） インフルエンザ菌

gBLNARのJPH002株は，0.5% yeast extract，2%の加熱処理した馬脱繊維血液，および15μg/mLのβ-NAD^+のsupplementを含むMH brothを用い，37℃で60分間の前培養を行った後film agarに接種した。灌流法で作用させる薬剤の作用濃度の設定は，肺炎球菌と同様である。TFLXは，小児に対し6mg/kgの投与で得られるCmax到達濃度の1μg/mLから1時間後の0.9μg/mL[16]とした。

また，薬剤作用後の形態学的変化の観察についても，肺炎球菌と同様に実施し，3時間後まで10秒間隔で撮影した。本論文においてはその中から30分ごとの画像を抽出し，比較した。

3. 透過型電子顕微鏡による形態変化の観察

gBLNARのJPH002株は，まず0.5% yeast extract，2%の加熱処理した馬脱繊維血液，および15μg/mLのβ-NAD^+のsupplementを含むMH broth（50mL）を用い，37℃で5%炭酸ガス培養を18時間実施した。培養後，その3mLをsupplementが入った新たなMH broth（100mL）中に接種し，37℃で180分間培養した。その後，それぞれの抗菌薬を加え，37℃で120分間培養した。それぞれの作用濃度は，動画撮影時と同一濃度である。

前固定は，0.1Mリン酸バッファー（pH 6.8）でpH調整した0.5%グルタルアルデヒド液にて室温で20分間細胞を固定した。細胞表面を固定された菌細胞は，1,400×g，5分間の遠心を行い回収した。細胞ペレットは再び新しい2.5%グルタルアルデヒド液に入れ，さらに4℃で120分間固定した。続いて2%の四酸化オスミウム液に交換し，4℃で1夜，後固定した[17]。被験菌はエタノール/アセトンシリーズで脱水処理を行った後，Quetol 812に包埋，超薄切片を作成後，6%酢酸ウラニウムで染色して透過型電子顕微鏡（JEOL JEM-1200 EXS型，日本電子（株））で観察した。

結果

1. 肺炎球菌に対する薬剤作用時の形態学的変化

gPRSPのME19株に対してAMPC（12.7μg/mL），CDTR（2.1μg/mL）およびTBPM（1.7μg/mL）を作用させた30分ごとの経時的な形態変化はFig. 1に示す。本菌に対する3薬剤の作用濃度とMICの比は，AMPCが作用濃度（12.7μg/mL）/MIC（1.0μg/mL）で12.7倍，CDTRのそれは作用濃度（2.1μg/mL）/MIC（1.0μg/mL）で2.1倍，TBPMが作用濃度（1.7μg/mL）/MIC（0.063μg/mL）で27.0倍である。また，Fig. 2には薬剤添加後の画面上の肺炎球菌細胞数を100として30分ごとの溶菌率を示した。

AMPC作用では，細胞は30分後までやや伸長化しながら膨化する像が確認され，40分後から徐々に溶菌像がみられ始めた。そして，60分後には14%，90分後には22%，120分後には32%の細胞が溶菌した。

Fig. 1の中央に示したCDTR作用では，薬剤作用30分後にはAMPCよりもさらに細胞の伸長化と増加が著明であった。菌数もむしろ一時的に増加していた。しかし，それ以降になると，そのよ