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

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 Chlamydia pneumoniae. 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. 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, we 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. and Jüven et al. 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 (PIV) 1, 2, and 3; adenovirus; rhinovirus (RV); enterovirus; coronavirus (OC43); human metapneumovirus (hMPV); and human bocavirus (HBoV). Human metapneumovirus (hMPV) and human bocavirus (HBoV) 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
respiratory viruses. The PCR methods appear to be more sensitive than culture and are less affected by specimen transport, and can 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. 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.

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 administering 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 Extragene 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 Jiang 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, 26 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