Simultaneous and Rapid Detection of Causative Pathogens in Community-acquired Pneumonia by Real-time PCR (1167)

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Introduction

Community-acquired pneumonia (CAP) is still a major threat to individuals, especially children and compromised hosts such as senior citizens and people with underlying chronic diseases. The main causative pathogens in CAP are Streptococcus pneumoniae, Haemophilus influenzae and Mycoplasma pneumoniae, but the rates change according to age and the underlying disease.1,2 Recently, Chlamydia pneumoniae and Legionella pneumophila have frequently been identified as causative pathogens of CAP.3–5 In clinical practice, empirical chemotherapy with broad spectrum antibiotics must be started based on clinical symptoms, chest X-rays, and clinical examinations, considering the severity of the symptoms.

On the other hand, the increase of resistant strains in the CAP pathogens is a worldwide health problem.6 One effective measure to prevent the new emergence of resistant bacteria is to use only the sufficient quantity of the most appropriate antibiotics to eliminate target pathogens. We have developed a method to simultaneously identify the six pathogens in CAP by real-time PCR with high sensitivity and specificity, thus reducing analysis time and improving cost performance, toward the goal of the rapid and precise detection of causative agents.7

In this paper, I will describe an outline of the new identification system using real-time PCR assay for six CAP pathogens, S. pneumoniae, H. influenzae, M. pneumoniae, C. pneumoniae, L. pneumophila, and Streptococcus pyogenes.

Methods

Extraction of DNA from clinical samples

Samples of nasopharyngeal and sputum from pediatric and adult patients who were diagnosed with CAP at their first visit to a hospital, were collected after obtaining informed consent from patients themselves or from their family members.

Firstly, the samples were suspended in 1.5 ml of PPLO broth and centrifuged at 2,000 × g for 5 min at 4°C to collect bacterial cells, together with epithelial and polymorphonuclear leukocyte cells. The supernatant was discarded, and the harvested pellet was resuspended in 150 μl of DNase- and RNase-free H2O. All DNA samples were extracted using EXTRAGEN II kit. The process of DNA extraction was completed in 20 minutes.

Identification of pathogens by real-time PCR

The six sets of bacterial specific primers and molecular beacon (MB) probes used in this real-time PCR have been reported by us.7 These primers and MB probes were designed on appropriate genes for each pathogen; the lytA gene was selected for S. pneumoniae, the mip and 16S rRNA genes for L. pneumophila, the slo and 16S rRNA genes for S. pyogenes, and the 16S rRNA gene for the other three pathogens were selected as a target, respectively.

As shown in Fig. 1, all of the MB probes were labeled with a fluorescent reporter, 6-carboxyfluorescein (FAM) at the 5’-end, and labeled with a black hole quencher 1 (BHQ-1) at the 3’-end.
The reporters and quenchers were connected to stem structure with short oligonucleotides. The central region of about 20 bp, which is shown as a loop, corresponds to the sequences of each target gene. The color development of FAM occurred by an attachment of the oligonucleotides to corresponding sequences of single stranded DNA at the denature stage.

The overall protocol of real-time PCR assay constructed by us is shown in Fig. 2. Reaction mixtures corresponding to six CAP pathogens were employed in six wells of one strip. After the addition of a 2 μl DNA extraction to the six wells, the strip was placed on the real-time PCR instrument (Thermal cycler Dice, TP800, TAKARA BIO INC.), and DNA amplification was immediately started at 40 cycles of PCR with conditions at 95°C for 15 sec, at 50°C for 30 sec, and at 75°C for 30 sec per cycle, followed by 95°C for 30 sec. The results for every one cycle of the PCR were displayed on the screen of a personal computer connected to a PCR instrument. All processes in this protocol were completed in 1.5 hours.

Results

Sensitivity and specificity of real-time PCR

The $C_t$ (threshold cycle) value for a positive
result was defined as the point at which the horizontal threshold line was crossed. The sensitivities of PCR assay for six CAP pathogens were high, from 2 copies for *S. pneumoniae* to 18 copies for *S. pyogenes* per reaction tube. The correlation coefficient between Ct and bacterial cell counts was high from $r = 0.9970$ of *S. pyogenes* to $r = 0.9992$ of *S. pneumoniae*.
The specificity of the six MB probe and primer sets were examined against 27 gram-positive and -negative microorganisms selected from laboratory stock cultures as amplification negative controls. From those organisms, no non-specific positive results were obtained.

Application of real-time PCR for clinical samples

Figure 3 shows the result of sputum collected from a typical CAP case in a young female adult. She was diagnosed with segmental pneumonia, having a severe cough, and hospitalized. The PCR results displayed on the PC screen suggested a mixed infection of *M. pneumoniae* and *S. pneumoniae*. Based on these results, medical treatment was conducted by combination chemotherapy.

Figure 4 shows the results of real-time PCR applied to pediatric patients (A) and adults (B) with CAP. The sensitivity and specificity of real-time PCR assay for *S. pneumoniae*, *H. influenzae*, *S. pyogenes*, and *M. pneumoniae* for 150 clinical samples was determined, comparing them with the results of conventional culture. Both the relative sensitivity and the specificity of this PCR was over 90% for all six pathogens.

Culture assay of *C. pneumoniae* has not been performed routinely in clinical laboratories, but has instead been determined by the antibody titer in acute phase and the convalescence phase. In all of *C. pneumoniae* cases identified serologically, the real-time PCR gave positive results.

Although the culture for sputum was negative, *L. pneumophila* pneumonia was identified by PCR in the case of an adult patient. Later, *L. pneumophila* serogroup 5 was actually detected in the water from the patient’s bathroom.

**Discussion**

One of the measures to decrease healthcare costs and to improve benefit for patients with bacterial infection is to identify the causative pathogens rapidly and precisely, thus enabling the most appropriate antibiotic to be selected at the beginning of hospitalization. In the case of patients who were treated by antibiotics prior to hospitalization, the culture method may sometimes give false-negative results.

By real-time PCR molecular assay, it is possible to detect microorganisms with high sensitivity and specificity, even if bacteria have been damaged by the antibiotics pretreatment. Basically, the simultaneous detection of the main CAP pathogens described here is desirable for rapid diagnosis of CAP and for the selection of the appropriate antibiotics.

To determine causative pathogens, sputum has been employed in the case of adults. However, expectoration is impossible for infants and children. Alternatively, nasopharyngeal secretions are readily obtainable from children with respiratory tract infections (RTIs), but the test results must be analyzed carefully because healthy children also carry *S. pneumoniae* and *H. influenzae* in nasopharyngeal secretions.

Our data demonstrates that real-time PCR with pathogen-specific MB can detect microorganisms in a few hours, and thus by this assay it is possible to assess the time course of empirical chemotherapy, thus supporting infection management.

Finally, we also expect that the real-time PCR technique described here could be expanded into a multiplex real-time PCR to detect several RTI causative viruses as a general diagnostic method for lower RTIs in the near future.

**References**


