PCR Assay for the Diagnosis of Pneumococcal Meningitis

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Abstract-
Objective: Streptococcus pneumoniae is not an uncommon causative pathogen of acute bacterial meningitis, for which a slight delay in the management may result in death or serious sequelae. The traditional method of bacterial identification by culture requires at least 1-2 days, whereas use of PCR assay may allow much earlier recognition and confirmation of the bacteria.

Materials and Methods: We designed a primer set targeting the amylomaltase gene (malM) of S. pneumoniae. Acute and convalescent cerebrospinal fluid (CSF) samples were collected from two patients of suspected acute bacterial meningitis. The acute CSF specimens were collected within the first hour the admission. The second specimens were collected during the convalescent stage after a 10-days treatment course with ceftriaxone. Polymerase chain reaction (PCR) assays were performed on the same day of sample collection.

Results: The specificity and sensitivity of the PCR assay were demonstrated, and the latter shows that the latter could detect as few as 12 copies of molecules. In both patients the PCR assay identified the bacteria in the pre-treatment CSF samples, but not in the post-treatment specimens. All PCR assay results obtained in the first day were consistent with the culture results that were obtained on the fourth day of incubation.

Conclusions: The use of PCR assays with the primer set targeting the malM gene would allow early recognition of the pneumococcal meningitis.

Key Words: Diagnosis, Pneumococcal meningitis, Polymerase chain reaction, PCR, Streptococcus pneumoniae

INTRODUCTION

Streptococcus (S.) pneumoniae (pneumococcus) is not an uncommon causative pathogen of acute bacterial meningitis. The traditional methods of diagnosis include smear, culture, and antigen detection. The direct smear examination is a rapid method but requires a minimum of 10⁴-10⁵ bacilli/ml. This would make the detection difficult for CSF samples which usually contain only a very low number of bacteria. With standard culture procedures, presumptive identification of S. pneumoniae usually takes 12 to 24 h, followed by biochemical confirmation tests. The culture procedures would usually yield negative results if antibiotics had been given previously. The optimal sensitivity for the practice of bacterial antigen detection, including latex agglutina-
tion (LA) and counter-immunoelectrophoresis, usually requires the presence of more than $10^3$ CFU of organisms per ml\(^3\), again a number unlikely to be achieved in CSF samples.

The recently developed polymerase chain reaction (PCR) method using different target genes provides a quick detection of \textit{S. pneumoniae}\(^7\). Hassan-King et al. detected the autolysin (lyt) gene of \textit{S. pneumoniae} in blood cultures from the patients by PCR, and demonstrated concordance between PCR and culture results\(^7\). The study from Cherian et al. furthermore showed that PCR-enzyme immunoassay targeting autolysin gene was a useful tool for the diagnosis of culture-negative meningitis\(^8\). The results from Dagan et al. also suggested PCR targeting pneumolysin gene was a sensitive test for the detection of \textit{S. pneumoniae}\(^9\). According to the data from Zhang et al., the PCR-based assays targeting penicillin-binding protein 2B gene prove useful to augment current methods for the detection of \textit{S. pneumoniae} bacteremia\(^10\). On the other hand, Radstrom et al. used a seminested PCR targeting 16S rRNA genes to detect \textit{Neisseria meningitidis}, \textit{Haemophilus influenzae} and \textit{S. pneumoniae}\(^11\).

Here we report two patients with pneumococcal meningitis. The etiologic diagnosis was made in six hours with PCR assay of the CSF using the primer developed in our laboratory.

**MATERIALS AND METHODS**

**Patients**

We studied two patients with acute bacterial meningitis. The first case was a 17 year-old man who suffered from head injury in 1995. He received craniotomy for removal of the hematoma. Subsequently, occasional CSF rhinorrhea was noted. He had recurrent streptococcal meningitis four times from 1996 to 1999. In January 2000, acute onset of changes in consciousness, fever and neck rigidity appeared. The patient was brought to the emergency room and admitted to the hospital the same day. The second case was a 48 year-old man who has a history of severe head injury when he was 20 years of age. He had no history of CSF rhinorrhea or meningitis. Meningeal signs appeared two weeks after an upper respiratory infection before current admission.

**CSF samples**

Two CSF samples, acute and convalescent, were collected from each patient by lumbar puncture. The acute CSF specimens were collected within the first hour after admission (in the same day that symptoms appeared) and the convalescent ones on the tenth day after the antibiotic treatment. In the secondary lumbar puncture, radioactive isotope counting by means of cotton packed into the nasal cavity for detecting CSF rhinorrhea was done at the same time. The CSF specimens were assayed by standard laboratory methods including cell counts, cultures (bacteria, fungus and \textit{Mycobacterium tuberculosis}), smears (bacteria, acid-fast bacilli, Indian ink) and serological tests (\textit{Treponema pallidum} hemagglutination test and cryptococcal antigen). A portion of the specimen was used for the PCR assay. The PCR assays were completed on the day of sample collection.

**DNA preparation**

The DNA was extracted and purified from 200 µL of CSF specimen with QIAamp blood kit (QIAGEN, Chatsworth, CA, USA) according to the manufacturer’s instructions. Purified genomic DNA was finally dissolved in 50 µL of TE buffer.

**PCR primers**

The PCR primer set was synthesized with an Applied Biosystems 394 DNA synthesizer. The PCR primer set included MPG3301F (5’-ATGTTATCATA-CAAG AATTTGTAC-3’) and MPG3529R (5’-CATCGTCGTAAGACCAAGGT-3’). The primer set targeting amyloamaltase gene (\textit{malM}) was based on our own design and previous reported from the other lab\(^12\). The expected amplicon had a size of 228 bp.

**PCR specificity**

To determine the specificity of the primer set, 105 copies (1 ng) of genomic DNA from different organisms including \textit{S. pneumoniae}, \textit{H. influenzae}, \textit{N. meningitidis}, \textit{Mycoplasma pneumoniae}, \textit{Escherichia coli} and human DNA were used as the template DNA.
PCR sensitivity

To determine the sensitivity of the primer set, a series of dilutions of the pneumococcal genomic DNA from 10^5 copies (1 ng) to 10^-1 copy (1 fg) were used as the template DNA.

PCR assays

The PCR reaction mixture (10 µL) contained 50 mM KCl, 10 mM Tris-HCl, pH 8.4, 1.5 mM MgCl₂, 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 1 µL (100 ng/µL) of each primer, 2.5 units of Taq DNA polymerase, and 1 µL of purified clinical or genomic DNA as the template. The PCR reaction was initiated by denaturation at 94 °C for 1 min, followed by 60 cycles of denaturation at 94 °C for 15s, annealing at 55 °C for 15s, and polymerization at 72 °C for 30s. Finally, the reaction was extended at 72 °C for 2 min. The negative control contained all components except the target DNA. The positive control contained all components and 1 ng of purified genomic DNA from S. pneumoniae. The PCR products separated on a 2% agarose gel were stained with ethidium bromide for analysis.

RESULTS

The target gene (malM) encodes amylomaltase of S. pneumoniae. The PCR primers (MPG 3301F and MPG 3529R) were designed corresponding to the gene sequence specific for S. pneumoniae. Their sequences are shown in the Materials and Methods section. In the study of specificity, the PCR assays showed that the primer set was specific for S. pneumoniae among the 6 different DNA (Fig. 1). The amplicon of 228 bp, as expected, was also confirmed by DNA sequencing (data not shown). To determine the assay sensitivity, a series of 10-fold dilutions of template DNA was used for the PCR assays. The results showed that the PCR assay sensitivity with their primer set is 10^2 copies of molecules (Fig. 2).

The laboratory data of CSF samples from both patients are shown in Table. The results of the stains (bacteria, acid-fast bacilli, Indian ink) and the serological studies (Treponema pallidum hemaglutination test and cryptococcal antigen) were all negative. The PCR results revealed the existence of pneumococcal DNA in the pre-
treatment CSF. The DNA was absent in the post-treatment specimens (Fig. 3). Only S. pneumoniae grew on the fourth day in the two first CSF cultures. Thus the PCR results were consistent with the culture data (Table).

### DISCUSSION

In this study, we designed the primer sets targeting the malM gene. We were able to detect 1000 fg of input DNA (corresponding to 100 CFU/ml) and shorten the time of recognizing the pathogen in the bacterial meningitis. Our results were less sensitive than primer sets targeting autolysin gene (3-50 CFU/ml), pneumolysin gene (10 CFU/ml) and penicillin-binding protein 2B gene (30 CFU/ml), but more sensitive than 16S rRNA gene (300 CFU/ml)\(^{15,11}\). Although our sensitivity was not the best, there was a good correlation between the PCR results (obtained in six hours on the first day of admission) and the culture data (obtained on the fourth day of admission). Nevertheless, it would be necessary to evaluate more cases to establish our PCR assay as a good screening test.

In recent years, resistant strains of S. pneumoniae have become a major problem worldwide. The routine dosages of cefotaxime or ceftriaxone used alone may not be sufficient to clear these resistant strains from the CSF promptly\(^{13}\). Therefore, the PCR test for pneumococci should identify not only the organism, but also its drug susceptibility\(^{14}\). The pneumococcal PCR assay that would also detect penicillin-resistance is being developed in our laboratory.

It is of interest that the PCR of the convalescent CSF, i.e. after 10 days of therapy, in both patients revealed no bacterial DNA. In the future this assay may be employed to determine the minimum necessary duration of the antibiotic therapy.

Because the etiologic diagnosis can be rapidly made (and in some cases, there is even no need for any invasive procedures), molecular diagnosis employing PCR is gaining clinical acceptance\(^{15}\). However, its major drawback has been the inability to investigate a large group of diverse potential etiologic agents simultaneously with one test\(^{16}\). The use of multiplex PCR or special biochips for simultaneous testing of multiple pathogens will be the future research direction in biotechnology\(^{17}\).

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REFERENCES