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 (*mal*M) 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 102 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 *mal*M gene would allow early recognition of the pneumococcal meningitis.

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

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INTRODUCTION

Streptococcus (S.) *pneumoniae* (pneumococcus) is not an uncommon causative pathogen of acute bacterial meningitis[1-3]. The traditional methods of diagnosis include smear, culture, and antigen detection. The direct smear examination is a rapid method but requires a minimum of 10^4-10^5 bacilli/ml. This would make the detection difficult for CSF samples which usually contain only a very low number of bacteria[4]. With standard culture procedures, presumptive identification of *S. pneumoniae* usually takes 12 to 24 h, followed by biochemical confirmation tests[5]. 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-

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tion (LA) and counter-immunoelectrophoresis, usually requires the presence of more than 10^3 CFU of organisms per ml[6], 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 *S. pneumoniae*[7-11]. Hassan-King et al. detected the autolysin (lyt) gene of *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 *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 *S. pneumoniae* bacteremia[10]. On the other hand, Radstrom et al. used a seminested PCR targeting 16S rRNA genes to detect *Neisseria meningitidis, Haemophilus influenzae* and *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 *Mycobacterium tuberculosis*), smears (bacteria, acid-fast bacilli, Indian ink) and serological tests (*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'-ATGTTATCACTACAAG AATTTGTAC-3') and MPG3529R (5'-CATCGTCGTAAAGACCAAGGT-3'). The primer set targeting amylomaltase gene (*mal*M) 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 *S. pneumoniae, H. influenzae, N. meningitidis, Mycoplasma pneumoniae, 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 105 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 $MgCl_2$, 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 (*mal*M) 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* hemagglutination test and cryptococcal antigen) were all negative. The PCR results revealed the existence of pneumococcal DNA in the pre-

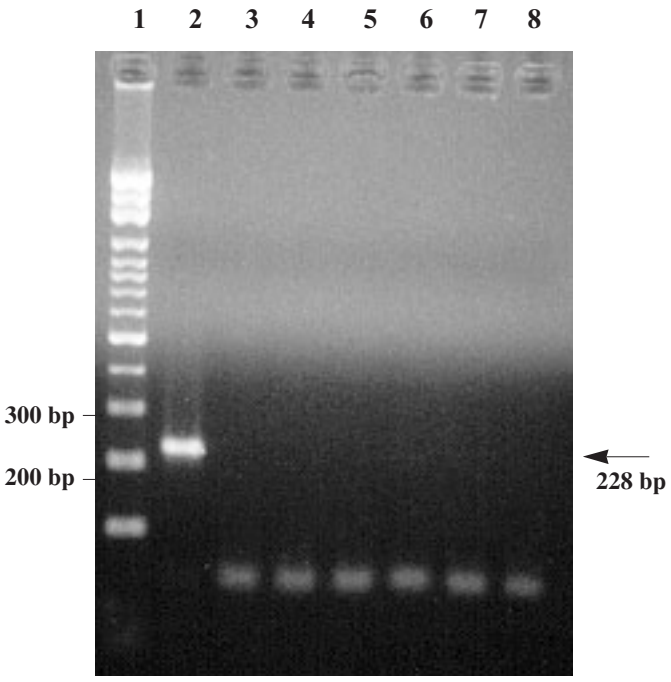

Figure 1. Determination of PCR specificity. Lane 1 is the 100 bp DNA markers. Lanes 2 to 6 are the results of PCR assay containing 106 copies of genomic DNA from *Streptococcus pneumoniae, Haemophilus influenzae, Neisseria meningitidis, Mycoplasma pneumoniae*, and *Escherichia coli*, respectively. Lane 7 is human DNA. Lane 8 is the negative control with no template added. The numbers on the left side indicate the size of DNA markers. The number on the right side indicates the size of the amplicon.

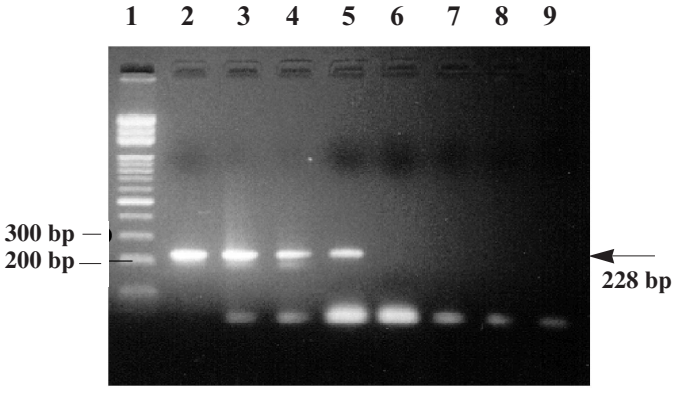

Figure 2. Determination of PCR sensitivity. Lane 1 is the 100 bp DNA markers. Lanes 2 to 8 are the PCR reaction mixtures containing serial dilutions of genomic DNA of *S. pneumoniae* in 105, 104, 103, 102, 101, 100, and 10-1,genomic DNA, respectively. Lane 9 is the negative control with no template added. The numbers on the left side indicate the size of DNA markers. The number on the right side indicates the size of the amplicon.

Table. *The laboratory data of CSF samples from two patients of pneumococcal meningitis*

Patient No.	Time	Appearance	Pressure (mmH_2O)	Glucose (mg/dL)	Protein (mg/dL)	WBC (/mL)	PMN (%)	Culture
1.	Pre-treatment	Colorless, slightly turbid	450	<10	630	11,000	97	*S. pneumoniae*
	Post-treatment	Colorless, clear	100	56	41	30	100	No growth
2.	Pre-treatment	Turbid	>400	17	385	3,050	86	*S. pneumoniae*
	Post-treatment	Colorless, clear	130	54	44	1	96	No growth

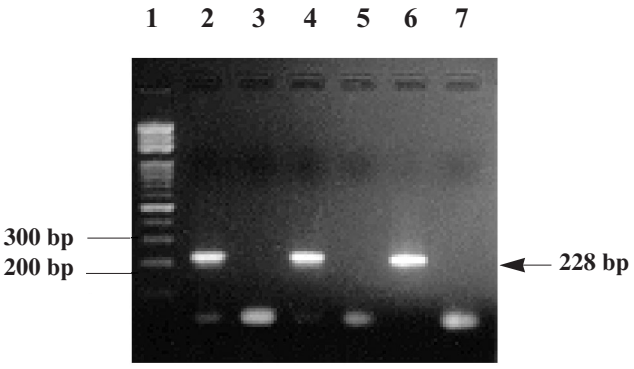

Figure 3. Clinical PCR assay. Lane 1 is the 100 bp DNA markers. Lanes 2 to 7 are the results of PCR assay targeting the amylomaltase gene of *S. pneumoniae* using MPG 3301F/MPG3529R primer set. The expected amplicon has a size of 228 bp. Lanes 2 to 7 are the pretreatment specimen from case 1, the post-treatment specimen from case 1, the pretreatment specimen from case 2, the post-treatment specimen from case 2, the positive control (1 ng of genomic DNA purified from *S. pneumoniae*), the negative control (no template added), respectively.

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 *mal*M 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)[7-11]. 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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