RAPID DETECTION OF BACTERIAL ATYPICAL PNEUMONIA AGENTS BY MULTIPLEX PCR

Pınar, A., Bozdemir, N., Kocagöz, T., Alaçam, R.
Hacettepe University, Faculty of Medicine, Department of Microbiology and Clinical Microbiology, Ankara, Turkey

SUMMARY

Approximately one third of community acquired pneumonia cases are caused by atypical pneumonia agents, Mycoplasma pneumoniae, Legionella pneumophila, and Chlamydophila pneumoniae (formerly Chlamydia pneumoniae). The laboratory diagnosis of these organisms is difficult and time-consuming by conventional microbiological techniques. Polymerase chain reaction (PCR) is one of the important tools which can circumvent this problem. A multiplex PCR assay was developed to achieve the diagnosis of these three organisms in a single tube. Primers used in PCR were selected in a way that they amplified different length DNA fragments from different agents but they all worked at the same amplification conditions. Therefore the organisms could be diagnosed according to the length of amplified products by agarose gel electrophoresis without using any hybridization probes. After development of the multiplex PCR method, totally 309 clinical samples which were sent to our laboratory for single-agent PCR, were also evaluated by this technique. The results showed that the multiplex PCR assay is a sensitive, useful, cheap, and rapid diagnostic tool for the management of pneumonia patients.

Key words: *Chlamydophila pneumoniae*, *Mycoplasma pneumoniae*, *Legionella pneumophila*, multiplex PCR, pneumonia

Address for correspondence: A. Pınar, Hacettepe University, Faculty of Medicine, Department of Microbiology and Clinical Microbiology, 06100, Sıhhiye, Ankara, Turkey. E-mail: pinar-a@tr.net

INTRODUCTION

Chlamydophila pneumoniae (formerly *Chlamydia pneumoniae*), *Mycoplasma pneumoniae*, and *Legionella pneumophila* are the important causes of community acquired pneumonia. Furthermore, respiratory tract infections are one of the leading causes of morbidity and mortality worldwide, especially in children (1).

Identification of these organisms by culture, flourescein antibody assays, and serology have several difficulties (2). Culture techniques require specialized laboratories and are expensive, time-consuming, and labor-intensive. Fluorescein antibody assays require technical expertise. Serology usually requires documentation of a rise in antibody concentration from an acute-phase to convalescent-phase blood sample. Development of a rapid method for the microbiological diagnosis of bacterial atypical pneumonia agents could result in less antibiotic therapy as well as more precisely tailored antibiotic therapies, resulting in reduced costs, fewer side effects, and a reduction of the emergence of resistance.

Currently available nucleic acid amplification techniques such as PCR are highly sensitive methods for the detection of nucleic acid sequences from viruses and bacteria in clinical specimens (3). These amplification techniques are particularly advantageous for the detection of fastidious or difficult-to-culture organisms. Most of the previous studies with PCR focused on the detection of a single bacterium; however, the diagnostic utility of PCR for a single infectious agent is limited, especially for pneumonia patients. Therefore we planned to develop a multiplex PCR assay for the diagnosis of bacterial atypical pneumonia agents.

MATERIALS AND METHODS

Preperation of DNA templates in order to develop multiplex PCR: Previously cultured microorganisms were suspended in TE (10 mM Tris pH: 8.0, 1 mM EDTA) buffer. These suspensions were mixed with sputum samples in order to get spiked samples. Spiked sputum samples were treated with 1 M DTT (dithiotreithol) for liquidification. The samples were centrifuged for 5 minutes at 12,000 *g*. Supernatant was discarded and sediment was resuspended in 1 ml TE buffer. This washing step was repeated two more times and at the end of the last step, sediment was suspended in 200 µl TE buffer. Tubes were placed on the 100 ˚C heat block and incubated for 30 minutes. After this incubation was completed, tubes were centrifuged at 12,000 *g* for 5 minutes and supernatant was transfered to the clean tubes. Tubes were kept at –20 ˚C until PCR.

Primers: For the detection of *L. pneumophila*, primers that amplify 630-bp fragment of mip gene were used (4). The primers MP-1 and MP-2 were used to amplify a 277-bp fragment of 16S rRNA gene of *M. pneumoniae* (5) and the primers HL-1 and HR-1 were used to amplify a 438-bp fragment of 474-bp *Pst*I fragment of cloned *C. pneumoniae* (6). After amplifications, 1.5% agarose gel electrophoresis at 100 V and ethidium bromide staining were used to visualize the PCR products.

Multiplex PCR: After some modifications, optimum amplification conditions were adjusted for multiplex PCR. Amplification was performed in MJR (PTC-200) thermal cycler as follows: Initial denaturation at 94 ˚C for 3 minutes, 42 cycles of denaturation (94 ˚C for 45 seconds), annealing (58 ˚C for 45 seconds), and polymerization (72 ˚C for 1 minutes), additionally 4 minutes

at 72 ˚C at the end of the cycles. Reaction mixture composition was as follows: Primers 20 pM each, *Taq* polymerase 0,7 U, KCl 50mM, Tris (pH 8.3) 10 mM, $MgCl_2$ 3,0 mM, dNTP 200 µM each in total volume of 25 µl.

Clinical samples: Totally 309 clinical samples which were sent to our laboratory for single-agent PCR, were included in this study. Clinical sample types were as follows: 171 throat swab, 37 bronchoalveolar lavage fluid, 37 urine, 33 blood, 17 sputum, 9 biopsy, 3 cerebrospinal fluid, and 2 thorasynthesis fluid. All these clinical samples were processed by proteinase K and phenol-chloroform extraction method as described previously (7).

Table 1. *The sample type distribution of multiplex PCR positive results*

	C. pneumoniae (+)	*M. pneumoniae* (+)	*L. pneumophila* (+)	Total
Throat swab	11	12	5	28
Blood	3	-	-	3
Urine	-	1	-	1
Sputum	-	1	-	1
BAL*	1	-	-	1
Total	15	14	5	34

*BAL: Bronchoalveolar lavage fluid

RESULTS

By using the same reaction mixture and conditions, three pathogens were amplified by PCR from spiked sputum samples. A 630-bp, 277-bp, and 438-bp products were seen after agarose gel electrophoresis when *L. pneumophila*, *M. pneumoniae* and *C. pneumoniae* infected sputum samples were amplified, respectively. Photograph of agarose gel electrophoresis can be seen in Fig. 1.

Totally 34 out of 309 clinical samples were found positive for at least one organism when evaluated by multiplex PCR protocol for *L. pneumophila*, *M. pneumoniae* and *C. pneumoniae.* Fifteen *C. pneumoniae,* 14 *M. pneumoniae*, and 5 *L. pneumophila* positive results were detected out of 34 positive samples. The sample types of positive results can be seen at Table 1. All of positive and negative results were confirmed by single-agent PCR protocols and all of the patients with positive results showed good outcome with appropriate antibiotic therapy.

DISCUSSION

Bacteria of the genus *Legionella* cause community-, travel-, and hospital-acquired pneumonia in humans. The first PCR systems for the detection of the genus *Legionella* were based on primers targeted at 5S rRNA gene, which amplified a wide range of *Legionella* spp. with specifity problems (8). 16S rRNA gene is more variable than 5S rRNA, hence it is difficult to design generic *Legionella* primers which targeted to this region. The primers designed by Jaulhac et. al. targeted to the conserved area of the *mip* gene. These primers can successfully amplify not only *L. pneumophila* but also *L. bozemaniae* and *L. micdadei* (4). Sensitivity of the PCR assay using these primers is acceptable for clinical use. Depending to these data, we used the primer set which amplifies 650-bp fragment of *mip* gene for developing a multiplex PCR assay.

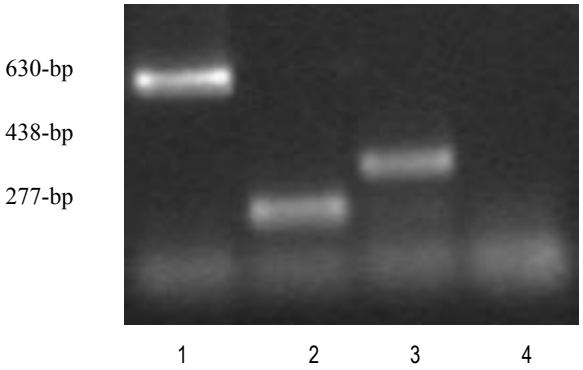

Fig. 1. *630-bp, 277-bp, and 438-bp products of the multiplex PCR (lane 1: L. pneumophila, lane 2: M. pneumoniae, lane 3: C. pneumoniae, lane 4: negative control).*

M. pneumoniae is a common etiologic agent of respiratory tract infections in humans and is responsible for 15 to 20% of all cases of pneumonia (9) and a wide range of mild to serious extrapulmonary complications (10, 11). Previously, diagnosis of infection with this organism was usually based on serology and culture (12, 13). PCR amplification of fragments of the P1 gene or the 16S rRNA gene was shown to be considerably more sensitive than culture for the detection of *M. pneumoniae* (5, 14, 15, 16). We used primers that sensitively amplify 16S rRNA gene fragment of *M. pneumoniae*.

C. pneumoniae is a major cause of acute respiratory disease in humans and is responsible for approximately 10% of cases of community-acquired pneumonia. Due to the difficulties with culturing and serologic analysis, a number of nucleic acid amplification assays for detecting *C. pneumoniae* have been developed (17). Current PCR methods are based on the amplification of a cloned *Pst*I fragment (6), genes encoding 16S rRNA (18, 19, 20), or the gene for the major outer membrane protein, known as *omp1* or *ompA* (21). We used the primers that targeted to cloned *PstI* fragment of *C.pneumoniae* encoding 16S rRNA genes.

In order to provide a technique for the rapid detection of bacterial atypical pneumonia agents, we developed a single--tube multiplex PCR. By this assay, three different pathogens of the respiratory tract can be detected within 1 day. Reaction conditions including $MgCl_2$, oligonucleotides, primer concentrations and temperature were optimized. We also performed this assay with clinical samples as well as spiked specimens. The results of clinical samples were also confirmed with single-agent PCR protocols. We did not perform a seperate sensitivity study with multiplex PCR technique, because all of the positive and negative results were comparable with single-agent protocols.

CONCLUSION

Molecular tests for laboratory diagnosis of infectious agents are getting more importance today. Molecular techniques are highly

sensitive and specific, and their costs are getting lower with extensive use. Introduction of standardized new methods into the field of molecular microbiologic diagnosis, will surely increase the health benefits of populations. Use of multiplex PCR for diagnosis of bacterial atypical pneumonia agents will probably result in fewer prescriptions of antibiotics and lower costs of diagnostic tests. Benefits of this method should be evaluated by larger clinical studies.

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