A Laboratory Comparison of Immunofluorescent Assay and Polymerase Chain Reaction (PCR) Methods for Detection of Pneumocystis jirovecii in Bronchoalveolar Lavage

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ABSTRACT

Pneumocystis jirovecii is one of the most common causative agents of Pneumocystis pneumonia (PCP) in immunocompromised patients. Reliable and rapid laboratory method for detection of the organism is crucial for diagnosis of PCP. In this study, the evaluation of detection techniques for Pneumocystis jirovecii in bronchoalveolar lavage (BAL) was performed. Immunofluorescent assay (IFA), single-step polymerase chain reaction (single-step PCR) and nested polymerase chain reaction (nested PCR) methods were compared.

This study was carried on sixty samples of BAL, from patients sent to the Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University. Twenty-three of the samples (38.3%) were found to be positive by IFA. Twenty-five of them (41.6%) were found to be positive by PCR, and 56 of them (93.3%) were found to be positive by nested PCR. As a result, the single-step and nested PCR, which are rapid and reliable diagnostic tests, can be used for the screening detection of this organism.

Keywords: Laboratory comparison, IFA, PCR, Pneumocystis jirovecii
is accurate as possible. Several different techniques for detecting *P. jirovecii* by using polymerase chain reaction (PCR) have been studied.\textsuperscript{15,17,18} Comparison and interpretation of results obtained in each technique can contribute to an evaluation of test performance (sensitivity, specificity) and agreement of each technique. The presence of a positive PCR product in a specimen that cannot be confirmed by other methods of detection presents a diagnostic problem. Such a situation might result from the recent administration of anti-*P. jirovecii* drugs or may represent subclinical infection. In the latter case, some patients have gone on to acquire PCP.\textsuperscript{19} In patients with positive PCR results in BAL or sputum but with negative smears, clinical management of the disease remains a challenge. In the present study the PCR techniques for detecting the micro-organism in bronchoalveolar lavage (BAL) were evaluated and compared to immunofluorescent assay.

**MATERIALS AND METHODS**

**Clinical specimens**

This study was approved by the Ethics Committee for Human Research at Faculty of Medicine Siriraj Hospital, Mahidol University. Sixty BAL specimens, from January to December, 2004, were submitted to the Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University for detection of *P. jirovecii*, were examined by both IFA and PCR.

BAL specimens were centrifuged at 1,000 rpm for 5 min to pellet cells and organisms. Drop of sediment were transferred to sterile glass slides, spread to form thin films, fixed in methanol, and used for IFA; the remaining portions of the sediment were kept for the PCR technique.

**Immunofluorescent technique**

The IFA using monoclonal mouse anti-*P. carinii* antibodies (clone 3F6; DAKO AS, Glostrup, Denmark) was performed with a commercially available murine monoclonal antibody labeled with fluorescein isothiocyanate that reacts with an antigenic epitope highly specific for *P. carinii* parasites.\textsuperscript{12,13} Briefly, BAL samples were mixed with 0.25% trypsin in phosphate buffer saline (PBS) and were incubated at room temperature for 5 min. Then, centrifuge preparations of trypsinized BAL fluids were made. Indirect immunofluorescence with acetone-fixed smears was performed by using monoclonal mouse anti-*P. carinii* antibodies (clone 3F6; DAKO AS, Glostrup, Denmark), followed by rabbit anti-mouse IgM-FITC labeled, according to the instructions provided by the manufacturer.

**PCR technique**

The BAL was centrifuged to pellet cells and organisms. To extract the *Pneumocystis* DNA in BAL, 200 µl of each resuspended sediment was used for DNA extraction with the QIAamp DNA Mini kit (Qiagen) according to the manufacturer’s recommendation. The primers used were specific for the amplification of mitochondrial ribosomal RNA sequences from the *P. jirovecii* DNA of rat and human origin (mt rRNA gene) and amplified at 346-bp and 120-bp sequences of the gene mt rRNA. The mt rRNA gene PCR assays were performed by a test developed as previously described.\textsuperscript{18,20}

Sequences were amplified using the single-step PCR and nested PCR assays. The single-step PCR round was done with primer pair pAZ102- E: 5'- GATGGCTGT TTTTCCAAGCCCA- 3' / pAZ102- H: 5'- GTTACGGTGCAA GTACTC- 3'.\textsuperscript{18} The product of the first round PCR was amplified with the *P. jirovecii* specific primer pair pAZ102- E: 5'- GATGGCTTGT TTTTCCAAGCCCA- 3' / pAZ102- L2: 5'- ATAAGGTGATAGTGTACAAAAG- 3'.\textsuperscript{18}

Cycling parameters were as follows: 94°C for 5 minutes; 94°C for 30 sec, 56°C for 30 sec; and 72°C for 30 sec for 40 cycles; and 72°C for 7 minutes by using the Perkin Elmer GeneAmp 2400 Thermal Cycler. To avoid contamination, each step (reagent preparation, extraction and amplification) was performed in different rooms with different sets of micropipettes and using filter tips. PCR mixtures and the extraction step were prepared in a laminarflow cabinet. To monitor for possible contamination, negative controls (ultra-pure distilled water) were included in each PCR step.

The PCR products were electrophoresed on 2% agarose gel to detect amplified products and to determine the size of the products. The expected sizes of PCR products were 346 bp by single-step PCR and 120 bp by nested PCR.

**Data analysis**

The agreement between the IFA, single-step PCR, and nested PCR tests was expressed as K-value (Kappa statistic), an index which compares the agreement against that which might be expected by chance. K-values between 0.4 and 0.6 indicate a moderate agreement, values between 0.6 and 0.8 indicate a good agreement, and values between 0.8 and 1.0 indicate a very good or high agreement.

**RESULTS**

Single-step PCR and nested PCR results are shown in Fig 1. The results of the 60 BAL specimens tested are given in Table 1. Agreement between IFA, single-step PCR, and nested PCR results of the laboratories was different. The highest agreement (kappa = 0.72) was found between test results of IFA and single-step PCR. Since thirty-three samples (55.0%) were negative by IFA but positive by nested PCR, no agreement (kappa = 0.09) was found between the results of IFA and nested PCR.

**DISCUSSION**

The primers described by Wakefield et al.\textsuperscript{18,20} were chosen because some investigators have shown that the mitochondrial rRNA gene PCR is the most specific and sensitive single-step PCR for the detection of *P. jirovecii*.\textsuperscript{15,21}

With a dramatic increase in the frequency of *P. jirovecii* pneumonia associated with human immunodeficiency virus infection, there has been a need for more rapid and less invasive diagnostic techniques. To make a diagnosis of *P. jirovecii* infection in immunodeficiency cases, it is important to perform traditional pathologic stains. The Wright-Giemsa stain and its modifications in a rapid manner (Diff-Quik) have a significantly lower sensitivity than other staining techniques.\textsuperscript{22,23} The most commonly employed cell wall stain is the silver stain. This modification is more sensitive and specific than other stains.\textsuperscript{24}
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TABLE 1. Results of IFA, single-step PCR, and nested PCR for 60 BAL specimens.

<table>
<thead>
<tr>
<th>IFA</th>
<th>Simple PCR result</th>
<th>Nested PCR result</th>
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<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Positive</td>
<td>20 (33.3)</td>
<td>3 (5.0)</td>
</tr>
<tr>
<td>Negative</td>
<td>5 (8.3)</td>
<td>32 (53.3)</td>
</tr>
</tbody>
</table>

REFERENCES

Pneumocystis jirovecii


