Evaluation of the sensitivity of multiplex PCR for the detection of

*Mycobacterium tuberculosis* and *Pneumocystis jirovecii*

in clinical samples

Sirirat Boondireke¹, Mathirut Mungthin², Peerapan Tan-ariya¹,

Pechara Boonyongsunthai³, Tawee Naaglor², Anan Wattanatum³,

Sompong Treewatchareekorn⁴, Saovanee Leelayoova²*

¹Department of Microbiology, Faculty of Science, Mahidol University

²Department of Parasitology, Phramongkutklao College of Medicine, 315 Ratchawithi Rd., Ratchathewi, Bangkok 10400, Thailand.

³Department of Medicine, Phramongkutklao Hospital, 315 Ratchawithi Rd., Ratchathewi, Bangkok 10400, Thailand.

⁴Department of Microbiology, Army Institute of Pathology, Royal Thai Army, Ratchawithi Rd., Ratchathewi, Bangkok 10400, Thailand.

*Corresponding author. Mailing address: Department of Parasitology, Phramongkutklao College of Medicine, 315 Ratchawithi Rd., Ratchathewi, Bangkok 10400, Thailand. Phone and fax: 662 354 7761. E-mail: s_leelayoova@scientist.com.
ABSTRACT

A multiplex polymerase chain reaction assay for the simultaneous detection of *Mycobacterium tuberculosis* and *Pneumocystis jirovecii* was developed using IS6110-based detection for *M. tuberculosis* and mitochondrial large subunit rRNA (mtLSU rRNA) gene for *P. jirovecii*. Ninety-five pulmonary blinded samples were examined using developed multiplex PCR assay, compared with single-nested PCR targeting IS6110 for *M. tuberculosis* and mtLSU rRNA for *P. jirovecii*. Of 95 pulmonary samples, developed multiplex-nested PCR could detect 36 cases for *M. tuberculosis*, 35 cases for *P. jirovecii* and 17 cases were diagnosed as a co-infection of *M. tuberculosis* and *P. jirovecii*. The sensitivity of multiplex-nested PCR in detecting *M. tuberculosis* and *P. jirovecii* was 92.1% and 81.4%, respectively, whereas the specificity in *M. tuberculosis* and *P. jirovecii* detection was 98.2% and 100%, respectively.
INTRODUCTION

Pulmonary tuberculosis (TB) and Pneumocystis pneumonia (PcP) are two of the most common opportunistic infections associated with AIDS (4) found worldwide including Thailand (2, 3, 6, 10). About one-third of the world population and one-third of people with HIV are infected with TB. The World Health Organization (WHO) reported globally 9.2 million new cases and 1.7 million deaths from TB occurred in 2006, of which 0.7 million cases and 0.2 million deaths were in HIV-positive people (21). At present, Pneumocystis pneumonia, caused by Pneumocystis jirovecii (also known as P. carinii f. sp. hominis), remains one of the most common AIDS-defining illnesses and is a frequent cause of morbidity and mortality in HIV-infected patients (7). Geographically, TB is the most common respiratory opportunistic infection in people with HIV worldwide, especially in the developing world (1, 4, 6, 8, 9, 12), whereas PcP is more prevalent in industrialized countries (4, 5, 13, 17). In Thailand, TB has been the most common whereas PcP is the second most common opportunistic infection in people with AIDS (12, 18). The total number of the two infections represents one half of opportunistic infections in AIDS cases. The infection of TB and PcP can clinically and radiologically mimic each other including a similar presentation in the patients, and cannot always be diagnosed by clinical presentation or sputum examination. In addition, co-infection in individuals may also occur. Therefore, accurate and rapid diagnosis is required. Molecular diagnosis is considered to be a reliable technique and essential to be developed or improved, to simultaneous diagnose TB and PcP. Having a developed technique for differential diagnosis between the two infections would contribute to the immediate treatment, controlling the diseases and decreasing the rate of transmission. The aim of the study was to develop a multiplex PCR technique for the detection of M.
*Mycobacterium tuberculosis* and *P. jirovecii* simultaneously in clinical samples. In this study, the development of multiplex PCR was involved with appropriate genes including optimum PCR mixture and PCR thermal profile. The multiplex PCR was applied to test its sensitivity and specificity with clinical specimens.

**MATERIALS AND METHODS**

**Sample collection**

A total of 95 sputum/bronchoalveolar lavage (BAL) samples obtained from patients of Phramongkutklao Hospital who had signs and symptoms suspecting TB or PCP were used in this study. These samples were diagnosed for *M. tuberculosis* using acid fast bacilli (AFB) stain and conventional *M. tuberculosis* culture. *P. jirovecii* was diagnosed using Toluidine Blue O stain and immunofluorescence assay (IFA). Samples were confirmed by PCR which were specific to each organism (16, 19, 20). Each sample was recorded as code number and kept at -20°C in order to use in a blind study of multiplex-nested PCR.

**DNA extraction**

Prior to DNA extraction, all samples were incubated at 75 to 90°C for 2-5 h to inactivate hazardous organisms. They were treated with N-acetyl-L-cysteine and lysis buffer and were subjected to DNA extraction using an automate extractor MagNa Pure Compact System and MagNa Pure Compact nucleic acid isolation kit (Roche Applied Science, Roche, Switzerland) according to the recommendations of the manufacturer. Final elutions of DNA were made in 100 µl and stored at -20°C until used.
For positive control, DNA was extracted from a positive culture of *M. tuberculosis* and *P. jirovecii* from a positive BAL sample, respectively. Extracted DNA was kept at -20°C and used as positive control throughout the study.

**Detection for TB and PcP using single-nested PCR**

Each sample was tested to confirm TB and PcP infection using single-nested PCR. PCR product was diluted 1:100 with distilled water, of which 1 µl was used as a template in the second round amplification. PCR products were analyzed using 2% agarose gel electrophoresis and visualized under UV light by UVitec BTS-15.M. The 100 bp DNA ladder (Promega, USA) was used as a standard marker.

**Single-nested PCR assay for IS6110 of TB**

PCR amplification was performed using primers previously described (16). The first round amplification using primer TB1 as a forward outer primer and TB4 as a reverse outer primer amplified a 302-bp region of repetitive sequence (Table 1). The reaction mixture consisted of de-ionized distilled water, Go Taq® Flexi buffer, 2 mM MgCl₂, 0.2 mM of each dNTPs, 25 pmol of each primer and 1.25 U Taq DNA polymerase (Promega, USA) and 1 µl DNA template. The protocol for IS6110 amplification was modified from a previous study (16), initiated with denaturation at 95°C for 5 min and then 40 cycles of 95°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec with a final extension of 72°C for 5 min.

In the second round amplification, the reaction mixture consisted of 25 pmol of each primer and other reagents similar to the first round amplification. A forward inner primer TB2 and a reverse inner primer TB3 amplified a 141-bp fragment (Table 1). The amplifying conditions used were the same as in the first round amplification with a total duration of 25 cycles.
Single-nested PCR assay for mtLSU rRNA gene of *P. jirovecii*

The amplification was performed using oligonucleotide primers published by Wakefield *et al.* (19, 20), as shown in Table 1. The first round amplification was performed using primer pAZ102-E and pAZ102-H as a forward and a reverse outer primer, respectively, which yielded a 346-bp fragment. The PCR mixture consisted of 1 µl of DNA template, de-ionized distilled water, Go Taq® Flexi buffer, 2 mM MgCl₂, 0.2 mM of each dNTPs, 25 pmol of each primer and 1.25 U Taq DNA polymerase. The condition, modified from a previous study (18), was as follows: denaturation of 94°C for 5 min, 40 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 1 min and extension at 72°C for 1 min. Final extension at 72°C for 5 min was performed.

In the second round amplification, the reaction mixture consisted of 25 pmol of each primer and other reagents similar to the first round amplification. A forward inner primer pAZ102-X and a reverse inner primer pAZ102-Y amplified a 260-bp fragment (Table 1) (20). The conditions used to amplify were the same as the first round amplification with a total duration of 25 cycles.

**Condition optimization of multiplex-nested PCR**

Multiplex-nested PCR was used to simultaneously amplify the two targets, IS6110 and mtLSU rRNA gene for *M. tuberculosis* and *P. jirovecii*, respectively. The first and second round amplification of the multiplex-nested PCR was performed using similar primers as the single-nested PCR for the detection of *M. tuberculosis* and *P. jirovecii*. To confirm the absence of cross-amplification between the selected primers, amplification of DNA mixture of both organisms was conducted. The optimization to adjust the parameters was performed to obtain a final condition that provided a good intensity of both amplicons, as well as the absence of non-specific
bands. Sequence analysis was also performed to confirm PCR products of both *M. tuberculosis* and *P. jirovecii* matched the theoretical amplicon sizes. Final conditions were initiated with a denaturation of 94°C for 5 min, 40 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 1 min and extension at 72°C for 1 min. Final extension was 72°C for 5 min. In the second round, the process was done using conditions similar to the first round amplification, with a total duration of 25 cycles. In each round, 25 µl of reaction mixture consisted of autoclaved de-ionized distilled water, Go Taq® Flexi buffer, 2 mM MgCl₂, 0.2 mM of each dNTP, 25 pmol of each primer and 1.25 U *Taq* DNA polymerase. In the first round PCR, one microlitre of DNA template comprised a mix of 0.5 µl (6 pg) *M. tuberculosis* DNA and 0.5 µl (18.19 pg) *P. jirovecii* DNA that was appropriate for dual bands.

**Detection limit of multiplex-nested PCR**

The multiplex-nested PCR assay's to detect artificially mixed DNA templates with varying ratios of *M. tuberculosis* and *P. jirovecii* were assessed. Titration experiments were conducted to determine the analytical sensitivity of the PCR for the detection of *M. tuberculosis* and *P. jirovecii* DNA.

**Sensitivity evaluation of developed multiplex-nested PCR**

All 95 blinded samples from the patients were examined using developed multiplex-nested PCR to test sensitivity and specificity. The efficiency of multiplex-nested PCR was evaluated using statistical analysis by means of sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). The results obtained from multiplex-nested PCR and single-nested PCR methods were compared with the calculation of 95% confidential interval using Stata/SE for Windows version 9.2 (StataCorp LP, College Station, TX).
RESULTS

Detection limit of multiplex-nested PCR

Each of the two pairs of oligonucleotide primers exclusively amplified the target gene of the specific microorganisms. The fragments obtained from multiplex-nested PCR optimization are shown in Figure. Using a multiplex-nested PCR assay could amplify and differentiate the two species, *M. tuberculosis* and *P. jirovecii*, in a single specimen. The expected sizes of *M. tuberculosis* and *P. jirovecii* were 141 bp and 260 bp, respectively. The detection limit of *M. tuberculosis* and *P. jirovecii* was approximately 0.30 fg and 0.28 fg, respectively.

Result comparison of multiplex-nested PCR and single-nested PCR.

Results obtained using single-nested PCR (gold standard) and multiplex-nested PCR are summarized (Table 2). Of 95 samples, 61 were positive for *M. tuberculosis* or *P. jirovecii* using single-nested PCR assay. Thirty eight samples (18 *M. tuberculosis* alone and 20 co-infections with *M. tuberculosis* and *P. jirovecii*) were positive for *M. tuberculosis* using IS6110-based detection. Of these samples, 16 were detected in the first and the second round amplification, 19 were detected in the second round alone, and another 3 were not detected in the second round, that contrast to the first round positive results. A total of 43 samples were positive for *P. jirovecii*, of which, 23 and 20 were *P. jirovecii* alone and co-infection with *M. tuberculosis* and *P. jirovecii*, respectively. Of these samples, 20 samples were detected in the first and the second round amplification, while another 23 samples were detected in the second round alone. Multiplex-nested PCR technique could identify 36 cases of TB (19 *M. tuberculosis* alone, 17 co-infection with *M. tuberculosis* and *P. jirovecii*) and 35 cases of PcP (18 *P. jirovecii* alone, 17 co-infection with *M. tuberculosis* and *P. jirovecii*).
Sensitivity and Specificity of multiplex-nested PCR

Using single-nested PCR as a gold standard method, the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of multiplex-nested PCR in detecting *M. tuberculosis* and *P. jirovecii* were individually determined, as shown in Table 3 and 4, respectively. The statistical determinations show the efficiency of this developed technique in the simultaneous detection of *M. tuberculosis* and *P. jirovecii*.

DISCUSSION

Numerous studies have reported molecular assay targeted *M. tuberculosis* and *P. jirovecii* separately. In this study, we describe the successful development of a multiplex-nested PCR for simultaneous detection of *M. tuberculosis* and *P. jirovecii*. The assay successfully amplified the positive controls and samples which nonspecific bands were not observed. The specificity of IS6110 in detecting the *M. tuberculosis*-complex (Mtc) using the specific sets of primer, TB1/TB4 and TB2/TB3, was confirmed by Se Thoe et al. in 1997 (16). These primers did not amplify non-Mtc strains (*M. fortuitum, M. intracellulare, M. kansasii, M. scrofulaceum, and M. smegmatis*). The specific primer set pAZ102-E/pAZ102-H was also confirmed for their specificity in detection by Wakefield AE et al. (19). These primers did not amplify other potential pulmonary pathogens (*Candida albicans*, other species of *Candida, Cryptococcus neoformans, Mycobacterium tuberculosis, Saccharomyces cerevisiae, Aspergillus nidulans*) and human DNA.

Clinical sample examination using single-nested PCR revealed 19 additional TB positive cases and 23 additional PcP positive cases in the second round from the first round amplification. This confirmed the improvement in sensitivity of nested PCR over single round PCR. A similar contribution also occurred in multiplex-nested
PCR. Moreover, the enhanced sensitivity in detection was obtained using multi-copy genes IS6110 and mtLSU rRNA. Most reports using IS6110-based detection have claimed sensitivities of over 75% and specificities approaching 100% (14). The sensitivity (92.1%) and specificity (98.2%) obtained from developed multiplex-nested PCR assay in detecting TB has proved to be reliable enough for diagnostic application. The sensitivity (81.4%) and specificity (100%) obtained in *P. jirovecii* detection using multiplex-nested PCR assay also indicated efficiency in diagnosis. The detection limits determined using diluted DNA solutions prior amplification were as little as 0.30 fg *Mtb* DNA and 0.28 fg *P. jirovecii* DNA. The amplification products of the lower DNA template amounts were below the limit of quantification. The detection limits of this multiplex-nested PCR showed the equivalent range for both *M. tuberculosis* and *P. jirovecii* compared to the previous studies using the same targets by single-nested PCR (11, 15). These showed efficiency in detection of two pathogens because the detection limits were low. However, the acquired sensitivities from evaluation were not as high as expected, especially in *P. jirovecii* detection. The probable reason for amplification of dual pathogens at the same time might compete with the use of PCR components (i.e., Mg²⁺ and dNTPs) between the process of each gene amplification and inhibition or reduction of PCR reactions in the first round amplification. This was due to the fact that the assay was developed by modification from two conditions and the conditions used in detecting TB alone was more sensitive than that in *P. jirovecii* detection.

In conclusion, we report the first simultaneous multiplex-nested PCR for the diagnosis and differentiation of TB and PcP. Rapid, cost-effective development of multiplex-nested PCR will be useful for the application in differential diagnosis of a
large number of clinical samples when compared with the single-nested PCR technique.

ACKNOWLEDGEMENT

This study was financially supported by Phramongkutklao Hospital’s Foundation under Her Royal Highness Princess Maha Chakri Sirindhorn’s Patronage and Phramongkutklao College of Medicine. We would like to thank Mr. Chavachol Setthaudom, Immunology Unit, Department of Pathology, Ramathibodi Hospital, Mahidol University.
Figure. Multiplex-nested PCR optimization. Lane M: 100 bp DNA ladder, Lane 1: negative control, Lane 2: positive TB DNA fragment, Lane 3: positive \textit{Pj} DNA fragment, Lane 4: Multiplex-nested PCR with the two species together—\textit{M. tuberculosis} (141 bp) and \textit{P. jirovecii} DNA fragment (260 bp).
<table>
<thead>
<tr>
<th>Primer</th>
<th>Oligonucleotide sequences (5’-3’)</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TB1/TB4</td>
<td>GTGCGGATGGTGCAGAGAT/CCTGATGATCGGCGATGAAC</td>
<td>302</td>
</tr>
<tr>
<td>TB2/TB3</td>
<td>AGCACGATTCCGGAGTGGGCA/TCACCGGATTTCTCGGTGCG</td>
<td>141</td>
</tr>
<tr>
<td>pAZ102-E/pAZ102-H</td>
<td>GATGGCTGTTCACAGCCCA/CTGTACGTTGCAAGTTACTC</td>
<td>346</td>
</tr>
<tr>
<td>pAZ102-X/pAZ102-Y</td>
<td>GTGAAATACAAATCGGACTAGG/TCACCTAATTATTTGGGAGC</td>
<td>260</td>
</tr>
</tbody>
</table>
Table 2. Results obtained by single-nested PCR and multiplex-nested PCR.

<table>
<thead>
<tr>
<th></th>
<th>Single-nested PCR</th>
<th>Multiplex-nested PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>18 \textit{Mtb}</td>
<td></td>
<td>16 \textit{Mtb}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 neg.</td>
</tr>
<tr>
<td>23 \textit{Pj}</td>
<td></td>
<td>17 \textit{Pj}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 \textit{Mtb} + \textit{Pj}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 neg.</td>
</tr>
<tr>
<td>20 \textit{Mtb} + \textit{Pj}</td>
<td></td>
<td>16 \textit{Mtb} + \textit{Pj}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 \textit{Mtb}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 \textit{Pj}</td>
</tr>
<tr>
<td>34 neg.</td>
<td></td>
<td>34 neg.</td>
</tr>
</tbody>
</table>

Total = 95

\textit{Mtb} = \textit{M. tuberculosis}, \textit{Pj} = \textit{P. jirovecii}, neg. = negative
Table 3. Results of multiplex-nested PCR compared with single-nested PCR for *M. tuberculosis* detection.

<table>
<thead>
<tr>
<th>Multiplex-nested PCR</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>%</td>
<td>N</td>
</tr>
<tr>
<td>Positive</td>
<td>35</td>
<td>36.84</td>
<td>1</td>
</tr>
<tr>
<td>Negative</td>
<td>3</td>
<td>3.16</td>
<td>56</td>
</tr>
<tr>
<td>Total</td>
<td>38</td>
<td>40.00</td>
<td>57</td>
</tr>
</tbody>
</table>

Sensitivity = 92.11% (95 % CI: 82.3-99.4), Specificity = 98.25%, PPV = 97.22%, NPV = 94.92%
Table 4. Results of multiplex-nested PCR compared with single-nested PCR for *P. jirovecii* detection.

<table>
<thead>
<tr>
<th>Multiplex-nested PCR</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N (%)</td>
<td>N (%)</td>
<td>N (%)</td>
</tr>
<tr>
<td>Positive</td>
<td>35 (36.84)</td>
<td>0 (0.00)</td>
<td>35 (36.84)</td>
</tr>
<tr>
<td>Negative</td>
<td>8 (8.42)</td>
<td>52 (54.74)</td>
<td>60 (63.16)</td>
</tr>
<tr>
<td>Total</td>
<td>43 (45.26)</td>
<td>52 (54.74)</td>
<td>95 (100.00)</td>
</tr>
</tbody>
</table>

Sensitivity = 81.40% (95% CI: 66.6-91.6), Specificity = 100%, PPV = 100%, NPV = 86.67%


14. **Sandhu, G. S., B. C. Kline, L. Stockman, G. D. Roberts, and M. E. Lewis.**


U.S. patent 5,731,150.


