

## Evaluation of Sensitivity of Multiplex PCR for Detection of *Mycobacterium tuberculosis* and *Pneumocystis jirovecii* in Clinical Samples<sup>∇</sup>

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**A multiplex PCR assay for the simultaneous detection of *Mycobacterium tuberculosis* and *Pneumocystis jirovecii* was developed using IS6110-based detection for *M. tuberculosis* and mitochondrial large-subunit (mtLSU) rRNA gene detection for *P. jirovecii*. Ninety-five pulmonary blinded samples were examined using the developed multiplex PCR assay, and the results were compared with those obtained by the single nested PCRs targeting IS6110 for *M. tuberculosis* and mtLSU rRNA for *P. jirovecii*. Of the 95 pulmonary samples tested, the multiplex nested PCR developed here could detect 36 cases of *M. tuberculosis* infection, 35 cases of *P. jirovecii* infection, and 17 cases of *M. tuberculosis* and *P. jirovecii* coinfections. The sensitivities of the multiplex nested PCR in detecting *M. tuberculosis* and *P. jirovecii* were 92.1% and 81.4%, respectively, whereas the specificities in detecting *M. tuberculosis* and *P. jirovecii* were 98.2% and 100%, respectively.**

Pulmonary tuberculosis (TB) and *Pneumocystis jirovecii* pneumonia are two of the most common opportunistic infections found in association with AIDS worldwide (4), including Thailand (2, 3, 6, 10). About one-third of the world's population and one-third of people infected with HIV are infected with *Mycobacterium tuberculosis*. The World Health Organization (WHO) reported that globally 9.2 million new cases of TB and 1.7 million deaths from TB occurred in 2006, and of these, 0.7 million cases and 0.2 million deaths, respectively, were in HIV-positive people (21). At present, *Pneumocystis* pneumonia, caused by *Pneumocystis jirovecii* (previously 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 infected with HIV worldwide, especially in the developing world (1, 4, 6, 8, 9, 12), whereas *P. jirovecii* pneumonia is more prevalent in industrialized countries (4, 5, 13, 16). In Thailand, TB has been the most common opportunistic infection in people with AIDS, whereas *P. jirovecii* pneumonia has been the second most common (12, 18). The total number of the two infections represents one-half of opportunistic infections in AIDS cases.

TB and *P. jirovecii* pneumonia can clinically and radiologically mimic each other, including having similar presentations in patients, and they cannot always be diagnosed by clinical presentation or sputum examination. In addition, coinfection

in individuals may also occur. Therefore, accurate and rapid diagnosis is required. The molecular means of diagnosis is considered to be a reliable technique, and it is essential that it be developed or improved to simultaneously diagnose TB and *P. jirovecii* pneumonia. Having a technique for differential diagnosis of the two infections would contribute to the ability to provide immediate treatment, controlling the diseases and decreasing the rates of transmission. The aim of the present study was to develop a multiplex PCR technique for the detection of *M. tuberculosis* and *P. jirovecii* simultaneously in clinical samples. In the present study, the development of a multiplex PCR involved selection of the appropriate genes, as well as the 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) fluid samples obtained from patients of Phramongkutklao Hospital who had signs and symptoms suspected of being TB or *P. jirovecii* pneumonia were used in this study. *M. tuberculosis* was detected in these samples using staining for acid-fast bacilli (AFB) and conventional *M. tuberculosis* culture. *P. jirovecii* was detected using toluidine blue O stain and immunofluorescence assay (IFA). The results for the samples were confirmed by a PCR specific for each organism (17, 19, 20). Each sample was recorded as a code number and kept at  $-20^{\circ}\text{C}$  in order to use in a blind study of the multiplex nested PCR.

**DNA extraction.** Prior to DNA extraction, all samples were incubated at  $75$  to  $90^{\circ}\text{C}$  for 2 to 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 automated MagNa Pure Compact extractor system and a 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  $100\ \mu\text{l}$  and were stored at  $-20^{\circ}\text{C}$  until use.

DNA was extracted from a culture of a BAL fluid sample positive for *M. tuberculosis* and one positive for *P. jirovecii* for use as positive controls. The

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TABLE 1. Primers used in single nested PCR assay and multiplex nested PCR assay

Primer	Oligonucleotide sequence (5'-3')	Size (bp)
TB1/TB4	GTGCGGATGGTTCGCAGAGAT CCTGATGATCGGCGATGAAC	302
TB2/TB3	AGCACGATTCGGAGTGGGCA TCACGGGATTCTTCGGTTCG	141
pAZ102-E/pAZ102-H	GATGGCTGTTTCCAAGCCCA GTGTACGTTGCAAAGTACTC	346
pAZ102-X/pAZ102-Y	GTGAAATACAAATCGGACTAGG TCACTTAATATTAATTGGGGAGC	260

extracted DNA was kept at  $-20^{\circ}\text{C}$  and was used as a positive control throughout the study.

**Detection of *M. tuberculosis* and *P. jirovecii* using single nested PCR.** Each sample was tested using single nested PCR to confirm the presence of *M. tuberculosis* and *P. jirovecii*. The PCR products were diluted 1:100 with distilled water, and 1  $\mu\text{l}$  was used as the template in the second-round amplification. The PCR products were analyzed using 2% agarose gel electrophoresis and were visualized under UV light by an UVitec BTS-15.M apparatus. A 100-bp DNA ladder (Promega) was used as a standard marker.

**Single nested PCR assay for IS6110 of *M. tuberculosis*.** PCR amplification was performed using primers described previously (17). 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 deionized distilled water, Go Taq Flexi buffer, 2 mM  $\text{MgCl}_2$ , 0.2 mM each deoxynucleoside triphosphate (dNTP), 25 pmol of each primer, 1.25 U Taq DNA polymerase (Promega), and 1  $\mu\text{l}$  DNA template. The protocol for IS6110 amplification was modified from that used in a previous study (17) and was initiated with denaturation at  $95^{\circ}\text{C}$  for 5 min, and then the mixture was subjected to 40 cycles of  $95^{\circ}\text{C}$  for 30 s, annealing at  $55^{\circ}\text{C}$  for 30 s, and extension at  $72^{\circ}\text{C}$  for 30 s and had a final extension of  $72^{\circ}\text{C}$  for 5 min.

In the second-round amplification, the reaction mixture consisted of 25 pmol of each primer and the other reagents used in the first-round amplification. Forward inner primer TB2 and reverse inner primer TB3 amplified a 141-bp fragment (Table 1). The amplification conditions used were the same as those used in the first-round amplification, with the total duration being 25 cycles.

**Single nested PCR assay for mtLSU rRNA gene of *P. jirovecii*.** Amplification of the mitochondrial large-subunit (mtLSU) rRNA gene of *P. jirovecii* was performed using the oligonucleotide primers published by Wakefield et al. (19, 20), as shown in Table 1. The first-round amplification was performed using primers pAZ102-E and pAZ102-H as the forward and reverse outer primers, respectively, and yielded a 346-bp fragment. The PCR mixture consisted of 1  $\mu\text{l}$  of DNA template, deionized distilled water, Go Taq Flexi buffer, 2 mM  $\text{MgCl}_2$ , 0.2 mM each dNTP, 25 pmol of each primer, and 1.25 U Taq DNA polymerase. The conditions, modified from those from a previous study (18), were as follows: denaturation at  $94^{\circ}\text{C}$  for 5 min and 40 cycles of denaturation at  $94^{\circ}\text{C}$  for 30 s, annealing at  $52^{\circ}\text{C}$  for 1 min, and extension at  $72^{\circ}\text{C}$  for 1 min. A final extension at  $72^{\circ}\text{C}$  for 5 min was performed.

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

**Condition optimization of multiplex nested PCR.** Multiplex nested PCR was used to simultaneously amplify the two targets, IS6110 and the mtLSU rRNA gene, for *M. tuberculosis* and *P. jirovecii*, respectively. The first- and second-round amplifications of the multiplex nested PCR were performed using primers similar to those used in 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 a mixture of DNA from both organisms was conducted. Optimization to adjust the parameters was performed to obtain a final condition that provided good intensities for both amplicons, as well as to provide no nonspecific bands. Sequence analysis was also performed to confirm that the

PCR products of both *M. tuberculosis* and *P. jirovecii* matched the theoretical amplicon sizes. Final conditions were initiated with denaturation at  $94^{\circ}\text{C}$  for 5 min and 40 cycles of denaturation at  $94^{\circ}\text{C}$  for 30 s, annealing at  $55^{\circ}\text{C}$  for 1 min, and extension at  $72^{\circ}\text{C}$  for 1 min. A final extension was carried out at  $72^{\circ}\text{C}$  for 5 min. In the second round, the process was done using conditions similar to those used in the first-round amplification, with the total duration being 25 cycles. In each round, 25  $\mu\text{l}$  of reaction mixture consisted of autoclaved deionized distilled water, Go Taq Flexi buffer, 2 mM  $\text{MgCl}_2$ , 0.2 mM each dNTP, 25 pmol of each primer, and 1.25 U Taq DNA polymerase. In the first-round PCR, 1  $\mu\text{l}$  of DNA template comprised a mix of 0.5  $\mu\text{l}$  (6 pg) *M. tuberculosis* DNA and 0.5  $\mu\text{l}$  (18.19 pg) *P. jirovecii* DNA, which was appropriate for the provision of dual bands.

**Detection limit of multiplex nested PCR.** The ability of the multiplex nested PCR assay to detect *M. tuberculosis* and *P. jirovecii* DNA when the DNA templates were present at various artificially mixed ratios was 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 the developed multiplex nested PCR.** All 95 blinded samples from the patients were examined using the multiplex nested PCR developed to test its sensitivity and specificity. The efficiency of the multiplex nested PCR was evaluated using statistical analysis for determination of the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). The results obtained from the multiplex nested PCR and single nested PCR methods were compared with the calculated 95% confidential intervals using the Stata/SE program 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 genes of the specific microorganisms. The fragments obtained from the multiplex nested PCR optimization are shown in Fig. 1. The multiplex nested PCR assay could amplify and differentiate the two species *M. tuberculosis* and *P. jirovecii* in a single specimen. The expected sizes of the *M. tuberculosis* and *P. jirovecii* products were 141 bp and 260 bp, respectively. The limits of detection of *M. tuberculosis* and *P. jirovecii* were approximately 0.30 fg and 0.28 fg, respectively.

**Comparison of multiplex nested PCR and single nested PCR results.** The results obtained using the single nested PCR (the "gold standard") and the multiplex nested PCR are summarized in Table 2. Of the 95 samples, 61 were positive for *M. tuberculosis* and/or *P. jirovecii* using the single nested PCR assay. Thirty-eight samples (18 with *M. tuberculosis* alone and

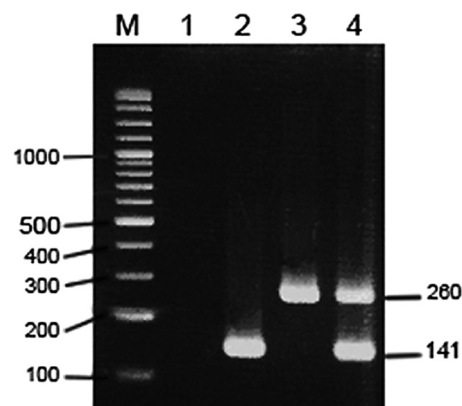


FIG. 1. Multiplex nested PCR optimization. Lane M, 100-bp DNA ladder; lane 1, negative control; lane 2, positive *M. tuberculosis* DNA fragment; lane 3, positive *P. jirovecii* DNA fragment; lane 4, multiplex nested PCR with the fragments of the two species *M. tuberculosis* (141 bp) and *P. jirovecii* DNA (260 bp) together. The numbers on the left and right of the gel are molecular sizes (in base pairs).

TABLE 2. Results obtained by single nested PCR and multiplex nested PCR

Sample no.	No. of samples with the indicated result	
	Single nested PCR	Multiplex nested PCR
1	18 <i>M. tuberculosis</i>	16 <i>M. tuberculosis</i> , 2 neg. <sup>a</sup>
2	23 <i>P. jirovecii</i>	17 <i>P. jirovecii</i> , 1 <i>M. tuberculosis</i> + <i>P. jirovecii</i> , 5 neg.
3	20 <i>M. tuberculosis</i> + <i>P. jirovecii</i>	16 <i>M. tuberculosis</i> + <i>P. jirovecii</i> , 3 <i>M. tuberculosis</i> , 1 <i>P. jirovecii</i>
4	34 neg.	34 neg.
Total	95	95

<sup>a</sup> neg., negative.

20 coinfecting 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 amplifications, 19 were detected in the second-round amplification alone, and another 3 were not detected in the second-round amplification, results that are in contrast to the positive results obtained in the first round. A total of 43 samples were positive for *P. jirovecii*; 23 and 20 of these samples were positive for *P. jirovecii* alone and coinfection with *M. tuberculosis* and *P. jirovecii*, respectively. Of these samples, 20 samples were detected in the first- and the second-round amplifications, while another 23 samples were detected in the second-round amplification alone. The multiplex nested PCR technique could identify 36 cases of TB (19 cases of *M. tuberculosis* infection alone, 17 cases of coinfection with *M. tuberculosis* and *P. jirovecii*) and 35 cases of *P. jirovecii* pneumonia (18 cases of *P. jirovecii* infection alone, 17 cases of coinfection with *M. tuberculosis* and *P. jirovecii*).

**Sensitivity and specificity of multiplex nested PCR.** Using single nested PCR as the gold standard method, the sensitivity, specificity, PPV, and NPV of the multiplex nested PCR in detecting *M. tuberculosis* and *P. jirovecii* were determined for each organism, as shown in Tables 3 and 4, respectively. The statistical determinations show the efficiency of the technique developed in the present study for the simultaneous detection of *M. tuberculosis* and *P. jirovecii*.

**DISCUSSION**

Numerous studies have reported on molecular assays that target *M. tuberculosis* and *P. jirovecii* separately. In this report,

TABLE 3. Results of multiplex nested PCR compared with those of single nested PCR for *M. tuberculosis* detection

Result by multiplex nested PCR	No. (%) of samples with the following single nested PCR result <sup>a</sup> :		
	Positive	Negative	Total
Positive	35 (36.84)	1 (1.05)	36 (37.89)
Negative	3 (3.16)	56 (58.95)	59 (62.11)
Total	38 (40.00)	57 (60.00)	95 (100.00)

<sup>a</sup> By use of gold standard 1 for the single nested PCR. Sensitivity, 92.11% (95% confidence interval, 82.3 to 99.4); specificity, 98.25%; PPV, 97.22%; NPV, 94.92%.

TABLE 4. Results of multiplex nested PCR compared with those of single nested PCR for *P. jirovecii* detection

Result by multiplex nested PCR	No. (%) of samples with the following single nested PCR result <sup>a</sup> :		
	Positive	Negative	Total
Positive	35 (36.84)	0 (0.00)	35 (36.84)
Negative	8 (8.42)	52 (54.74)	60 (63.16)
Total	43 (45.26)	52 (54.74)	95 (100.00)

<sup>a</sup> By use of gold standard 2 for the single nested PCR. Sensitivity, 81.40% (95% confidence interval, 66.6 to 91.6); specificity, 100%; PPV, 100%; NPV, 86.67%.

we describe the successful development of a multiplex nested PCR for the simultaneous detection of *M. tuberculosis* and *P. jirovecii*. The assay successfully amplified the positive controls and samples for which nonspecific bands were not observed. The specificity of IS6110 in detecting the *M. tuberculosis* complex (MTC) using the primer specific sets TB1/TB4 and TB2/TB3 was confirmed by Se Thoe et al. in 1997 (17). These primers did not amplify non-MTC strains (*M. fortuitum*, *M. intracellulare*, *M. kansasii*, *M. scrofulaceum*, and *M. smegmatis*). The detection specificity of specific primer set pAZ102-E/pAZ102-H was also confirmed by Wakefield et al. (19). These primers did not amplify DNA from other potential pulmonary pathogens (*Candida albicans*, other species of *Candida*, *Cryptococcus neoformans*, *Mycobacterium tuberculosis*, *Saccharomyces cerevisiae*, *Aspergillus nidulans*) or human DNA.

Clinical sample examination using a single nested PCR revealed 19 additional TB-positive cases and 23 additional *P. jirovecii* pneumonia-positive cases in the second-round amplification compared with the numbers revealed from the first-round amplification. This confirmed the improvement in sensitivity of the nested PCR over that of the PCR with single-round amplification. A similar contribution of the multiplex nested PCR also occurred. Moreover, the enhanced sensitivity of detection was obtained using multicopy genes IS6110 and mtLSU rRNA. Most reports of studies using IS6110-based detection have claimed sensitivities of over 75% and specificities approaching 100% (14). The sensitivity (92.1%) and specificity (98.2%) of the multiplex nested PCR assay developed in the present study for detecting *M. tuberculosis* have proved that the assay is reliable enough for diagnostic application. The sensitivity (81.4%) and specificity (100%) of the multiplex nested PCR assay for detecting *P. jirovecii* also indicated its efficiency for diagnosis. The detection limits determined using DNA solutions diluted prior to amplification were as little as 0.30 fg *M. tuberculosis* DNA and 0.28 fg *P. jirovecii* DNA. The amplification products obtained with smaller DNA template amounts were below the limit of quantification. The detection limits of this multiplex nested PCR showed ranges for both *M. tuberculosis* and *P. jirovecii* equivalent to those obtained by single nested PCR in previous studies using the same targets (11, 15). These detection limits showed the efficiency of the method for the detection of the two pathogens because they were low. However, the sensitivities acquired from the evaluation were not as high as expected, especially for *P. jirovecii* detection. The probable reason for this is that amplification of two pathogens at the same time might cause competition for

the components of the PCR mixture (i.e., Mg<sup>2+</sup> and dNTPs) between the processes of amplification of each gene and inhibition or reduction in the amounts of products from the PCRs in the first-round amplification. This was due to the fact that the assay was developed by modification of two conditions, and the conditions used in detecting *M. tuberculosis* alone were more sensitive than those used in detecting *P. jirovecii*.

In conclusion, we report on the first multiplex nested PCR for the simultaneous detection and differentiation of TB and *P. jirovecii* pneumonia. The rapid, cost-effective development of the multiplex nested PCR will result in an assay more useful than the single nested PCR technique for application to differential diagnosis for a large number of clinical samples.

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