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Detection of Mycobacterium tuberculosis complex in formalin-fixed, paraffin-embedded tissue by Multiplex Polymerase Chain Reaction Assay

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Mycobacterium;
C-PCR;
M-PCR;
Formalin-Fixed;
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ABSTRACT

Tuberculosis (TB) is a common infectious disease and a serious public health problem in Thailand. The causative agents of TB are a group of closely related bacteria known as the Mycobacterium tuberculosis complex (MTBC). One accepted method for the diagnosis of tuberculous infection is the detection of their DNA by conventional polymerase chain reaction (C-PCR) assay. However, this method is complicated and time-consuming, and thus unsuitable for mass screening. A simpler and faster multiplex PCR (M-PCR) assay has been developed to overcome these problems. The objective of this study is to prove that detection of MTBC DNA from formalin-fixed, paraffin-embedded (FFPE) tissue by M-PCR assay can be acceptable in comparison to the previous C-PCR assay. Paraffin-embedded tissue samples of one hundred and fifteen suspected cases of tuberculosis referred to the Institute of Pathology, Department of Medical Services were retrieved for DNA extraction. IS6110 and B-globin gene were examined by M-PCR and C-PCR assays for the detection of M. tuberculosis complex. The results of the M-PCR assay agreed with the C-PCR assay (K=1.00, 95% CI 1.00-1.00). In conclusion, M-PCR assay is a simpler, faster, and less costly method that can be an efficient and effective alternative to C-PCR assay for the DNA detection of M. tuberculosis complex from formalin-fixed, paraffin-embedded (FFPE) tissue.

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Introduction

Tuberculosis (TB) is the crucial cause of death from infectious diseases worldwide and a serious public health problem in Thailand[1,2]. The causative agents of TB are a group of closely related bacteria known as the *M. tuberculosis* complex (MTBC)[1,3].

Diagnosis of MTBC infection has been implemented by multiple methods. Culture is a standard method but it takes more than eight weeks to complete[4,5]. Sputum smear for acid-fast bacilli (AFB) is easy, fast, and inexpensive, but its efficiency is limited by relatively low sensitivity and cannot distinguish MTBC from non-tuberculous mycobacteria (NTM)[6,7]. For the tissue section, Ziehl-Neelsen staining and careful search for acid-fast bacilli should be made to diagnose tuberculosis. Its efficiency is also limited. Acid-fast bacilli are frequently missed under light microscopy on formalin-fixed, paraffin-embedded tissue[8].

Currently, nucleic acid amplification test (NAAT)-based assays are being actively conducted for the detection of MTBC[4,6,9]. Conventional polymerase chain reaction (C-PCR) assay is generally reliable but has limitations because of two separate reactions between the IS6110 gene target and the beta-globin gene target.

Multiplex polymerase chain reaction (M-PCR) refers to the use of PCR to amplify several different DNA sequences simultaneously as if performing many separate PCR reactions all together in one reaction[10-12]. M-PCR can be performed in a single PCR reaction. This decreases the duration and risk of contamination. In addition, the M-PCR assay uses lesser amount of reagents causing lower cost than the C-PCR. We aimed to evaluate that this more beneficial method can be accepted as the C-PCR assay.

Materials and methods

Clinical Samples

One hundred and fifteen formalin-fixed paraffin-embedded (FFPE) tissue samples suspected of tuberculosis that were referred to the Institute of Pathology, Department of Medical Services were retrieved for DNA extraction. The study was approved by the Ethics Committee of the Institute of Pathology (IOP-KM-R63-001). The pathologist reexamined the histomorphology of all samples and marked the area suspected of the tuberculous lesion.

DNA extraction of FFPE tissues

Each tissue sample was manually micro-dissected from paraffin-embedded blocks. Ten µm thick ribbon sections were put in a microcentrifuge tube. Paraffin was removed from the tissue sections with xylene and rehydration with 100% ethanol. DNA was extracted from FFPE tissues and purified using a QIAamp DNA FFPE Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions[13]. DNA quantity was determined by NanoDrop spectrophotometry (NanoDrop Technologies, Wilmington, DE). The DNA solution was adjusted to a concentration of 500 ng/µl and stored at -40 °C for further use in the following process.

Detection of M. tuberculosis complex in FFPE tissue by C-PCR Assay

The acceptable C-PCR reaction for the detection of *M. tuberculosis* complex in FFPE tissues was performed by using two tubes in each case. One was used for the IS6110 gene target and β-globin gene was performed as internal control. The first tube (25 µl in total volume) for the detection of IS6110 gene-specific MTBC gene contained 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP (New England Biolabs, USA), Amplitaq gold DNA polymerase 0.625 Unit (Applied Biosystems, USA), DNA template 500 ng/µl, adjusted volume with distilled water and 0.4 µM oligonucleotide primers: IS6110-F; 5'-CCT-GCG-AGC-GTA-GGC-GTC-GG-3' and IS6110-R; 5'-CTC-GTC-CAG-CGC-CGC-CGC-TTC-GG-3'. The second tube (25 µl in total volume) for the detection of DNA quality contained 1XPCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP (New England Biolabs, USA), Amplitaq gold DNA polymerase 0.625 Unit (Applied Biosystems, USA), DNA template 500 ng/µl, adjust the volume with distilled water and 0.4 µM Oligonucleotide primer: β-globin-F; 5-'ACA-CAA-CTG-TGT-TCA-CTA-GC-3' and β-globin-R; 5'-CAA-CTT-CAT-CCA-CGT-TCA-CC-3'. Reactions in both tubes were

amplified performed in the T100 thermocycler (Bio-Rad, USA) under the following conditions: an initial denaturation step at 95 °C for 10 min, followed by 40 cycles at 95 °C for 45 s, 63 °C for 45 s, 72 °C for 45 s, and finally 10 min at 72 °C. After amplification, the amplified products with a loading volume of 10 µl were analyzed by 6% polyacrylamide gel electrophoresis at 140 V for 45 min. Gels were stained with SYBR Green I Nucleic Acid Gel Stain (1:400, Lonza, USA) for 30 min. Two separate lanes on the electrophoresis gel were evaluated. One lane was for the MTBC. The MTBC-positive reaction showed a band at 123 bp. The other lane was for DNA quality. The good DNA quality showed a band at 110 bp. (Figure 1).

Detection of M. tuberculosis complex in FFPE tissue by M-PCR Assay

M-PCR reaction for the detection of *M. tuberculosis* complex in FFPE tissue was performed using a single tube in each case. The single-tube reaction had two main genes composed of IS6110 gene and β-globin gene. The single tube reaction (25 µl in total volume) contained 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP (New England Biolabs, USA), Amplitaq gold DNA polymerase 0.625 Unit (Applied Biosystems, USA), DNA template 500 ng/µl, 0.4 µM IS6110-F primer, 0.4 µM IS6110-R primers, 0.2 µM B-globin-F primer, 0.2 µM B-globin-R primer, and adjustable volume with distilled water. The following amplification reaction and analysis, the procedures were the same as the C-PCR assay. Each case needed only one lane on the electrophoresis gel for evaluation. The MTBC-positive reaction showed two bands at 123 bp and 110 bp. The MTBC-negative reaction showed only one band at 110 bp in case of good DNA quality.

Minimum DNA concentration for the detection of MTBC by M-PCR assay

DNA from the MTBC-positive case detected by M-PCR assay was diluted to various concentrations to evaluate the minimum DNA concentration that can be used for the detection of MTBC by M-PCR assay. The diluted DNA samples were 500, 300, 200, 100, 50, 25, 10 and 5 ng/µl. Distilled water was used as a negative control. All different DNA concentration samples of the MTBC-positive case were performed by the same M-PCR assay. Methods for amplification reaction and analysis were the same as previously mentioned in all samples. The minimum appropriate concentration for the detection of MTBC showed two bands at 123 bp and 110 bp. The inappropriate concentration showed only one band at 110 bp. or without any band.

Statistical analysis

Results of the detection of MTBC from the 115 formalin-fixed, paraffin-embedded specimens by M-PCR and C-PCR techniques were compared and were evaluated for significance using the Kappa statistics. The k-value was more than 0.81. It was considered to be significant and indicating that both methods provided almost perfect results. Statistics were carried out using SPSS software (version 19).

Results

Demographic of patients' characteristics

The demographic characteristics are presented in Table 1. Of the 115 patients, the median age was 45 years old. The male to female ratio was 1.3:1. The dominant organ was lymph node 29.6% (34/115). The most common histologic features of the sample (53.9%) were chronic inflammation and necrosis.

Table 1 Patients' characteristics of 115 samples

Characteristics	N (%)
Total patients	115
Median age (range)	45
< 20 years	13 (11.3%)
20 - 39 years	33 (28.7%)
40 - 59 years	37 (32.2%)
60 - 79 years	29 (25.2%)
80 - 99 years	3 (2.6%)
Gender	
Male	65 (56.5%)
Female	50 (43.5%)
Organs	
Lung	15 (13.0%)
Brain	2 (1.7%)
Intestine	24 (20.9%)
Gallbladder	2 (1.7%)
Skin	10 (8.7%)
Synovitis	2 (1.7%)
Lymph node	34 (29.6%)
Bone	11 (9.6%)
Soft tissue	5 (4.3%)
Others	10 (8.7%)
Histological classification no granulomatous	34 (29.6%)
Chronic, Inflammation, Necrotic	62 (53.9%)
Caseation, granuloma, Langhans giant cells	19 (16.5%)

M-PCR and C-PCR analysis of M. tuberculosis complex detection in FFPE tissue specimens

Results of M-PCR and C-PCR analysis of MTBC detection in FFPE tissue were the same. Twenty-six samples (22.61%) were positive for MTBC-DNA and 89 samples (77.39%) were negative for MTBC-DNA. All samples (115 samples, 100%) had good DNA quality (positive for B-globin-DNA). The comparison of their results by kappa analysis (Table 2) showed that the M-PCR assay had a very good agreement with the C-PCR assay (K=1.00, 95% CI 1.00-1.00). This means that the M-PCR assay is an acceptable assay as the reliable C-PCR assay. Duration for the M-PCR assay is approximately four hours shorter than the C-PCR assay. In addition, the M-PCR assay uses a lesser amount of reagents and materials than the C-PCR assay. This means that the M-PCR assay for the detection of MTBC in FFPE is faster and less expensive than the C-PCR assay.

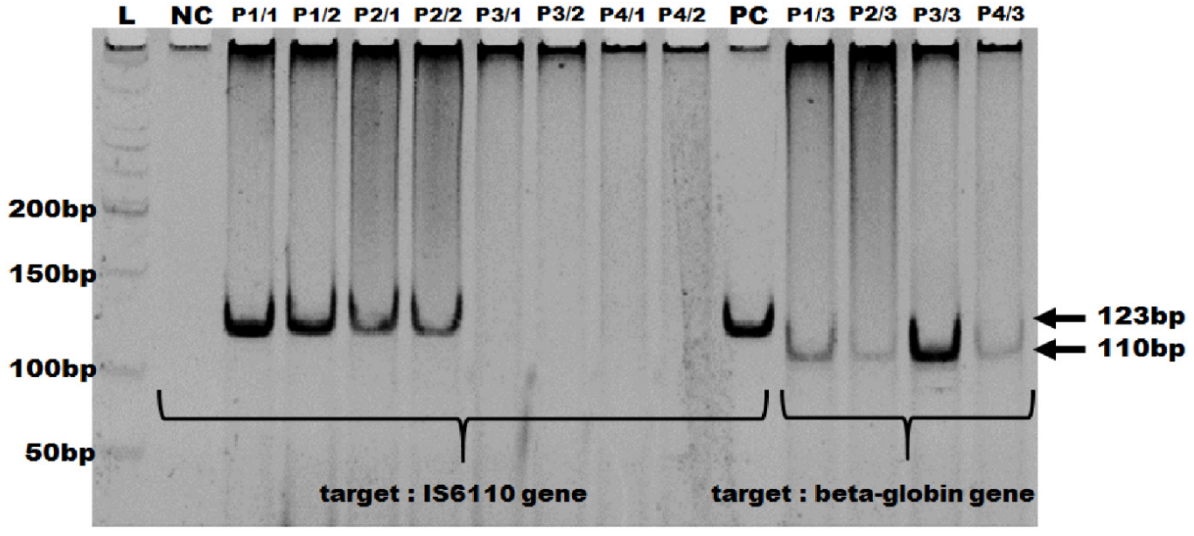

Figure 1 C-PCR assay of IS6110 and ß-globin gene for detected MTBC. Lane L: 50 bp DNA ladder. Lane NC: Negative control (IS6110 gene). Lane P1/1-P1/2 and P2/1-P2/2: By duplication reaction with clinical sample number 1 and 2 showed MTB infection; Lane P3/1-P3/2 and P4/1-P4/2: By duplication reaction with clinical sample number 3 and 4 showed no MTB infection. Lane PC: Positive control (IS6110 gene); show PCR product at 123 bp. Lane P1/3, P2/3, P3/3 and P4/3: clinical sample number 1, 2, 3 and 4 were test DNA quality show PCR product at 110 bp, respectively.

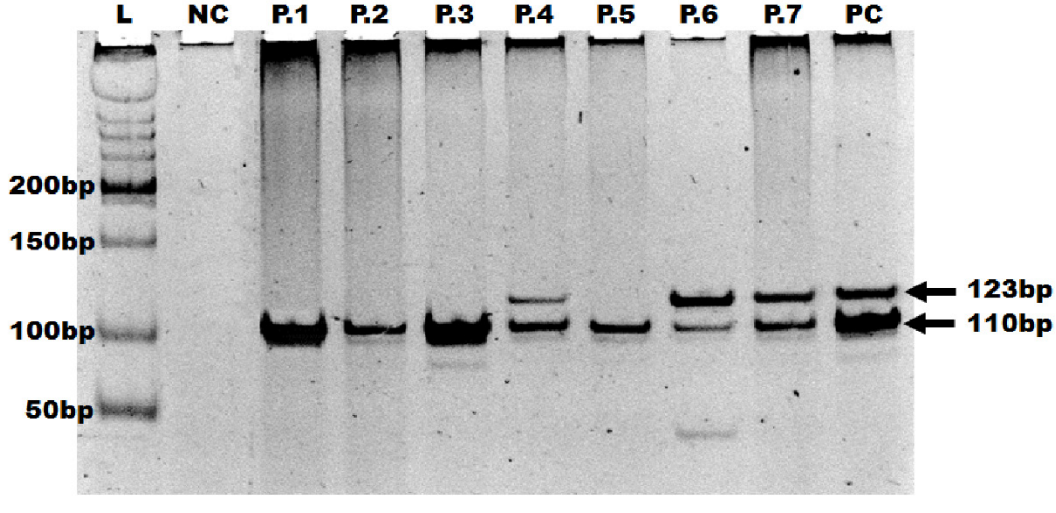

Figure 2 M-PCR assay distinguished IS6110 and beta-globin genes for detected MTBC. Lane L: 50 bp DNA ladder. Lane NC: Negative control. Lane P.1, P.2, P.3 and P.5: clinical sample number 1, 2, 3 and 5 showed no MTB infection. Lane P.4, P.6 and P.7: clinical sample number 4, 6 and 7 showed MTB infection. Lane PC: Positive control; showed PCR products at 123 bp and 110 bp.

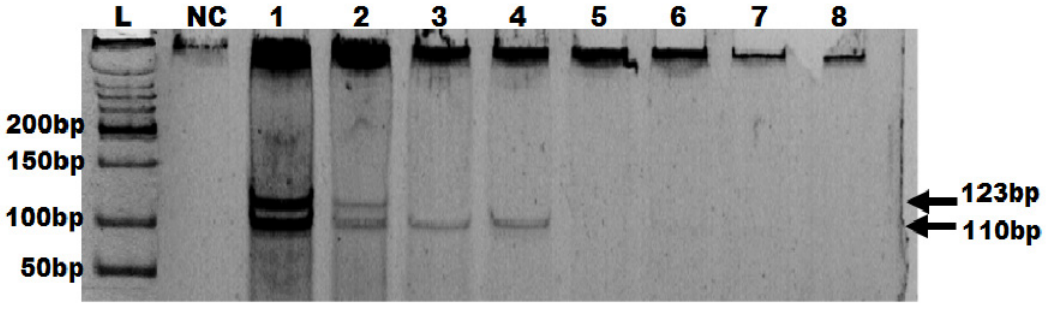

Figure 3 Minimum DNA concentration of detection by M-PCR Assay. A representative gel is shown. Dilutions of DNA template of patients with Positive-MTBC (from 500, 300, 200, 100, 50, 25, 10 and 5 ng/µl). Lane L: 50 bp DNA ladder. Lane NC: Negative control. Lane 1–8: corresponded to PCR products from 500 ng/µl to 5 ng/µl.

Table 2 Pairwise comparison and agreement analyses between C-PCR and M-PCR

Method	Sample (N)	Result				K (95%CI)	Duration (hours)
		IS6110		B-globin			
		Positive	Negative	Positive	Negative		
C-PCR	115	26	89	115	0	1.0 (1.0-1.0)	8
M-PCR	115	26	89	115	0		4

Discussion

Nucleic acid amplification test (NAAT)-based assays are recommended by the WHO as initial tools for cases suspected to have TB[14,15]. The polymerase chain reaction (PCR) for the detection of MTBC in formalin-fixed paraffin-embedded (FFPE) tissue has been widely performed[9,16]. It can give faster results than the culture and more sensitivity than the histochemical staining on the FFPE tissue.

MTBC has several DNA-regions, such as the intergenic spacer from the 16S or 23S rRNA genes, MPB64, insertion sequences 6110 (IS6110) that have been selected as NAAT PCR targets[17,18]. IS6110 is an insertion element that is found exclusively within the MTBC. It can be detected from all species of the *M. tuberculosis* complex (MTBC)[9,19,20] and has been reported to be widely used for the diagnosis of mycobacterial infection in clinical specimens[18,19,21]. To assess the specimen quality, the human beta-globin gene was included as an internal control to check for inhibition, nucleic acid extraction, and the amplification processes[9,16].

Most of the PCR based studies had reported the use of a single target like IS6110 for the diagnosis of MTB[10,22], without checking an internal control for the assessment of the DNA quality[10,22]. In addition, many PCR based studies had reported the use of two separate targets by C-PCR assay. One target was IS6110 for the diagnosis of MTB. The other was the beta-globin gene for the evaluation of internal control[9,16]. The other method, multiplex polymerase chain reaction (M-PCR) can amplify several different DNA sequences simultaneously and can be performed in a single PCR reaction. This method uses lesser amount of reagents and a shorter duration than the two separate reactions of the C-PCR assay.

To prove that M-PCR assay, the shorter duration method and lower-cost testing than C-PCR assay can be used to detect MTBC in FFPE tissue as the C-PCR assay; 115 samples of DNA extracted from the FFPE tissue were used to detect MTBC by both methods. Their results showed a superior consistency between the C-PCR assay and M-PCR (K = 1.0), which means that there was the acceptance of the similarity between the

two methods. Even though, the C-PCR method is a single target in a single reaction tube (first tube; IS6110 gene target and second tube; ß-globin gene target), which can be amplified to a PCR at the same condition. In contrast, M-PCR allows for simultaneous amplification of multiple target sequences in a single tube. However, the C-PCR method was limited and requires a larger amount of material and time.

Thus, the M-PCR proposed in this study is an effective and reliable diagnostic tool for accurate detection of *M. tuberculosis* complex infection in routine formalin-fixed, paraffin-embedded (FFPE) tissue. In addition, we also tested that the minimum concentration that can be used to detect the positivity for MTBC in the FFPE tissue is 300 ng/µl.

Conclusion

In conclusion, the M-PCR assay can be used for the detection of *M. tuberculosis* complex in FFPE tissue, by performing the IS6110 gene and B-globin gene in a single tube. Compared to the C-PCR assay, both showed the same testing results, but the M-PCR assay is easier to perform, faster, and more cost-effective. This method has a great potential to improve the clinicians' ability for early and rapid diagnosis of MTBC, thus ensuring early treatment and preventing further transmission of disease.

Clinical implication

- The single-tube reaction had two main genes composed of IS6110 gene and ß-globin gene detection of MTBC in FFPE tissue.
- Minimum concentration that can be used to detect the positivity for MTBC in the FFPE tissue is 300 ng/µl.

Conflicts of interest

The authors declare no conflict of interest.

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