Detection and differentiation of *Mycobacterium tuberculosis* complex and Nontuberculous Mycobacteria in Liquid Cultures and Sputum Specimens by Real-time PCR

การตรวจและจำแนกเชื้อมัยโคแบคทีเรียก่อวัณโรคและเชื้อมัยโคแบคทีเรียไม่ก่อวัณโรคในอาหารเลี้ยงเชื้อเหลวและตัวอย่างเสมหะด้วยเรียลไทม์พีซีอาร์

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ABSTRACT

The detection and differentiation of *Mycobacterium tuberculosis* complex (MTC) from nontuberculous mycobacteria (NTM) using effective and rapid method is a primary importance for infection control and selection of the appropriate antimicrobial therapy. This study aimed to develop a real-time PCR assay for detection and differentiation of MTC and NTM. Forty liquid culture positive specimens, 20 acid fast bacilli (AFB) smears positive, 20 AFB smears negative sputum, 10 *Mycobacterium* reference strains and 5 strains of other bacteria were tested by the real-time PCR. An AFB staining and culturing were used as reference methods. It was found that the developed real-time PCR could detect and differentiate MTC and NTM from positive liquid culture media and sputum specimens.

บทคัดย่อ

การตรวจและจำแนกเชื้อมัยโคแบคทีเรียก่อวัณโรคจากเชื้อมัยโคแบคทีเรียไม่ก่อวัณโรคโดยใช้วิธีที่มีประสิทธิภาพและรวดเร็วเป็นพื้นฐานที่สำคัญสำหรับการควบคุมการติดเชื้อและการเลือกยาต้านจุลชีพที่เหมาะสมในการรักษา การศึกษานี้ได้พัฒนาเรียลไทม์พีซีอาร์เพื่อตรวจและการจำแนกเชื้อมัยโคแบคทีเรียก่อวัณโรคและเชื้อมัยโคแบคทีเรียไม่ก่อวัณโรค โดยทดสอบกับ ตัวอย่างอาหารเลี้ยงเชื้อเหลวที่มีผลการเลี้ยงเชื้อเป็นบวก 40 ตัวอย่าง ตัวอย่างเสมหะที่มีผลการย้อมสีทนกรดเป็นบวก 20 ตัวอย่าง, ตัวอย่างเสมหะที่มีผลการย้อมสีทนกรดเป็นลบ 20 ตัวอย่างมัยโคแบคทีเรียสายพันธุ์อ้างอิง 10 สายพันธุ์และแบคทีเรียสายพันธุ์อื่นๆ 5 สายพันธุ์ ทั้งนี้ได้ใช้การย้อมแบคทีเรียทนกรดและการเลี้ยงเชื้อในอาหารเลี้ยงเชื้อเป็นวิธีอ้างอิง วิธีตรวจด้วยเรียลไทม์พีซีอาร์ที่พัฒนาใหม่นี้สามารถใช้ตรวจและจำแนกกลุ่มเชื้อมัยโคแบคทีเรียก่อวัณโรคและเชื้อมัยโคแบคทีเรียไม่ก่อวัณโรคในอาหารเลี้ยงเชื้อเหลวและในตัวอย่างเสมหะ

Key Words: *Mycobacterium tuberculosis* complex, Nontuberculous mycobacteria, Real-time PCR
คำสำคัญ: มัยโคแบคทีเรียก่อวัณโรค มัยโคแบคทีเรียไม่ก่อวันโรค เรียลไทม์พีซีอาร์

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Introduction

Mycobacterial pathogens are categorized into 2 groups including *Mycobacterium tuberculosis* complex (MTC) and nontuberculous mycobacterium (NTM). MTC is causative agent of tuberculosis (TB) which is a global public health problem. Moreover, NTM causes mycobacteriosis which is a major health problem in immunocompromised patients and HIV-infected patients (Miguez-Burbano et al., 2005). The increase in mycobacterial diseases has stimulated the development of the rapid, sensitive, and accurate diagnostic methods for detection and differentiation of mycobacteria.

Nowadays, many methods have been developed for the diagnosis of the mycobacterial pathogen (Neonakis et al., 2008). Early presumptive diagnosis of mycobacterial infection may be suggested by patient's history, clinical symptom and radiological finding. The initial diagnosis of mycobacterial pathogen by microscopic examination of Ziehl-Neelson stained bacterial smear is rapid, simple and low cost but this method gave low sensitivity (Chakraborty et al., 2009). Culture and biochemical test is gold standard method for diagnosis of tuberculosis but it is a time consuming process (Morcillo et al., 2008).

Recently, real-time PCR assay was developed for rapid, sensitive and specific for detection and differentiation of mycobacteria (Rindi et al., 2004 and Torres et al., 2003). These techniques based on fluorescent dye or fluorophores have been used to detect and differentiate MTC by examination of amplification curves or measure melting characteristics and it can monitor the progress of the PCR processes as it occurs in real time. However, real-time PCR technique based on fluorescent dye are low specificity and sometime cause false positive. Therefore specific fluorogenic probes have been used to increase specificity of the real-time PCR assay. Real-time PCR assay based on fluorogenic probes was more specific and sensitive for detection and differentiation of mycobacteria (Miller et al., 2001; Shrestha et al. 2003). In this study, we developed the real-time PCR for detection and differentiation of MTC and NTM from liquid cultured bacteria and clinical sputum specimens.

Objective of the study

The aim of this study was to develop the real-time PCR for detection and differentiation of MTC and NTM from liquid cultured bacteria and sputum specimens.

Methodology

Samples and isolates

A Forty MGIT BACTEC culture positive specimen, 20 acid fast bacilli (AFB) smear positive sputum and 20 AFB smear negative sputum specimens were obtained from the tuberculosis clinic of Srinagarind Hospital and The office of Disease Prevention and Control 6th, Khon Kaen, Thailand.

Ten mycobacterial reference strains and 5 bacterial reference specimens used for evaluation of the real-time PCR were shown in Table 1.

This study was approved by the Khon Kaen University Ethics Committee for Human Research (approval number HE521199).

DNA preparation

Bacterial DNA was purified using QIAGEN DNeasy® blood and tissue kit (QIAGEN, Hilden, Germany). Bacterial cells were lysed by lysozyme and incubated at 37°C for 30 min. Proteinase K was added

to the cells, and incubated at 56°C for 30 min. Chromosomal DNA was precipitated with absolute ethanol, and DNA was purified by using a QIAGEN DNeasy® blood and tissue kit column. The DNA was stored at -20°C.

The DNA of each specimen was purified using the same kit as above. Sputum samples were decontaminated with N-acetyl-L-cysteine-NaOH. The chromosomal DNA was extracted and purified by QIAGEN DNeasy® blood and tissue kit (QIAGEN, Hilden, Germany) following above protocol.

Table 1 List of reference mycobacteria and non mycobacteria used for evaluation of the real-time PCR

Species	Strains
M. tuberculosis (3)	H37Rv, H37Ra, H37Rvj
M. bovis	BCG Pasteur
M. kansasii	302
M. szulgai	JATA
M. xenopi	ATCC 19250
M. intracellulare	ATCC 13950
M. flavescens	ATCC 23035
Streptococcus pneumoniae	Clinical specimen
Staphylococcus aureus	Clinical specimen
Klebsiella pneumoniae	Clinical specimen
Haemophilus influenzae	Clinical specimen
Nocardia	Clinical specimen

Primers and probes design

Primers and probes (TIB MOLBIOL, Berlin, Germany) were designed by using all mycobacterial internal transcribed spacer (ITS) regions between 16S-23S rRNA sequences currently available at www.ncbi.nlm.nih.gov/Entrez/ and were compared with previously published sequences of primers and probes. The design was done delicately to avoid self-complementarity and formation of dimer and hairpins, which was evaluated by Oligoanalyzer 3.1 software.

Forward (5'- CCA ACA CAC TGT TGG GTC) and reverse primers (5'-AGA ATA TTG CAC AAA GAA CAC G) were used to amplify 159 base pair of the ITS region of *Mycobacterium* species. The specific probes, LC53 (5'-GTG GTG TTT GAG AAC TGG ATA GTG-fluorescein) LC64 (5'-LightCycler Red 640- TGC GAG CAT CAA TGG ATA CGC TGC CGG -Phosphate) were used to capture the variable in polymorphic sequence of ITS region for differentiation MTC form NTM.

Real-time PCR assay

The condition of real-time PCR was optimized by using the LightCycler® FastStart DNA Master Hybprobe Kit (catalog no. 12 239 722 001; Roche Applied Science, Germany). The real-time PCR mixture contained 2 µl of 10x LightCycler FastStart DNA Master Hybprobe, 2 mM $MgCl_2$, 0.5 µM of each primer, 0.2 µM of each probe and 5 µl of DNA extract. Nuclease-free water added to a final volume of 20 µl. Each set of the assays included negative control which its reaction contained all substances except DNA template, and positive control which template DNA from *M. tuberculosis* H37Rv was used. The real-time PCR was performed on the LightCycler Real-time system, version 2.0 (Roche Applied Science, Germany). The PCR program setting included 1 cycle of initial phase at 95 °C for 10 min; 45 cycles phase of amplification, consisting of a 95°C denaturation for 10 sec, a 55°C annealing for 10 sec, and a 72°C extension for 20 sec; a melting phase from 45 to 80°C with a temperature transition rate of 0.1°C/sec; and final cooling phase at 40°C for 10 sec. The quantity of amplified product was measured through channel modes of energy emitted at 640 nm. The total time for amplification, detection and analysis was approximately 90 min for up to 32 samples per run.

Melting curve analysis

The temperature at which the hybridization probes dissociated from their specific target nucleotides was determined by melting curve analysis. This allowed for differentiation between species based on differences in the avidity of the hybridization probes for the complementary sequences in the amplified DNA. The melting curve for each sample was analyzed by the manual melting temperature (Tm) function of the LightCycler 4.05 software (Roche Applied Science, Germany).

Determination of Sensitivity and specificity

The specificity was tested against DNA extract from mycobacterial reference strains and other bacterial strains (Table 1). Moreover, the sensitivity and specificity of the real-time PCR for detection and differentiation of MTC and NTM were tested with the extracted DNA from the cells growth in liquid culture medium and sputum specimens. AFB staining combined with culturing and biochemical test were used as reference methods.

Results

The developed real-time PCR was specific to the ITS region between 16S -23S rRNA sequences of all tested mycobacteria (data not shown) but not for non-mycobacteria (Figure 1). This method can distinguish MTC from NTM by using melting temperature (Tm). An average Tm of MTC was 62°C, NTM group 1 and 2 was 55°C and 54°C, respectively (Figure 2). Based on Runyon classification (Runyon, 1959), the NTM group 1 categorized by the Tm consisted of photochromogens and rapid growing mycobacteria while the NTM group 2 consisted of scotochromogens and non-chromogens. Forty positive-liquid culture specimens were also found all positive with real-time PCR which had the Tm at 64°C which were identified as MTC (Figure 3). Eighteen of 20 sputum specimens that showed positive with AFB stain and culture were found positive with the real-time PCR with 64°C Tm and identified as MTC. But 2 samples were negative with real-time PCR and culture method (Figure 4A). Twenty AFB stain negative sputum specimens were found all negative with the real-time PCR (Figure 4B). The sensitivity of the real-time PCR was 100% (40/40) from liquid culture. While the sensitivity of the real-time PCR from sputum specimens was 90% (18/20). The specificity of real-time PCR was 100% (5/5) from reference non mycobacteria strains and 100% (20/20) from AFB stain negative sputum specimens.

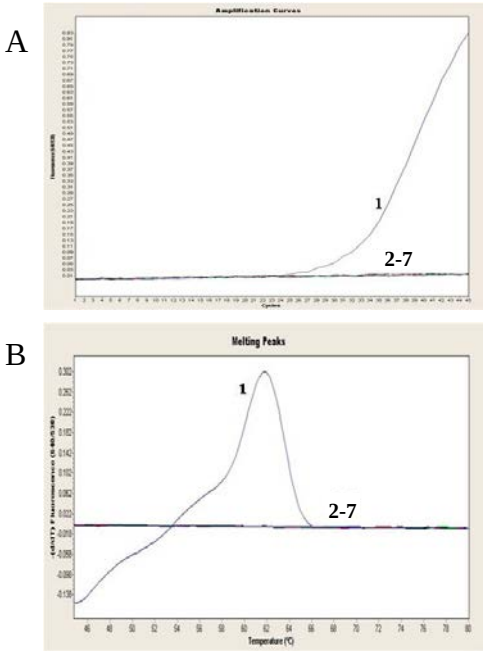


Figure 1 Amplification curve (A) and melting peak (B) of mycobacteria and non-mycobacteria including: *M. tuberculosis* H37Rv, *Nocardia*, *Streptococcus pneumonia*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Haemophilus influenza* and Negative control

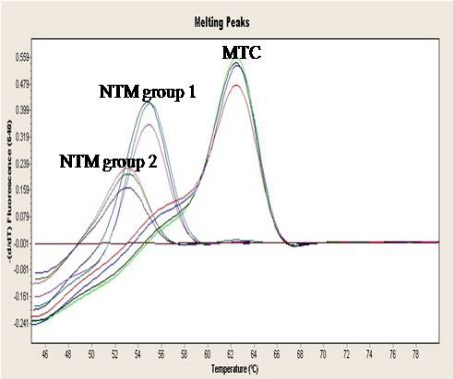
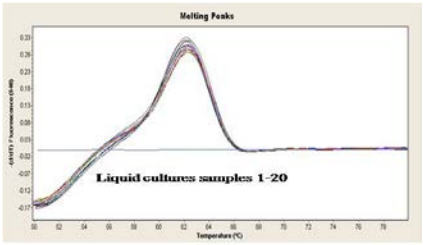


Figure 2 Melting peaks of reference strains show ability of real-time PCR for differentiation MTC from NTM; MTC consisted of *M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra, *M. tuberculosis* H37Rvj and *M. bovis* BCG Pasteur; NTM group 1 consisted of *M. kansasii* 302, *M. chelonae* ATCC 23016 and *M. flavescens* ATCC 23035; NTM group 2 consisted of *M. szulgai* JATA 3201, *M. xenopi* ATCC 19250 and *M. intracellulare* ATCC 13950.

A



B

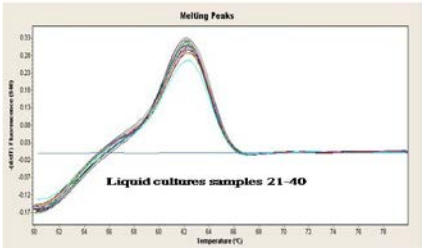
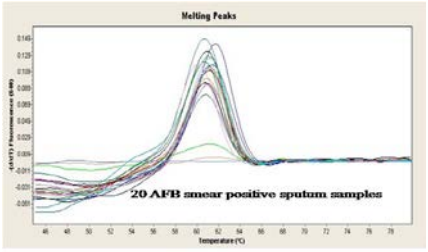


Figure 3 Melting peaks of 40 positive liquid culture media. The melting peak of samples 1-20 (A) and samples 21-40 (B).

A



B

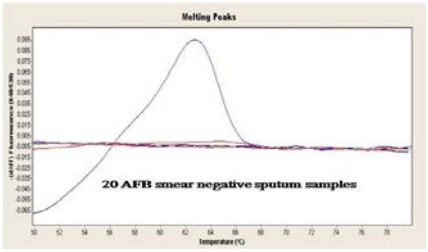


Figure 4 Melting peaks of sputum specimens. The melting peak of 20 AFB smear positive sputum samples (A) and 20 AFB smear negative sputum samples (B).

Discussion and Conclusions

A rapid, specific and sensitive diagnostic method to detect and identify MTC from NTM is needed for control of the infection and successful treatment. The real-time PCR technique for detection and identification of mycobacteria was widely studied (Miller et al., 2002; Shrestha et al., 2003). The method is sensitive and specific for detection and differentiation of MTC and NTM.

A previous study the real-time PCR using SYBR Green I dyes and specific fluorescent probes based on amplification of the internal transcribed spacer (ITS) region of mycobacteria were tested with 135 acid-fast bacillus (AFB) smear-positive respiratory specimens and in 232 BacT/ALERT MP (MP) culture bottles of respiratory specimens. The sensitivity and specificity of this assay were 98.1% and 100%, respectively for the AFB smear-positive specimens. For the MP culture bottles specimens, this assay showed 100% sensitivity and 100% specificity (Miller et al., 2002).

Real time PCR hybridization probe based on amplification of 16S rRNA of mycobacteria using melting curve analysis was used to identification of *M. tuberculosis*, *M. kansasii, M. avium*, *M. intracelluare*, *M. marinum* and rapid growing mycobacteria with the mean melting temperature (Tm) at 64.35°C, 59.20°C, 57.82°C, 54.46°C, 58.91°C and 53.09°C, respectively, when it was tested with 18 reference strains and 168 clinical mycobacterial isolates (Shrestha et al., 2003).

In this study, the real-time PCR using hybridization probes targeted at ITS region between 16S-23S rRNA gene of mycobacteria was developed. This assay was evaluated for detection and differentiation of MTC from NTM by measuring the differential Tm characteristics. The real-time PCR assay was tested with 10 mycobacterium reference strains, 5 strains of other bacteria, 40 liquid cultures positive, 20 acid fast bacilli (AFB) stain positive sputum, 20 AFB stain negative sputum specimens. The real-time PCR could differentiate MTC and NTM from liquid culture medium and sputum specimens. The detection and differentiation of MTC and NTM in sputum specimens the real-time PCR assay gave negative in 2 of 20 acid fast bacilli (AFB) stain positive sputum which were negative with culture assay. From the result we suggested that the sputum samples had a few bacilli less than the limit detection of real-time PCR or the bacilli in the sample were not mycobacteria. This assay could not identify the mycobacteria into the species level, but potential differentiate non-chromogens and scotochromogens from photochromogens and rapid growing mycobacteria. Moreover, in this study we evaluated the developed real-time PCR assay with fewer samples. For further study we will evaluate the developed real-time PCR assay with many samples. In order to identify mycobacteria into species level, another target sequence with higher discriminatory power for amplification and probes detection are needed (Pinsky et al., 2008; Foongladda et al., 2009).

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