Molecular detection of viable *Mycobacterium tuberculosis* complex in clinical specimens

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การตรวจหาความมีชีวิตของเชื้อ *Mycobacterium tuberculosis* complex ในสิ่งส่งตรวจของผู้ป่วยด้วยเทคนิคทางอณูชีววิทยา

น.ส.สุธิดี เพ็ชรสงค์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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สุธิดี เพ็ชรสงค์ : การตรวจหาความมีชีวิตของเชื้อ *Mycobacterium tuberculosis* complex ในสิ่งส่งตรวจของผู้ป่วยด้วยเทคนิคทางอณูชีววิทยา. (Molecular detection of viable *Mycobacterium tuberculosis* complex in clinical specimens) อ.ที่ปรึกษาหลัก : รศ.ดร. พญ.กนิษฐา ภัทรกุล, อ.ที่ปรึกษาร่วม : อ.ดร. พญ.สุวัชรีพร โรจน์ชีวพันธ์,รศ. นพ.กมล แก้วกิติณรงค์

วัณโรคเป็นปัญหาสาธารณสุขที่สำคัญเนื่องจากมีอุบัติการณ์ทั่วโลกและอัตราตายสูงขึ้น โรคนี้มีสาเหตุมาจาก *Mycobacterium tuberculosis* complex (MTBC) โดยติดต่อผ่านระบบทางเดินหายใจ จากการสูดหายใจเอาฝอยละอองที่มีเชื้อ องค์การอนามัยโลกแนะนำการตรวจวิเคราะห์หาเชื้อในห้องปฏิบัติการทั่วไป ได้แก่ การย้อมสีทนกรดตรวจหา acid-fast bacilli (AFB) การเพาะเชื้อ การตรวจทางอณูชีววิทยาเพื่อตรวจหาดีเอ็นเอของเชื้อ การทราบผลการตอบสนองต่อการรักษาให้เร็วที่สุดมีความจำเป็นเพื่อลดการแพร่กระจายของโรคและการดื้อยา การเพาะเชื้อถือเป็นวิธีมาตรฐานในการตรวจหาความมีชีวิตของเชื้อ แต่วิธีนี้ยุ่งยาก ราคาแพง อาจต้องใช้เวลานานถึง 8 สัปดาห์ วิธีการตรวจหา AFB และการตรวจทางอณูชีววิทยาเพื่อตรวจหาดีเอ็นเอของเชื้อเป็นวิธีที่รวดเร็วที่ใช้ในการตรวจหาเชื้อในสิ่งส่งตรวจของผู้ป่วย แต่ไม่สามารถแยกระหว่างเชื้อเป็นและเชื้อตายได้ การศึกษาก่อนหน้านี้พบว่าการตรวจหา ribosomal RNA precursor (pre-rRNA) สามารถใช้เป็นสารบ่งชี้ทางชีวภาพ (biomarker) ของเชื้อแบคทีเรียที่มีชีวิตได้ การศึกษานี้จึงมีวัตถุประสงค์เพื่อประเมินวิธีตรวจหา pre-rRNA ของเชื้อ MTBC ด้วยวิธี RT-PCR (pre-rRNA MTBC) ในการใช้เป็นวิธีใหม่เพื่อตรวจหาเชื้อ MTBC ที่มีชีวิตในสิ่งส่งตรวจของผู้ป่วย ผลการศึกษาพบว่าวิธีการตรวจ pre-rRNA MTBC ในสิ่งส่งตรวจที่ได้จากการเตรียมตัวอย่างเพาะเชื้อ MTBC ตามวิธีดั้งเดิมของ WHO ทั้งหมด 564 ตัวอย่าง เมื่อเทียบกับการเพาะเชื้อมีความไวเท่ากับร้อยละ 33.33 ในสิ่งส่งตรวจทั้งหมด และร้อยละ 52.77 ในสิ่งส่งตรวจที่ให้ผล AFB เป็นบวกตั้งแต่ 1+ ขึ้นไป มีค่าความจำเพาะและค่าการพยากรณ์ผลบวกเท่ากับร้อยละ 100 เมื่อเตรียมตัวอย่างด้วยวิธีดัดแปลง (วิธีที่ 2) โดยทำการบั่นล้าง NaOH ซึ่งอยู่ในน้ำยาที่ใช้ลดการปนเปื้อนเชื้อในตัวอย่างออกก่อน แล้วจึงทำการทดสอบในสิ่งส่งตรวจจำนวน 45 ตัวอย่าง พบว่ามีความไวเท่ากับร้อยละ 57.14 ในสิ่งส่งตรวจทั้งหมด และร้อยละ 76.92 ในสิ่งส่งตรวจที่ให้ผล AFB เป็นบวกตั้งแต่ 1+ ขึ้นไป และมีค่าความจำเพาะและค่าการพยากรณ์ผลบวกเท่ากับร้อยละ 100 ซึ่งผลที่ได้เทียบเท่ากับการย้อมสี fluorescein diacetate (FDA) การเตรียมตัวอย่างด้วยวิธีดัดแปลง (วิธีที่ 2) จึงเพิ่มความไวในการตรวจหาเชื้อ MTBC ที่มีชีวิตในสิ่งส่งตรวจของผู้ป่วยโดยเฉพาะตัวอย่างที่ให้ผลการย้อมสี AFB เป็นบวก โดยสรุป วิธี pre-rRNA MTBC สามารถนำมาใช้ในการตรวจหาเชื้อ MTBC ที่มีชีวิตในสิ่งส่งตรวจของผู้ป่วยได้อย่างรวดเร็ว

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Suthidee Petsong : Molecular detection of viable *Mycobacterium tuberculosis* complex in clinical specimens. Advisor: Assoc. Prof. KANITHA PATARAKUL, M.D.Ph.D Co-advisor: SUWATCHAREEPORN ROTCHEEWAPHAN, M.D.,Ph.D,Assoc. Prof. KAMON KAWKITINARONG, M.D.

Tuberculosis (TB) is the global health concern because of the rising incidence and mortality. TB is caused by *Mycobacterium tuberculosis complex* (MTBC) and transmitted via inhalation of infectious droplet nuclei circulating in the air. The World Health Organization (WHO) recommends the use of Acid-fast (AFB) smear microscopy, MTBC culture, and molecular detection of MTBC DNA for diagnosis of TB. The response to treatment should be acquired as soon as possible to reduce disease transmission and drug resistance. Although MTBC culture is a gold standard, it is laborious, expensive, and may take up to 8 weeks. The AFB staining and molecular detection of MTBC DNA are faster, but they cannot differentiate live and dead MTBC. Ribosomal RNA precursor (pre-rRNA) was previously used as a molecular biomarker to indicate viable bacteria. Therefore, this study aimed to evaluate pre-rRNA MTBC detection by RT-PCR (pre-rRNA MTBC) as a new method to detect viable MTBC in clinical specimens. The total number of 564 clinical specimens were processed by the conventional WHO protocol of decontamination and concentration before RNA extraction. The results showed that pre-rRNA MTBC detection had a sensitivity of 33.33% for all specimens and 52.77% for positive AFB specimens. The specificity and positive predictive value (PPV) is 100%. When 45 specimens were prepared by the modified protocol to remove NaOH in the decontamination solution and washing before used, the sensitivity increased to 57.14% for all specimens and 76.92% for positive AFB specimens and the specificity and positive predictive value (PPV) is100%, which were comparable to those of the Fluorescein diacetate (FDA) vital staining. The modified method of sample preparation (protocol 2) can improve sensitivity of pre-rRNA MTBC for detection viable MTBC, especially in positive AFB specimens. In summary, pre-rRNA MTBC RT-PCR can be used for rapid detection of viable MTBC in clinical specimens.

Field of Study:	Medical Microbiology	Student's Signature .....
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Suthidee Petsong

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Abbreviations

AFB	Acid-fast bacilli
BSC	Biological safety cabinet
CFU/mL	Colony forming units per milliliter
DNA	Deoxyribonucleic acid
DST	Drug susceptibility testing
FDA	Fluorescein diacetate
FN	False negative
FP	False positive
cDNA	Complementary DNA
gDNA	Genomic DNA
h	Hour
MDR-TB	multidrug-resistant tuberculosis
MGIT	Mycobacterium growth indicator tube
Min	Minute
MTBC	*Mycobacterium tuberculosis* complex
NAAT	Nucleic acid amplification test
NALC	N-acetyl-L-cysteine
NaOH	Sodium hydroxide

NPV	Negative predictive value
NTM	Non-tuberculous mycobacteria
PBS	Phosphate-bufferred saline
PCR	Polymerase chain reaction
pre-rRNA	Ribosomal RNA precursor
PPV	Positive predictive value
RNA	Ribonucleic acid
RT-PCR	Reverse transcription PCR
TB	Tuberculosis
Tm	Melting temperature
TN	True negative
TP	True positive
v/v	volume per volume
WHO	World health organization
Wks	Weeks
LOD	Limit of detection

CHAPTER I
INTRODUCTION

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* complex (MTBC), remains a critical health issue worldwide (1).The major system involvement is the lung (pulmonary TB), however TB can occur outside the lung (extrapulmonary TB) such as peritonitis, lymphadenitis, colitis, meningitis, and pericarditis. Pulmonary TB is transmitted via inhalation of infectious droplet nuclei circulating in the air when active TB patients cough or sneeze. TB cases are commonly found in HIV infected patients, handicapped, diabetic patients, smokers, and alcoholic patients (2). TB has been one of the world's health concerns since the rising number of HIV/AIDS cases (2). In 2016, 600,000 new cases resisted rifampin, of which 490,000 patients experienced multidrug-resistant TB (MDR-TB) reported worldwide (3). In 2017, 10 million people developed tuberculosis reported in all countries and age groups (4). The latest report of the global situation of tuberculosis in 2020 revealed 10 million people around the world developed TB in 2019 (5). The death of tuberculosis patients with non-HIV infection reached 1.4 million cases and the death of 208,000 HIV patients were related to tuberculosis Moreover, approximately 500,000 patients were diagnosed with MDR-TB(5).

The sputum acid-fast bacilli (AFB) smear and *Mycobacterium* conventional culture are used for diagnosis and follow-up of patients receiving anti-TB drugs to determine treatment outcome. World Health Organization (WHO) recommends the laboratory diagnosis of TB by using AFB smear microscopy, mycobacterial culture, *Mycobacterium* identification, drug

susceptibility testing, and molecular testing (6). Patients whose sputum AFB smear remains positive after treatment for 3 months should be investigated for mycobacterial culture as a gold standard test to determine viability of MTBC (7). However, MTBC culture is laborious, and may take up to 8 weeks. The response to treatment should be acquired as soon as possible to reduce disease transmission and identify drug resistance. The AFB staining and molecular detection of MTBC DNA are faster than the culture method, but these methods are unable to differentiate live and dead MTBC. ((6), (8-10)).

Fluorescein diacetate (FDA) vital or live/dead staining (7, 11) is a fast bacterial viability assay that is currently available for *Mycobacterium.* However, FDA staining has some limitations including false negative results due to low esterase activity in some bacilli and inability to differentiate between MTBC and NTM. In addition, a working FDA solution must be prepared daily because fluorescent dye can fade rapidly (7).

Molecular viability testing (MVT) to detect rRNA precursor (pre-rRNA) (12), a new method to differentiate viable from dead organisms, is based on the ability of viable bacteria to rapidly synthesize species-specific pre-rRNA in response to brief nutritional stimulation. Detection of pre-rRNA of 16S rRNA gene designed specific for MTBC and analyzed by reverse-transcription PCR (RT-PCR) (13, 14) may be more specific and sensitive than FDA staining (12).

Therefore, my project aimes to evaluate MTBC-specific pre-rRNA detection by RT-PCR as a new method to detect viable *M. tuberculosis* complex in clinical specimens.

CHAPTER II
OBJECTIVE

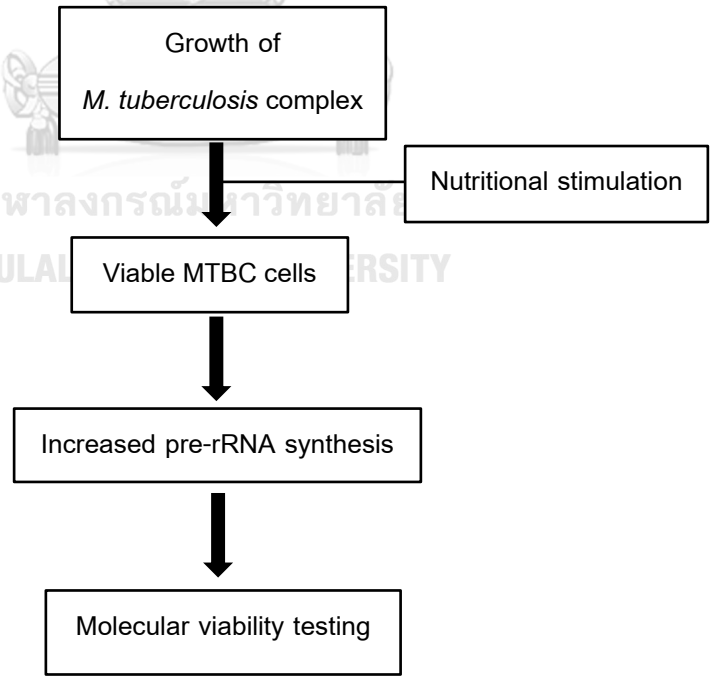
Hypothesis

RT-PCR of pre-rRNA gene can be used to detect viable of *M. tuberculosis* complex in clinical specimens.

Objectives

To evaluate the RT-PCR of pre-rRNA for detection of viable *M. tuberculosis* complex in clinical specimens.

Conceptual Framework



CHAPTER III
REVIEW OF RELATED LITERATURES

Mycobacterium tuberculosis complex and tuberculosis disease

M. tuberculosis complex (MTBC) are obligate aerobic, non- motile, rod- shaped bacteria. MTBC is a genetically homogeneous group of *M. tuberculosis, M. bovis, M. bovis* BCG, *M. africanum*, *M. canetti*, *M. caprae*, *M. microti*, *M. pinnipedii*, *M. mungi*, and *M. orygis*. Among others, *M. tuberculosis* is the most common cause of tuberculosis in humans. Mycobacteria have unique cell wall structures different from other bacteria (15). The cell wall of mycobacteria contains several complex lipids (C_{60}—C_{90}) called mycolic acids, which make the cell wall hydrophobic and strengthen mycobacteria to survive in a dry environment and resist disinfectants and penetration of several antibiotics. The special characteristic of mycobacterial cell wall is useful for detection of the organisms with basic laboratory techniques such as AFB staining (16).

Tuberculosis is a granulomatous disease caused by *M. tuberculosis* complex. The risk of TB disease depends on the host's immune status. TB is a contagious disease with complex pathogenic mechanisms. Although the lung is the main organ system involvement, MTBC can spread outside the lung causing extrapulmonary TB. (3),(17, 18). MTBC may be present for a long period in asymptomatic hosts, in which MTBC evade killing by the host's innate immunity to a state of paucibacillary TB (a low bacterial load of MTBC causing direct smear negative and culture positive cases) or latent TB (19). When hosts become

immunocompromised, MTBC can spread via the lymphatic system causing active TB (19, 20)

Criteria for diagnosis of tuberculosis/guideline for diagnosis of TB

The diagnosis of TB is complicated. TB disease should be suspected in persons who have the following symptoms (Table 1).

Table 1. Symptoms of pulmonary and extrapulmonary TB diseases, from Diagnosis of TB Disease by Center for Disease Control and Prevention. (https://www.cdc.gov/tb/education/corecurr/pdf/chapter4.pdf; access date May 12, 2019)

Symptoms of Pulmonary TB Disease (TB disease usually causes one or more of the symptoms)	Symptoms of Possible Extrapulmonary TB Disease (Depends on the part of the body that is affected by the disease)
Cough (especially if lasting for 3 wks or longer) with or without sputum production	TB of the kidney may cause hematuria
Coughing up blood (hemoptysis)	TB meningitis may cause headache or confusion
Chest pain	TB of the spine may cause back pain
Loss of appetite	TB of the larynx can cause hoarseness
Unexplained weight loss	Loss of appetite
Night sweats	Unexplained weight loss
Fever	Night sweats
Fatigue	Fever
	Fatigue

Methods for identification of MTBC and limitations for diagnosis and follow up after treatment

Due to the capability of transmission of MTBC and the spread of TB infection, the World Health Organization (WHO) recommends the Good Clinical Laboratory Practice (GCLP) of Mycobacteriology laboratory to handle all clinical specimens from suspected TB patients. MTBC can be found in sputum, gastric lavage fluids, cerebrospinal fluid, urine, and tissues. MTBC may be alive in heat-fixed smears and can be aerosolized during inoculation of culture media. Clinical specimens used for detection of TB cases including MTBC cultures and drug susceptibility testing shoud be appropriately managed under precautions in order to reduce the risk of aerosolization (6).

The biosafety containment and training exercises are mandatory for handling specimens from suspected TB patients. The WHO recommends a standard requirement of Mycobacteriology laboratory including facilities and equipment, biosafety practices, personal protective equipment, and personal protection-training and monitoring. All activities involving clinical specimens or mycobacterial isolates must be operated at least in a biological safety cabinet class II (BSC class II), along with wearing N95 or equivalent respirators such as Powered Air Purifying Respirator (PAPR), gloves, and gown (6).

There are several methods to identify MTBC and non-tuberculous mycobacteria (NTM) in clinical specimens including microscopic examination, mycobacterial culture, and molecular detection as follows:

Acid-fast bacilli (AFB) stain

AFB stain is the microscopic detection of the acid-fast organisms in clinical specimens or culture materials. By Kinyoun or Ziehl-Neelsen staining, the acid-fast organisms with high mycolic acid content in the cell wall are stained red against the blue background. All acid-fast organisms can be counted and visualized under a bright-field microscope at a low limit of detection about 5,000 – 10,000 bacilli load per milliliter (mL). The results of AFB stain are reported according to a standard scoring system published by WHO/IUATLD system. This investigation cannot distinguish viable from non-viable acid-fast organisms and identify individual species (6, 21, 22).

Table 2. The grading scale of WHO/IUATLD for acid-fast bacilli visualized by bright-field microscopy after Kinyoun or Ziehl-Neelsen staining.

Bright field microscopy at 1000x magnification	Result	
No acid-fast found in at least 100 fields	-	Negative
1-9 AFB per 100 fields	Scanty (exact no./100)	Positive
10-99 AFB per 100 fields	1 +	Positive
1-10 AFB per field in at least 50 fields	2 +	Positive
More than 10 AFB per field in at least 20 fields	3 +	Positive

Fluorescence stain

Fluorescence stain is the microscopic detection of the organisms in clinical specimens or culture materials. The organisms are stained fluorescent bright yellow (Auramine O stain), yellow-red (Auramine/Rhodamine stain), or orange-red (Acridine Orange) according

to the type of fluorescent dyes against the dark background. The fluorescent bacilli which have a low limit of detection about 5,000 – 10,000 bacilli load per milliliter (mL). The results of fluorescence stain are reported in a standard scoring system published by WHO/ IUATLD system. This investigation cannot distinguish viable from non-viable acid-fast organisms and identify individual species similar to AFB stain (6, 21, 22)

Table 3. The grading scale of WHO/IUATLD for acid-fast bacilli visualized at 200-250x magnification after fluorescence staining.

Fluorescence in 200-250x magnification 1 length = 30 fields (300 HPF)	Result	
No acid-fast found per 1 length	-	Negative
1-29 AFB per 1 length	Scanty (Exact no./100)	Positive
30-299 AFB per 1 length	1 +	Positive
10-100 AFB per 1 field on average	2 +	Positive
More than 100 per 1 field on average	3 +	Positive

Table 4. The grading scale of WHO/IUATLD for acid-fast bacilli visualized at 400x magnification after fluorescence staining.

Fluorescence in 400x magnification 1 length = 40 fields. (200 HPF)	Result	
No acid-fast found per 1 length	-	Negative
1-19 AFB per 1 length	Scanty (Exact no./100)	Positive
20-199 AFB per 1 length	1 +	Positive
5-50 AFB per 1 field on average	2 +	Positive
More than 50 per 1 field on average	3 +	Positive

Mycobacterial culture

WHO recommends mycobacterial culture as the gold standard for diagnosis of TB disease. It is based on the growth of live MTBC in specific culture media. Respiratory specimens are the most frequent clinical samples received by the laboratory. These non-sterile specimens usually contain numerous bacterial flora. The main purpose of mycobacterial culture is to promote the growth of MTBC, which are slow-growing. Overgrowth of bacterial flora can inhibit the growth of mycobacteria. Therefore, bacterial flora must be decontaminated from all non-sterile specimens before culture (6, 9, 22).

Specimen processing: decontamination and concentration

WHO recommends "NaOH/NALC-Na Citrate Solution and Phosphate Buffer method" for processing of non-sterile specimens to decontaminate bacterial flora and liquify the clinical specimens (22).

N-acetyl-L-cysteine (NALC) is a mucolytic agent used to digest the mucous and debris in non-sterile specimens. Sodium hydroxide (NaOH) at a low final concentration is a decontaminating agent. Sodium citrate is a stabilizing agent of NALC. Phosphate Buffer Saline (PBS), is a neutralizing and homogenizing agent to neutralize NaOH and decrease the viscosity of specimens. The digestant-decontaminant solution (NaOH/NALC-Na Citrate Solution) must be prepared daily because NALC can be rapidly degraded within 24 hours (h).

Liquid culture media method

The method uses the Mycobacterium Growth Indicator Tube (MGIT) to determine the signal of mycobacterial growth by time to detection (TTD) taken for culture (6). The MGIT tube has a nutritional-rich medium to promote mycobacterial growth from the clinical specimens after decontamination and concentration process and contains additional antibiotics to prevent growth of other bacteria. After incubation, the mycobacteria replicate and accumulate in the tube. Due to mycobacterial metabolism, oxygen in the tube is used and its level declines leading to an increase of free-form fluorescence emitting. The fluorescence signal can be detected by UV light and complex algorithms of BACTEC MGIT 960 system instrument. The advantage of the MGIT system has been developed for enhancing growth and reducing the duration of a solid culture method. However, the culture-negative result or no mycobacterium isolated can be reported after incubation until 8 wks. for the primary solid culture (6, 9, 22).

Solid culture method

The method is used to isolate mycobacteria on an egg-potato based media such as Lowenstein-Jensen (LJ) medium and Ogawa medium (22, 23), or an oleic acid-albumin agar based medium such as Middlebrook 7H10 agar and Middlebrook 7H11 agar (23, 24). This method can provide visible appearance of colonies after incubating at an optimal condition (37°C ± 1°C), which is not seen in MGIT liquid culture method. The egg-potato base solid medium has a malachite green as an inhibitor of bacterial contaminants that can overgrow on the medium surface leading to the delay or disappearance of mycobacterial growth (22).

Moreover, the solid culture medium is beneficial to isolate colonies for the drug susceptibility test (DST) (6, 9, 22, 24). The use of the solid culture method can be divided into dual categories as follows:

Primary solid culture

Clinical specimens are processed by decontamination and concentration before incubation at an optimal condition until 8 wks. Primary solid culture is positive if there are colonies growing on the media and the mycobacterial culture is identified following a laboratory protocol (22).

Secondary solid culture

The pellet from a positive signal MGIT tube is inoculated to a secondary solid medium. The advantage of MGIT system is to get the colony growth on a solid medium before the growth on a primary solid medium. However, duration of detection is still long because of the natural slow-growing of mycobacteria (22).

The mycobacterial culture is a gold standard for detection of live mycobacteria in the clinical specimens. However, it is a complicated method that requires biosafety practice and takes a long period of incubation.

Molecular detection of *M. tuberculosis* complex (MTBC)

Due to the long period of MTBC culture, WHO recommends the methods to reduce time to detection by rapid genotypic detection of MTBC DNA and mutations associated with drug-resistance in the clinical specimens, such as Line probe assay (LPA)(25, 26) and Xpert MTB/RIF(27). Other methods of MTBC DNA detection apart from the recommendation can be used up to the laboratory policy such as commercial real-time PCR assay, conventional PCR, and in-house PCR (6, 9).

Line probe assay (LPA)

This method is used to identify MTBC DNA and mutations associated with drug-resistance directly in the clinical specimens or culture material samples, such as colonies on solid media and pellets from positive signal MGIT tubes. The regions of *rpoB*, *inhA,* and *katG* genes are targets to detect the first line drug-resistant MTBC, and the regions of *gyrA, gyrB, rrs,* and *eis* genes are targets to detect the fluoroquinolones and second-line drug-resistant MTBC (25, 26, 28). Biotinylated primers are used to label PCR products that hybridize with specific oligonucleotide probes on the nitrocellulose strip (29-31). The post-hybridization reactions can be detected by the colorimetric banding pattern using the GT-Blot 48 hybridization system instrument (6, 9).

Xpert MTB/RIF assay

This method uses an automated real-time PCR amplification system and cartridge-based nucleic acid amplification test (NAAT) to detect MTBC and its resistance to rifampin by *rpoB* gene amplification directly in sputum in less than 2 h (27, 32). It is highly

sensitive, easy to perform, and requires biosafety precaution similar to the smear preparation for microscopic examination of TB suspected patients (27, 33) .

Conventional PCR

This method is to identify MTBC DNA by using specific primers to the region of the insertion sequence *IS6110* gene that is highly specific for MTBC strain(31). The PCR reaction includes DNA template extracted from the clinical specimens, the specific primers (*IS6110*)(34, 35), Taq polymerase enzyme, and deoxyribonucleoside triphosphate (dNTPs) solution. All these solutions contain in the PCR tube and the reaction is done by a PCR Thermocycler instrument, which generates repetitive cycles of several temperatures (6, 9, 35). The process of PCR reaction consists of the following elements as follow:

Denaturation

This step is to raise the temperature to 94° C to break the hydrogen bond to separate double-stranded DNA into single-stranded DNA.

Annealing

The second step is to lower the temperature to around 40° C - 70° C. The specific primers hybridize to the target regions of the single-stranded DNA and the polymerase binds to the area of primer-single stranded template complex. Then, the DNA synthesis begins and the targets are amplified. If the temperature is too low, the specific primers cannot bind to the DNA strand specifically. On the other hand, if the temperature is too high, the specific primers cannot bind the target regions.

Elongation or Extension

This step is used for synthesis of the complementary strand by at a temperature optimal for the DNA polymerase enzyme. The optimal temperature is approximately 72°C. The dNTPs in the reaction solution are added for synthesis of the new DNA strand complementary to the DNA template in the 5' to 3' direction. The new DNA products increase in double at each cycle step and become new templates of the next cycle. The cycles are repeated several times. In the final step, numerous DNA products are achieved. So the DNA product can be detected by agarose gel electrophoresis.

The advantages and disadvantages of different detection methods for diagnosis of TB recommended by WHO are summarized in Table 5.

Table 5. The advantage and disadvantage of detection methods for diagnosis of TB recommended by WHO from Implementing tuberculosis diagnostics, WHO (6).

Method	Advantage	Disadvantage
AFB Stain	Present acid-fast bacilli in clinical specimens Simple and fast Cheap	Less sensitive (require high TB load > 10^5 CFU/mL) Cannot differentiate viable and dead *Mycobacteria*
Fluorescence stain	Fast screening	not specific for acid-fast organisms Cannot differentiate viable and dead *Mycobacteria*
Mycobacterial culture	Increase the number of TB cell detection (more than 10^2 CFU/mL) The gold standard for the viability of MTBC cells	Require enriched media Requires at least 4 wks of incubation
Molecular detection of MTBC DNA	Rapid and specific detection of TB in clinical specimens	Cannot differentiate viable and dead cells

Even though microscopic detection and molecular detection of MTBC DNA are rapid assessment, they are unable to differentiate between live cells and dead cells. On the other hand, MTBC culture is used as a standard method to identify viable MTBC but takes a long time. Therefore, rapid viability tests are useful to detect viable MTBC in clinical specimens.

Microscopic viability test

There are many viable stains used to determine viable microorganisms. One of the most common vital stains is fluorescein diacetate (FDA) using 0.5% potassium permanganate (0.5% $KMnO_4$) as a counterstain.

Fluorescein diacetate (FDA) vital staining

FDA vital staining is used to detect viable mycobacteria by fluorescence microscopy in sputum samples to follow up on the response of TB treatment (10). It is a rapid test (within 1 h) to predict positive culture. Patients with positive viability microscopy after 2 months of treatment may need drug susceptibility testing (DST) for diagnosis of probable drug-resistant tuberculosis (11).

FDA is a vital stain based on the principle of intracellular FDA hydrolysis. Only living cells can actively convert the nonfluorescent FDA by esterase activity into fluorescein, the green-fluorescent compound as an indicator of viability (7, 8, 11, 36).

In a previous study in 2019, Sumona Datta et al. (36) has studied fluorescein diacetate vital staining in sputum smear- positive microscopy for investigating pulmonary tuberculosis patients. They found FDA microscopy that had high sensitivity and repeatable results indirectly the sputum specimens. These results also similar to the study of Swapna Kanade et al., in 2016 and A. Van Deun et al., in 2012 showed that FDA microscopy can be used for the rapid detection of tuberculosis patients. especially high grading of AFB stain (7, 36) and low evidence of NTM infection (7, 11).

Molecular viability test

The analysis of bacterial ribosomal RNA precursor (pre-rRNA) (Fig.1) has been applied to differentiate live cells from dead cells (12-14) because pre-rRNA is highly abundant in viable bacterial cells in response to nutritional stimuli. Detection of species-specific pre-rRNA from nutritionally stimulated samples and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay was used to detect viable bacteria, such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *M. tuberculosis* complex (12-15, 37). It was also modified for the rapid detection of rifampin- and ciprofloxacin-resistant *M. tuberculosis* strains after brief *in vitro* antibiotic exposure that can affect RNA synthesis (37).

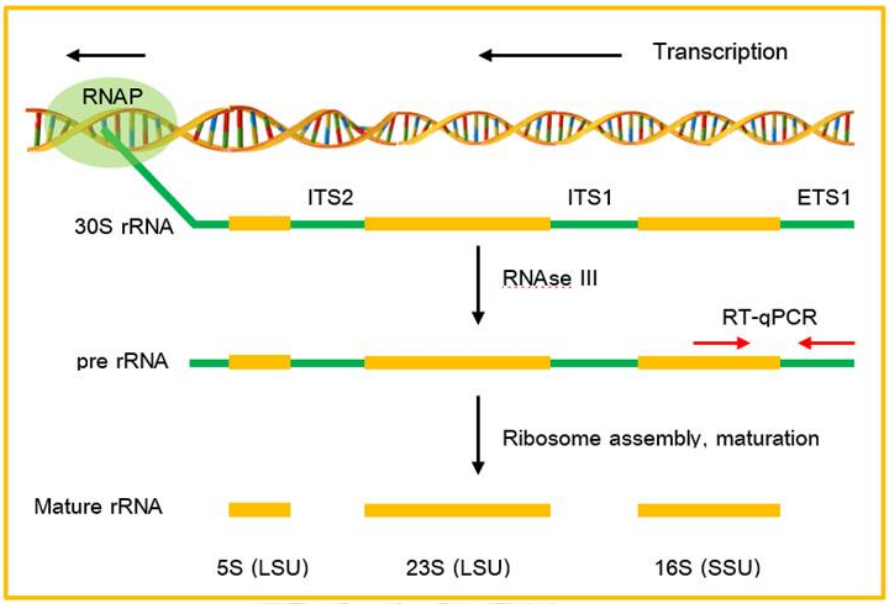


Figure 1. rRNA synthesis and maturation of bacteria. RNA polymerase (RNAP), which has been reading from the right to left derived from the shown diagram is generated long transcript (30S rRNA) consisting of 3 rRNA subunits that can be spread of external transcribed spacer (ETS) and internal transcribed spacers (ITS). The transcript is promptly transformed by RNase activity and other endonucleolytic exercises to pre- RNA subunits with leader and tail sequences. The leaders and tails are divided into exonucleolytic procedures which are linked to ribosome assembly and the initial protein synthesis. RT-PCRs can be designed to target the pre-rRNA exclusively or to straddle a pre-rRNA-mature rRNA junction for amplification the 5' leader region (ETS1) is specifically helpful for Molecular viability test (MVT) due to its species specificity and abundance of activated transcription. In plus pre-rRNA sequences (e.g., ITS1) is eligible to be detected by RT-PCR primers (12).

Nutritional stimulation: use of optimal growth medium

Do J.S. (2014) studied on the growth medium of which stimulated pre-rRNA of slow-growing mycobacteria by applying the growth medium Middlebrook 7H9 broth medium to stimulate pre-rRNA of *M. avium* by using specific primers and probe. Moreover, RT-PCR and qPCR were used to determine the increase of pre-RNA (38).

Detection of pre-rRNA in live cells after nutrional stimulation

Live cells were activated to increase a metabolic activity that caused transcription of pre-rRNA with a greater number than gDNA (38). A previous study detected pre-rRNA from the milk sample which was stimulated by the Middlebrook 7H9 broth medium at the temperature of 37°C overnight. It was found that the number of pre-rRNA was greater than gDNA 5 up to over 10 fold in all samples (38).

The capability of pre-rRNA to differentiate live and dead cells

Moreover, there was the study proved that pre-rRNA was able to identify live cells and dead cells by the use of suspension of *M. avium* spiked into the sample of milk. All samples were analyzed to detect pre-rRNA, gDNA, and culture. All samples of pre-pasteurization showed clearly positive for pre-rRNA, consistent with positive culture. The pasteurized samples were unable to detect pre-rRNA and culture showed no growth. In contrast, gDNA was positive in the samples either pre-or post-pasteurization. Therefore, pre-rRNA could differentiate between live and dead cells (Figure 2) (38).

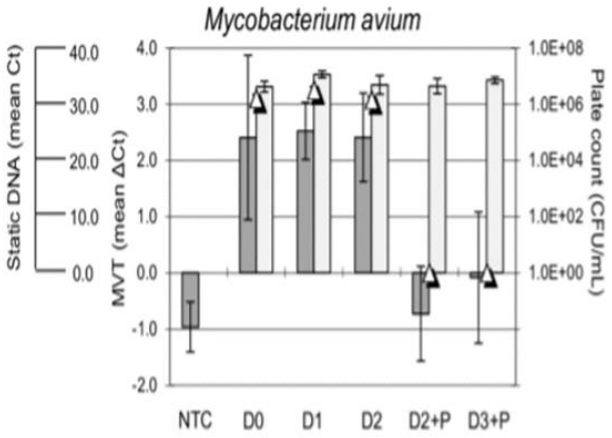


Figure 2. Molecular viability test of *M. avium* in milk sample. The dark bar represents the result of pre-rRNA, the light bar represents the result of gDNA, and the triangle represents the result of culture (figure from Do JS, et al. Biosynthetic enhancement of the detection of bacteria by the polymerase chain reaction. PLoS One. 2014;9(1): e86433,(38)).

Optimal condition for pre-rRNA stimulation

Weigel K. M. (2013) studied on the optimal period for stimulating pre-rRNA of *M. tuberculosis* complex H37Ra by incubation in human serum at 37°C for 30 days. Afterwards, all samples were centrifuged and resuspended in Middlebrook 7H9 broth medium and incubated at 37°C for stimulation pre-rRNA, called nutritional stimulation. After stimulation, the samples at 0, 1, 2, 4, and 24 h, the number of pre-rRNA was increased compared with the unstimulated result. The result of pre-rRNA: gDNA ratio is shown in Figure 3. When the samples were nutritionally stimulated by Middlebrook 7H9 broth medium for 4 h, the pre-rRNA was greatly increased (13).

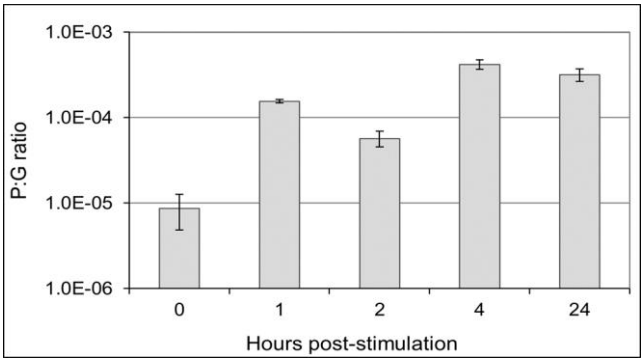


Figure 3. Ratiometric pre-rRNA analysis of *M. tuberculosis* H37Ra cells in serum. The pre-rRNA: gDNA ratio was determined after nutritional stimulation at 0, 1, 2, 4, and 24 h. (figure from Weigel KM, et al. Molecular viability testing of bacterial pathogens from a complex human sample matrix. PLoS One. 2013;8(1): e54886,(13))

Cangelosi, G. A. (2010) conducted the study to find appropriate time to stimulate pre-rRNA. *M. avium*, representing slow-growing mycobacteria, was spiked into the water sample and stimulated by Middlebrook 7H9 broth medium at 37° C for 2, 4, 7, and 24 h and then analyzed for the existing pre-rRNA. After nutritional stimulation, pre-rRNA was highly increased within 4-24 h of stimulation as shown in Figure 4. Therefore, 4 h incubation was sufficient for nutritional stimulation (14).

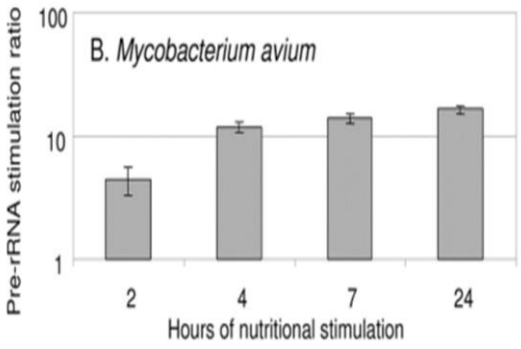
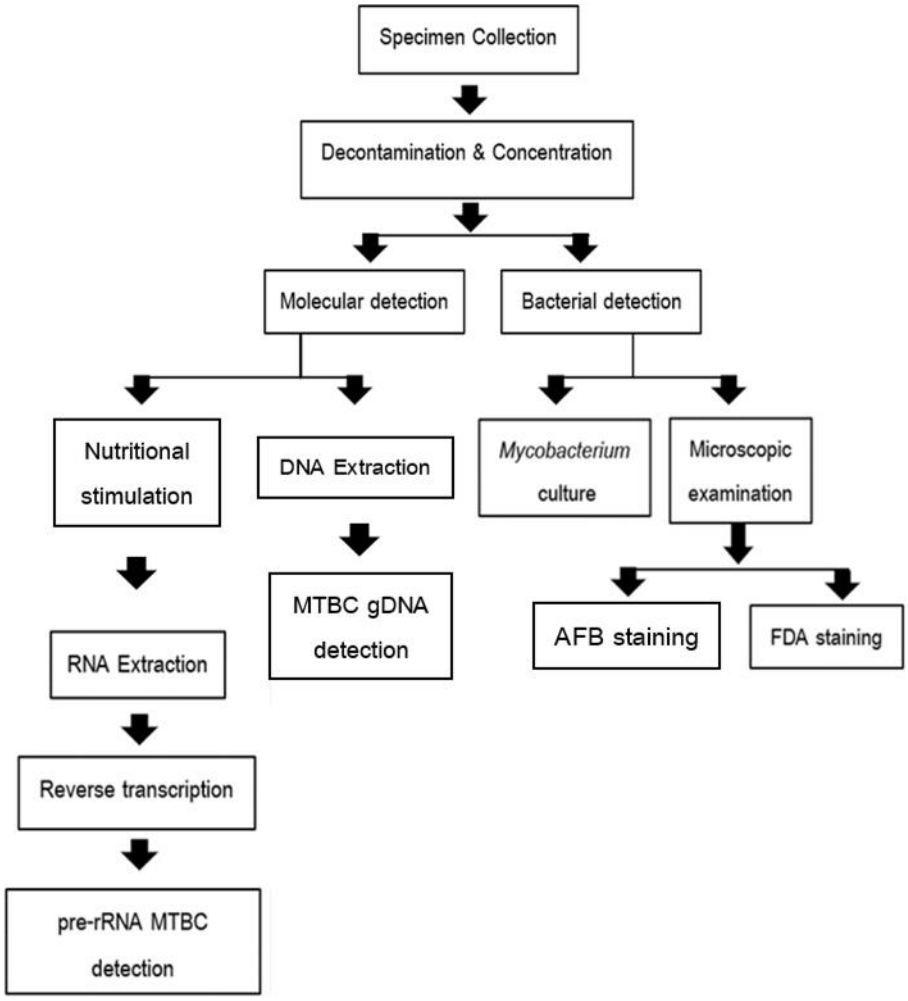


Figure 4. Hours of nutritional stimulation of *M. avium* in water (figure from Cangelosi GA, et al. Molecular detection of viable bacterial pathogens in water by ratiometric pre-rRNA analysis. Appl Environ Microbiol. 2010;76(3):960-962,(14)).

CHAPTER IV
MATERIALS AND METHODS

Experimental design



Materials and methods

Clinical specimens

The 609 clinical specimens used in this study are listed as follows: sputum (577 samples), BAL fluid (9 samples), endotracheal aspirates (8 samples), pus (10 samples), body fluid (2 samples), and tissues (3 samples). All clinical specimens except tissue were concentrated by conventional WHO protocol. Large tissues were homogenized by sterile pestle and were resuspended with PBS before culture.

Bacterial strains and culture conditions

All *Mycobacterium* reference strains in Table 6 were inoculated in Ogawa medium and were incubated at 37 °C until visible colony appeared. For the preparation of the RT-PCR detection, all strains were spiked into Middlebrook 7H9 broth and incubated at 37°C for 4 h to stimulated pre-rRNA MTBC before extraction.

Table 6. Bacterial strains and culture conditions.

Organism	Growth condition	Temperature
M. tuberculosis ATCC 27294	Colony growth on Ogawa medium and Middlebrook 7H9 broth	37°C
M. kansasii ATCC 12478		
M. godornae ATCC 14470		
M. avium ATCC 15769		
M. fortuitum ATCC 684		

Design of the study

This study collected 609 remaining clinical samples after routine mycobacterial cultures performed at the Mycobacteriology laboratory, King Chulalongkorn Memorial Hospital KCMH, Thailand. All clinical specimens were collected from patients suspected of tuberculosis.

This study was approved by the Ethical Committee of the Faculty of Medicine, Chulalongkorn University (IRB No. 424/63).

Conventional mycobacterial culture

The preparation of digestant-decontaminant solution

The digestant-decontaminant solution was made from 6% NaOH and 2.9% sodium citrate in separate stock solutions. When preparing a working solution, the equal volume of NaOH and sodium citrate was mixed in a sterile flask. Therefore, the final concentration of NaOH in the digestant-decontaminant solution was 3% NaOH (22).

Decontamination and concentration

After the digestant-decontaminant solution was prepared, at least 3 mL to the maximum volume of 10 mL of the specimen were placed into a 50 ml tube. If the volume of the specimen were higher than 10 mL, the specimen must be centrifuged to achieve 10 mL before the decontamination proceeds. After that, an equal volume of the digestant-decontaminant solution was added into the specimen tube (1:1 v/v). The final concentration of NaOH at this stage was 1.5% NaOH. Then, the suspension was mixed for around 15-30 seconds until it became liquefied and then incubated for 15 min. After that, a sterile PBS solution (pH 6.8) was added into the suspension to become 40 mL of total volume and the tube was mixed by inverting gently

and spun using a refrigerated centrifuge at 3,000 xg for 15 min. The supernatant was gently discarded carefully without disturbing the sediment. If the volume of pellet in the final step was less than 2.5 mL, sterile PBS was added to ensure the final volume of 2.5 mL and the pellet was resuspended gently by pipetting up and down (6, 9, 22).

Isolation and identification

Five-hundred microliters of the pellet suspension were inoculated in liquid media (MGIT, Becton Dickinson, USA) and 100 – 200 µl were inoculated into two solid medium tubes each (Ogawa media, In-house) and then incubated in the MGIT 960 system (Becton Dickinson, USA) and 37ºC incubator, respectively. The growth of mycobacteria was observed weekly up to 8 weeks for solid media and 6 weeks for liquid media before reporting negative culture. Colonies from positive culture of solid or liquid media were examined by Kinyoun acid-fast staining and MPT64 TB Ag rapid (SD-Bioline, Korea) (39). *M. tuberculosis* complex identification was confirmed by the GenoType MTBC line probe assay kit (HAIN-Lifescience, Germany). The susceptibility of *M. tuberculosis* complex isolates to streptomycin, isoniazid, rifampin, and ethambutol was performed using MGIT 960 SIRE kit (Becton Dickinson, USA). All procedures were performed under a biological safety cabinet class II that was certified annually.

The remaining precipitates from the conventional mycobacterial culture were stored at -80°C before the microscopic and molecular viability tests were performed.

Conventional microscopic examination

Fifty to one-hundred microliters of the remaining precipitate from the conventional mycobacterial culture were smeared on a glass slide and fixed on a 67-72° C slide warmer for at least 2 hours in a biological safety cabinet class II. Then, Kinyoun acid-fast staining following routine protocol was performed (22). The smear was examined by 1000x magnification using a light microscope (Olympus BX-50, Japan).

M. tuberculosis complex DNA detection by real time PCR

All clinical specimens were decontaminated with NaOH following routine protocol (Seegene, Korea). The samples were mixed by vortexing and allowed to sit at room temperature for 10-15 min. The samples were centrifuged at maximum speed at 1300 xg, the supernatant was discarded, and the pellet was resuspended in 200 µl PBS. DNA was extracted from the concentrated specimens using a MagLEAD 12 gC Nucleic acid extraction system (Precision System Science, Japan). Extracted DNA was amplified at IS6110 and MPB64 as specific targets using the Anyplex MTB/NTM Real-time kit (Seegene, Korea). The amplicons were detected by real-time PCR instruments according to manufacturer instruction (31). This method was performed by Molecular bacteriology laboratory, KCMH.

Microscopic viability test by Fluorescein diacetate (FDA) staining

The conventional WHO protocol protocol 1) : After decontamination and concentration, 50 - 100 µl of the remaining precipitate was smeared and air-dried on glass slides and fixed on a 67-72°C slide-warmer for at least 2 h in a biological safety cabinet class II.

The modified protocol (protocol 2): After decontamination and concentration, 50 - 100 µl of the remaining precipitate was washed with nuclease-free water, centrifuged at max speed, and the supernatant was discarded to remove NaOH from the precipitated specimens. Then, the precipitate was smeared and air-dried on glass slides and fixed on a 67-72°C slide warmer for at least 2 h in a biological safety cabinet class II.

The fixed smears were prepared for FDA staining. The FDA stock solution (Sigma-Aldrich) was used to prepared fresh FDA working solution by added 1 mL of the stock solution into 5 mL Acetone mix well and transfer the solution for 0.5 mL into 4.5 mL of PBS – 0.05% Tween 80. Air-dried smears were covered with a working solution of FDA for 30 minutes, washed with distilled water, and decolorized with 0.5 % acid alcohol for 3 minutes. Next, the slides were washed with water, counterstained with 0. 5 $KMnO_4$, and then examined under 400x magnification with 455 nm light-emitting diode (LED) microscope (Zeiss Primo Star iLED, Germany) (7, 8, 11, 12). The positive FDA smear was the presence of bright yellow or bright green slender bacilli on a dark background.

For the reading of FDA staining, the slides were prepared in triplicate for 3 technicians to evaluate the interobserver variation.

Molecular viability test: Analysis of ribosomal RNA precursors RT-PCR for *M. tuberculosis* complex (pre-rRNA MTBC)

Limit of detection (LOD) of pre-rRNA RT-PCR for *M. tuberculosis* complex detection

The limit of detection was performed in duplicate by using spiked *M. tuberculosis* ATCC 27294 with 1:10 serial dilution in PBS. The dilution started with McFarland 0.5 corresponding to approximately 10^7 CFU/mL of viable MTBC. Each dilution (100 µL) was also cultured on two agar plates to determine the average colony count for accurate concentration of MTBC.

Nutritional stimulation of pre-rRNA in *M. tuberculosis* complex

The conventional WHO protocol (protocol 1): After decontamination and concentration, 400 µL of the remaining precipitate was enriched in Middlebrook 7H9 broth for 4 h at 37°C (13),(14).

The modified protocol (protocol 2): Before nutritional stimulation, 400 µL of the remaining precipitate was washed with nuclease-free H_2O, centrifuged at max speed, and the supernatant was completely discarded to remove NaOH in the specimens and transferred to Middlebrook 7H9 broth for 4 h at 37°C (13),(14).

pre-rRNA of *M. tuberculosis* complex analysis

After nutritional stimulation, the suspension was concentrated by centrifugation. The pellet was transferred to the process of RNA extraction. RNA was extracted using the Tough-to-Lyse samples of bacteria, yeast, plant) (Mechanical Lysis, Monarch Total RNA Miniprep Protocol, NEB) according to manufacturer instruction. This procedure was performed under Biological safety cabinet class II respectively. The concentration of extracted RNA was

measured by NanoDrop (Denovix, USA). After that, extracted RNA was treated by Dnase I to remove genomic DNA (gDNA) contamination and checked for the clearance of gDNA by real-time PCR. The extracted RNA was converted to cDNA using iScript™ Reverse Transcription Supermix for qRT-PCR *(BIO-RAD)* according to manufacturer's instruction. cDNA products were used as templates for real-time PCR using specific primers to target MTBC. The pre-rRNA forward primer was the species-specific sequence of External transcribed spacer (ETS1: 5'-TACCTTTGGCTCCCTTTTCC-3', while the reverse primer was a semi-conserved sequence of the mature 16S rRNA: 5'-GCCCGCACGCTCACAGTTAAG-3'(13). The reaction mix contained the final volume of 1X buffer, 200nM each primer and cDNA template by 7.3 µL maximum added (Table 7). The amplicons are detected by SYBR Green-based real-time instrument (12, 13, 17). The positive results were interpreted when Ct value at 30 cycles, at mean Tm 85.15°C. Positive controls were live MTBC and MTBC genomic DNA. Negative controls were dead MTBC and nuclease-free water (Table 8).

Table 7. The components of real-time PCR set up per reaction.

Component	1 Reaction
2X buffer Luna Universal qPCR Master Mix	7.5 µL
pre-rRNA MTBC Forward Primer	0.1 µL
pre-rRNA MTBC Reverse Primer	0.1 µL
Template cDNA	7.3 µL
Total	15 µL

Table 8. Positive control and negative controls used for RT-PCR analysis per reaction. The controls replaced the template DNA used in the reaction of Table 7.

Component	Source	Control	Concentration	Volume
Live MTBC	MTBC growth in Middlebrook 7H9 broth	Positive control analysis	1 ng/ µL	3 µL
Dead MTBC	Autoclaved MTBC growth in Middlebrook 7H9 broth	Negative control analysis	-	3 µL
MTBC gDNA	Known MTBC DNA	Positive control for qPCR system	1 ng/ µL	3 µL
Nuclease free water	Nuclease free water	Negative control for RNA elution system	-	3 µL

Data analysis and statistics.

The results of all samples were used to calculate sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) (40). Mycobacterial culture was the gold standard to confirm viability. Because the clinical specimens prepared by protocol 1 and 2 were different, the results of these two sets of specimens were analyzed separately.

	Positive culture	Negative culture
Positive test result	True positive (TP)	False positive (FP)
Negative test result	False negative (FN)	True negative (TN)

Sensitivity = TP/(TP+FN)

Specificity = TN/(TN+FP)

PPV = TP/(TP+FP)

NPV = TN/(FN+TN)

CHAPTER V
RESULTS

Microscopic viability test: Fluorescein diacetate (FDA) staining

The positive result of FDA staining in the specimens was found as green-yellow bacilli or rod-shaped bacteria on a black background as shown in Figure 5A and was reported as "Positive for FDA stain". In contrast, the specimen that showed no bacilli or rod-shaped bacteria in green-yellow shades on a black background (Figure 5B) was reported as "Negative for FDA stain".

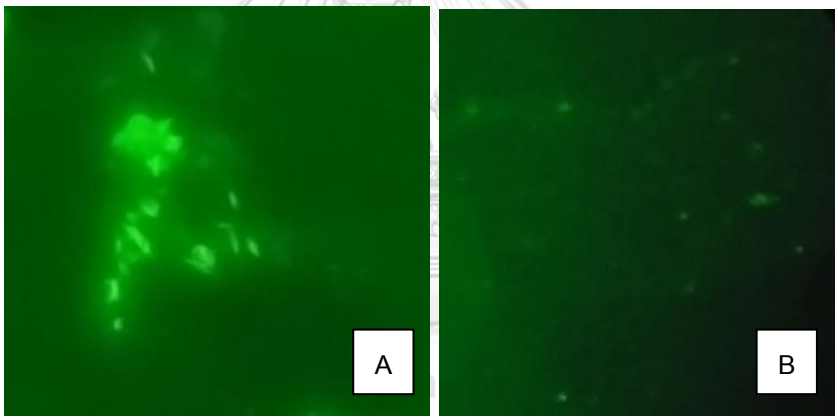


Figure 5. Interpretation of Fluorescein diacetate (FDA) staining. Positive for FDA stain (A) in comparison with Negative for FDA stain (B).

The interobserver variation of FDA vital staining

Interpretation of FDA staining performed by 3 technicians showed high variation (Table 9) . In some purulent specimens, the thick background caused FDA crystal to cover the mycobacterial cells resulting in difficult examination and interpretation In addition, the procedures of FDA staining were complicated because the freshly prepared solution was required every time before testing.

Table 9. Interobserver variation of Fluorescein diacetate (FDA) stain. After FDA staining, ten slides smeared with known AFB positive samples were examined and interpreted by 3 different technicians.

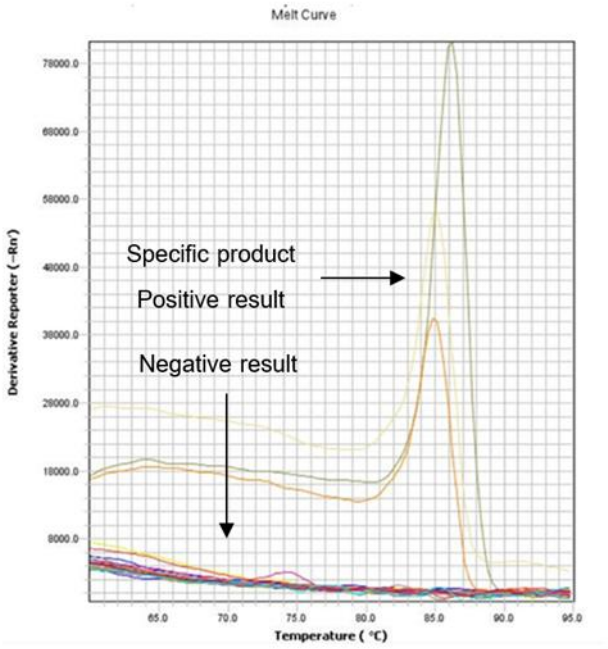
No.	AFB stain	FDA stain		
	(Kinyoun)	Technician 1	Technician 2	Technician 3
1	Positive 1+	**Positive**	Negative	+/-
2	Positive 1+	**Positive**	Negative	Negative
3	Positive 1+	**Positive**	Negative	**Positive**
4	Positive 3+	**Positive**	**Positive**	**Positive**
5	Positive 2+	**Positive**	+/-	Negative
6	Positive 1+	Negative	**Positive**	Negative
7	Scanty	Negative	Negative	Negative
8	Scanty	Negative	Negative	Negative
9	Scanty	Negative	Negative	Negative
10	Positive 2+	**Positive**	**Negative**	+/-

Molecular viability test: Analysis of ribosomal RNA precursors (pre-rRNA) RT-PCR results

Specificity of primers used to detect *M. tuberculosis* complex

The primers used in this study, the forward primer was the species-specific sequence of External transcribed spacer (ETS1: 5'-TACCTTTGGCTCCCTTTTCC-3', while the reverse primer was a semi- conserved sequence of the mature 16S rRNA: 5'-GCCCGCACGCTCACAGTTAAG-3' (13). The primers were used in SYBR Green-based pre-rRNA RT-PCR. The specificity of amplification was distinguished based on the melting temperature (Tm) of PCR products. Amplification of MTBC cDNA from clinical samples, positive control (live *M. tuberculosis* ATCC 27294), and MTBC genomic DNA with specific primers has an average of Tm at 85.15° C as a positive result, while the negative control showed no amplification (dead *M. tuberculosis* ATCC 27294) (Figure 6A). In case of NTM, the results showed no amplification or non-specific amplification with different Tm (Figure 6B and Table 10). Therefore, the primers used in this study are specific and can detect only MTBC.

A



B

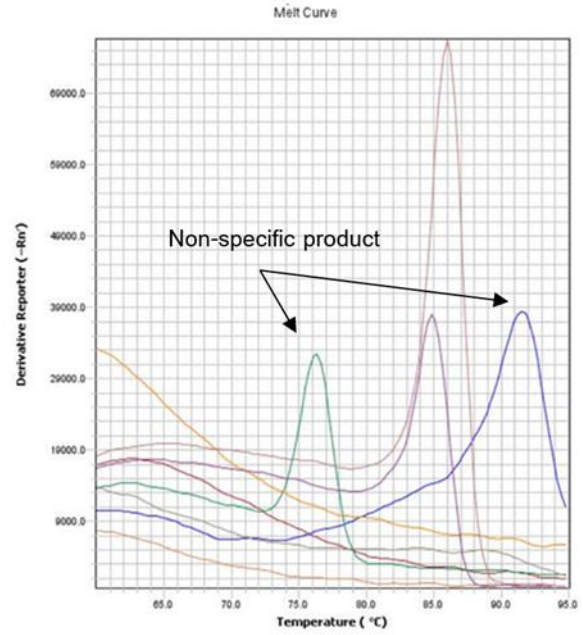


Figure 6. SYBR Green-based pre-rRNA RT-PCR detection. Positive amplification of MTBC cDNA from positive clinical samples (orange), positive control (yellow), and MTBC gDNA (gray) (6A). No amplification (orange, gray, pale orange) and non-specific amplification (green and

dark blue) in NTM and positive control (brown and purple) (6B). Five NTM species were used to test specificity of primers for pre-rRNA MTBC and *M. tuberculosis* ATCC 27294 was used as a positive control.

Table 10. Specificity of primers used in pre-rRNA RT-PCR reaction to detect *Mycobacterium* spp.

Organism	Description	Pre-rRNA
M. kansasii ATCC 12478	Photochromogen mycobacteria	Negative
M. godornae ATCC 14470	Scotochromogen mycobacteria	Negative
M. avium ATCC 15769	Non-photochromogen mycobacteria	Negative
M. fortuitum ATCC 684	Rapid growing mycobacteria	Negative
M. abscessus lab strain	Rapid growing mycobacteria	Negative
M. tuberculosis ATCC 27294	Positive control	Positive

Pre-rRNA RT-PCR detection to differentiate known live and dead *M. tuberculosis* complex

Two biological replicates of either live MTBC, *M. tuberculosis* ATCC 27294 culture in Middlebrook 7H9 broth, or dead MTBC, autoclaved *M. tuberculosis* ATCC 27294, were used for pre-rRNA RT-PCR analysis. As expected, positive Pre-rRNA RT-PCR detection was shown both samples of live MTBC, but negative in both samples of dead MTBC (Table 11). Therefore, Pre-rRNA RT-PCR could differentiate between live and dead MTBC.

Table 11. Detection of pre-rRNA RT-PCR in live and dead *M. tuberculosis* complex.

No.	Description	Viability	
		pre-rRNA MTBC	MTBC culture
1	Live MTBC 1	**Positive**	**Positive**
2	Live MTBC 2	**Positive**	**Positive**
3	Dead MTBC 1	Negative	Negative
4	Dead MTBC 2	Negative	Negative

Duration of nutritional stimulation of pre-rRNA synthesis in live *M. tuberculosis* complex obtained from clinical specimens

To determine appropriate duration to induce pre- rRNA synthesis in MTBC, the remaining precipitates of positive AFB specimens were stimulated by Middlebrook 7H9 broth medium at 37°C for 4, 24, 48, and 72 h and then pre-rRNA of MTBC was detected by RT-PCR. pre-rRNA was detected since 4 h and up to 72 h of stimulation as shown in Table 12. All of these specimens were positive by mycobacterial culture and gDNA MTBC. Therefore, 4-h incubation was sufficient for nutritional stimulation of pre-rRNA synthesis in clinical specimens.

Table 12. Hours of nutritional stimulation for live *M. tuberculosis* complex in clinical specimens.

Hours of Nutritional stimulation	pre-rRNA MTBC
	Positive AFB specimens
4	Positive
24	Positive
48	Positive
72	Positive

Limit of detection (LOD) of pre-rRNA RT-PCR for *M. tuberculosis* complex

This experiment showed that pre-rRNA RT-PCR results were positive when live *M. tuberculosis* complex diluted at 1:10 – 1:10000 were used in the reaction (Table 12). Therefore, the limit of detection of pre-rRNA MTBC was approximately 10^3 CFU/mL by performed in replicates.

Table 13. The limit of detection of pre-rRNA RT-PCR of *M. tuberculosis* complex.

Conventional preparation for clinical specimens (Protocol 1).

No	Description	pre-rRNA MTBC	MTBC Culture	Average Colony count/100 μL
1	Dilution 1:10 (10^6 CFU/mL)	Positive	Positive	>300
2	Dilution 1:100 (10^5 CFU/mL)	Positive	Positive	>300
3	Dilution 1:1000 (10^4 CFU/mL)	Positive	Positive	278 ($2.78x10^3$ CFU/mL)
4	Dilution 1:10000 (10^3 CFU/mL)	Positive	Positive	67 ($6.7x10^2$ CFU/mL)
5	Dilution 1:100000 (10^2 CFU/mL)	Negative	Positive	13 ($1.3x10^2$ CFU/mL)
6	Dilution 1:1000000 (10^1 CFU/mL)	Negative	Positive	1 (10^1 CFU/mL)

Initially, 564 clinical specimens were decontaminated according to the conventional WHO protocol (protocol 1) before nutritional stimulation of pre-rRNA followed by RNA extraction and pre-rRNA RT-PCR. Both AFB-positive and AFB-negative samples were included in the experiment. In addition, genomic DNA of all samples were used for detection of MTBC by real-time PCR (gDNA MTBC). The results of pre-rRNA viability test were compared to those of FDA vital staining using mycobacterial culture as a gold standard for identification of viable MTBC as shown in Table 14 and Table 15.

Viability and non-viability test results of 564 clinical specimens prepared by the conventional WHO protocol (protocol 1)

Of the total 564 clinical specimens, 57 samples were positive for MTBC culture and 507 samples were negative for MTBC culture. Pre-rRNA MTBC and FDA staining were correctly detected 19 and 27 samples of positive MTBC culture, respectively. FDA staining could detect 8 more samples of positive MTBC culture than pre-rRNA MTBC, all of which were positive AFB score $\geq$ 1+. Pre-rRNA MTBC was unable to detect 17 AFB-positive samples (7 AFB score 1+, 1 AFB score 3+, and 9 AFB score scanty) and 34 positive gDNA MTBC that subsequently found positive for MTBC culture. All 36 samples of positive FDA staining were positive AFB (24 AFB score 1+, 1 AFB score 2+, and 11 AFB score 3+), of which 9 turned out to be NTM. Four positive gDNA MTBC (1 AFB score 1+, 1 AFB score scanty, and 2 AFB negative) were negative for both pre-rRNA MTBC and FDA staining, which were consistent with negative MTBC culture, indicating dead MTBC. Four positive and 466 negative MTBC culture were not detected by any viability and non-viability tests.

Table 14. Viability and non-viability test results of 564 clinical specimens prepared by the conventional WHO protocol (protocol 1): The culture " - " were indicated non-relevant results.

Viability test						Non-viability test			Total
pre-rRNA	FDA stain		*Mycobacterium* culture			gDNA MTBC	AFB stain (Kinyoun)		
Positive	Positive	Negative	MTBC	NTM	Negative	Positive	Positive	Negative	
19	19	0	19	-	-	19	19	0	19
0	8	26	34	-	-	34	17	17	34
0	1	0	-	1	-	1	1	0	1
0	8	24	-	32	-	0	11	21	32
0	0	4	-	-	4	4	2	2	4
0	0	4	-	-	4	0	4	0	4
0	0	4	4	-	-	0	0	4	4
0	0	466	-	-	466	0	0	466	466
TOTAL									564

Table 15. Comparison of pre-rRNA MTBC and FDA staining for detection of viable MTBC in 564 clinical specimens prepared by the conventional WHO protocol (protocol1).

Testing of all samples (564)*	Pre-rRNA MTBC result		FDA staining result	
	Positive (19)	Negative (545)	Positive (36)	Negative (528)
AFB result	+ (19) (Scanty = 0, ≥ 1+ = 19)**	+ (35) (Scanty = 17, ≥ 1+ = 18)	+ (36) (Scanty = 0 ≥ 1+ = 36)	+ (18) (Scanty = 17 ≥ 1+ = 1)
	- (0)	- (510)	- (0)	- (510)
gDNA MTBC result	+ (19)	+ (39)	+ (28)	+ (30)
	- (0)	- (506)	- (8)	- (498)
MTBC culture result	+ (19)	+ (38)	+ (27)	+ (30)
	- (0) (No growth = 0, NTM = 0)	- (507) (No growth = 474, NTM = 33)	- (9) (No growth = 0 NTM = 9)	- (498) (No growth = 474, NTM = 24)

* number of samples were shown in parenthesis in each category as indicated in the table

** AFB score

Diagnostic accuracy of pre-rRNA MTBC and FDA staining (protocol 1)

The results of 564 samples prepared by the conventional method (protocol 1) were analyzed to compare sensitivity, specificity, PPV, and NPV of pre-rRNA MTBC with those of FDA staining. In addition, only 37 AFB (Kinyoun) positive specimens were independently analyzed.

Table 16. Diagnostic accuracy of pre-rRNA MTBC and FDA staining: sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) of pre-rRNA MTBC, and FDA staining of all specimens and AFB (Kinyoun) positive specimens using MTBC culture as a gold standard for viability.

Protocol 1	All Clinical specimens (AFB Positive & Negative)				AFB (kinyoun) positive specimens			
	Sensitivity	Specificity	PPV	NPV	Sensitivity	Specificity	PPV	NPV
pre-rRNA	33.33%	100%	100%	93.03%	52.77%	100%	100%	51.43%
FDA	47.37%	98.22%	75%	94.32%	75%	50%	75%	50%

MTBC was isolated in 57 specimens prepared by protocol 1, of which 19 were positive by pre-rRNA MTBC and 27 were positive by FDA staining. Based on mycobacterial culture as a gold standard method, pre-rRNA MTBC had 33.33% sensitivity, 100% specificity, 100% PPV, and 93.03% NPV. Sensitivity, specificity, PPV, and NPV of FDA staining were 47.37%, 98.22%, 75%, and 94.32%, respectively (Table 16). In case of AFB positive specimens, sensitivity of pre-rRNA MTBC and FDA staining increased to 52.77% and 75%, respectively. Although specificity and PPV of pre-rRNA MTBC in AFB-positive samples remained 100%, its NPV reduced to 51.43%. In comparison with the results of all samples, specificity and NPV of FDA staining in AFB-positive samples decreased to 50% and 50%, respectively (Table 16).

Modified preparation for clinical specimens (Protocol 2)

To improve sensitivity of pre-rRNA MTBC, the preparation method of clinical specimens were modified to minimize the effect of NaOH on the degradation of RNA, i.e., the NaOH-containing decontamination solution was completely discarded, and the remaining precipitate was washed before use (protocol 2).

To demonstrate that the modified protocol (protocol 2) was able improve sensitivity, the limit of detection of samples obtained from protocol 1 and 2 was compared using spiked *M. tuberculosis* ATCC 27294 with 1:10 serial dilution in sputum. The dilution started with McFarland 0.5 corresponding to approximately 10^7 CFU/mL of viable MTBC.

LOD of MTBC spiked into sputum samples in the modified method (protocol 2) was approximately 10^6 CFU/mL, which was 10-fold lower than that of protocol 1 (10^7 CFU/mL) (Table 17). The LOD was performed in replicates. Thus, pre-rRNA MTBC of samples prepared by the modified method (protocol 2) might improve sensitivity compared to those prepared by the conventional method (protocol 1).

Table 17. The limit of detection pre-rRNA MTBC for *M. tuberculosis* complex spiked in the sputum samples and prepared by the conventional WHO protocol 1 and the modified protocol (protocol 2).

Description	Protocol 1			Protocol 2		
	pre-rRNA MTBC	MTBC culture	Average Colony count	pre-rRNA MTBC	MTBC culture	Average Colony count
0.5 McFarland	Positive	Positive	>300	Positive	Positive	>300
Dilution 1:10 (10^6 CFU/mL)	Negative	Positive	>300	Positive	Positive	>300
Dilution 1:100 (10^5 CFU/mL)	Negative	Positive	>300	Negative	Positive	>300
Dilution 1:1000 (10^4 CFU/mL)	Negative	Positive	117 ($1.17x10^3$ CFU/mL)	Negative	Positive	132 ($1.32x10^3$ CFU/mL)
Dilution 1:10000 (10^3 CFU/mL)	Negative	Positive	12 ($1.2x10^2$ CFU/mL)	Negative	Positive	18 ($1.8x10^2$ CFU/mL)
Dilution 1:100000 (10^2 CFU/mL)	Negative	Positive	1 (10^1 CFU/mL)	Negative	Positive	3 (10^1 CFU/mL)
Dilution 1:1000000 (10^1 CFU/mL)	Negative	Positive	0	Negative	Positive	1 (10^1 CFU/mL)

Next, additional 45 clinical specimens were collected and separately prepared by this modified protocol (protocol 2), and further examined for viable MTBC by viability and non-viability tests, similar to those prepared by protocol 1.

Viability and non-viability test results of 45 clinical specimens prepared by the modified preparation for clinical specimens (protocol 2)

The results of specimens prepared by the modified protocol 2 are shown in Table 18 and 19. Of the 45 clinical specimens, 35 specimens were reported positive by MTBC culture, 39 specimens were positive by gDNA detection, and 10 specimens were negative for MTBC culture. Pre-rRNA MTBC and FDA staining were correctly detected 20 samples of positive MTBC culture. Of these 20 positive pre-rRNA MTBC specimens, 18 specimens were AFB score $\geq$ 1+ (8 AFB score 1+, 8 AFB score 3+, 2 AFB score 2+), and 2 specimens were AFB score scanty. FDA staining could detect 2 more samples of positive MTBC culture than pre-rRNA MTBC. Fifteen specimens were reported negative for pre-rRNA (6 AFB score 1+, 3 AFB score 3+, 1 AFB score 2+, and 5 AFB score scanty). Twenty of 35 positive gDNA MTBC were subsequently found positive for MTBC culture. NTM were isolated in 5 specimens. All 23 samples of positive FDA staining were positive AFB score $\geq$ 1+ (10 AFB score 1+, 10 AFB score 3+, and 3 AFB score 2+) 3 of which turned out to be NTM. Four specimens were detected only by gDNA MTBC (1 AFB score 1+, 1 AFB score 3+, 1 AFB scanty, and 1 AFB negative) but were negative for pre-rRNA MTBC, FDA staining and MTBC culture, indicating dead MTBC. One sample was positive for both FDA staining and Kinyoun smear but was negative for other techniques including mycobacterial culture suggesting dead NTM or other related organisms.

Table 18. Viability and non-viability test results of 45 clinical specimens prepared by the modified preparation for clinical specimens (protocol 2): The culture " - " were indicated non-relevant results.

Viability test						Non-viability test			Total
pre-rRNA	FDA stain		*Mycobacterium* culture			gDNA MTBC	AFB stain (Kinyoun)		
Positive	Positive	Negative	MTBC	NTM	Negative	Positive	Positive	Negative	
20	18	2	20	-	-	20	20	0	20
0	2	13	15	-	-	15	6	9	15
0	3	2	-	5	-	0	5	0	5
0	0	4	-	-	Neg	4	3	1	4
0	0	1	-	-	Neg	0	1	0	1
TOTAL									45

Table 19. Comparison of pre-rRNA MTBC and FDA staining for detection of viable MTBC in 45 clinical specimens prepared by the modified protocol (protocol 2).

Total samples (45)*	Pre-rRNA MTBC		FDA staining	
	Positive (20)	Negative (25)	Positive (23)	Negative (22)
AFB	+ (20) (Scanty = 2 ≥ 1+ = 18)**	+ (15) (Scanty = 5 ≥ 1+ = 10)	+ (23) (Scanty = 0 ≥ 1+ = 23)	+ (12) (Scanty = 7 ≥ 1+ = 5)
	- (0)	- (10)	- (0)	- (10)
gDNA MTBC	+ (20)	+ (19)	+ (20)	+ (19)
	- (0)	- (6)	- (3)	- (3)
MTBC culture	+ (20)	+ (15)	+ (20)	+ (15)
	- (0) (No growth = 0, NTM = 0)	- (10) (No growth = 5, NTM = 5)	- (3) (No growth = 0, NTM = 3)	- (7) (No growth = 5, NTM = 2)

* number of samples were shown in parenthesis in each category as indicated in the table

** AFB score

Diagnostic accuracy of pre-rRNA MTBC and FDA staining (protocol 2)

Table 20. Diagnostic accuracy of pre-rRNA RT-PCR for MTBC and FDA staining: sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) of pre-rRNA MTBC, and FDA staining of all specimens and AFB (Kinyoun) positive specimens using MTBC culture as a gold standard for viability.

Protocol 2	All Clinical specimens (AFB Positive & Negative)				AFB (kinyoun) positive specimens			
	Sensitivity	Specificity	PPV	NPV	Sensitivity	Specificity	PPV	NPV
pre-rRNA	57.14%	100%	100%	40%	76.92%	100%	100%	60%
FDA	57.14%	70%	86.96%	31.82%	76.92%	66.66%	86.96%	50%

MTBC was isolated in 35 specimens prepared by protocol 2, of which 20 specimens were positive by pre-rRNA and 22 specimens were positive by FDA stain. By using the modified protocol 2, the sensitivity of pre-rRNA MTBC and FDA staining increased to 57.14%, which was highter than that of samples from the convential WHO method (Figure 7A). In addition, pre-rRNA MTBC had 100% specificity, 100% PPV, and 40% NPV. FDA stain had 70% specificity, 86.96% PPV, and 31.82% NPV (Table 20). For only Kinyoun smear-positive samples prepared by protocol 2, the sensitivity of pre-rRNA MTBC and FDA staining increased to 76.92 (Table 20 and Figure 7B). Moreover, pre-rRNA MTBC in AFB-positive samples had 100% specificity, 100% PPV, and 60% NPV, while FDA staining had 66.66% specificity, 86.96% PPV, and 50% NPV (Table 20)

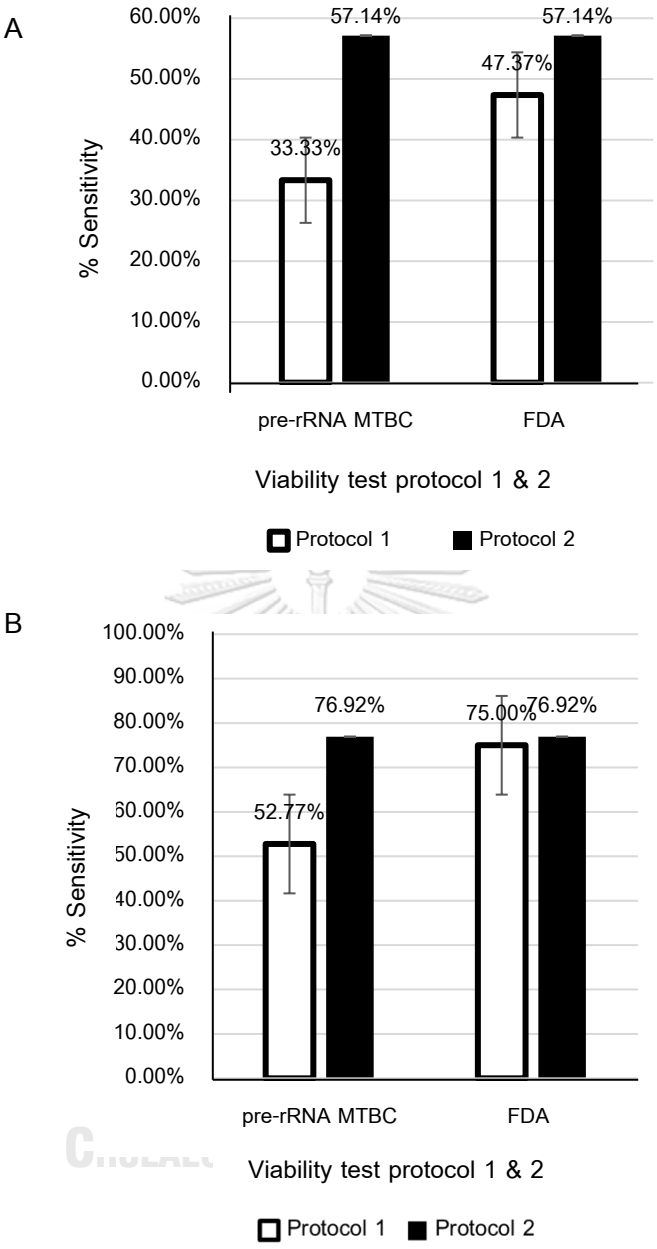


Figure 7. The comparison of the sensitivity of pre-rRNA MTBC and FDA staining to detect viable MTBC in all clinical specimens (A) and in AFB (Kinyoun) positive specimens (B) prepared by the conventional WHO protocol (protocol 1) and the modified protocol (protocol 2).

The combination of pre-rRNA MTBC and FDA staining

The combination of pre-rRNA MTBC and FDA staining can enhance sensitivity for detection of viable MTBC compared to pre-rRNA MTBC or FDA staining alone as shown in Table 21 and Figure 8.

Table 21. Enhanced accuracy of viability tests. Sensitivity and specificity of FDA staining after combination with pre-rRNA MTBC in all specimens and AFB positive specimens prepared by the conventional WHO method (protocol 1) and the modified method (protocol 2).

Protocol 1	All clinical specimen (AFB positive and negative)					AFB (kinyoun) positive specimen			
	Sensitivity	Specificity	PPV	NPV		Sensitivity	specificity	PPV	NPV
pre-rRNA + FDA	50.94%	98.24%	75%	95.08%		75%	50%	75%	50%
Protocol 2	**All clinical specimen (AFB positive and negative)**					**AFB (kinyoun) positive specimen**			
	Sensitivity	Specificity	PPV	NPV		Sensitivity	specificity	PPV	NPV
pre-rRNA + FDA	59.46%	70%	88%	31.82%		78.57%	66.66%	88%	50%

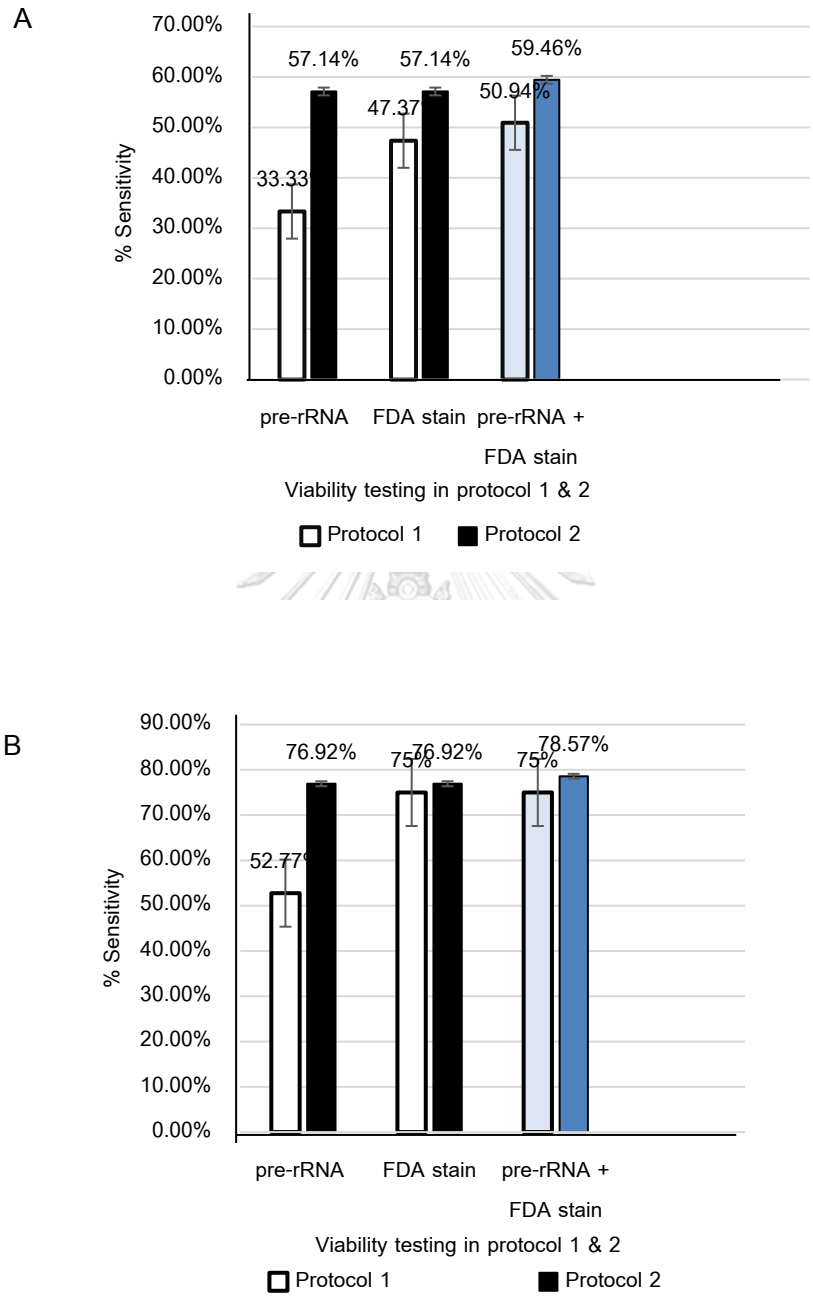


Figure 8. Enhanced accuracy of pre-rRNA after combination with FDA stain in all clinical specimens (A) and AFB Positive specimens (B). Sensitivity of pre-rRNA MTBC after combination with FDA staining in all specimen (Figure 8A) and Positive AFB specimens (Figure 8B) prepared

by the conventional WHO method (protocol 1, the light blue bar) and the modified method (protocol 2, the dark blue bar) compared with pre-rRNA alone and FDA alone.

CHAPTER VI
DISCUSSION

Viability of MTBC in clinical specimens is important to determine the response of treatment to anti-tuberculosis drugs. Although mycobacterial culture for MTBC is a gold standard for viable MTBC, it is not useful for early detection of viability due to slow growing MTBC. This study aimed to use pre-rRNA MTBC detected by RT-PCR as a biomarker to rapidly and specifically identify viable MTBC in clinical specimens. The pre-rRNA MTBC detection was compared with FDA staining for their accuracy in clinical specimens. Initially, clinical samples were collected and prepared by the conventional WHO method (protocol 1) before RNA extraction.

Previous studies found that pre-rRNA detection was more sensitive than genomic DNA detection in slow-growing mycobacteria (12, 38). However, using the conventional method for preparation (protocol 1) of 564 samples, the sensitivity of pre-rRNA MTBC method seemed to be low (33.33%). Therefore, each step of the pre-rRNA MTBC detection method was reviewed to assess the possible cause of poor sensitivity. Regarding the amount of extracted RNA used in each PCR reaction, the maximum volume of cDNA per reaction was already used (Table 7). However, it was possible that NaOH remained in the specimens after the step of decontamination in the mycobacterial culture procedure might cause RNA degradation. Therefore, the sample preparation was modified to get rid of NaOH in the decontamination solution (protocol 2). Additional 45 samples were washed with nuclease-free water to remove excess NaOH from precipitated specimens before the nutritional stimulation step. Therefore,

the modified sample preparation method (protocol 2) was able to improve sensitivity of pre-rRNA detection to 57.14% in all specimens (Figure 7A) and up to 76.92% in AFB positive specimens (Figure 7B), which was comparable to the FDA stain staining.

LOD of pre-rRNA RT-PCR using MTBC serially diluted in sputum samples prepared by protocol 1 and 2 was approximately 10^7 and 10^6 CFU/mL, respectively (Table 17). This finding was consistent with the results of most clinical samples with positive pre-rRNA MTBC were AFB score at least 1+ (Table 14 and Table 18) equivalent to MTBC load up to 50,000 – 500,000 bacilli per 1 mL of specimens (44).

Another reason that our pre-rRNA detection had lower sensitivity than gDNA to detect MTBC, which was discordant with the previous study (12, 14), might be the result of endogenous RNAse activity and exogenous RNAse in clinical specimens especially respiratory samples (41) (42). Moreover, molecular processing including DNase treatment, RNA conversion, and real-time amplification could lead to RNA degradation (41) and loss of RNA. Therefore, the yield of RNA and higher sensitivity of pre-rRNA detection might be obtained if the specimens were stored in the RNA protection/stabilization reagent (41, 43).

FDA staining had higher sensitivity in AFB positive specimens (75% and 76.92% in protocol 1 and 2, respectively) as shown in Table 16 and 20. However, its sensitivity in this study was lower than 96% sensitivity reported in the previous study (7) because all specimens in that study had higher number of MTBC (AFB grade 2+and 3+) while our study mostly had AFB 1+ specimens. In addition, in this study the limit of detection of FDA staining was similar to that of

pre-rRNA (up to 50,000 – 500,000 bacilli per 1 mL of specimen approximately 10^6 CFU/mL). However, the FDA staining procedure had to use freshly prepared working FDA reagent. The visualization was difficult to interpret, required high experience technicians, and showed very high interobserver variation (Table 9). In case of thick smear, large crystals of FDA dye may obscure the bacilli leading to false-negative results under microscopic observation. Moreover, the FDA staining did not differentiate between MTBC and NTM, so it requires further confirmatory tests such as additional MTBC-specific PCR and mycobacterial culture.

Pre-rRNA RT-PCR for MTBC can overcome the limitations of FDA staining for detection of viable MTBC in clinical specimens, especially in AFB positive smear. Pre-rRNA MTBC is very specific, having 100% specificity and 100% PPV. Therefore, positivity of pre-rRNA MTBC almost always indicates viable MTBC in the clinical specimens, which will be remarkably useful for appropriate treatment of tuberculosis. However, pre-rRNA MTBC has limitations because RNA is susceptible to degradation. Therefore, this method must be done promptly by avoiding gDNA contamination. In addition, RNA extraction and RT-PCR detection are complicated methods. The first step before extraction needs extra steps of centrifugation to concentrate sample yields and washing to eliminate NaOH excess and adding the specimens to an RNA protection/stabilization reagent (41, 43). Moreover, it needs an additional 4 h for nutritional stimulation to induce the metabolic activity of the MTBC cell to express the pre-rRNA pool in the samples.

In conclusion, RT-PCR of pre-rRNA gene can be used to detect viable *M. tuberculosis* complex in clinical specimens. The modified method of sample preparation (protocol 2) can improve accuracy of pre-rRNA MTBC for detection of viable MTBC, especially in AFB positive specimens.

Practical application of pre-rRNA MTBC detection

In patients with tuberculosis, if clinical specimens remain positive for AFB staining and PCR MTBC, viability tests are required to differentiate dead and viable MTBC. Pre-rRNA MTBC detection is extremely useful and rapid to determine MTBC viability in the clinical specimens, especially in samples with positive AFB staining, because PPV to predict positive MTBC culture is 100%. In contrast, the negative result of pre-rRNA MTBC cannot exclude the possibility of viable MTBC.

APPENDIX

Media and Reagent

Ogawa medium (3000 mL)

KH_2PO_4	10	g
Sodium glutamate	10	g
Deionized water	1,000	mL
Glycerine	60	mL
2% Malachite green	60	mL
Both yolks and whites egg	2,000	mL

1.1 Mixed all ingredients then autoclave at 121°C, 15 min.

1.2 Prepared an egg by clearing and soaking 70% alcohol for an hour.

1.3 Beated into a sterile beaker, blending magnetic stirrer for well-mixed, then adding a solution from 1.1 for mixed well along with pouring malachite green to reach the dark green, blending an hour while gently dropping. (If the color is light, it must be added.)

1.4 Aliquoted into the test tube at 6 mL for each.

1.5 Arranged slant in the oven at a temperature of 75°C for 2 hours, chilling in the fridge.

Ogawa + 5% Blood medium (3000 mL)

KH_2PO_4	10	g
Sodium glutamate	10	g
Deionized water	1,000	mL
Glycerine	60	mL
2% Malachite green	60	mL
Both yolks and whites egg	2,000	mL

1.1 Mixed all ingredients then autoclave at 121°C, 15 min.

1.2 Prepared an egg by clearing and soaking 70% alcohol for an hour.

1.3 Beated into a sterile beaker, blending magnetic stirrer for well-mixed, then adding a solution from 1.1 for mixed well along with pouring malachite green to reach the dark green, blending an hour while gently dropping. (If the color is light, it must be added.)

1.4 Added 5% sheep blood into the solution and gently blending.

1.5 Aliquoted into the test tube at 6 mL for each.

1.6 Arranged slant in the oven at a temperature at 75°C for 2 hours, chilling in the fridge.

Middlebrook 7H9 broth (400 mL)

Middlebrook 7H9 powder	4.7	g
Deionized water	900	mL
Glycerol	2	mL
OADC Enrichment supplement	100	mL

1.1 Weighted Middlebrook 7H9 powder and mixed with Deionized water.

1.2 Simmered ingredients then adjusting pH 7.2 ± 2.

1.3 Adjusted the solution to pH 6.6 ± 2.

1.4 Transfered to steaming for pasteurization at 121°C, 15 min.

1.5 Chilled at room temperature and adding OADC Enrichment supplement.

1.6 Aliquoted in the sterile test tube 4 mL for each by using a sterile dispenser.

AFB stain by Kinyoun method

Basic Fuchsin	40	g
Methanol (95%)	100	mL
Phenol (Crystal use)	80	g
Deionized water	1000	mL

1.1 Added 40 g Basic Fuchsin into 95% Methanol 100 mL, drop the magnetic stirrer into the beaker, and adjust the shaker at medium blending speedily until homogenized. And leave it overnight.

1.2 Added Phenol (crystal use) 80 g into 1 L of Deionized water, and blending all mixture.

1.3 Taked liquid phenol from 3.2 mixes with Basic fuchsin from 3.1

1.4 Taked the solution filter throughout filter papers and store the flow-through solution at room temperature by aliquoting into the amber bottle.

3% Acid alcohol (1 L)

HCL	30	mL
95% Methanol	970	mL

Added methanol into the sterile beaker, gently pouring HCl, and mix well before storing it at room temperature by aliquoting into the amber bottle.

Methylene blue

Methylene blue	3	g
Deionized water	1000	mL

Mixed well, drop the magnetic stirrer into the beaker and adjust the shaker at medium blending speedily until homogenized. Then taking the solution filter throughout filter papers. Stored the flow-through solution at room temperature.

PBS – 0.05% Tween 80 (100 mL)

Tween 80	50	ul
PBS	100	mL

Mixed well and store at room temperature.

Fluorescein diacetate (FDA) stock solution (5 mg/mL)

FDA powder	5	mg
Acetone	1	mL

one mililiter of each stock was distribute to cryovial and store at -20°C avoiding the light.

Fluorescein diacetate (FDA) fresh working solution

FDA stock solution	1	mL
Acetone	5	mL

Mixed well and take the solution for 0.5 mL add to 4.5 mL of PBS – 0.05% Tween 80. The FDA working solution must be prepared immediately before use.

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