การจำแนกสปีชีส์ของเชื้อมัยโคแบคทีเรีย โดยการหาลำดับเบสของชิ้นส่วน 16S rDNA จากตัวอย่างเลือดที่เพาะในอาหารเหลว

นางสาวอัญชลี ละอาด

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SPECIES IDENTIFICATION OF MYCOBACTERIA BY SEQUENCING OF AMPLIFIED 16S rDNA FROM HEMOCULTURES

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อัญชลี ละอาด : การจำแนกสปีชีส์ของเชื้อมัยโคแบคทีเรีย โดยการหาลำดับเบสของชิ้นส่วน 16S RDNA จากตัวอย่างเลือดที่เพาะเลี้ยงในอาหารเหลว (SPECIES IDENTIFICATION OF MYCOBACTERIA BY SEQUENCING OF AMPLIFIED 16S RDNA FROM HEMOCULTURES) อาจารย์ที่ปรึกษา : รศ.ดร. สมหญิง ธัมวาสร, อาจารย์ที่ปรึกษาร่วม : นายนิพนธ์ อุดมสันติสุข, 85 หน้า. ISBN 974-03-1723-5.

การหาลำดับเบสของชิ้นส่วนยีน 16S rRNA (16S rDNA) เป็นวิธีที่ใช้กันอย่างแพร่หลายในการจำแนกสปีชีส์ของเชื้อมัยโคแบคทีเรียในสิ่งส่งตรวจ ความล้มเหลวของการเพิ่มปริมาณ DNA ด้วยเทคนิค PCR มักจะเกิดจากสารยับยั้ง (inhibitors) ในปฏิกิริยา การเพิ่มจำนวนชิ้นส่วน 16S rDNA จากตัวอย่างเลือดที่เพาะเลี้ยงในอาหารเหลวในเบื้องต้นก็ล้มเหลวเนื่องมาจากสาเหตุเดียวกัน ได้ใช้วิธีแยกและสกัด DNA 5 วิธีเพื่อกำจัดสารยับยั้ง พบว่า การใช้ด่างชะล้างประกอบกับการใช้ความร้อนแยกสลายเชื้อมัยโคแบคทีเรีย (alkali wash with heat lysis) ให้ผลเป็นอย่างดี เปรียบเทียบผลการทดลองของการหาลำดับเบสของชิ้นส่วน 16S rDNA กับวิธีที่ใช้อยู่เป็นประจำ (conventional method) และการใช้ AccuProbe จาก 381 ตัวอย่างของเลือดที่เพาะเลี้ยงในอาหารเหลวใน MB/BacT พบสัญญาณบวก 73 ตัวอย่าง (19.16%) 69 ตัวอย่างที่สัญญาณบวกพบการเจริญของเชื้อที่ติดสีทนกรด (acid fast bacilli positive) และ 4 ตัวอย่างให้ผลลบกับการย้อมสีทนกรด (acid fast bacilli negative) จาก 69 ตัวอย่างที่พบเชื้อติดสีทนกรด 66 ตัวอย่างสามารถจำแนกเชื้อได้ 67 isolates และ 3 ตัวอย่างไม่สามารถเพาะเลี้ยงเชื้อได้บนอาหารแข็ง วิธีที่ใช้อยู่เป็นประจำ และ AccuProbe สามารถจำแนกเชื้อเป็น 4 สปีชีส์ คือ *M. tuberculosis, M. avium complex, M. scrofulaceum* และ *M. simiae* เชื้อที่เจริญบนอาหารแข็ง 4 ตัวอย่างไม่สามารถจำแนกสปีชีส์ได้ และ 1 ตัวอย่างให้ผลการทดสอบผิดพลาด การหาลำดับเบสของชิ้นส่วน 16S rDNA ได้ผลการทดสอบเป็น 9 สปีชีส์ คือ *M. tuberculosis* complex, *M. avium, M. intracellulare, M. scrofulaceum, M. simiae, M. ulcerans, M. haemophilum, M. interjectum* และ *M. triplex* ผลการจำแนกสปีชีส์ด้วยวิธีการดังกล่าวให้ผลที่สอดคล้องกันยกเว้น 1 isolate คือ วิธีที่ใช้อยู่เป็นประจำให้ผลเป็น *M. xenopi* ขณะที่ผลการทดสอบด้วยการหาลำดับเบสของชิ้นส่วน 16S rDNA ให้ผลเป็น *M. scrofulaceum* ทั้งนี้เป็นสิ่งที่พบได้เนื่องจาก *M. scrofulaceum* และ *M. xenopi* มีลักษณะปรากฏที่คล้ายคลึงกัน

การศึกษาครั้งนี้พบว่า การหาลำดับเบสของชิ้นส่วน 16S RDNA เพื่อจำแนกสปีชีส์ของเชื้อมัยโคแบคทีเรีย เป็นวิธีการที่น่าเชื่อถือ มีความรวดเร็ว (ให้ผลการทดสอบภายใน 3 วัน) แม่นยำ และสามารถนำมาประยุกต์ใช้ในงานประจำของห้องปฏิบัติการที่ทันสมัยได้

ภาควิชาสหสาขาวิชาจุลชีววิทยาทางการแพทย์	ลายมือชื่อนิสิต…….……..
สาขาวิชาชีวิทยาทางการแพทย์	ลายมือชื่ออาจารย์ที่ปรึกษา…….……..
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THESIS ADVISOR : ASSOC. PROF.SOMYING TUMWASORN, Ph.D.,
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PCR sequencing of the gene coding for 16S rRNA (16S rDNA) is a well established method used to identify mycobacteria in clinical samples. A common technique problem with PCR is amplification failure due to the presence of PCR inhibitor (s). Initial attemp to amplify mycobacterial 16S rDNA from hemocultures failed because of this reason. Five DNA extraction methods were used for purification of DNA and removal of inhibitor (s) from hemoculture. Alkali wash and heat lysis was found to be the best suit method for preparation of mycobacterial DNA from hemoculture. The results of sequencing of amplified 16S rDNA were compared with those of conventional method and AccuProbe (Gen-Probe, Inc., San Diego, Calif.). Out of 381 hemoculture in MB/BacT instrument, 73 samples (19.16%) were flagged positive. Sixty-nine flagged positive hemocultures were acid-fast bacilli (AFB) positive and 4 samples were acid-fast bacilli (AFB) negative. Of these 69 AFB positive samples, 66 grew 67AFB and 3 could not grow AFB on solid media. Identification by conventional method and AccuProbe revealed 4 different species as follows: M. tuberculosis, M. avium complex, M. scrofulaceum and M. simiae. Four isolates from 4 samples were unidentified and one isolate was mis-identified. Identification by 16S rDNA sequencing demonstrated 9 different species as follows: M. tuberculosis, M. avium. M. intracellulare, M. scrofulaceum. M. simiae, M. ulcerans, M. haemophilum. M. interjectum, and M. triplex. The results of species identification by these methods were concordant except one isolate identified as M. scrofulaceum with 16S rDNA analysis was identified as M. xenopi with conventional methods. This was not uncommon as M. scrofulaceum phenotypically resembles M. xenopi .

This study concludes that direct sequence analysis of amplified 16S rDNA is a promising, rapid (within 3 days) and accurate method for species determination of mycobacteria. This method might also be applicable for routine identification of mycobacteria from hemocultures in advanced laboratory.

Department of Medical Microbiology	Student's signature……
Field of student Medical Microbiology	Advisor's signature……
Academic year 2001	Co-advisor's signature……

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ABBREVIATION

A	adenosine
AIDS	acquired immunodefficiency syndrome
bp	base pair
C	cytidine
^{o} C	degree celsius
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate
ddATP	dideoxyadenosine 5'-triphosphate
ddCTP	dideoxycytidine 5'-triphosphate
ddGTP	dideoxyguanosine 5'-triphosphate
ddTTP	dideoxythymidine 5'-triphosphate
DDW	double distilled water
ddNTPs	dideoxynucleotide-tri-phosphate
dGTP	deoxyguanosine 5'-triphosphate
DNA	deoxynucleic acid
dNTPs	deoxynucleotide-tri-phosphate
dTTP	deoxythymidine 5'-triphosphate
DW	distilled water
EDTA	ethylenediamine tetraacetic acid
et al.	et alii

g	gram
G	guanosine
GLC	gas liquid chromatography
HCl	hydrochloric acid
HIV	human immunodeficiency virus
HPLC	high performance liquid chromatography
hr	hour
i.e	id est
M	molar
M.	Mycobacterium
mg	milligram
$MgCl_2$	magnesium chloride
min	minute(s)
ml	millilitre
mM	millimolar
mmol	millimolar
NaOH	sodium hydroxide
NTM	non-tuberculous mycobacteria
PCR	polymerase chain reaction
pmol	picomol
RNA	ribonucleic acid
RFLP	restriction fragment length polymorphism
rRNA	ribosomal ribonucleic acid
16S rRNA	sixteen subunit ribonucleic acid
SDS	sodium polyanetholesulfonate

sec	second
16S rDNA	sixteen subunit deoxynucleic acid
T	thymidine
TAE	Tris-acetate-EDTA
Taq	Themus aquaticus
TB	tuberculosis
Tris	Tris-(hydroxymethyl)-aminoethane
U	unit
ug	micrograme
ul	microliter
uM	micromolar
UV	ultraviolet
V	voltage
WHO	World Health Organization

CHAPTER I

INTRODUCTION

Disease's caused by species of the genus *Mycobacterium* are major sources of morbidity and mortality in the world today, particularly in developing and tropical country (1,2,3). Tuberculin surveys have shown that it is likely that one third of the world's population has been infected with the tubercle bacillus. There are approximately 100 million new cases of TB (4,5). World Health Organization announced in 1994, about 3 millions people died of tuberculosis and, unless global control programs are adequately funded and developed, this mortality rate could rise to 4 millions annually by the year 2004 (6).

Since the mid-1980s it has been evidenced that the HIV/AIDS pandemic is having devastating effect on the prevalence of tuberculosis. About 50 percents of HIV-positive persons infected with the tubercle bacilli develop the disease over a life span. Thus some 8 percents of HIV-positive individuals infected by the tubercle bacillus develop overt tuberculosis annually, a 20-fold higher rate than in the HIV-negative groups (7).

The emergence of AIDS epidemic and the growing rate of iatrogenic immunosuppression have rapidly increased the incidence of disease caused by nontuberculous mycobacteria (NTM), such as *Mycobacterium avium* complex, *Mycobacterium terrae* complex, *Mycobacterium fortuitum* and *Mycobacterium*

chelonae (8,9,10). In United States *Mycobacterium tuberculosis*, *Mycobacterium bovis, Mycobacterium avium* complex and *Mycobacterium kansasii* acount for > 90 % of pathogenic mycobacterial isolate recover in public health laboratory (11). They cause disease mainly in patients with previous tuberculosis or pre-existing lung disease in apparently healthy individual and can cause life-threatening infections in immunocompromised patients. The significance of isolation of NTM in the laboratory often remains unclear. Infections caused by NTM often require different treatment regimens. Diseases caused by NTM are relatively resistant to anti-tuberculous chemotherapy, which make species identification and sensitivity testing crucial in treatment of such infection (12,13,14).

Routine diagnostics of mycobacterial infection in clinical sample is based on the presence of acid-fast-stained bacilli on microscopy and is confirmed by culture and biochemical testing. However, microscopy is hampered by a low sensitivity and does not contribute to species diagnosis. In addition, many culture are slowly growing and identification to the species level can take up to 4-8 weeks (15). A combination of solid and liquid media is currently regarded as a "gold standard" for primary isolation of mycobacteria, and turnaround times are not exceeding 21-30 days after specimen collection (16,17). Furthermore, biochemical identification is based on phenotypic characteristics and may not be highly reproducible. The limited number of species in the phenotypic characteristics data base further restricts the method (18).

Recently developed techniques provide more reliable means of species identification in comparison with conventional testing. High performance liquid chromatography (HPLC) and gas liquid chromatography (GLC) which are used to differentiate species by lipid analysis are rapid but need standardized growth condition (19,20). AccuProbe (Gen-Probe, Inc., San Diego, Calif.) is an ideal choice for the rapid detection of *M. tuberculosis*, *M. avium* complex, *M. kansasii* and *M. gordanae* but requires a well-grown culture and testing with several probes and covers only a narrow range of mycobacterial species (21,22). PCR-restriction fragment length polymorphism analysis of the *hsp*65 gene is relatively new technique that is increasingly being used for the differentiation of mycobacteria (23,24). Sequence-based identification, such as with the 16S rRNA (15,25,26,27), *recA* (28), *rpoB* (29), or *dnaJ* (30) gene is more definitive and allow analyses of phylogenic relationships.

The 16S rRNA gene is the most widely accepted gene used for bacterial identification. It has contributed greatly to the discovery of new species of the *Mycobacterium* genus, and it continues to serve as an important tool as an alternative to phenotypic identification method (31,32,33). The direct sequencing of amplified DNA from the 16S rRNA gene of *Mycobacterium* carries 2 hypervariable domains that correspond to *E. coli* positions 129-267 and 430-500 (18,26,27). These domains contain species-specific sequence that can be used to identify, to the species level, almost all mycobacteria of clinical relevance.

The methods of identification in current use require cultured mycobacteria. Culture from clinical samples is hampered by the slow growth of mycobacteria.

Inoculation on solid media requires a mean incubation time of 4 weeks before sufficient growth is obtained to enable identification to begin, while the use of liquid cultures improves the clinical laboratory's ability to isolate mycobacteria more rapidly and also increases the recovery rate of mycobacterial isolation. More rapid methods for the identification of cultured mycobacteria are the analysis of lipid composition, the use of species-specific DNA or RNA probes, restriction fragment length polymorphism (RFLP) determination and DNA sequence analysis of certain amplified genes.

The 16S rRNA genes has been identified as a target which allows the detection and identification of mycobacteria by using polymerase chain reaction amplification. Sequence analysis of the amplified 16S rRNA gene has been employed for species identification of mycobacteria in clinical specimens with several advantages such as the shorter turnaround time, high discrimination power for diffentiation and the ability to detect non-cultivable mycobacteria.

In this study, sequencing of the PCR amplified 16S rDNA fragments will be used for the detection and identification of mycobacteria in hemocultures.

CHAPTER II

OBJECTIVE

1. To develop method for identification of mycobacteria from hemocultures by polymerase chain reaction (PCR) and sequencing technique.

2. To study the prevalence of *Mycobacterium* species from hemocultures in King Chulalongkorn Memorial hospital.

CHAPTER III

LITERATURE REVIEW

GENERAL CHARACTERISTICS

Mycobacteria are slender, slightly curved or straight rod-shaped organisms of 0.2 to 0.4 x 2 to 10 um in size. They are nonmotile and do not form spores. No aerial hyphae are grossly visible. The cell wall structure has a very high lipid content (Figure 1); thus, mycobacteria cells resist staining with commonly used basic aniline dyes at room temperature. Mycobacteria do take up dye with increased staining time or with application of heat; however, they resist decolorization with up to 3% hydrochloric acid, and some also resist decolorization with 95% ethanol; usually weakly Gram positive. These characteristics, referred to as *acid fastness* and *acid-alcohol fastness*, respectively, are basic to distinguishing mycobacteria from other genera and species (34,35,36,37).

Figure 1. Diagrammatic section of the mycobacterial cell wall (34)

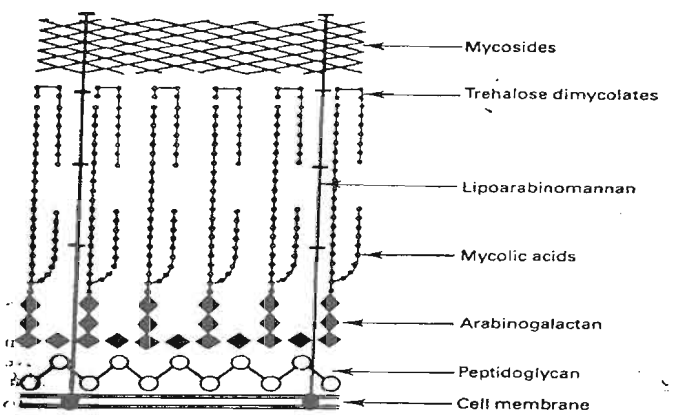


Fig. 2.3 Diagrammatic section of the mycobacterial cell wall

Mycobacteria are strictly aerobic and growth is slow or very slow; visible colonies appear in 2-60 days at optimum temperature. Colonies are often pink, orange, or yellow, especially when exposed to light, pigment is not diffusing, surface commonly dull or rough. The most rapidly growing species generally grow on simple media in 2 to 3 days and most mycobacteria associated with disease require 2 to 6 weeks of incubation on complex media. Some species are fastidious, requiring special supplements (e.g., *M. paratuberculosis*), or are noncultivable (*M. leprae*). Widely distributed in soil and water; some species are obligate parasites and pathogens of vertebrates (35,36,37,38).

CLASSIFICATION

In 1882, Robert Koch was the first to establish the casual relationship between the tubercle bacilli and the disease tuberculosis (TB). The generic name *Mycobacterium* was introduced by Lehmann and Neumann in the first edition of their 'Atlas of Bacteriology' published in 1896 (39).

The mycobacteria are included in Section 16 of Volume 2 of *Bergy's Manual of Systemic Bacteriology* (39). *Mycobacterium* is the only genus of the family *Mycobacteriaceae*. The distinguishing characteristics of this genus include acid fastness, the presence of mycolic acid, and the appropriate DNA G+C content (66 to 71%) (40).

For the most part, mycobacteria can be divided into two major groups based on fundamental differences in epidemiology and association with disease:

those belonging to the *Mycobacterium tuberculosis* complex (*M. tuberculosis, M. bovis, M. africanum, M. microti* and *M. canettii*), *M. leprae* and those referred to nontuberculous mycobacteria (NTMs).

Runyon (1959) drew attention to the role of 'anonymous mycobacteria' in human lung disease and placed the responsible strains into four groups according to their speed of growth and pigmentation. These groups are:

I . photochromogens (yellow pigment formed in the light)

II. scotochromogen (yellow pigment formed in the dark)

III. non-photochromogen

IV. rapid growers

Mycobacteria are divisible into the rapid growers, slowers and those not yet cultivated *in vitro*. The 'approved lists of bacterial names'(34) contain 41 species of mycobacteria (Table 1.1). A few apparently distinct cultivable species, such as *M. diernhoferi*, were omitted from the lists and several have been described subsequently but most of these are of little or no clinical significance. In addition, a number of very slowly growing or non-cultivable species, mostly isolated from AIDS patients, have been identified by ribotyping. *Mycobacterium genavense* is the most frequently encountered member of this group (33). Other species delineated by this technique include *M. confluentis*, *M. intermedium* and *M. interjectum* (41). Species described or reintroduce after the publication of the approved lists are shown in Table 1.2 (34).

Table 1.1. The species of mycobacteria in the 'approved lists' (34)

Slowly growing

M. tuberculosis	*M. bovis*	*M. africanum*
M. microti	*M. kansasii*	*M. marinum*
M. simiae	*M. asiaticum*	*M. gordonae*
M. scrofulaceum	*M. szulgai*	*M. paratuberculosis*
M. intracellulare	*M. lepreamurium*	*M. avium*
M. malmoense	*M. haemophilum*	*M. farcinogenes*
M. triviale	*M. terrae*	*M. nonchromogenicum*
M. ulcerans	*M. gastri*	*M. xenopi*

Rapid growing

M. chelonae	*M. fortuitum*	*M. phlei*
M. smegmatis	*M. aurum*	*M. gadium*
M. neoaurum	*M. flavescense*	*M. gilvum*
M. komossense	*M. senegaiense*	*M. parafortuitum*
M. thermoresistible		

Non-cultivable

M. leprae

Table 1.2. Other validly described species of mycobacteria (34)

Slowly growing

M. shimoidei *M. celatum*

Rapid growing

M. diernhoteri *M. agri*

M. archense *M. chubuense* *M. austroafricanum*

M. rhodesiae *M. tokaiense* *M. shinshuense*

M. porcium *M. fallax* *M. pulveris*

M. sphagni

Non-cultivable (or very fastidious growth)

M. genavense *M. confluentis* *M. intermedium*

M. interjectum

CULTURE MEDIA AND ISOLATION METHODS

Mycobacteria are strictly aerobic and grow more slowly than most bacteria pathogenic for humans. The generation time of mycobacteria is more than 12 hours, that of *M. tuberculosis* having the longest replication time at 20 to 22 hours. The growth of mycobacteria is enhanced by an atmosphere of CO_2 between 5 and 10% in primary isolation cultures. Mycobacteria require a pH between 6.5 and 6.8 for the growth medium and they grow better at higher humidity.

The many different media available for the recovery of mycobacteria from a clinical specimen are variations of three generation types (Table 2) : inspissated egg medium, serum albumin agar medium, and liquid medium. Within each general type, there are nonselective formulations and formulation that has been selective by the addition of antimicrobial agent. Because some isolates do not grow on a particular agar and each type of culture offers certain advantages, a combination of culture media is generally recommended for primary isolation (36).

Table 2. Suggested media for cultivating of mycobacteria from clinical specimens (42)

Solid

Agar-based

1. Middlebrook 7H10
2. Middlebrook 7H11
3. Mitchison's setective 7H11

Egg-based

1. Wallenstein
2. Löwenstein-Jensen (LJ) with RNA
3. L-J with pyruvic acid

Liquid

1. BACTEC 12B medium
2. Middlebrook 7H9 Broth
3. Septi-Chek AFB

There are several different systems to culture and detect the growth of mycobacteria in liquid media. The most commonly employed system are summarized in Table 3.

Table 3. Commonly used commercial liquid media systems to culture and detect the growth of mycobacteria (37)

SYSTEM	BASIC PRINCIPLE(S) OF DETECTION
BACTEC 460 TB (Becton Dickinson Diagnostic Systems, Cockeysville, Md.)	Culture media contains ^{14}C-labeled palmitic acid. If present in the broth, mycobacteria metabolize the ^{14}C-labeled substrates and release radioactively labeled $^{14}CO_2$ in the atmosphere, which collects above the broth in the bottle. The instrument withdraws this CO_2-containing atmosphere and measures the amount of radioactivity present. Bottles that yield a radioactive index, called a *growth index*, greater than or equal to 10 are considered positive
Septi-Chek AFB System (Becton Dickinson Diagnostic Systems)	Biphasic culture system made up of a modified Middlebrook 7H9 broth with a three-sided paddle containing chocolate, egg-based, and modified 7H11 solid agars. The bottle is inverted regularly to inoculate the solid media. Growth is detected by observing the three-sided paddle
Mycobacteria Growth Indicator Tube (MGIT) (Becton Dickinson)	Culture tube contains Middlebrook 7H9 broth and a fluorescent compound embedded in a silicone sensor. Growth is detected visually using an ultraviolet light. Oxygen (O_2) diminishes the fluorescent output of the sensor; therefore, O_2 consumption by organisms present in the medium are detected as an increase in fluorescence. This system is conducive to possible automation
Continuous Growth Monitoring Systems	
ESP Culture System II Accumed International, Inc., Chicago, Ill.	Organisms are cultured in a modified Middlebrook 7H9 broth with enrichment and a cellulose sponge to increase the culture's surface area. The instrument detects growth by monitoring pressure changes that occur as a result of O_2 consumption or gas production by the organisms as they grow
BACTEC 9000 MB (Becton Dickinson)	Organisms are cultured in a modified Middlebrook 7H9 broth. The instrument detects growth by monitoring O_2 consumption by means of a fluorescent sensor

Recently, three different automated, continuously monitored systems initially designed for growth and detection microorganism in blood, have been adapted for mycobacterial cultures. The BACTEC 9000 series MB system (Becton Dickinson, USA) uses the fluorescence quenching-based oxygen sensor

to detect growth. The technology used in the ESP II system (AccuMed International, Westlake, Ohio) is based on detection of pressure changes in the headspace above the broth medium in the sealed bottle resulting from gas production or consumption due to growth of microorganisms. The MB/BacT system (Organon Teknika, Durham, N.C.) employs a colorimetric carbon dioxide sensor in each bottle to detect the growth of mycobacteria. Each system includes a broth similar to 7H9 supplemented with various growth factors and antimicrobial agents. It is recommended that a broth-based system be used for primary mycobacterial isolation to favor rapid detection, increase recovery rate of positive cultures (35).

The MB/BacT system (Organon Teknika, Durham, N.C.) is a fully automated, rapid, nonradiometric system for the culture of mycobacteria from clinical specimen. The MB/BacT system employs a colorimetric sensor and reflect light to monitor the presence and production of carbon dioxide (CO_2) dissolved in the culture medium. If mycobacteria are present in the test sample, CO_2 is produced as the organisms metabolize the substrate in the culture. When growth of the microorganisms produces CO_2, the color of the gas-permeable sensor at the bottom of each culture bottle change from dark green to bright yellow. The lighter color results in an increase of reflectance units monitored by the system (43).

The MB/BacT system consists of a bottle containing basic broth (Middlebrook 7H9; Organon Teknika), casein, glycerol, and sodium polyanetholesulfonate (SPS) in purified water. Bottle contains 29 ml of media

and is prepared with an atmosphere of CO_2 in oxygen under vacuum. MB/BacT enrichment fluid consists of bovine serum albumin, sodium chloride, oleic acid, and saponin in purified water (43).

Medium selection for the isolation of mycobacterial and the culture reading schedule is usually based on personal preference and/or laboratory tradition. Both should be optimized for the most rapid detection of positive cultures and identification of mycobacterial isolates. The variety of media and methods available today is sufficient to permit laboratories to develop a system that is optimal for patient and administrative needs (35).

IDENTIFICATION

1. MICROSCOPIC EXAMINATION

The examination of direct smears for mycobacteria is important for several reasons. Although the smear is not as sensitive as culture techniques and requires approximately 10^4 bacilli per milliliter of sample to be positive, smear examination provides an easy, rapid, presumptive diagnosis of mycobacterial disease.

The large amounts of lipids present in the cell wall of mycobacteria render them impermeable to the dyes used in the Gram stain. In fact, when stain with the Gram stain, the mycobacteria vary from Gram-positive to "Gram-ghost" or "Gram-neutral" bacilli (44). Mycobacteria are able to form stable complexes with

certain arylmethane dyes (dyes with aromatic methane rings) such as fuchsin and auramine O. Once these complexes are form, they are very resistant to decolorization with acid alcohol or strong mineral acids and are thus termed acid-fast. The Ziehl-Neelsen, Kinyoun, and fluorochrome acid-fast staining techniques are used in mycobacteriology (35,39).

2. PHENOTYPIC CHARACTERISTICS

Colony morphology, growth rate, optimum growth temperature, and photoreactivity are phenotypic characteristics that may help speciate mycobacteria.

2.1 Colony morphology

Colonies of mycobacteria are generally distinguished as having either smooth and soft or rough and friable appearance. Colonies of *M. tuberculosis* that are rough often also exhibit a prominent patterned texture referred to as cording (curved strands of bacilli); this texture is the result of tight cohesion of the bacilli. Colonies of *M. intracellulare* may appear to have a dense center, looking like a "fried egg" (36). Colonial characteristics of mycobacteria are summarizes in Table 4 (35).

2.2 Growth rate and recovery time

Growth rate and recovery time depend on the species of mycobacteria but are also influenced by the media used, the temperature of incubation, and the initial inoculum size. The range in recovery time is wide from 3 to 60 days.

Mycobacteria are generally categorized as having visible growth in less than or more than 7 days. Rapid growers are able to produce colonies in less than 7 days upon subculture to a nonselective madia. The inoculum should be sufficiently small to produce isolated colonies. Microscopic agar examination for microcolonies allows earlier detection of growth (Table 4) (35).

2.3 Temperature

The optimum temperature are ranged at with a mycobacterial species may grow may be extremely narrow, especially at the time of initial incubation. Growth in relation to temperature can usually be adequately determined by observing the primary cultures or subcultures at 37° or 30° C. When more definitive identification is needed, isolates should be incubated at 24°, 30°, and 42° C. Growth at these temperatures is interpreted as in Table 5 (39).

2.4 Photoreactivity

Mycobacterium have classically been categorized according to their photoreactive characteristics. Some mycobacteria produced carotenoid pigments. Mycobacteria are classified into three groups based on the production of pigment. Those species produced carotene pigment upon exposure to light are referred to as photochromogens. Those species produced

Table 4. Growth characteristics of commonly isolated mycobacteria (35)

Organism	Growth rate (days)	Pigmentation production		Colony morphology on Middlebrook 7H10 agar
		Light	Dark	
M. abscessus	3-7		Buff	Rounded,smooth, matte, periphery entire or scalloped, no branching filaments, some colonies are wrinkle
MAC	10-21		Buff or yellow	Thin, transparent, smooth, entire and round; some colonies rough and wrinkled
M. chelonae	3-7		Buff	Rounded,smooth, matte, periphery entire or scalloped, no branching filaments, some colonies are wrinkle
M. fortuitum groups	3-7		Buff	Circular, convex, wrinkled, or matte, branching filaments on periphery are obvious
M. gordonae	10-25		Yellow to orange	Round, smooth, convex
M. kansasii	10-21	Yellow	Buff	Aised and smooth; some are rought and wrinkled; carotene crystals numerous after exposure to light
M. marinum	5-14	Yellow	Buff	Round smooth
M. mucogenicum	3-7		Buff	Round smooth, highly mucoid
M. scrofulaceum	10-14		Yellow	Smooth, moist, yellow and round
M. simiae	7-14	Yellow	Buff	Smooth, domed, and slightly pigmented
M. terrae complex	10-21		Buff	Round, smooth, glisteding, and sometimes colorless
M. tuberculosis complex	12-28		Buff	Flat, rought, spreading to irregular periphery
M. xenopi	28-42	Yellow	Buff	Small,domed, yellow, smooth or rought; resembles a miniature bird's nest

pigment in the dark are referred to as scotochromogens. Color may range from pale yellow to orange. Nonphotochromogens are nonpigmented in the light and dark or have only a pale yellow, buff, or tan pigment that does intensify after light exposure Table 4 (35).

Table 5. Growth of Mycobacteria regarding to temperature (39)

Growth Rate	Temperature	Organism
Slow (≥ 2 wk)	Growth at 35° – 37 °C but none at 24 °or 42 °	*M. tuberculosis* or *M. bovis*
	Growth at 35 ° -37 °C and 42 ° C but none at 24 °C	*M. xenopi*, some *M. avium complex*
	Growth at 35 ° -37 °C, slower at 24 ° C, negative at 42 °C	*M. kansasii*
	Growth at 32 ° and 24 °C in 2 wk, none or poorly at 35 ° - 37 ° C	*M. marinum*
	Growth at 32 °C in 2-4 wk, at 25 ° or 35 °C in 4-8 wk, no growth at 37 °C	*M. haemophilum*
Slow (≥ 3 wk)	Growth at 32 °C, but none at 24 °C or 35 ° -37 °C	*M. ulcerans*
	Growth at 35 ° -37 °C	*M. malmoense*

3. BIOCHEMICAL IDENTIFICATION

The biochemical tests are based on the enzymes that organism possesses, the substance that their metabolism produce, and the inhibition of their growth on exposure to selected biochemical reagent. Table 6 (35) gives biochemical test profiles for the most commonly encountered species. Selected key tests, those useful for identifying the suspected species, are performed on the isolate based on the preliminary grouping in Table 7 (37). Biochemical typing is commonly employed for species identification but is time-consuming, and is unable to differentiate some closely related species.

Because culture based identification using convention biochemical tests may take weeks after sufficient growth is observed. Furthermore, biochemical identification is based on phenotypic characteristics may not be highly reproducible, complicated, and non-reproducible (18).

Table 6. Distinctive properties of cultivable mycobacteria encountered in clinical specimens (35)

Runyon group	Complex[a]	Species	Clinical significance[b]	Growth rate[c] at				Colony type			Niacin	Susceptibility to T2H[e] (5 μg/ml)	Nitrate reduction
				45°C	37°C	31°C	24°C	Usual colony morphology[d]	See fig. no.	Pigmentation[f]			
		M. ulcerans	1	-	-	S	-	R		N	-	-	-
	TB	*M. tuberculosis*	1	-	S	S	-	R	2, 3A, B, C, E, G	N	+	-	+
		M. bovis	1	-	S		-	Rt		N	-	+	-
I		*M. marinum*	2		±	M	M	S/SR	3D	P	±	-	-
		M. kansasii	2		S	S	S	SR/S	3F, H	P	-	-	+
		M. simiae	3–2	-	S			S		P	+	-	-
		M. asiaticum		-	S		S			P	-	-	-
II	*M. scrofulaceum*	*M. scrofulaceum*	3–2		S	S	S	S		S	-	-	-
		M. szulgai	1		S	S	S	S or R		S/P	-	-	+
		M. gordonae	4		S		S	S		S	-	-	-
		M. flavescens	4		M		M	S		S/	-	-	+
		M. xenopi	3	S	S			Sf		S	-	-	-
III	*M. avium*	*M. avium*	2	-/+	S		±	St/R		N	-	-	-
		M. intracellulare	2	-/+	S		±	St/R		N	-	-	-
		M. gastri	4		S		S	S/SR/R		N	-	-	-
		M. malmoense	1		S	S	S	S		N	-	-	-
		M. haemophilum	1	-	-	S[g]	S	R		N	-	-	-
		M. nonchromogenicum	4		S		S	SR		N	-	-	+
	M. terrae	*M. terrae*	4		S		S	SR		N	-	-	+
		M. triviale	4		M		S	R		N	-	-	+
IV		*M. fortuitum*	4–3	-	R		R	St/Rf		N	-	-	+
	M. fortuitum	*M. chelonae*	4–3	-	R		R	S/R		N	V	-	-
		M. phlei	4	R	R		R	R		S			+
		M. smegmatis	4	R	R		R	R/S		N			+
		M. vaccae	4		R		R	S		S			+

Table 6. Distinctive properties of cultivable mycobacteria encountered in clinical specimens (35) - continue

Species	Semiquantitative catalase (>45 mm)	68°C catalase	Tween hydrolysis, 5 days	Tellurite reduction	Tolerance to 5% NaCl	Iron uptake	Arylsulfatase, 3 days	MacConkey agar	Urease	Pyrazinamidase, 4 days	Agglutination tests available
M. ulcerans	−	+	−		−		−		−	−	
M. tuberculosis	−	−	−[A]	∓	−	−	−	−	+	+	
M. bovis	−	−	−	∓	−	−	−	−	+	−	
M. marinum	−	−	+	∓	−	−	∓[f]	−	+	+	+
M. kansasii	+	+	+	∓	−	−	−	−	+	−	+
M. simiae	+	+	−	+	−	−	−		+	+	+
M. asiaticum	+	+	+	−	−	−	−		−	−	
M. scrofulaceum	+	+	−	∓	−	−	V	−	+	±	+
M. szulgai	+	+	∓[h]	±	−	−	V	−	+	+	+
M. gordonae	+	+	+	−	−	−	V	−	−	∓	+
M. flavescens	+	+	+	∓	+	−	−	−	+	+	
M. xenopi	−	+	−	∓	−	−	+	−	−	V	+
M. avium	−	±	−	+	−	−	−	∓	−	+	+
M. intracellulare	−	±	−	+	−	−	−	∓	−	+	+
M. gastri	−	−	+	∓	−	−	−	−	+	−	
M. malmoense	−	±	+	+	−	−	−		V	+	
M. haemophilum	−[f]	−[f]	−	−	−	−	−	−	−	+	
M. nonchromogenicum	+	+	+	−	−	−	−	V	−	V	
M. terrae	+	+	+	−	−	−	−	V	−	V	
M. triviale	+	+	+	−	+	−	∓	−	−	V	
M. fortuitum	+	+	V	+	+	+	+	+	+	+	+
M. chelonae	+	V	V	+	V[f]	−	+	+	+	+	+
M. phlei	+	+	+	+	+	+	−	−			
M. smegmatis	+	+	+	+	+	+	−	−			
M. vaccae	+	+	+	+	V	+	−	−			

[a] Plus and minus signs indicate the presence and absence,; v, variable;+, usually present;-/+ usually absent.

[b] R, rough; S, smooth; SR, intermediate in rough; t, thin or transparent; f, filamentous extension.

[c] P, photochromogen; S, scrotochromogen; N, nonchromogenic.

[d] Urease test perform by the method of Steadham.

[e] Probe identifies *M. tuberculosis* complex.

[f] Requires hemin as growth factor.

[g] Arylsulfatase reaction at 14 days is positive.

[h] Young cultures may be nonchromogenic or possess only pale pigment that ma intensify with age.

[i] Includes *M. fortuitum, M. perigrinum, M. fortuitum* third biovariant complex.

Table 7. Key biochemical reactions to help distinguish mycobacteria belonging to the same mycobacterial group (37)

MYCOBACTERIAL GROUP	KEY BIOCHEMICAL TESTS
M. tuberculosis complex	Niacin, nitrate reduction, susceptibility to TCH if *M. bovis* is suspected
Photochromogens	Tween 80 hydrolysis, nitrate reduction, pyrazinaminidase, 14-day arylsulfatase, urease, niacin
Scotochromogen	Permissive growth temperature, Tween 80 hydrolysis, nitrate reduction, semi-quantitative catalase activity, urease, 14-day arylsulfatase
Nonphotochromogen	Heat-resistant and semi-quantitative catalase activity, nitrate reduction, Tween 80 hydrolysis, urease, 14-day arylsulfatase, tellurite reduction, acid phosphatase activity
Rapidly growing	Growth on MacConkey agar, nitrate reduction, Tween 80 hydrolysis, 3-day arylsulfatase, iron uptake

4. CHROMATOGRAPHY

Aanlysis of mycobacterial cell wall fatty acid composition by gas liquid chromatography (GLC) and high performance liquid chromatography (HPLC) is recognized as a useful tool for the identification of mycobacteria (46). Mycolic acids are high-molecular-weight fatty acid that are present in the cell wall of a restricted number of bacterial genera. The mycolic acids of the genus *Mycobacterium* contain the maximun number (60 to 69) of carbon atom. Species identifications made with high performance liquid chromatography (HPLC) have been shown to agree well with biochemical and probe identifications. Chromatography is rapid and highly reproducible but the initial equipment cost is high (47).

5. MOLECULAR METHODS

Nucleic acid technologies give offer perhaps the best potential for rapid and definitive identification of mycobacteria, as well as enabling determination of evolutionary relationships between and within different species (27,26). DNA hybridization is used to identify some of the more common mycobacterial species isolated on solid culture media or broth culture. Of importance, these tests can be performed with the sufficient growth from primary cultures. Nonisotopically labeled (i.e., acridinium ester-labeled) DNA probe specific for mycobacterial ribosomal RNA (rRNA) sequence are commercially available (GenProbe, San Diego, Calif) (37). Nucleic acid probes are available for the *M. tuberculosis* complex, *M. avium* complex, *M. kansasii* and *M. gordonae* and doubtless others

complex, *M. avium* complex, *M. kansasii* and *M. gordonae* and doubtless others will become available. These can be used to identify culture from conventional and radiometric vial (35). The accuracy of these is very high but not absolute. Cultures of *M. celatum* and *M. terrae* have, for example, been miss identified as *M. tuberculosis*. Thus identification by use of probes must be followed up by conventional confirmatory tests (34).

Method based on the polymerase chain reaction (PCR) amplification of specific genes such as the 16S rRNA, recA, rpoB, dnaJ or 65-KDa genes combined with molecular analyses of the PCR product have been evaluated for differentiation and identification of mycobacterial species. Rogall et al. (26), Amin et al.(68), and Lawrence et al. (22) evaluated a scheme for identification of clinical isolates of Mycobacterium species by nucleic acid sequence analysis of the 16S rRNA gene and concluded that sequencing analysis of the this target gene is an exact way to speciate mycobacteria in the routine laboratory. Blackwood et al. (28) indicated that the *recA* gene is a good choice for use in molecular systemic study and species identification of mycobacteria. Anyhow, this target is less conserved at the nucleic acid level than the 16S rRNA gene. Kim at al. (29) chose *rpoB* gene as a target for the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and concluded that this method was rapid, cost effective and efficient for the identification of mycobacteria in clinical microbiology laboratory. Takewaki et al. (30) used PCR-RFLP technique with *dnaJ* gene and their result showed that this method generated easy, rapid and precise identification of mycobacterial species. Plikalytis et al. (23), and Taylor et al. (24) identified an acid fast isolated by using mycobacterial 65-kDa heat shock

protein gene. They reported that PCR-RFLP of this gene appeared to be a reliable method, more accurate and more rapid than conventional identifications. However, PCR-RFLP based on *rpoB* gene, *dnaJ* gene, and 65-kDa heat shock protein gene are still cumbersome since they require several enzyme digestions for species identification and the results are not easy to interpret for species identification due to the limit size difference of DNA fragments after digestion.

5.1 The polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is a technique for the *in vitro* amplification of specific DNA sequences by the silmultaneous primer extension of complementary strands of DNA. DNA polymerase carries out the synthesis of a complementary strand of DNA in the 5' to 3' direction using a single-stranded template, but starting from a double-stranded region. This is the primer extension reaction (Figure 2) and is a basis for a variety of the labelling and sequencing techniques. The cycle, which only takes a few minutes, is repeated many times so that after many cycles there may be a million-fold replication of the target DNA (Figure 3) (48).

Figure 2. Primer extension. DNA polymerase extends a primer by using a complementary strand as a template (48)

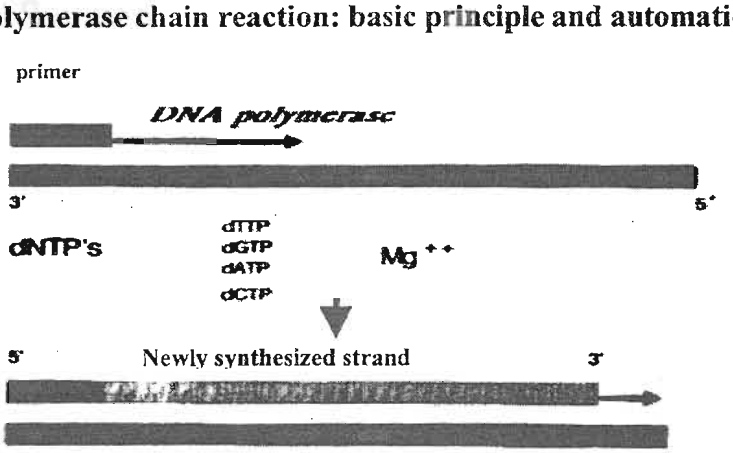
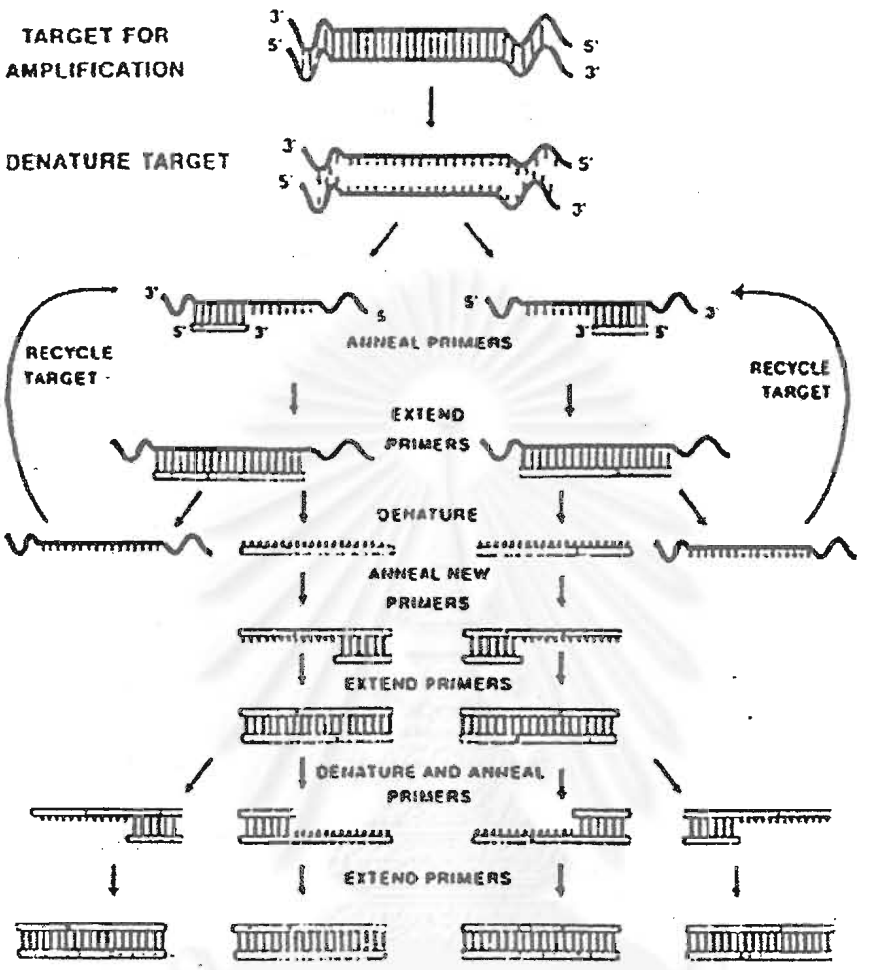


Figure 3. Schematic diagram of PCR (49)



5.2 DNA Sequencing (Dideoxy sequencing) (50)

The dideoxy enzymatic method as originally developed by Sanger F. utilizes *E. coli* DNA polymerase I to synthesize a complementary copy of a single-stranded DNA copy of a single-stranded DNA template. After primer Figure 2 Primer extension. DNA polymerase extends a primer by using a complementary strand as a template (49) is annealed to DNA template, the deoxynucleotide added to the growing chain is selected by base-pair matching to the template DNA. Chain growth involves the formation of a phosphodiester bridge between the 3'-hydroxyl group at the growing end of the primer and the

5'-phosphate group of the incoming deoxynucleotide. Overall chain growth is in the 5' ⟶ 3' direction.

The Sanger sequencing method capitalizes on the ability of *E. coli* DNA polymerase I to use 2', 3'-dideoxynucleotides as a substrates. When a dideoxynucleotide is incorporated at the 3' end of the growing primer chain, chain elongation is terminated selectively at G, A, T or C because the primer chain now lacks a 3'-hydroxyl group (Figure 4) (50).

Automate sequencer; thermal cycle sequencing is a method of dideoxy sequencing in which a small number of template DNA molecules are repetitively utilized a generate a sequencing ladder, A dideoxy sequencing reaction mixture (template, primer, dNTPs, ddNTPs, and a thermostable DNA polymerase) is subjected to repeated rounds of denaturation, annealing and synthesis steps, similar to PCR using a commercially available thermal cycling machine (51). In practice, automate sequencing that use fluorescent-based chemistry can provide accurate sequence data within 24-48 hr.

Figure 4. Dideoxynucleotide and Sanger sequencing principle (50)

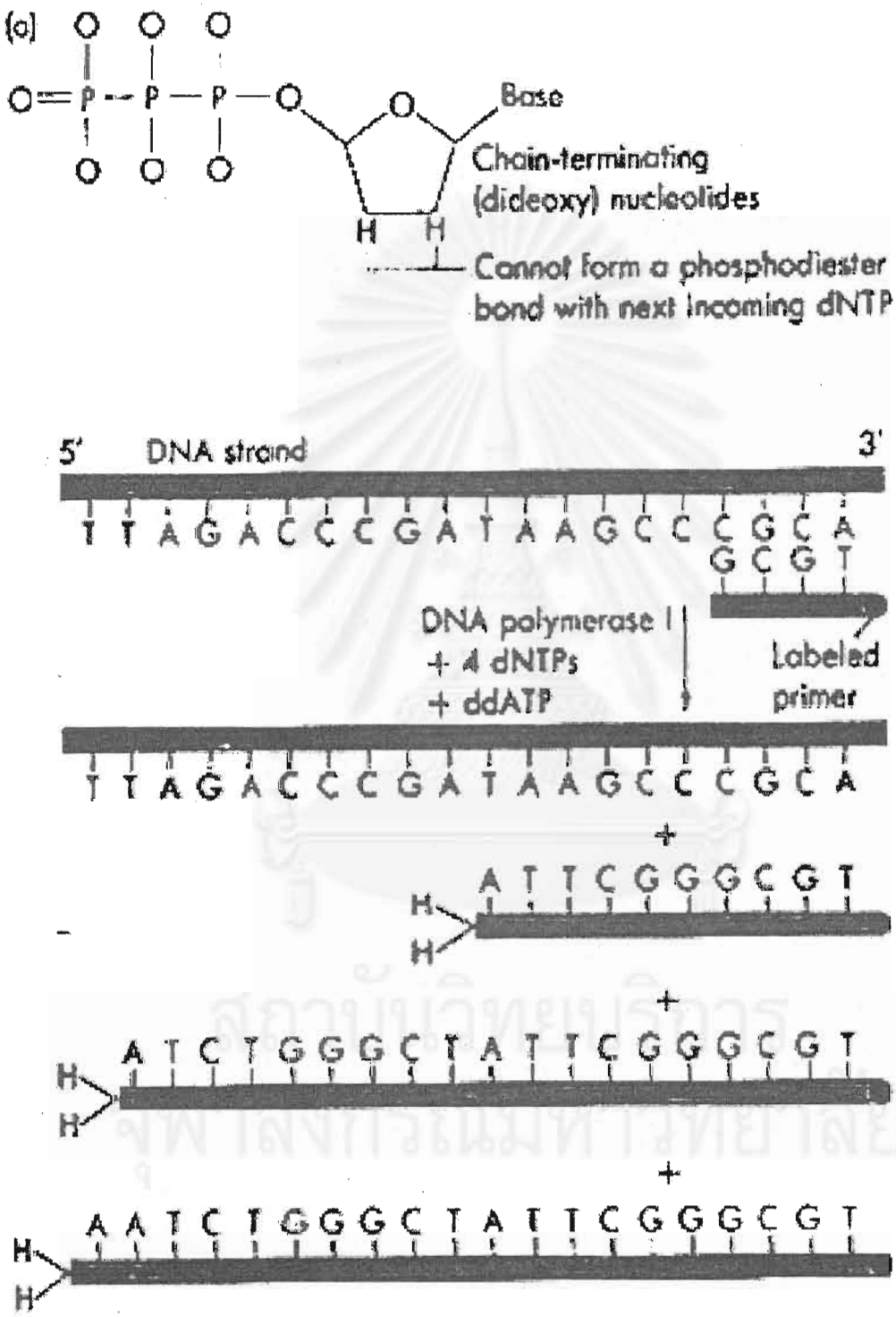
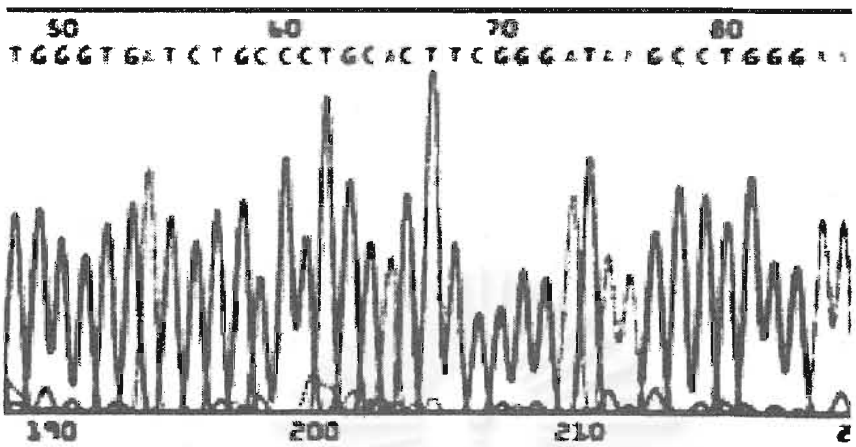


Figure 5. Chromatogram of sequencing by automate sequencer



6. STRAIN-TYPING SYSTEM

Epidemiological studies of tuberculosis can be strengthened by the application of strain-typing system. DNA fingerprinting of *M. tuberculosis* has proven to be a powerful epidemiological tool (35). The DNAs of vast majority of *M. tuberculosis* strains contain IS6110, whose number (0 to > 25 copies) and location are unique to individual strains (52).

7. SEROLOGY

The history of serologic testing in mycobacterial disease parallels developments in immunological testing. A lack of antigen specificity and the weakness of antibody response to illness have been insurmountable obstacle. The use of antigen tests is not be recommended at this times. Some disadvantages are: the need for very specific, purified antigens, immunosuppressed patients may not

amount and antibody response and immune complexes may form and inhibit detection of an antibody (34).

EPIDEMIOLOGY AND SPECTRUM OF DISEASE

Epidemiology

In the past 110 years after Robert Koch discovered the tubercle bacilli, TB has climbed the lives of at least 200 million people. The World Health Organization (WHO) Global Tuberculosis Programme reports that about one-third of the world's population is infected with tuberculosis. By estimate, there are about 7-8 million new cases each year worldwide. About three millions humans die every year of tuberculosis even today (53,54). In Thailand, TB was fifth ranked among over all mortality rate reported in 1995. The annual risk of infection in 1997 is estimated to be 1.40%, while approximately 100,000 new TB patients of which 50,000 cases are smear positive develop each year (55).

In the last few years, there has been a change in the spectrum of mycobacterial disease, which the incidence of infection due to mycobacteria other than tuberculous increased significantly. This trend can be attributed to a number of factors, primarily the increasing numbers of immunocompromise patients susceptible to opportunistic mycobacteria (27,56). The incidence of mycobacterial infection in patient with AIDS continues to climb, with *M. avium* complex accounting for the vast majority of these disseminated infections (10). *M. avium* complex infection occurs in 15 to 24% of AIDS patients monitored in

clinic. The incidence of the incidence of *M. tuberculosis* infection in patients with AIDS ranges from 4 to 21% depending on geographical region (57).

In King Chulalongkorn Memorial Hospital has increased incidence of NTM infection was 1.79%, 2.65%, 4.41% and 5.30% from 1997 to 2000 respectively. While, in 2000 the used of liquid culture could isolated 15.09% of mycobacteria from 439 blood samples by MB/BacT. There were *M. tuberculosis* 39.06%, *M. avium* complex 56% and NTM other than MAC 4%. Suwanagool S. has been reported the incidence of opportunistic infection in AIDS patients from Mar 1997 to December 1998 in Sirirag Hospital was 24.56% for *M. avium* complex and 21.05% for *M. tuberculosis* from 171 samples by Bactec 9240 system. In Tuberculosis Division, Thailand has been announced that the incidence of opportunistic infection in AIDS patients was 0.3% for *M. avium* complex and 0.1% for other NTM. (58).

Other mycobacterium species have been reported as infrequent or rare cause of disseminated infection in immunocompromised patients. These include *M. kansasii, M. scrofulaceum, M. fortuitum, M. chelonae, M. gordonae, M. xenopi, M. asiaticum, M.flavescens, M. malmoense, M. szulgai, M.terrae* (11), *M. haemophilum* (59), *M. simiae* (60), *M. marinum* (61), *M. leprae* (62), *M. genavense* (33), *M. interjectum* (41), and *M. septicum* (32).

Spectrum of diseases

Tuberculosis may mimic other diseases such as pneumonia, neoplasm or fungal infection. Common presenting symptoms include low-grade fever, night sweats, fatigue, anorexia (loss of appetite, and weight loss). If patient presents with pulmonary tuberculosis, a productive cough is usually present, along with fever, chills, aches, and sweating (37). The spectrum of disease caused by the most commonly encounter NTM is summarized in Table 8 (39).

CHEMOTHERAPY

Although a variety of antimicrobial agents are available for the treatment of mycobacterial disease, not all agents are suitable for treating all types of infections. The situation is confounded further by the need to treat mycobacterial infection with a mixture of agents to improve efficiency; it is essential to give at least two drugs (34), to prevent resistance, or to overcome intrinsic resistance. The antimicrobial agents that are used in treatment of mycobacterial infection are discussed below (Table 9). Although a variety of antimicrobial agents for the treatment of mycobacterial disease, not all agents are suitable for treating all types of infections. Furthermore, in the face of antimicrobial resistance, the choice of alternative therapies can be problematic and clinical experience become a prevailing factor. For other uncommon mycobacterial infections, the physician is not infrequently faced with a dilemma in choosing a treatment regimen because of o lack of clinical precedence or unclear efficiency. The situation is confounded further by the need to treat mycobacterial infections with a mixture of agents to improve efficiency, to prevent resistance, or to overcome intrinsic resistance (35)

Table 8. Clinical significance of Atypical mycobacteria (39)

Species	Environmental Sources	Clinical Significance
M. avium-intracellulare complex	Soil, water (including drinking water), birds and other animals (especially chicken, swine, and cattle), foods such as meat, milk, and eggs	Chronic pulmonary disease, local lymphadenitis, bone and joint disease, disseminated disease, skin and soft tissue infections including abscesses and corneal infections, rarely genitourinary disease; disseminated disease in patients with AIDS; also responsible for the most important mycobacterial diseases in animals
M. fortuitum-chelonae	*M. fortuitum* is found almost everywhere in the environment including water, soil, and dust; the habitat of *M. chelonae* is not known for certain, although water may be a source	Disseminated disease, cutaneous lesions, pulmonary disease and a variety of miscellaneous infections; infection is often preceded by traumatic or surgical events
M. genavense	Natural reservoir unknown (? water supply)	Disseminated disease in AIDS patients
M. haemophilum	Unknown	Skin lesions, lymphadenitis
M. kansasii	Natural reservoir unknown; has been recovered from tap water and rarely from tissues of cattle and swine; has not been recovered from soil or dust	Chronic pulmonary disease, bone and joint disease, disseminated disease, cervical lymphadenitis, rarely genitourinary disease
M. malmoense	Unknown	Chronic pulmonary disease
M. marinum	Found in fresh and salt water as a result of contamination from infected fish and other marine life; has been cultivated from water of natural and constructed swimming pools and aquariums; also recovered from rough surface of swimming pools	Cutaneous disease
M. scrofulaceum	Soil, water (including tap water), raw milk, other dairy products, oysters	Cervical lymphadenitis in children, less commonly chronic pulmonary disease in adults occasionally disseminated disease in children
M. simiae	Found in monkeys imported from India (*Macaeus rhesus*) and isolated from tap water in a hospital in Tucson, Arizona, but these isolates were not associated with disease	Only rarely associated with chronic pulmonary disease, osteomyelitis, and disseminated disease with renal involvement
M. szulgai	Although the distribution of this organism appears widespread, little information is available in regard to its epidemiology	Chronic pulmonary disease; extrapulmonary disease is uncommon but has included infections of elbow, cervical lymphadenitis, and cutaneous infections
M. ulcerans	Environmental source seems likely, but has not been recovered outside the human body	Buimsdale ulcer, Buruli ulcer
M. xenopi	Hot and cold water taps, hot water generators and storage tanks of hospitals, birds	Chronic pulmonary disease

CHAPTER IV

MATERIAL AND METHODS

1. Patient and clinical specimens

Three hundreds eighty-one blood samples were collected from patients who were suspected of having disseminated mycobacterial infection from January to September 2000. Five-milliliters of peripheral blood were inoculated into MB/BacT blood culture bottle and examined with MB/BacT 240 instrument (Organon Teknika).

2. Media and culturing methods

The MB/BacT system (Organon Teknika) consists of a bottle containing basic broth Middlebrook 7H9 (0.47% w/v), casein (0.1% w/v), glycerol (1.0% w/v), and sodium polyanetholesulfonate (SPS) (0.025% w/v) in purified water. Bottles contain 29 ml of media and are prepared with an atmosphere of CO_2 in oxygen under vacuum.

Before specimen incubation, bottles were supplemented with 0.5 ml of MB/BacT blood culture bottles enrichment, which consists of bovine serum albumin (14.5% w/v), sodium chloride (2.5% w/v), oleic acid (0.174% w/v), and saponin (4.4% w/v) in purified water (43).

After inoculation, bottles were introduced into MB/BacT ™ 240 instrument (Organon Teknika) and incubated at 37° C for 6 weeks. When MB/BacT blood culture bottles were flagged positive by the instrument, a growth

was regarded as positive and smear was made to confirm the presence of mycobacteria (43).

3. Microscopy and subculturing

Smear were stained with Kinyoun's method to detect mycobacteria. A total of 50 ul of specimen was used to subculture onto 1% Ogawa medium and incubated at 37^{o} C for recovery of mycobacterial colonies. Ogawa slants were visually inspected for growth on the slant and smear from suspected colonies were made.

4. Identification of mycobacteria

Routine biochemical methods and the AccuProbe culture confirmation test (Gen-probe Inc., San Diego, Calif) were employed for the identification of isolates.

4.1 Conventional biochemical test (35)

4.1.1 Niacin accumulation test (paper strip method)

4.1.1.1 Inoculum

Culture (> 3 week old) on solid medium showing heavy growth.

4.1.1.2 Reagent

Agent-impregnated paper test strips are commercially available(Niacin Test Strip; Remel, Lenexa, Kans.).

4.1.1.3 Procedure

The direction supplied with the strips should be followed. Sterile distilled water of 1.0 ml was added to the solid medium. The tube was placed

horizontally so that the fluid covers the entire surface, and left for al least 15 min for the extraction of niacin. The extract of 0.6 ml was removed to a 12-by 75-mm test tube. The strip was then inserted and the tube was sealed immediately. Then, left the tube for 15 min at room temperature, which occasion agitation. The result was observed the color of the liquid in the tube against a white background, which indicated positive as a yellow color in the liquid (not on the strip) .

4.1.1.4 Control

M. tuberculosis as positive control, and MAC and uninoculated medium as negative control

4.1.2 Nitrate reduction

4.1.2.1 Culture

Culture on solid medium should be 3 to 4 weeks old except for rapid grower, which may to be 2 to 4 weeks

4.1.2.2 Reagent

(i) A 1:2 dilution of concentrated (HCl). (ii) 0.2% aqueous solution of sulfanilamide. (iii) 0.1% aqueous 0.01 M solution of $NaNO_3$ in 0.002 M phosphate buffer pH7 (Nitrate broth, Difco), (v) Powdered zinc.

4.1.2.3 Procedure

Steriled distilled water of a few drop was placed in a screw-capped tube (16 by125 mm). One loopful of mycobacterial growth was inoculated in the tube. $NaNO_3$ solution 2 ml was added and the mixture was shaken and incubated for 2 h in a water bath at 37^{o} C. Reagent ii of 1 drop, regent ii of 2 drops and reagent iii of 2 drops were added into the tube. The test was examined the solution immediately for the development of a pink-to-red color contrasting with

the reagent control. The powder zinc was added to all the negative tubes to reduce nitrate to nitrite.

4.1.2.4 Control

M. tuberculosis as positive control, and MAC and uninoculated medium as negative control

4.1.3 Urease (Wayne method)

4.1.3.1 Inoculum

Active growing colonies from solid media

4.1.3.2 Reagent

Mix 1 part of urea agar base concentrate with 9 parts of sterile distilled water. Do not add agar. Dispense 4 ml amount into 16- by 125 mm screw-cap tubes, and store at 4^{o} C.

4.1.3.3 Procedure

A three-mm loopful of growth was emulsified in a tube of substrate and incubated for 3 days at 37^{o} C. The test was observed for a pink or red color as positive result.

4.1.3.4 Control

M. scrofulaceum as positive control and *M. gordonae* as negative control.

4.1.4 Tween hydrolysis

4.1.4.1 Inoculum

Active growing colonies from solid media

4.1.4.2 Reagent

(i) 100 ml of 0.067 M phosphate buffer (pH7), (ii) 0.5 ml of Tween 80, (iii) 0.1% Aqueous neutral red. Mix the three reagents in order. Dispense this substrate in a 4 ml amount in 16-by 125 ml screw-cap tube, and autoclave at 121^{o} C for 10 min. Store in the dark at 4^{o} C for no more than 2 weeks.

4.1.4.3 Procedure

A three-mm loopful of growth was emulsified in a tube of substrate and incubated for 3 days at 37^{o} C without shaken the tube. The tube was observed for a pink or red color after 1, 5, and 10 day. The result was recorded the number of days required for the first appearance of pink or red color. A negative result was indicated by the substrate remaining amber-colored after 10 days.

4.1.4.4 Control

M. kansasii as positive control and MAC as negative control.

4.1.5 Heat-stable (68^{o} C) catalase test

4.1.5.1 Inoculm

Well-developed, isolated colonies from solid media

4.1.5.2 Reagents

Freshly prepared mixture of 10% Tween 80 and 30% H_2O_2. Phosphate buffer (0.067 M, pH6)

4.1.5.3 Procedure

Several colonies was suspened in a 0.5 ml of phosphate buffer in a screw-cap tube. The tube was placed for 20 min in a 68^{o} C water bath and left at room temperature. Tween-H_2O_2 mixture was added of 0.5 ml in the tube and

observed bubbles (positive). The tube was hold for 20 min before discarding as negative.

4.1.5.4 Control

M. kansasii as positive control and *M. tuberculosis* as negative control.

4.1.6 Sodium chloride tolerance

4.1.6.1 Inoculum

Barely turbid suspension

4.1.6.2 Substrate

Ogawa medium contain 5% NaCl. Ogawa without salt should be used for a control)

4.1.6.3 Procedure

Bacterial suspension of 1 ml was inoculated in the media, and incubated at 37^{o} C. The culture was read the mycobacterial growth or no growth at 4 weeks.

4.1.6.4 Control

M. fortuitum as positive control (growth) and *M. tuberculosis* as negative control (no growth).

4.1.7 Tellurite reduction

4.1.7.1 Inoculum

Several colonies were suspended in 5 ml of 7H9 broth. The tube was incubated for 7 days before test procedure.

4.1.7.2 Reagent

A 0.2% aqueous solution (0.1 g in 50 ml distilled water) of potassium tellutite. Dispense in 2-to5 ml amounts, and autoclave.

4.1.7.3 Procedure

The tellurite solution of 2 drops was added to each culture, and incubated at 37° C. The cultures was examined daily for 4 days or more. A jet black precipitate was shown as positive result .

4.1.7.4 Control

MAC as positive control and *M. kansasii* as negative control.

4.2 AccuProbe (Gen-Probe, Inc., San Diego, Calif.) (66)

The AccuProbe was performed according to the instructions supplied by the manufacturer. The protocol consisted the following steps. For lysis, one loopful of mycobacterial colonies was added to 200 ul of specimen dilution buffer in a lysing tube, and the mixture was sonicated for 15 min in a model 1200 water bath sonicator (Branson Ultrasonics Corporation, Danbury, Conn.) at room temperature. Next step, the tube was incubated at 95° C for 15 min. Chemiluminescent acridinium ester-labeled DNA probes were used to specifically detect the mycobacterial DNA. One hundred microliters from the lysing tube were add to the reaction tube of specific probe and the tube was incubated at 60° C for 15 min in a water bath to allow hybridization. After addition of selection reagent (300 ul), the tube was vortex and incubate for an additional 10 min. After the tube was cooled at room temperature for at least 5 min, the result was read in a luminometer. Sample producing signals greater than or equal to the cutoff value 30,000 relative light units were considered positive and signal less than cutoff value were considered negative.

5. Preparation of DNA from hemocultures for analysis by PCR

Initially, hemocultures with flagged positive and Kinyoun smear positive for acid-fast bacilli were tested by PCR-sequencing. Flagged-negative hemocultures were discarded.

All methods for the preparation of mycobacterial DNA from positive hemocultures were performed in a class IIA biological safety cabinet, according to the biosafety guideline recommended by the Centers for Disease Control and Prevention (Atlanta, Ga.) for laboratory work with mycobacterial isolates. Samples for DNA extracts were stored at -20^{o} C in a sealed container until they were required for PCR, and the addition of sample extracts to PCR reagent was performed in a class IIA biological safety cabinet different from the one used for DNA extraction of specimens.

One millilitre of positive hemoculture broth was transferred to 1.5 ml eppendorf tubes, and centrifuged at 12,000 x g for 5 min. The supernate was discarded and the pellet was collected for extraction of DNA.

5.1 Pretreatment of hemocultures (64).

Lysis buffer consisting of 1% Triton X-100 in 20 mM Tris-HCl (pH 8.3) buffer was added to the pellet until a total volume of 1.5 ml was reached. The sample was centrifuged for 5 min at 12,000 x g and was again treated with lysis buffer and centrifuged. The supernatant was then removed and the pellet was collected for DNA extraction.

5.2 Extraction of DNA

In pilot study, twenty samples were processed with five DNA extraction methods to achieve the best method for preparation of mycobacterial DNA from hemocultures which each specimen was prepared all five methods. Statistical analyses were performed by Npar tests, McNemar test and Cochran test, when appropriate p values ≤ 0.05 were considered to be statistically significant.

5.2.1 Method A: Lysis buffer method (65, 73)

The pellet from positive hemocultures was washed once with 1% Triton X-100 solution containing 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA, sonicated with ultrasonic bath for 15 min at room temperature and was then incubated in 150 ul of the same solution for 30 min at 100^{o} C. After incubation, the sample tubes was centrifuged 12,600 x g for 3 min and 125 ul of the supernatant was transferred into another labelled tube and stored at -20^{o} C until further used.

5.2.2 Method B: Proteinase K and phenol-chloroform method (51)

Ten microlitres of Proteinase K solution (1 mg of proteinase K per ml, 5% Triton X-100, and 200 mM Tris-HCl [pH 8.3]; 10X) were added to the pellet from positive hemocultures and the mixture was incubated at 60^{o} C overnight. The enzyme was inactivated by boiling for 15 min. The protein was extracted by adding 1 of total volume of phenol-chloroform-isoamyl alcohol (25:24:1) into the mixture and mixed extensively on the vortex mixer. The tube was centrifuged at 12,000 rpm for 15 sec. The aqueous phase was transferred to a new eppendorf tube, followed by adding 1 volume of chloroform-isoamyl alcohol (24:1) and was

centrifuged at 12,000 rpm for 15 sec. Then, the aqueous phase was transferred to a new eppendorf tube, followed by adding 0.1 of total volume of 3M sodium acetate and 2 of total volume of cold absolute ethanol. The solution was mixed and DNA was precipitated at –70° C for 30 min. DNA was pelted by centrifugation at 12,000 rpm for 10 min at 4° C and the supernatant was discarded. The pellet was washed once with 1 ml of cold 70% ethanol. After centrifugation and supernatant discarded. The final DNA was dissolved in Tris-EDTA buffer (TE buffer) and stored at –20° C.

5.2.3 Method C: Boom's method (66)

Briefly, 100 ul of the pretreated sample was pipetted into a reaction vessel containing 900 ul of guanidinium thiocyanate (GuSCN; Fluka Chemie AG, Buchs, Switzerland) containing lysis buffer (500 mM GuSCN, 50 mM Tris-HCl, 20 mM EDTA, 0.1 mM Triton X-100) and 20 ul of diatom (diatoms; Sigma Chemical Co., St. Louis, Mo.) suspension (10 g of high-purity diatomaceous earth in 50 ml of H_2O and 500 ul of 32% HCl). The mixture was immediately vortexed for 5 sec. After being held for 10 min at room temperature, the mixture was vortexed again and centrifuged in an eppendorf microcentrifuge at 12,000 x g for 15 sec, and the supernatant was removed by suction. The diatom-nucleic acid pellet was washed twice with washing buffer (120 g of GuSCN in 100 ml of 0.1 M Tris-HCl pH 6.4), twice with 70% ethanol, and once with acetone. After acetone supernatant was removed, the vessels were dried at 56° C for 10 min with lids open. The nucleic acid binding to the diatom in the vessel was eluted by incubation in 100 ul of an aqueous low-salt buffer (1 mM EDTA in 10 mM Tris-

HCl [pH 8.0]) at 56^{o} C for 10 min, centrifuged at 10,000 for 2 min and collected the supernatant at –20^{o} C prior PCR.

5.2.4 Method D: QIAmp silica column purification (69)

The DNA was purified according to the manufacturer's directions by using the QIAmp blood kit (Qiagen Corporation, Germany) In this method, DNA absorbs to silica in the presence of a chaotrope, was washed with buffer, and eluted from the column in 0.1 ml of 10 mM Tris-0.1 mM EDTA buffer at pH 8.5.

5.2.5 Method E: Alkali wash and heat lysis method (67)

The pretreated sample was added to 1.4 ml of alkali solution wash solution (5M NaOH and 0.5 M sodium citrate) in a 1.5 ml eppendorf tube and left shaking on shaker at 100 rpm for 10 min at room temperature. The tube was centrifuged at 13,000 x g for 5 min to deposit the bacterial cell and the pellet was washed with 0.5 ml of 0.5 M Tris-HCl (pH 8.0) and was centrifuged as described before. This step was repeated at least once. Proteinase K was added to the pellet to a final concentration of 0.3 ug/ml, incubated at 55^{o} C for 30 min and was centrifuged as described before . After the final centrifugation, the cell pellet was resuspended in 0.1 ml of distilled water, heated at 95^{o} C for 30 min in a heating box and stored in a sealed container at –20^{o} C prior PCR.

6. DNA amplification

PCR was done as described by Rogall et al. (26), with primers pA (5' AGA GTT TGA TCC TGC CTC AG 3') and pI (5' TGC ACA CAG GCC ACA

AGG GA 3'). Amplification was performed in 50 ul mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 200 uM each dNTPs (dATP, dCTP, dGTP, and dTTP), 100 pmol of each of two primers and 2.5 U of *Taq* polymerase (Qiagen Corporation, Germany), and 50 ul of mineral oil (Sigma, St. Louis, Mo.) was added to each vial before the 10 ul sample was placed under the oil layer. The reaction were performed in 0.5 ml eppendorf tube with target DNA on a Hybaid OmniGene Thermal cycles. The thermal profile involved 36 cycles of denaturation at 93° C for 1 min, primer annealing at 65° C for 2 min, and extension at 72° C for 6 min. The amplified product of the 16S rDNA gene was about 1030 bp.

All samples were tested in duplicate. Part of each sample was spiked with *M. tuberculosis* DNA to detect inhibitors in the sample. Positive and negative control were included in each run.

7. Detection of amplification product

Five microliters of PCR product were mixed with 3 ul gel loading buffer (20% ficoll, 0.05% bromophenol blue), analyzed by electrophoresis on 1.5% agarose gel. The electrophoresis was carried out at 80 volts for 30 min. The gels were stained with 0.1 ug of ethidium bromide per ml for 15 min, and the bands were visualized by UV transillumination. The positive result of PCR showed a single band of 1030 bp fragment for 16S rRNA gene compared with the 1000 bp molecular size marker.

8. Sequencing of the 16S rDNA gene

An ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystem) was used for the sequencing of the PCR product (67). Sequencing was performed on 30-90 ng of purified PCR product using QIAquick PCR Purification Kit; Qiagen Corporation, Germany (the DNA concentration was determined by measuring the A_{260} of 1 corresponds to a DNA concentration of 50 ug/ml). The sequencing reaction required 4 ml of Premix, 3.2 pmol of sequencing primer; primer pB: 5' TAA CAC ATG CAA GTC GAA CG 3' corresponding to *Mycobacterium* 16S rDNA position 50-70 (26), and 150 ng of PCR product template in a total volume of 10 ul. The sequencing reaction was performed using Perkin Elmer GeneAmp PCR system 9600 with cycling parameters were 25 cycles of 96^{o} C for 30 sec, 55^{o} C for 10 sec, 60^{o} C for 4 min and hold 4^{o} C until ready to purify. The products were purified with ethanol/sodium acetate precipitation before capillary electrophoresis was run for sequence analysis.

The amount 300 bp 16S rDNA fragment corresponding to hypervariable region A of *Mycobacterium* was analyzed in an ABI Prism^{TM} 310 Automate Sequencer (PE Applied Biosystem) and the DNA sequences were then compared with sequence by DNA database the Basis Local Alignment Search Tool (BLAST) (70).

Table 9. Antimycobacterial agents ranked by clinical utility and candidacy for *in vitro* susceptibility testing (39)

Mycobacterium species	Antimycobacterial agent			
	Primary first choice	Secondary or second choice	Tertiary or third choice	Primary resistance likely
M. tuberculosis *M. africanum* *M. bovis*	INH, RMP,PZA Streptomycin, EMB	Ciprofloxacin, ofloxacin,sparfloxacin, rifapentine	Rifabutin, amikacin, levofloxacin, cycloserine	
M. leprae	Clarithromycin, dapsone, RMP	Ethionamide, prochronamide, minoclycin		
M. avium complex	Arithromycin, clarithromycin, EMB	Amikacin, ciprofloxacin, rifabutin	Streptomycin, cycloserine, ethionamide	INH, PZA
M. chelonae *M. fortuitum* *M. abscessus* *M. mucogenicum* *M. smegmatis*	Amikacin, cefoxitin, ciprifloxacin, clarithromycin, sulfonamide, doxycycline	Cefmetazole, imipenem, ofloxacin, tobramycin		INH, PZA, RMP, streptomycin, EMB, clofaximide
M. kansasii	RMP, INH, EMB	Clarithromycin	Amikacin, streptomycin, rifabutin, ciprofloxacin	PZA
M. scrofulaceum	Lymphadenitis (surgical excision without chemotherapy)	Arithromycin, clarithromycin		INH, PZA
M. marinum	Doxycycline or minocyclin, EMB, RMP, sulfonamide	Amikacin, ciprofloxacin, clarithromycin, rifabutin		INH, PZA
M. haemophilum *M. malmoense* *M.terrae* *M. szulgai* *M. xenopi* *M. ulcerans*	Clarithromycin, EMB, RMP	Amikacin, ciprofloxacin, INH, rifabutin, streptomycin		PZA

CHAPTER V

RESULT

1. Cultures and identification by conventional method and AccuProbe

Seventy-three of 381 hemocultures (19.16%) were flagged positive by the MB/BacT instrument. Of these positive hemocultures, 69 samples (18.11%) were AFB positive and 4 samples (1.04%) were AFB negative (Table 10). Of the 69 AFB positive samples, 67 acid-fast bacilli were isolated from 66 positive hemocutures (1 specimen grew 2 AFB) and 3 positive samples could not grow AFB on solid media. Four flagged positive, AFB negative hemocultures were subcultured onto Ogawa medium and all grew AFB which were identified to be 1 *M. tuberculosis* and 3 *M. avium* complex. Identification was performed by routine biochemical method and AccuProbe culture confirmation tests. The result of species identification of the isolates from positive hemocultures is shown in Table 11. There were 12 *M. tuberculosis* and 59 non-tuberculous mycobacteria (NTM). Out of 59 NTM, 51 were *M. avium* complex, 2 were *M. scrofulaceum*, 1 was *M. simiae*, 1 was *M. xenopi* and 4 were not identified. One specimen had mixed cultures of *M. tuberculosis* and *M. avium* complex. The average time for detection of mycobacteria from hemocultures was 17.45 days with MB/BacT. Isolation time for each species is shown in Table 12. Several biochemical tests and AccuProbe were tested after incubation of cultures on solid media for ≥ 21 days. The average times for identification of *Mycobacterium* species from active growing colonies are shown in Table 13.

Table 10. Number of hemocultures with indicated signal result

No. of hemocultures	No. of signal positive (%)		No. of signal negative (%)
	AFB positive	AFB negative	
381	69 (18.11)	4 (1.04)	308 (80.83)

Table 11. Results of biochemical test and AccuProbe for identification of mycobacterium species

Number of isolates	Biochemical tests[A]									AccuProbe[B]	Identification
	Colony Morphology Tellurite Reduction	Pigmention[D]	Nitrate	Niacin	Urease	Tween Hydrolysis	68° C catalase	Tolerance to 5% NaCl			
12	R	N	+	+	ND	ND	-	ND	ND	+	*M. tuberculosis*
51	S	N	-	-	ND	ND	-	ND	+/-	+	*M. avium* complex
2	S	S	-	-	+	-	+	-	ND	NA	*M. scrofulaceum*
1	S	S	-	-	-	-	+	-	ND	NA	*M. xenopi*
1	S	P	-	+	+	-	+	-	ND	NA	*M. simiae*
2	R	N	-	-	-	-	+	-	ND	NA	Unidentified
1	S	N	-	-	-	-	-	-	+	NA	Unidentified

1	S	N	-	-	+	-	+	-	ND	NA	Unidentified

A; -, negative; +, positive, +/- usually positive; ND, not done

B; Accuprobe are *M. tuberculosis* complex, *M. avium* complex, *M. kansasii, M. gordonae*

C; colony morphology: S, smooth;R, rought

D; Pigmentation: N, nonphotochromogen; S, scotochromogen; P, photochromogen

Table 12. Detection times of mycobacteria in hemoculture by MB/BacT

Microorganisms (No.)	**Times (mean) for detection (days)**
M. tuberculosis (12)	15.5 (6.5-36.0)
M. avium complex (51)	13.0 (4.0-25.5)
M. scrofulaceum (2)	33.5 (25, 42)
M. xenopi (1)	8.3
M. simiae (1)	20.8
Other NTM (4)	24.0 (13.7- 31.7)

Table 13. Average number of days for identification of mycobacteria from colonies on solid media

Microorganism	**Average No. of days (mean)**
M. tuberculosis	**3**
***M. avium* complex**	**14**
M. scrofulaceum	**35**
M. xenopi	**45**
M. simiae	**30**

2. Comparison of five DNA extraction methods for sample preparation from hemocultures

A comparison of five DNA extraction methods in pilot study are shown in Table 14. The result found that method B and D failed to extract the mycobacterial DNA, method A and method C yielded low positive result and method E was successful in preparation of mycobacterial DNA from hemocultures (69). The method E, alkali wash with heat lysis was significantly superior to those five DNA extraction methods ($p < 0.001$). The comparison of preparation of mycobacterial DNA for PCR from blood culture fluid between alkali wash with heat lysis and Boom's method demonstrated that alkali wash with heat lysis method was significantly more effective than Boom's method ($p = 0.008$). Alkali wash and heat lysis method was the best method which was then chosen for amplification of mycobacterial DNA from hemocultures.

Table 14. Comparison of five extraction methods for sample preparation from AFB-positive hemocultures

No. of hemocultures	No. of PCR-positive samples (%) by method :				
	A	B	C	D	E
20	2 (10)	0 (0)	12 (60)	0 (0)	20 (100)

3. PCR results of DNA was prepared by alkali wash and heat method

Specific PCR products were detected in all 69 AFB-positive hemoculture by using DNA prepared by the alkali wash and heat lysis method (Table 15). The PCR primers directed the synthesis of an approximately 1 kb gene fragment containing the 5' part of the gene coding for 16S rDNA of *Mycobacterium* (Figure 8).

Table 15. PCR of DNA was prepared by alkali wash and heat lysis method from 69 AFB-positive hemocultures

No. of hemocultures	No. of PCR-positive samples (%)
69	69 (100)

4. 16S rDNA sequencing results compared with biochemicals and AccuProbe

The 69 clinical isolates were identified to species level by biochemical tests and by direct sequencing analysis of 16S rDNA. Using biochemical profiles, the isolates could be identified to belong to 5 different species. All AFB positive hemocultures were identified using direct sequence analysis and were found to represent 9 different species, 12 *M. tuberculosis* and 57 non-tuberculous mycobacterial species which included 44 *M. avium*, 4 *M. intracellulare*, 3 *M. scrofulaceum*, 2 *M. ulcerans*, 1 *M. simiae*, 1 *M. haemophilum*, 1 *M. interjectum*, and 1 *M. triplex* (Figure 8- 16). Four flagged positive hemocultures with AFB-negative were not amplified 16S rDNA but were identified the *Mycobacterium* species by biochemical tests and AccuProbe from active growing colonies from solid media. One isolate was *M. tuberculosis* and 3 isolates were *M. avium* (3 were positive by AccuProbe of *M. avium* complex and also positive by AccuProbe for *M. avium)*.

The DNA sequence was examined to the published sequence in the GenBank for the species identification. Comparing the different sequences for the same species, which observed between 97 to 100% for most of them. Identities below 100% may have resulted from variability in some position or ambiguities in the published sequence (68, 70). However, for one identity as low as 92% was observed as *M. tuberculosis* complex so this isolate was compared to another public sequence

database such as The Ribosomal Differentiation of Medical Microorganisms (RIDOM) and also was the same result (Table 16).

Most established mycobacterial species show a unique signature sequence in hypervariable region A. Member of *M. tuberculosis* complex, i.e., *M. tuberculosis, M. bovis, M. africanum, M. microti, M. canetti* exhibit an identical 16S rRNA sequence reflecting the fact that these texa have to be separated at asubspecific or infrasubspecific level (18). The closely related species *M. ulcerans* and *M. marinum* show a nearly homologous 16S rRNA gene sequence. Hypervariable region A and B do not allow one to distinguish between the two texa, but sequence determination of nucleic acid positions outside these two regions allow proper identification (18). Unusual isolate include in this study either failed definite phenotypic identification or was misidentified as other species by biochemical test (*M. scrofulaceum* was identified as *M. xenopi*) this finding of 16S rDNA sequence is consistent to *M. scrofulaceum*. One isolate was identified as *M. triplex* by genotyping and unidentified by biochemical tests. *M. triplex* is a new species of slowing, nonpigmented *Mycobacterium* and biochemical is similar to *M. avium* complex (35). This isolate was identified by sequencing of 16S rDNA of which give 100% identity to *M. triplex*.

Table 16. Results of genotypic identification, biochemical tests and AccuProbe for the isolates that gave typical results

No. of isolates	Biochemical tests	AccuProbe	16S rDNA sequencing	Identity with GenBank (%)
12	*M. tuberculosis* (12)[A]	*M. tuberculosis* complex (12)[A]	*M. tuberculosis* complex (10)	92, 97-100
2	NG (2) [B]	NG (2) [B]	[C]	98, 100
51	*M. avium* complex (51)	*M. avium* complex (51)	*M. tuberculosis* (2)	97-100
			M. avium (44) [C]	98-100
2	*M. scrofulaceum* (2)	*M. scrofulaceum* (2)	*M. intracellulare*(4)	97, 99
1	*M. xenopi* (1)	*M. xenopi* (1)	*M. scrofulaceum* (2)	98
1	*M. simiae* (1)	*M. simiae* (1)	*M. scrofulaceum* (1)	99
2	Unidentified (2)	Unidentified (2)	*M. simiae* (1)	98, 100
1	NG (1)	NG (1)	*M. ulcerans* / *M. marinum* (2)	100
1	Unidentified (1)	Unidentified (1)	*M. haemophilum* (1)	97

1	**Unidentified (1)**	**Unidentified (1)**	***M. interjectum* (1)** ***M. triplex* (1)**	**100**

A; 1 specimens was amplified16S rDNA amplified corresponding to *M. avium* but 2 AFB were identified by biochemical tests to both *M. tuberculosis* and *M. avium* complex

B; NG, no growth

C; Four flagged positive hemocultures with AFB-negative were not amplified 16S rDNA but were identified the *Mycobacterium* species by biochemical tests and AccuProbe from active growing colonies from solid media. One isolate was *M. tuberculosis* and 3 isolates were *M. avium* (3 were positive by the probes of *M. avium* complex and *M. avium).*

Table 17. Results of *Mycobcaterium* species from hemocultures

No. of isolates (Total 74)	Species of mycobacteria (%)
14	***M. tuberculosis* (18.91)**
51	MAC (68.91) - *M. avium* (63.51) - *M. intracellulare* (5.71)
9	NTM other than MAC (12.16) - *M. scrofulaceum* (4.05) - *M. simiae* (1.35) -*M. ulcerans* (2.70) - *M. haemophilum* (1.35) - *M. interjectum* (1.35) - *M. triplex* (1.35)

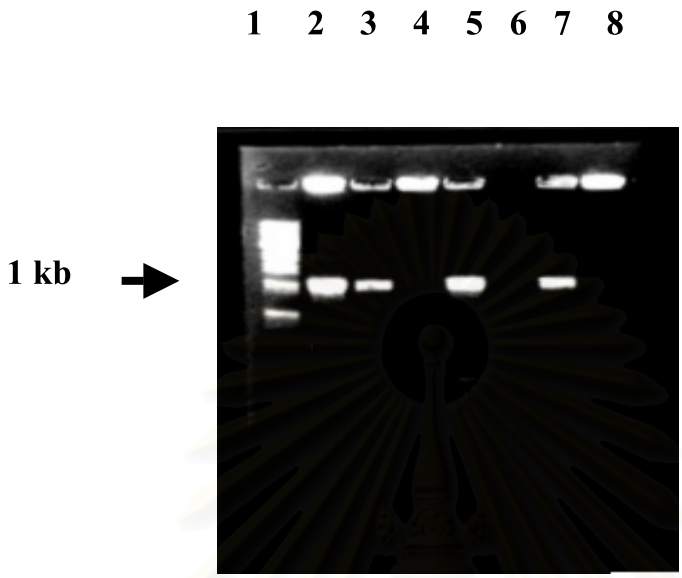


Figure 6. Amplification of mycobacterial DNA from hemocultures by five extraction methods. Agarose gel electrophoresis of PCR product amplified with 16S rDNA gene primer pA and pI.

Lane 1, 1000-bp DNA ladder;

Lane 2, mycobacterial DNA- positive control

Lane 3, lysis buffer method (method A)

Lane 4, proteinase K and phenol-chloroform method (method B)

Lane 5, Boom method (method C)

Lane 6, QIAGEN blood kit method (method D)

Lane 7, alkali wash and heat lysis method (method E)

Lane 8, negative control (DDW)

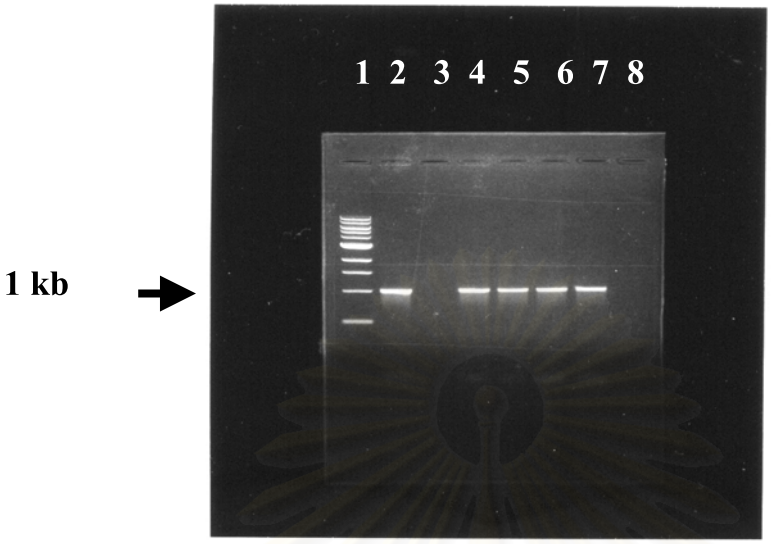


Figure 7. PCR-mediated synthesis of 1000 bp fragment of the gene coding for 16S rDNA of *Mycobacterium*, using alkali wash and heat lysis and primer combination pA and pI from hemocultures.

Lane 1, 1000-bp DNA ladder

Lane 2, mycobacterial DNA- positive control

Lane 3, negative control (DDW)

Lane 4-7, AFB positive hemocultures using alkali wash and heat lysis method

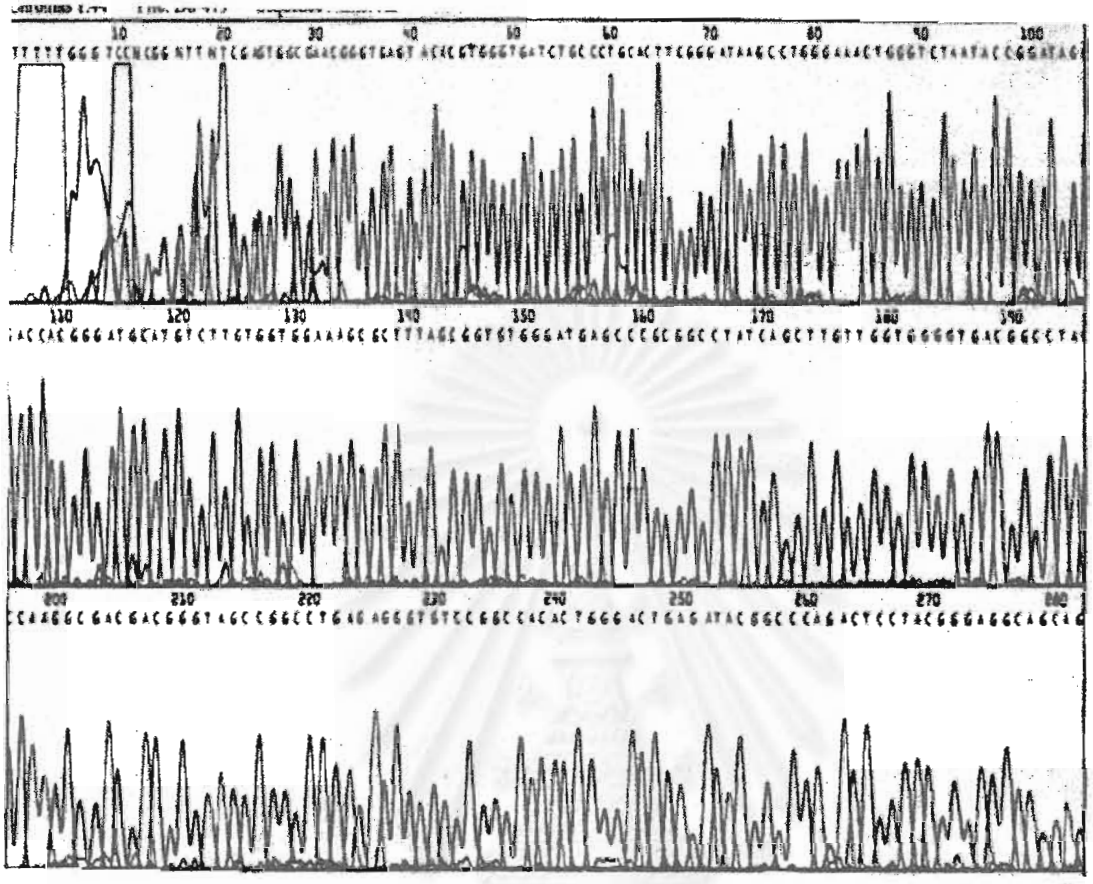


Figure 8. Chromatogram of sequencing by automate sequencer of *M. tuberculosis* complex

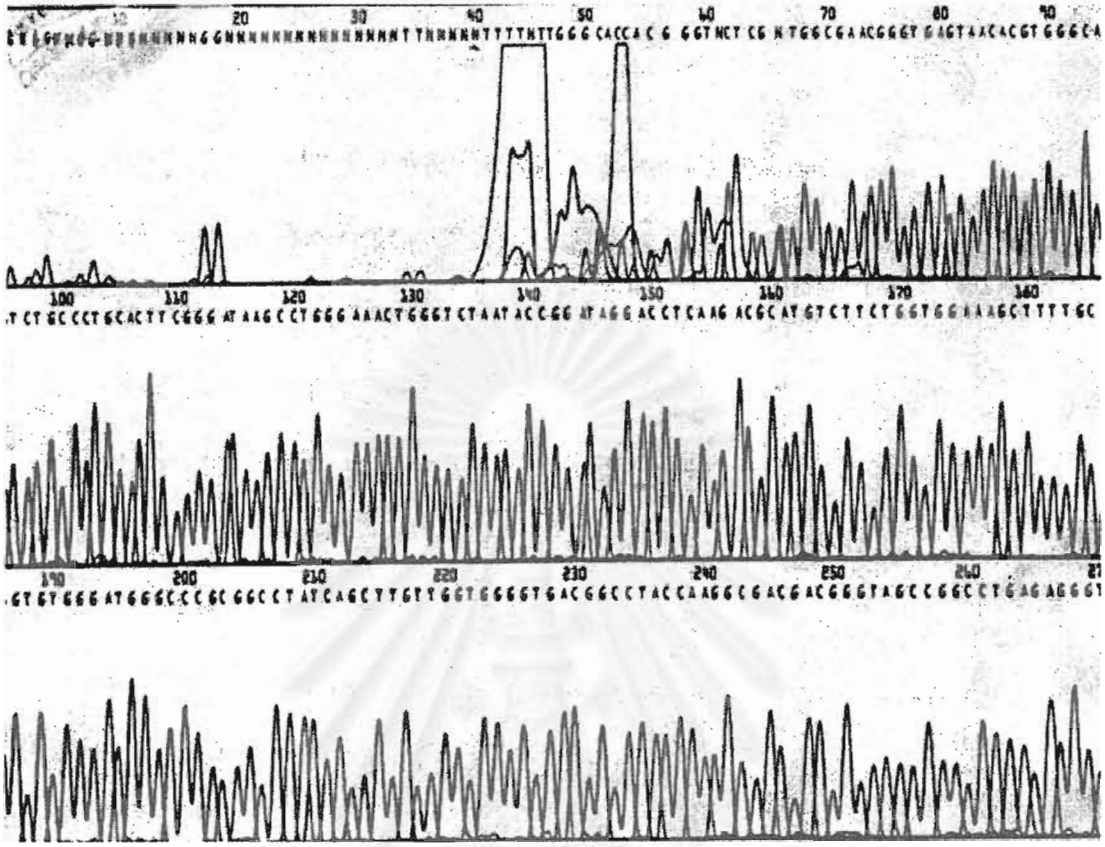


Figure 9. Chromatogram of sequencing by automate sequencer of *M. avium*

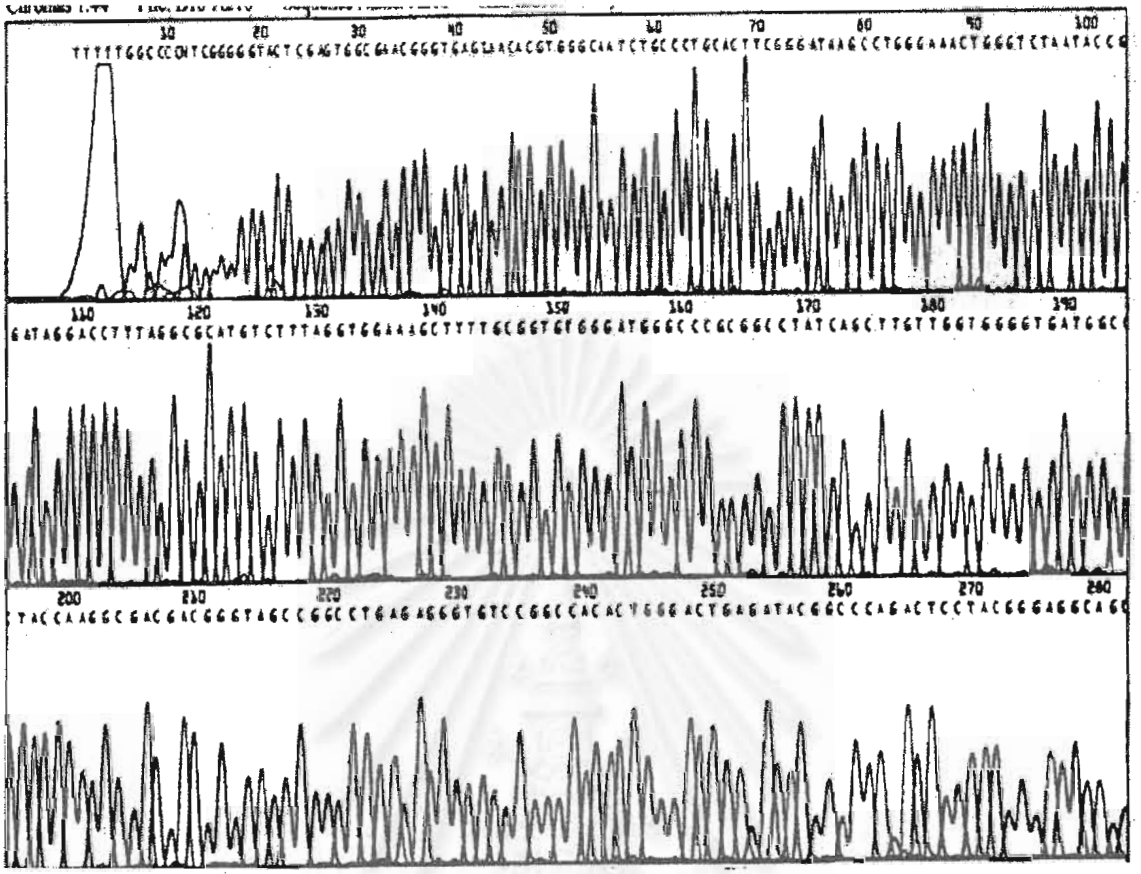


Figure 10. Chromatogram of sequencing by automate sequencer of *M. intracellulare*

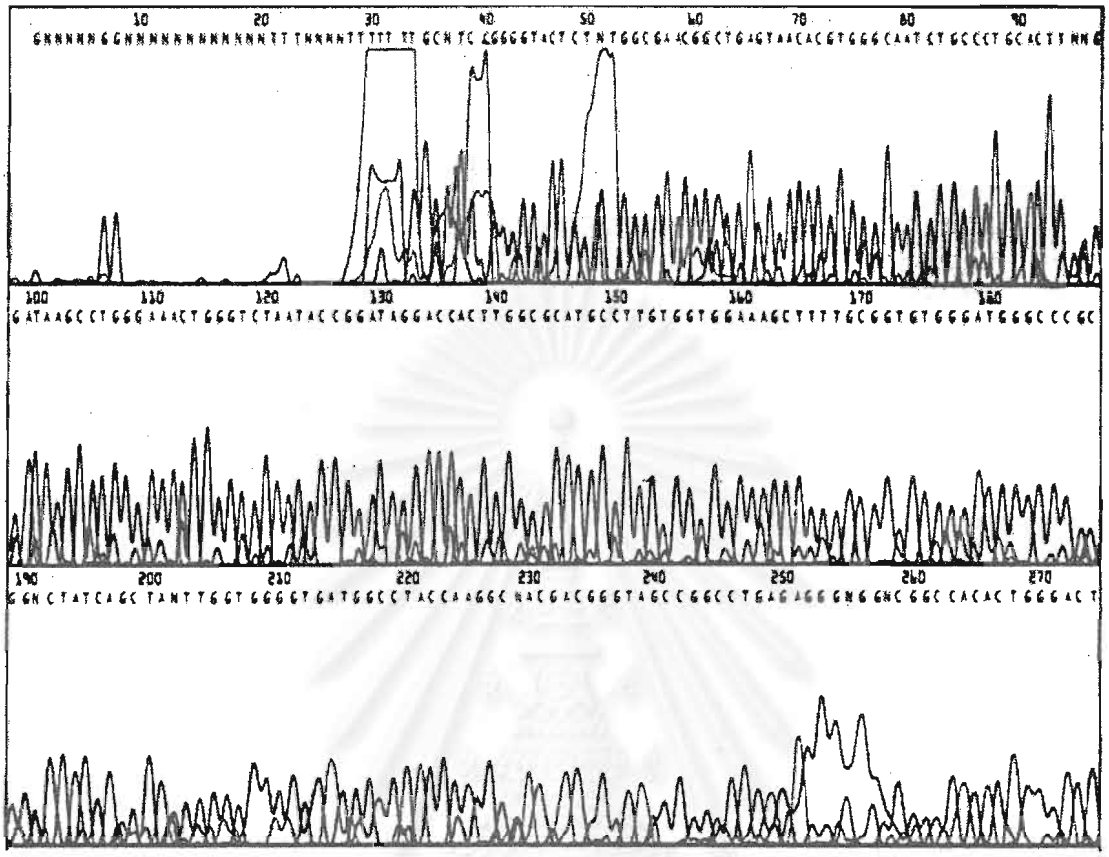


Figure 11. Chromatogram of sequencing by automate sequencer of *M. scrofulaceum*

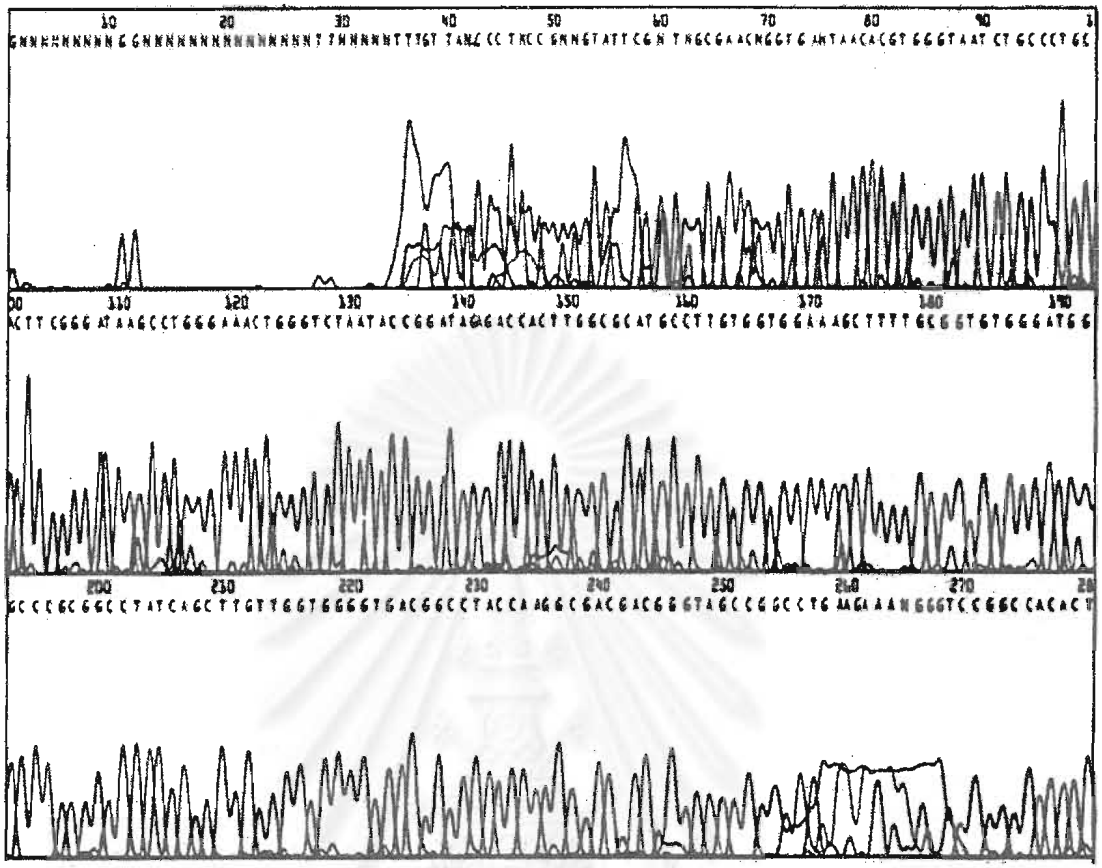


Figure 12. Chromatogram of sequencing by automate sequencer of *M. simiae*

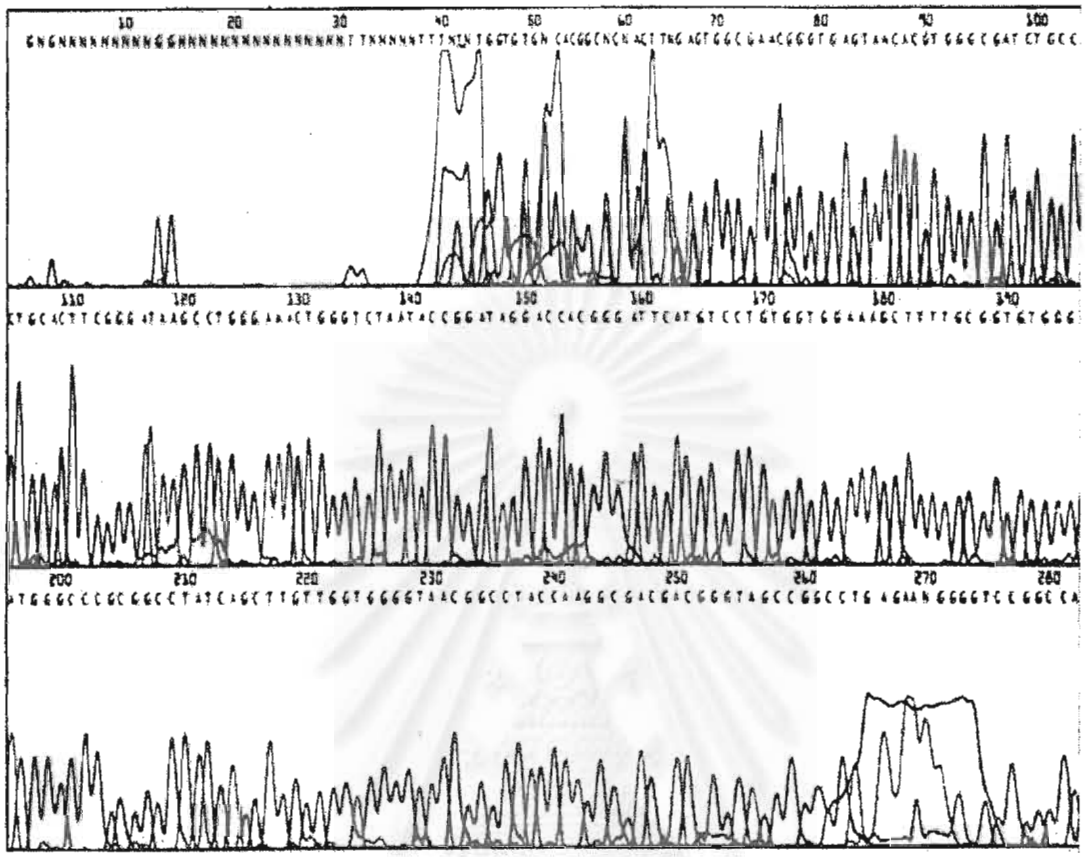


Figure 13. Chromatogram of sequencing by automate sequencer of *M. ulcerans/ M. marinum*

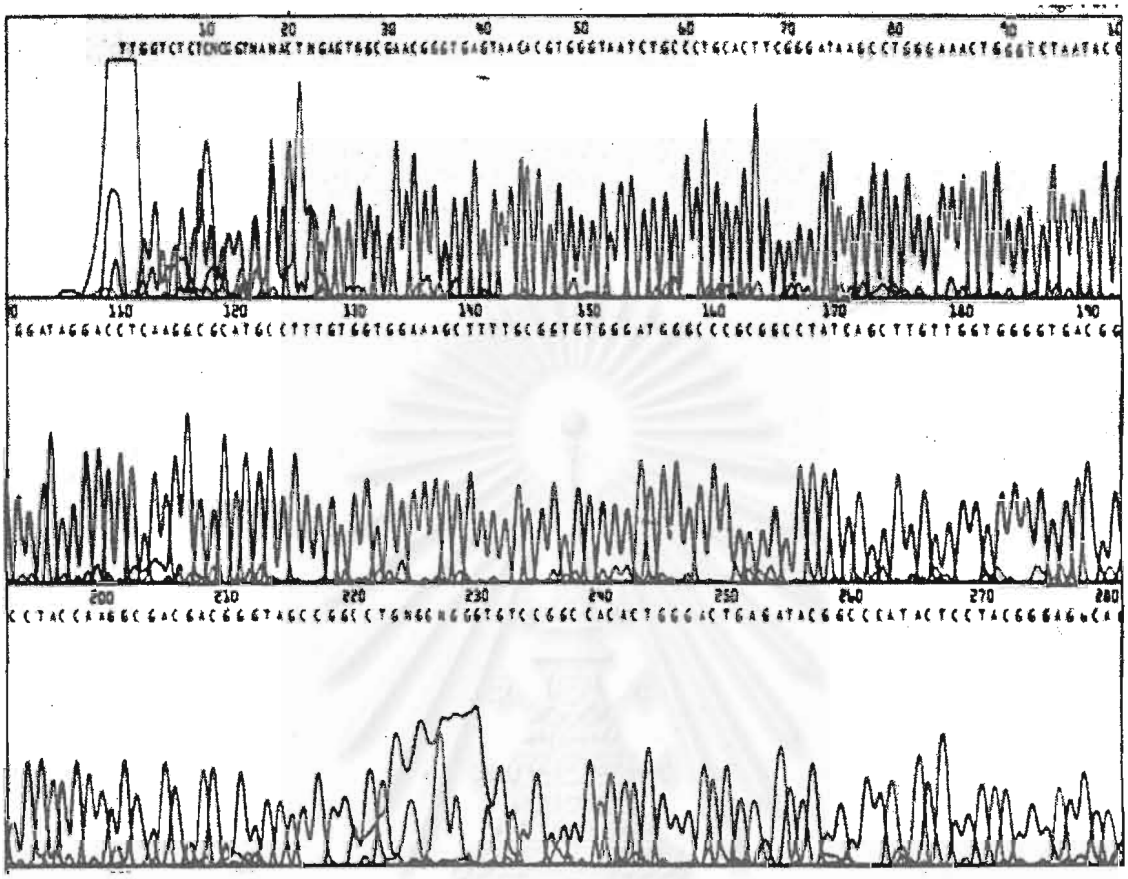


Figure 14. Chromatogram of sequencing by automate sequencer of *M. haemophilum*

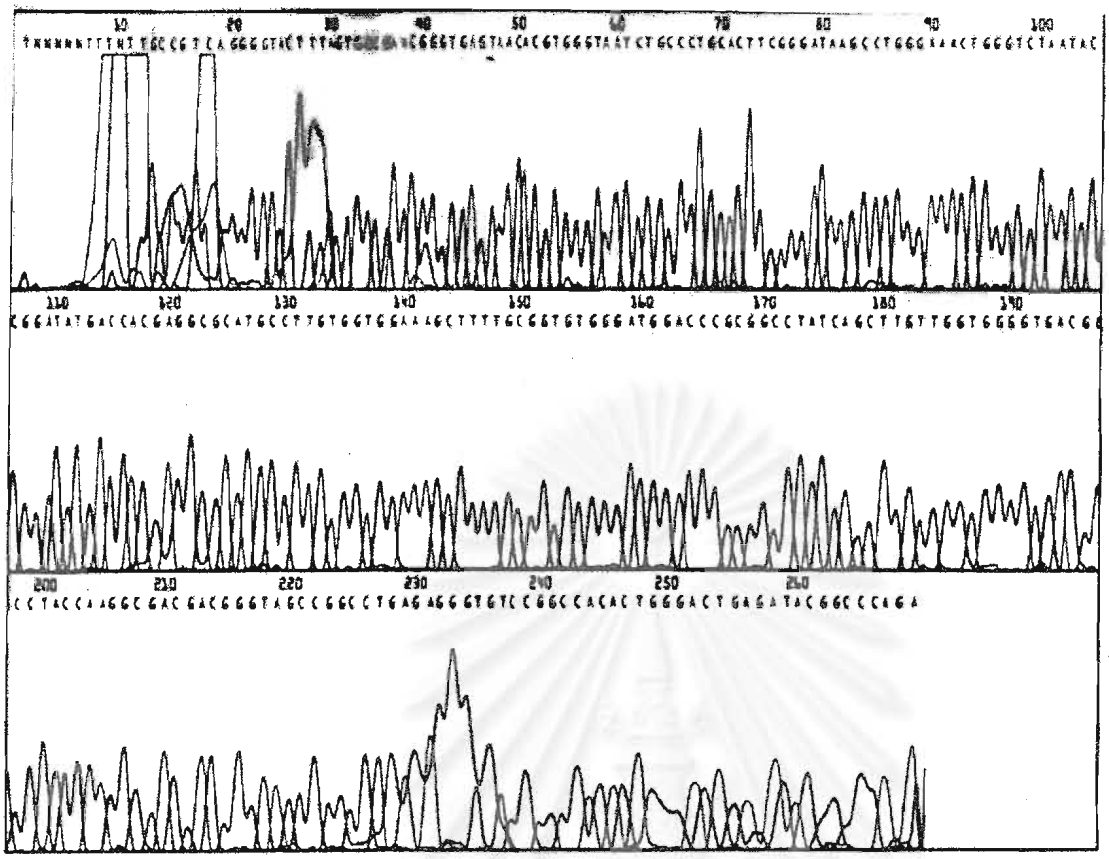


Figure 15. Chromatogram of sequencing by automate sequencer of *M. interjectum*

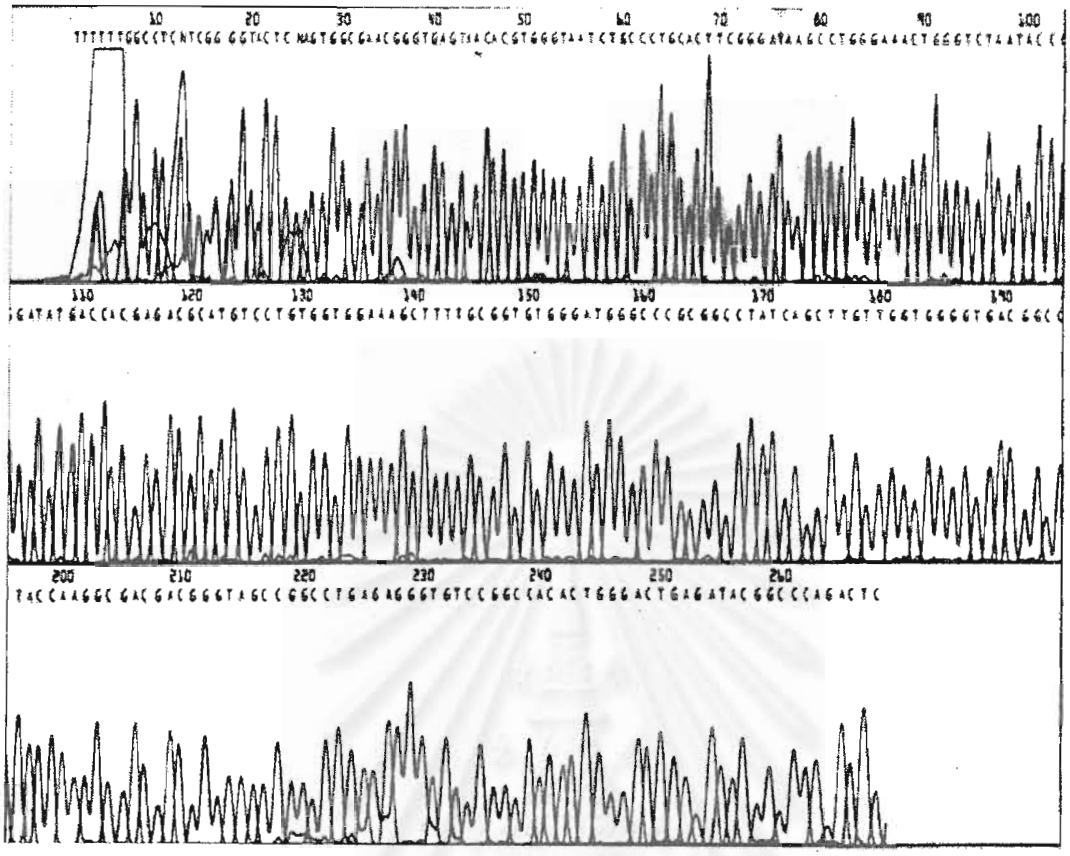


Figure 16. Chromatogram of sequencing by automate sequencer of *M. triplex*

CHAPTER VI

DISCUSSION

Mycobacterial infections are a major clinical problem in immunocompromised patients, particularly those with AIDS. The most prevalent disseminated mycobacterial infections in AIDS patient are those caused by MAC, which occur at a high frequency during the late stage of disease or are detected autopsy, and *M. tuberculosis* or other mycobacteria. Identification of the causative agent is thus important for patient management, antimicrobial treatment, and epidemiology (71). Identification of mycobacteria in the clinical laboratory still remains fastidious, difficult, and time-consuming. The morphological, cultural and biochemical tests used for identification required knowledge and well-trained laboratory technique (72).

Identification of mycobacteria in the clinical laboratory still remain fastidious, difficult, and time-consuming procedure. The morphological, cultural and biochemical tests used for identification required knowledge and well-trained laboratory technique (72).

DNA amplification method using PCR targeting 16S rRNA and direct sequencing is extensively used to identify and classify different species (15,25,26,27). In this study, an important development in the rapid isolation of mycobacteria from blood was the used of MB/BacT blood culture bottles (liquid media) for primary culture. This method had turnaround times of 17.45 days after specimen collection. In order to process for species identification, the primary culture was further cultivated on solid media and performed biochemical tests.

This needs 15-45 days for the whole process. PCR-sequencing of primary culture can shorten the time of species identification to 3 days.

However, the application of PCR for the identification of mycobacteria in hemoculture fluid specimens is a problem because of the complicated method used to extract genomic DNA for analysis. Another problem associated with the use of nucleic acid amplification technique with blood specimens is the frequent failure of amplification due to the presence of inhibitors which may interfere with the activity of the reaction (69) and the poor sensitivity of detection mycobacteria by PCR may be due to cell inadequacy or loss of DNA during purification.

Accordingly, the preparation of mycobacterial DNA for PCR from hemoculture was compared by five methods. It was found that lysis buffer method (method A) yielded low positive result. The problem may be from incomplete lysis of the tubercle bacilli from clinical specimens. Sonication and specimens heating (73) did not help to achieve complete cell lysis. Proteinase K and phenol-chloroform (method B) and QIAGEN blood kit method (method D) failed to extract the mycobacterial DNA. David et al. (69) found that phenol-chloroform failed to remove SPS and SPS bind to silica in the presence of chaotropes and elutes with water, just like DNA. Boom's method (method C) did not result in the complete removal of SPS and other problems may be from blood samples which were old or frozen. This method could not be used because of lysis of erythrocyte which releases hemoglobin and the DNA binding capacity of the diatom was limiting (75).

This study demonstrated the superiority of DNA extraction method by alkali wash and heat lysis (method E). The combination of centrifugation, an alkali wash with heat lysis appeared to be better suited for the routine laboratories because it is simple, sensitive and economical. For hemocultures, washing of cell pellets by centrifugation and resuspension are variably successful in removing SPS because SPS may bind to the hemoglobin and erythrocyte membranes so multiple wash cycle are required (75). Heat lysis is a simple method for releasing DNA for PCR from mycobacterial cells and alkali; NaOH was used principally to lyse and wash out the human erythrocytes and leukocyte components from the remaining mycobacterial cell pellet prior to heat treatment. It is also widely used to harvest mycobacteria for identification by culture or PCR (67).

The identification results obtained by biochemical and direct sequence analysis of the 16S rRNA hypervariable region A were identical in similarity 98.63% (72/73) from hemocultures. Most of the discrepant result between 16S rRNA gene sequence analysis and conventional methods resulted in species belonging to the same group or to a phenotypically closely related species.

For the discrepant result; one isolate, identified as *M. scrofulaceum* with 16S rRNA analysis, was identified as *M. xenopi* with conventional methods. This was not surprising as *M. scrofulaceum* phenotypically resembles *M. xenopi* (35). Differentiation between these species was possible by the observation of growth at 37^{o} C and 42^{o} C since *M. xenopi* grows better at 42^{o} C whereas *M. scrofulaceum* grows better at 37^{o} C. Four flagged positive, AFB-negative hemocultures were identified into species by biochemical tests and AccuProbe

from active growing colonies from solid media. One isolate was *M. tuberculosis* and 3 isolates were *M. avium* (positive by the probes of *M. avium* complex and *M. avium)*. These isolates were not included in species identification by DNA sequencing. Since three isolates could not grow on solid media. Therefore they were not identified with conventional methods but were identified with sequence analysis. They were 2 *M. tuberculosis* complex and 1 *M. haemophilum* as shown in Table 16. These two isolates of *M. tuberculosis* complex may not be *M. tuberculosis* as they could not grow on Ogawa medium. Unfortunately, the viable hemoculture samples were not available for further confirmation by conventional method. Four isolates were identified by genotyping but unidentified with conventional method. They were 2 *M. ulcerans*, 1 *M. interjectum* and 1 *M. triplex*. Culture on solid media from one specimen contained more than one mycobacterial species but identification with gene analysis could identify one mycobacterial species (Tables 11 and 16). This finding has important implication for mycobacterial species identification in clinical specimens by molecular methodologies. If PCR amplification of the 16S rRNA gene is performed alone, all of the species present may not be detected if one species is predominant (76).

16S rRNA sequences do not vary greatly within a species and they are identical in some species, such as *M. tuberculosis* complex, *M. avium* and *M. paratuberculosis*, *M. marinum* and *M. ulcerans* and also *M. kansasii* and *M. gastri.* For further differentiation, one may use phenotypic characteristics for *M. tuberculosis* complex, *M. avium* and *M. paratuberculosis*, *M. marinum* and *M. ulcerans.* In addition, two high-copy-number insertion sequences, IS*2404* and IS*2606*, have recently been reported in *M. ulcerans* and significantly, these

elelements are not present in *M. marinum*(78). *M. kansasii* and *M. gastri* can be differentiated by using 16S-23S rRNA gene internal transcribed spacer sequence base analysis for identification (77).

16S rRNA sequence analysis, which was introduced recently in the clinical laboratory, was performed by concentration on two previously characterized regions: region A, corresponding to *E.coli* positions 129 to 267, and region B corresponding to *E.coli* positions 430 to 500. While region A has been used for routine identification with primer pB (26), the additional analysis of region B is especially useful for isolates with show no unique sequence in region A. The mycobacterial 16S rRNA gene carries a species-specific sequence with in the hypervariable region A, which is sufficient, in most cases for identification of different mycobacterial species (68). However, some isolates do not show unique sequence in region A , hence analysis of region B is needed. For example, *M. triviale*, *M. shimoidei* and *M. lepraemurium* have the same sequences position 129-214 in region A so sequencing must be used in region B for mycobacterial identification(15).

Differentiation of mycobacteria to the species level is currently done by time-consuming evaluation of phenotypic and biochemical characteristics. Additional methods such as high-performance liquid chromatography (HPLC) or thin layer chromatography are limited by the need for standardized growth conditions (46, 47). AccuProbe (Gen-Probe, Inc., San Diego, Calif.) is a rapid method but is requires several probes and covers only a limited rang of mycobacterial species (35, 37). Sequencing of the 16S rRNA gene is a powerful

technique of differentiating species, a rapid method for results within 3 days (PCR and sequencing with automate sequencer), specific and highly sensitive. However, the instruments are expensive for routine use in many clinical laboratories.

CHAPTER VII

CONCLUSION

The preparation of mycobacterial DNA for polymerase chain reaction amplification and sequencing analysis from hemoculture fluid specimens by alkali wash and heat lysis method was significantly more effective than either lysis buffer, proteinase K and phenol-chloroform, QIAGEN blood kit or Boom's method. Out of 381 hemoculture in MB/BacT instrument, 73 samples (19.16%) were flagged positive. Sixty-nine flagged positive hemocultures were acid-fast bacilli (AFB) positive and 4 samples were acid-fast bacilli (AFB) negative. Of these 66 grew 67 AFB and 3 could not grow AFB on solid media. Identified by conventional method and AccuProbe revealed 4 different species as follows: *M. tuberculosis, M. avium complex, M. scrofulaceum* and *M. simiae*. Four isolates from 3 samples were unidentified and one isolate was mis-identified. Identification by 16S rDNA sequencing demonstrated 9 different species as follows*: M. tuberculosis, M. avium. M. intracellulare, M. scrofulaceum. M. simiae, M. ulcerans, M. haemophilum. M. interjectum* and *M. triplex*. *M avium* complex was the most prevalent pathogen, followed by *M. tuberculosis* and other non-tuberculous mycobacteria from hemocultures. The polymerase chain reaction-based sequencing strategy demonstrated that this technique is highly sensitive and specific, reliably differentiates *Mycobacterium* species which are difficult to identify by classical method. It also provides information of the taxonomic relatedness of new species which may not be identify by other technologies. The notion that sequence-based methodologies will take their places in routine clinical laboratories in an increasing reality. The initial cost of

equipment, i.e., automate sequencer, can quickly be recovered with saving in personel, time and ultimately in health care costs.

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APPENDIX I

REAGENT, MATERIALS AND INSTRUMENTS

A. REAGENT

Absolute ethanol	(Merck, U.S.A)
Acrytamide / bisacrylamide	(Biorad, U.S.A)
Agarose (ultrapure)	(Amresco, U.S.A)
Ammonium persulfate	(Biorad, U.S.A)
Boric acid	(Merck, Germany)
Developer	(Kodak, Japan)
Ethidium bromide	(Amresco, U.S.A)
EDTA	(Amresco, U.S.A)

B. MATERIALS

X-ray film (Kodak, Japan)

C. INSTRUMENTS

Hybaid OmniGene thermal cycler	(Hybaid, England)
Sonicator	(Branson, USA)
Water bath	(Memmert, USA)

Luminometer (Gen-Probe, USA)

APPENDIX I (CONTINUE)

Perkin Elmer GeneAmp PCR system 9600 (Perkin Elmer, USA)

ABI Prism™ Automate Sequencer (Perkin Elmer, USA)

APPENDIX II

REAGENTS AND PREPARATIONS

1. 0.5 M EDTA, pH 8.0

Disodium ethylene diamine tetraacetate.2H2O	186.1 g
DDW	800.0 ml

Adjust pH to 8.0

Adjust volume to 1,000 ml

2. 1 M Tris-HCl, pH 8.0

Tris (ultrapure)	121.1 g
DDW	800.0 ml
Adjust to pH 8.0 by adding conc. HCl	42.0 ml

Sterilize by autoclaving

3. 50 x Tris-acetate buffer (TAE)

Tris (ultrapure)	242.0 g
Glacial acetic acid	57.1 g

0.5 M EDTA pH 8.0	100.0	ml

Adjust the volume to 1,000 ml with DDW

Sterilize by autoclaving

4. 5M NaOH

NaOH	200	g

Adjust the volume to 1,000 ml with DDW

5. 0.5 M $Na_2C_5O_7.2H_2O$

$Na_2C_5O_7.2H_2O$	135.5	g

Adjust the volume to 1,000 ml with DDW

6. Diatom suspension (Boom method)

Cellite (Jasen Chimica)	10	g
32% Hcl	50	ul
DDW	50	ml

7. Lysis buffer (Boom method)

GUSCN	120	g
0.1 M Tris-HCl	100	ml
Triton X	2.6	g

8. Washing buffer (Boom method)

GUSCN	120	g
0.1 M Tris-HCl	100	ml

9. Elution buffer (Boom method)

1 M Tris-HCl pH 8	100	ul
0.25 M EDTA	40	ul
DDW	9.86	ml

10. Lysis buffer (Lysis beffer method)

1% Triton X 100	0.2	ml
20 mM Tris-HCl	0.2	ml
DDW	19.6	ml

11. 3 M Sodium acetate

Sodium acetate. $3H_2O$	408.1	g
DDW	800	ml

Adjust pH to 5 with glacial acid

Adjust volume to 1,00 ml

APPENDIX III

REAGENTS FOR AGAROSE GEL ELECTROPHORESIS

1. 10 mg/ml Ethedium bromide

Ethidium bromide	1	g
DDW	100	ml

Stir on a magnetic stirrer for several hours to ensure that the dye has dissolved.

Wrap the container in aluminum foil or transfer to a dark bottle and store at 4oC.

2. 1.5% Agarose gel

Agarose (ultrapure)	0.3	g
1 x TAE	20.0	ml
10 mg/ml Ethidium bromide	1.0	μl

BIOGRAPHY

Miss Anchalee La-ard was born on April 29, 1974 in Bangkok, Thailand. She graduated with bachelor degree of science in Kasetsart university in 1996.

Now she works as a Microbiologist at faculty of medicine in Chulalongkorn university.