CHAPTER III

LITERATURE REVIEW

GENERAL CHARACTERISTICS

Mycobacteria are aerobic, non-spore forming, nonmotile, slightly curved or straight rods, 0.2 to 0.6 x 1.0 to 10 μm in size. No aerial hyphae are grossly visible. They have cell walls with a high lipid content that include waxes (Figure 1). These waxes have characteristics mycolic acids with long, branched chains. The lipid content of the cell wall excludes the usual aniline dyes used to stain bacteria. Mycobacteria are not therefore readily using the Gram stain method; however, mycobacteria are usually considered gram positive. Special staining methods are used to promote the uptake of dye and, once stained, mycobacteria are not easily decolorised; that is, they retain the stain even when washed with acid-alcohol solutions. Their resistance to decolorization is termed 'acid fastness', hence the term acid-fast bacilli (AFB).

Colony morphology varies among the species, ranging from smooth to rough and from nonpigmented (nonphotochromogens) to pigmented. Colonies of the latter are regularly or variably yellow, orange, or pink, usually due to carotenoid pigments. A natural division exists between slowly and rapidly growing species of mycobacteria. The most slowly growing one requires more than 7 days to produce colonies on solid media from a dilute inoculum under ideal culture conditions. Rapid growers, by definition, require less than 7 days but may also take several weeks to appear on primary culture from clinical specimens [40]. A few species are fastidious, requiring special supplements (e.g., *M. haemophilum*), or are noncultivable (*M. leprae*).

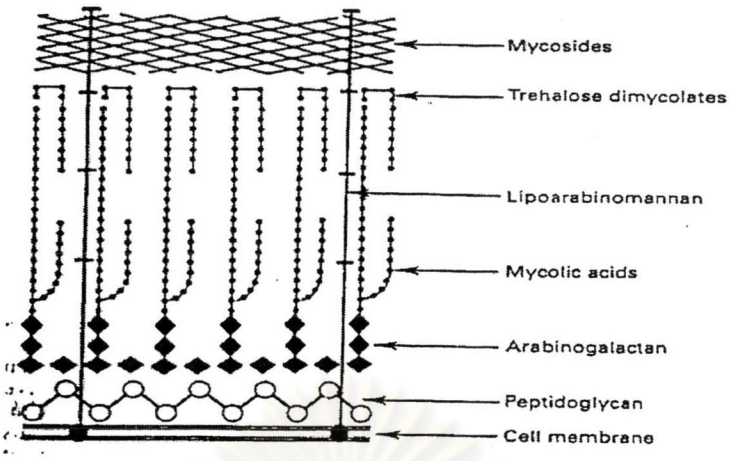


Figure 1. Diagrammatic section of the mycobacterial cell wall [41]

CLASSIFICATION

Mycobacteia were among the first bacteria to be described to specific disease. In 1874, Armauer Hansen identified a rod-shaped bacillus (*Bacillus leprae*) in a tissue biopsy from a lepromatous leprosy patient and suggested that it was the aetiological agent of leprosy. In 1882, Robert Koch identified a rod-shaped bacillus (*Bacterium tuberculosis*) as the causative agent of tuberculosis and formulated Koch'postulates for establishing a causal relationship between a suspected pathogen and a given disease. In 1886, these species were subsequently renamed *Mycobacterium leprae* and *Mycobacterium tuberculosis*, respectively, and placed in the genus *Mycobacterium*.

The genus *Mycobacterium* is currently the only genus in the family *Mycobacteriaceae*, Order *Actinomycetales*. The minimal standards for including a species in this genus are :

(1) acid-alcohol fastness (i.e. resist decolorization by acidified alcohol after being stained with a basic fuschin dye)

(2) the presence of mycolic acid containing 60-90 carbon atoms which are cleaved to C_{22}-C_{26} fatty acid methyl esters by pyrolysis and

(3) a G+C content of the DNA of 61-71 mole%, except for *M. leprae* (55%) [42]

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 (NTM).

The term nontuberculous mycobacteria (NTM) and *M. leprae* includes all other mycobacterial species that do not belong to *M. tuberculosis* complex. In 1959 Runyon classified NTM into four groups according to their speed of growth and pigmentation. These groups are:

I. photochromogen (yellow pigment formed in the light)
II. scotochromogen (yellow pigment formed in the light or dark)
III. non-photochromogen (nonpigment in both the light and dark)
IV. rapid growers

Mycobacteria are divisible into the rapid growers, slow growers and those not yet cultivated in vitro (Table 1) [40, 42, 43]. In addition, 'No- Name' mycobacteria have been discovered [40]. There were a few reports of novel NTM that cause disease in humans but have not been assigned to a particular species yet. For instance, a mycobacterium resembling *M. kansasii*, although nonphotochromogenic, was isolated from an AIDS patient with acute lymphadenitis [44]. Evoking the same clinical manifestation in a child, another novel mycobacterium related to *M. triplex* was described by Harza et al.[45].

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[46].

In detection as few as 10^1 as 10^2 viable organisms/ml, specimen culture is more effective than smear. Media available for the recovery of mycobacteria include nonselective and selective on33es, the latter containing one or more antibiotics to prevent overgrowth by contaminating bacteria or fungi. Broth media are preferred for a rapid initial isolation of mycobacteria [40]. The many different media available for the recovery of mycobacteria from a clinical specimen are variations of three generation types (Table 2) : egg medium, serum albumin agar medium, and liquid medium.

Egg-based medium contain whole eggs or egg yolk, potato flour, salts, and glycerol and are solidified. These media have a good buffer capacity and a long shelf life (several months when refrigerated) and support good growth of most mycobacteria. Also, materials in the inoculum or medium that are toxic to mycobacteria are neutralized. Disadvantages of these media include variations from batch to batch depending on the quality of the eggs used, difficulties in discerning colonies from debris, and the inability to achieve accurate and consistent drug concentrations for susceptibility testing. When egg-based media become contaminated , they may liquefy. In contrast to egg-containing media, agar based media are chemically well defined. Agar –based media are transparent and provide a ready means of detecting early growth of microscopic colonies easily distinguished from inoculum debris. Colonies may be observed in 10 to 12 days, in contrast to 18 to 24 days with egg-based media. Agar-based media can be used for susceptibility testing. They do not readily support the growth contaminants; however, the plates are expensive to prepare and their shelf life is relatively short (1 month in the refrigeration). Care should be exercised in preparation, incubation, and storage of the media, because excessive heat or light exposure may result in deterioration and in the release of formaldehyde, which is toxic to mycobacteria. The addition of antimicrobial agents may be helpful in eliminating the growth of contaminating organisms. If a selective medium is used for a particular specimen, it should not be used alone but should be used in conjunction with a nonselective agar or egg-based medium [40].

Table 1 The species of mycobacteria [40, 42, 43]

Slowly growing

M. tuberculosis	*M. bovis*	*M. africanum*	*M. shimoidei*
M. microti	*M. kansasii*	*M. marinum*	*M. cookii*
M. celatum	*M. simiae*	*M. asiaticum*	*M. gordonae*
M. interjectum	*M. scrofulaceum*	*M. szulgai*	*M. paratuberculosis*
M. canettii	*M. intracellulare*	*M. lepreamurium*	*M. avium*
M. malmoense	*M. haemophilum*	*M. farcinogenes*	*M. hiberniae*
M. triviale	*M. terrae*	*M. nonchromogenicum*	*M. conspicuum*
M. ulcerans	*M. gastri*	*M. xenopi*	*M. branderi*
M. genavense	*M. intermedium*	*M. heckeshornonse*	*M. heidelbergense*
M. lentiflavum	*M. triplex*	*M. tusciae*	*M. doricum*
M. kubicae	*M. palustre*	*M. lacus*	*M. shottsii*
M. botniense			

Rapid growing

M. chelonae	*M. fortuitum*	*M. phlei*	*M. peregrinum*
M. smegmatis	*M. aurum*	*M. gadium*	*M. immunogenum*
M. neoaurum	*M. flavescense*	*M. gilvum*	*M. mageritense*
M. komossense	*M. senegalense*	*M. parafortuitum*	*M. alvei*
M. thermoresistible	*M. confluentis*	*M. mucogenicum*	*M. septicum*
M. goodii	*M. wolinskyi*	*M. brumae*	*M. chitae*
M. diernhoferi	*M. agri*	*M. vaccae*	*M. duvalii*
M. archense	*M. chubuense*	*M. austroafricanum*	*M. gilvum*
M. rhodesiae	*M. tokaiense*	*M. shinshuense*	*M. komossense*
M. porcium	*M. fallax*	*M. pulveris*	*M. madagascariense*
M. sphagni	*M. methylovorum*	*M. moriokaense*	*M. obuense*
M. poriferae	*M. shanghaiense*	*M. yannanense*	*M. hossiacum*
M. novocastrense	*M. elephantis*	*M. holsaticum*	*M. chlorophenolicum*
M. frederiksbergense	*M. hodleri*	*M. murale*	*M. vanbaalenii*

Non-cultivable

M. leprae

Table 2. Suggested Media for cultivation of Mycobacteria from clinical specimen*[47]

Solid

Egg-based

1. Lowenstein-Jensen (L-J)
2. L-J Gruft
3. L-J with pyruvic acid
4. L-J with iron

Agar-based

1. Middlebrook 7H10 and Middlebrook 7H10 selective
2. Middlebrook 7H11 and Middlebrook 7H11 selective
3. Middlebrook biplate (7H10 / 7H11S agar)

Liquid

BACTEC 12B medium

Middlebrook 7H9 broth

Septi-Check AFB

Commercially supplied broth for continuously monitoring system for mycobacteria

*For optimal recovery of mycobacteria, a minimum combination of liquid medium and solid media is recommended.

Broth media may be used for both primary isolation and subculturing of mycobacteria. Cultures based on liquid media yield significantly more rapid results than do those based on solid media. At present, a number of elaborate culture systems marketed for the isolation of mycobacteria are available commercially (Table 3).

Medium selection for the isolation of mycobacteria and culture reading schedules are usually based on personal preference and/or laboratory tradition. Addition of a solid medium is advantageous for the detection of strains which occasionally do not grow in liquid medium, aids in the detection of mixed mycobacterial infections, and can serve as a back-up for broth cultures, if contaminated. All positive cultures, even if identified directly from the broth, must be subcultured to solid media to detect mixed cultures and to correlate direct identification results with colony morphology [40]

Table 3. Commonly used commercial liquid media systems to culture and detect the growth of mycobacteria [47]

System	Basic Principle of detection
BACTEC 460 TB (Becton Dickinson Diagnostic Systems, Cockeysville, Md.)	Culture media containing ^{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 collect 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-Cek AFB System TB (Becton Dickinson Diagnostic Systems)	Biphasic culture system made up of a modified Middlebrook 7H9 broth with 1 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-side 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 conductive to possible automation
Continuous Growth Monitoring Systems	
ESP Culture System II Accumed International, Inc., Chicago, III.	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

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 common Gram stain is not suitable for mycobacteria. They may be gram-invisible, may appear as clear zones or 'ghosts', or may appear as beaded gram-negative rods, particularly rapidly growing mycobacteria. 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 acidfast [40]. There are three commonly used staining methods to detect acid fast bacilli (AFB): the two carbolfuchsin-basedstains, Ziehl-Neelsen and Kinyoun, and fluorochrome stains. Both carbolfuchsin methods stain the mycobacterial cells red against the methylene blue counter stain. A fluorochrome stain, auramine O or auramine-rhodamine, is the screening method recommended for laboratories with a fluorescent (ultraviolet) microscope [48]. In practice, the fluorochrome stain is more sensitive than the carbolfuchsin stain, even when read at lower magnification, probably because the fluorochrome-stained smears are easier to read. The overall sensitivity of the smear has been reported to range from 22 to 80% [49]. The specificity of the smear for the detection of mycobacteria is very high. Other factors influencing smear sensitivity include the type specimens examined, staining techniques, the experience of the reader, the patient population being evaluated, and whether the smear has been done with or without pretreatment (indirect versus direct smear) [40].

2. PHENOTYPIC CHARACTERISTICS

2.1 Growth rate and Preferred Growth temperature

Growth rate refers to the length of time required to form mature, isolated colonies visible without magnification on solid media (Table 4). Mycobacteria forming colonies within 7 days are termed rapid growers, while those requiring longer periods are termed slow growers [50]. The cultures are incubated at 35 to 37°C. Some species have special nutrient or temperature requirements for growth. Cultures are observed at 5 to 7 days and weekly thereafter for visible colonies. Growth in relation to temperature can usually be adequately determined by observing cultures at 37 and 30°C. When more definitive identification is needed, isolates should be incubated at 24, 25, 32, 35, 37, and 42°C. Growth at these temperatures is interpreted as in Table 5 [51]

2.2 Pigmentation and Photoreactivity

Some mycobacteria produce carotenoid pigments without light, whereas others require light (photoactivation) for pigment production. The terms photochromogen, scotochromogen, and non-photochromogen are used to distinguish the *M. tuberculosis* complex (MOTT) based on their pigmentation and photoreactivity. Photochromogens produce nonpigmented colonies when grown in the dark and pigmented colonies only after exposure to light. Scotochromogens produce deep yellow to orange pigmented colonies when grown in either the light or the dark (some strains show an increased pigment production on continuous exposure to light) . Nonchromogens are nonpigmented in both the light and dark or have only a pale yallow, buff or tan pigment that does not intensify after light exposure. These responses to light exposure were originally delineated to aid in the identification of NTM. Members of the MTBC, however, are considered nonchromogens, and pigmented mycobacteria may be preliminarily reported as NTM [50] as shown in Table 4.

Table 4. Colony morphology and growth characteristics of mycobacteria encountered in the clinical laboratory [52]

Mycobacterium species	Colony morphology	Colony pigment	Growth rate* (wk)	comments
M. tuberculosis	Rough	N(buff)	4-6	
M. bovis	Rough; thin or transparent	N(colorless to buff)	4-6	
M. avium complex	Smooth; small, thin, transparent, or large opaque, domed; ± rough	N	4-6	Growth may required 8 wk; colonies of some strains come lightly pigmented with prolonged incubation
M. scrofulaceum	Smooth, globoid	S	4-6	Pigmented varies from light yellow to deep orange
M. kansasii	Rough, β-carotene crystals	P	4-6	Rare strains N or S
M. fortuitum-chelonae	Smooth or Rough	N	≤1	
M. xenopi	Smooth, filamentous extensions ("bird nest")	S	4-6	Growth at 42°C
M. szulgai	Smooth or Rough	S, 37°C; P, 25°C	4-6	
M. malmoense	Smooth, dysgonic	Colerless	2-3	
M. simiae	Smooth	P+	4-6	
M. marinum	Wrinkled, shiny; smooth, hemispherical; (rarely), rough, dry	P	2-3	Optimal growth, 31-33°C
M. haemophilum	Rough; ± smooth	N	4-6	Optimal growth, 20-32°C
M. gordonae	Smooth	S	4-6	
M. thermoresistible	Smooth or Rough	P	≤1	Optimal growth, 37-45°C pigment is yellow-orange, becoming brown
M..terrae-triviale	Smooth (*M. terrae*); rough (*M. triviale)*	N	4-6	
M. nonchromogenicum	Intermediate in roughness	N	4-6	
M. flavescens	Smooth	S	2-3	
M. smegmatis	Rough; ± smooth	N (buff)	≤1	

*Average, on solid medium.

+ Pigment production often requires prolonged exposure to light.

N = Nonphotochromogen; S = Scotochromogen; P = Photochromogen; ± occasional strains have the indicated morphology

Table 5. Growth of mycobacteria regarding to temperature [51]

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*

2.3 Colony morphology

The colonial morphology of an isolate may provide useful clues to its identification. Isolated colonies are required for optimum characterization of colony morphology. Some isolates are uniform with regard to their colonial morphology, whereas others may exhibit considerable variability. Important features of colony morphology (which may vary significantly depending on age and growth conditions) include: surface characteristics, colony shape, opacity, and color. Colonial characteristics of mycobacteria are summarized in Table 4.

3 BIOCHEMICAL IDENTIFICATION

The biochemical tests are based on the enzyme the organisms posses, the substances that their metabolisms produce, and the inhibition of their growth on exposure to selected biochemicals reagents. Table 6 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 [47]. 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 conventional biochemical tests may take weeks after sufficient growth is observed. Furthermore, biochemical identification based on phenotypic characteristics may not be highly reproducible, complicated, and non-reproducible [11].

Table 6. Distinctive properties of cultivable mycobacteria encountered in clinical specimens [52]

Runyon group	Complex[a]	Species	Clinical significance[c]	Growth rate[d] at				Colony type			Niacin	Susceptibility to T2H[g] (5 μg/ml)	Nitrate reduction
				45°C	37°C	31°C	24°C	Usual colony morphology[e]	See fig. no.	Pigmentation[f]			
	TB	*M. ulcerans*	1	–	–	S	–	R		N	–	–	–
		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[h]	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*	*M. fortuitum*	4–3	–	R		R	Sf/Rf		N	–	–	+
		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 [52] - continued

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	−	−	−[h]	∓	−	−	−	−	+	+	
M. bovis	−	−	−	∓	−	−	−	−	+	−	
M. marinum	−	−	+	∓	−	−	∓[g]	−	+	+	+
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]	−	−	−	−	−	−	−	−	−	+	
M. nonchromogenicum	+	+	+	−	−	−	−	V	−	V	
M. terrae	+	+	+	−	−	−	−	V	−	V	
M. triviale	+	+	+	−	+	−	∓	−	−	V	
M. fortuitum	+	+	V	+	+	+	+	+	+	+	+
M. chelonae	+	V	V	+	V[i]	−	+	+	+	+	+
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, nonchromogen.

[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 [47]

Mycobacterial group	Key biochemical tests
M. tuberculosis complex	Niacin, nitrate reduction, susceptibility to TCH (Thiophene-2-carboxylic acid hydrazide) 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

The mycolic acid pattern of the cell wall generally varies with the species. Thus, mycolic acid analyses can be a useful tool for mycobacterial identification. Two direct approaches may be used for the analysis of mycolic acids: thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). A third method, gas-liquid chromatography (GLC), in which mycolic acids are investigated in the form of their cleavage products, is also used.

In HPLC analysis, the mycolic acids are separated on the basis of their polarity and the carbon chain length, with the more polar and shorter eluting first. In contrast to TLC, the identification of eluted compounds is unimportant; the arrangement of major peaks in the chromatogram, the position on the basis of retention times, and the height in comparison with other peaks are the only pieces of information needed. Each species is characterized by a pattern with a particular number, position, and height of peaks. The visual comparison of HPLC chromatograms with ones of known species is still the most reliable identification procedure. With GLC, not only mycolic acids but the whole lipid component of the cell wall is analysed [43].

5 MOLECULAR METHODS

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.

In 1992, Plikaytis and colleagues [53]developed a PCR-resrticion endonuclease analysis (PRA) method for the rapid identification of mycobacteria to the specific level. This technique was based on PCR of a sequence of the gene encoding the 65-kDa heat shock protein (*hsp65*), followed by restriction enzyme digestion [53]. In 1993, Telenti, et al. [20] presented a modification of this method using a smaller frafment (439 bp) and only two restriction endonucleases, *Bst*EII and *Hae*III [20]. The method has been extensively used for mycobacterial identification [20, 54-56] *M. tuberculosis* is easily differentiated from the NTM by a charcteristic band on *Hae*III restriction endonuclease

digestion. However, members of the MTBC are not discriminated by their PRA. By contrast, most NTM can be recognized by their PRA patterns. Several alleles have been identified in *M. gordonae* [57], *M. kansasii* [58, 59], *M. abscessus*, *M. chelonae*, and *M. peregrinum*[49], indicating that the molecular clock of the *hsp65* gene is faster than that of 16S rDNA. In addition, the other gene sequences for PRA, such as the *rpoB* gene coding for the RNA polymerase [32], *dnaJ* gene [23], and the 16S-23S rRNA gene spacer [60]. The advantages of PRA are that identification is largely independent of growth rate and requirements, equipment is not very expensive, and the method is relatively rapid and identified most mycobacterial species including some not identified by phenotypic method and/or HPLC. The disadvantages are that it requires knowledge of PCR, the results are not easy to interpret for species identification due to the limit size difference of DNA fragments after digestion, and is a relatively complex procedure. Furthermore, it is not commercialized or Food and Drug Administration (FDA) approved, and it requires a significant amount of in-house validation.

AccuProbe (Gen-Probe, Inc., San Diego, Calif.) specific for MTBC, MAC (as well as separate probes for *M. avium* and *M. intracellulare*), *M. kansasii*, and *M. gordonae* are FDA approved and commercially available. The current total test time for the assay is within 2 h [61] . Briefly target 16S rRNA is released from the organism by sonication. The labeled DNA probe combines with the organism's rRNA to form a DNA-rRNA hybrid. The labeled product is detected in a luminometer. Tests with DNA probes can be performed using isolates from solid media or from broth cultures. Combining probes with a broth culture system has the advantage of optimizing rapid detection and identification of mycobacteria present in clinical samples [62].

It has been shown that specificity is 100% when testing mycobacterial colonies. Sensitivity, however, varies with the species or species complexes: 95.2 to 97.2% for MAC, 100% for MTBC, 100% for *M. gordonae*, and 97.4 to 100% for *M. kansasii* [63-65]. Later studies using AccuProbe on more than 11,000 positive BACTEC (Becton Dickinson) cultures also showed 100% specificity and >85 to 100% sensitivity for all species tested [61, 66]. Advantage of this test include the simplicity and rapidity with which mycobacteria can be identified. The use of a nonradioactive precedure and the extended shelf life of the chemiluminescent probes offer the potential for widespread

application in most clinical laboratory settings [63, 67] but requires a well-growth culture and testing with several probes, covers only a narrow range of mycobacterial species, and high costs.

Recently a new DNA probe kit (INNO-LiPA Mycobacteria; Innogenetics, Ghent, Belgium), available only in Europe at present. It is based on the reverse hybridization principle, in which the mycobacterial 16S-23S rRNA spacer region is amplified by PCR. INNO-LiPA was developed for the detection of *Mycobacterium* spp. and identification of members of the *M. tuberculosis* complex, *M. kansasii, M. xenopi, M. gordonae, M. avium, M. intracellulare, M. scrofulaceum*, and *M. chelonae* [12, 36]. Biotinylated amplicons are subsequently hybridized with probes immobilized as parallel lines on a membrane strip. The addition of streptavidin labeled with alkaline phosphatase and a chromogenic substrate results in a purple-brown precipitate on hybridized lines. The kit may be applied to strains subcultured on solid or in liquid media. The main advantage of the kit is that a range of several species can be identified by a single PCR assay and, unlike the AccuProbe, it does not require a tentative selection of the adequate probe. The test is performed in 6 h, including the preliminary PCR amplification. It requires several time-consuming washes. An automated machine, Auto-LiPA (Tecan Trading AG, Mannedorf, Switzerland), which runs the washes and ensures the gentle shaking necessary for several steps of the procedure greatly contributes to time saving and ease of amplification in clinical laboratories. However, the cost of this apparatus may hamper its introduction into laboratories [50].

Kox et al.[68] developed a PCR assay based on DNA coding for the 16S subunit of rRNA, combined with IS*6110*-based PCR assay for detection of *M. tuberculosis* in a multiplex PCR assay and analyzed in a reverse cross-blot hybridization assay. They found this technique identified *M. tuberculosis* and the most important opportunistic mycobacteria. Tanaka et al. [69] chose multiplex PCR to detect only IS*6110* in isolates identified as MTC and IS*1245* was found only in the *M. avium* isolates, and concluded that multiplex-PCR assay may be used in a routine diagnosis and especially for detection of these organisms in mixed cultures.

To reduce the time taken for the identification and species determination of *M. avium* and *M. intracellulare* to a few days. Various approaches have been described for

the identification of MAC by PCR. Fries et al.[70, 71] used primers and probes to detect and identify a species sequence of *M. avium* after in vitro culture of the organism. Boddinghaus et al. [31] amplified a 16S rRNA gene of mycobacteria at the genus level and identified the species of MAC by hybridizing the amplified product with species-specific oligonucleotide probes. By comparison, Wilton and Cousins [37] described a sensitive multiplex PCR which specifically amplified DNA fragments of different sizes from the MPB70 gene of *M. tuberculosis* and from the 16S rRNA gene of members of the genus *Mycobacterium, M. avium*, and *M. intracellulare* in a single-tube assay without using a hybridization step. The multiplex PCR can also be used to detect and identify mycobacteria directly in blood specimens and blood cultures at an earlier stage of growth (GI, <20 U) [38].

Kim et al. [19] used a simplified multiplex PCR assay, basically a duplex PCR (DPCR) assay, to differentiate *M. tuberculosis* complex and NTM by using a single gene, the RNA polymerase β-subunit-encoding gene (*rpoB*). They founded (i) *rpoB* nucleotides specific for *M. tuberculosis* complex or NTM are invariably constant. Therefore, false-positive or -negative results due to sequence variations do not occur. (ii) DPCR yields only one product, irrespective of the *Mycobacterium* species tested. Even the coexistence of *M. tuberculosis* and NTM can be detected by the presence of two different PCR products in a single reaction mixture. (iii) Moreover, the (136-bp) *rpoB* DNAs of NTM can be further analyzed by RFLP or by direct sequencing to supplement species identification.

The availability of DNA-sequencing technologies constituted a great benefit for mycobacterial identification, owing to the peculiar slow growth of these organisms. Recent improvements in automation of terget amplification and sequence analysis led to practical implememtation of DNA sequencing in the clinical laboratory. The polymorphism of several conserved genes has been investigated for identifying mycobacterial species, such as the gene encoding the 32-kDa protein [72], the *dnaJ* gene [23], the *sod* gene encoding the superoxide dismustase [73], the *gyrB* gene coding for the gyrase subunit B [74], the *rpoB* gene, and the ITS 16S-23S sequence [60, 75, 76]. The most widely used targets are the *hsp65* gene [28, 49] and 16S rDNA [30, 31, 77].

The 16S rRNA gene is highly conserved in all prokaryotes and is an essential constituent of bacterial ribosomes [31, 78]. Therefore, bacterial 16S rRNA gene is widely used for phylogenetic analyses [75, 78-80]. Unfortunately, in the genus *Mycobacterium*, this gene has few polymorphic sites. In fact, some mycobacterial species have identical 16S rRNA (16S rDNA) sequences and others share a high degree of sequence homology [30, 75] . This hampers the design of probes that can be applied for a panel of clinically relevant species [75]. Rogall et al.[30] and Amin et al.[77] 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.

The internal transcribed sequence (ITS) between the 16S and 23S rDNA shows more nucleotide polymorphism than the 16S rDNA, but is still unable to differentiate between very closely related mycobacterial species such as those belonging to members of the *M. tuberculosis* complex [81].

Polymerase chain reaction (PCR)-linked reverse hybridization is one of the methods that could fulfill these requirements. Basically, the amplified product is hybridized with oligonucleotides probe immobilized on a micro-titer well plate or membrane strip, and detected using a colorimetric system. Commercial kits using a micro-titer well plate or a line probe assay are available and their sensitivities and specificities are being continuously evaluated [82-84]. The 16S rRNA gene [27, 83-85] and 16S-23S rRNA spacer region [43, 84] are most widely used for the PCR linked reverse hybridization method.

Rossi, et al.[25] were developed and evaluated a PCR-hybridization technique based on the amplification of a 16S rRNA gene sequence using pan-*Mycobacterium* primers followed by hybridization of the amplification products to *M. tuberculosis*- and *M. avium*-specific probes, and concluded that the PCR-hybridization technique can also be used to detect and identify *M. tuberculosis* and *M. avium* from particular biological samples (formalin-fixed, paraffin-embedded tissues) and especially from blood specimens at an early stage of growth. Kox et al. [39]and Sanguinetti et al.[86] evaluated PCR amplification of 16S rRNA gene sequences and a subsequent reverse cross-hybridization

assay with species-specific probes for the identification of mycobacteria and concluded that this technique was able to identify a wide variety of clinically relevant mycobacteria.

Recently, partial *rpoB* sequences suitable for the mycobacteria identification were reported [32]. Hong et al.[26] developed new *M. tuberculosis*-specific probes, *rpoB* oligonucleotides, used to identify *M. tuberculosis* and evaluated the efficiency of *M. tuberculosis*-specific *rpoB* probes. They concluded that *rpoB* probes can satisfy two major concerns associated with the diagnosis of mycobacterial infections, (i) rapid and precise identification of mycobacterial species at the gene level, and (ii) the determination of rifampin resistance among *M. tuberculosis* isolates.

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 simultaneous 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) [87].

Figure 2. Primer extension. DNA polymerase extends a primer by using a complementary strand as a template [87]

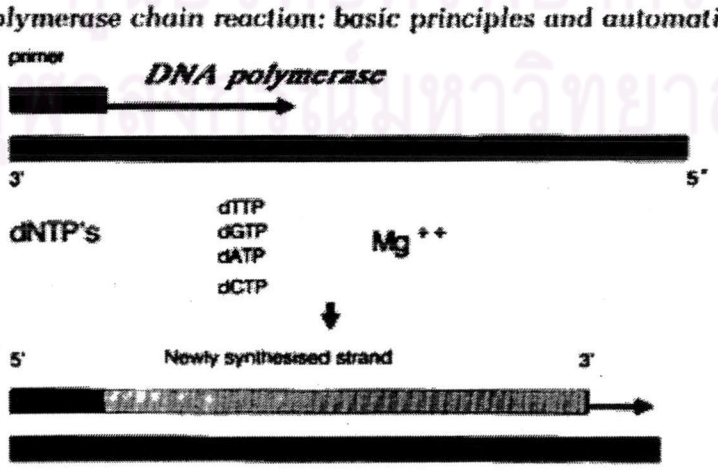
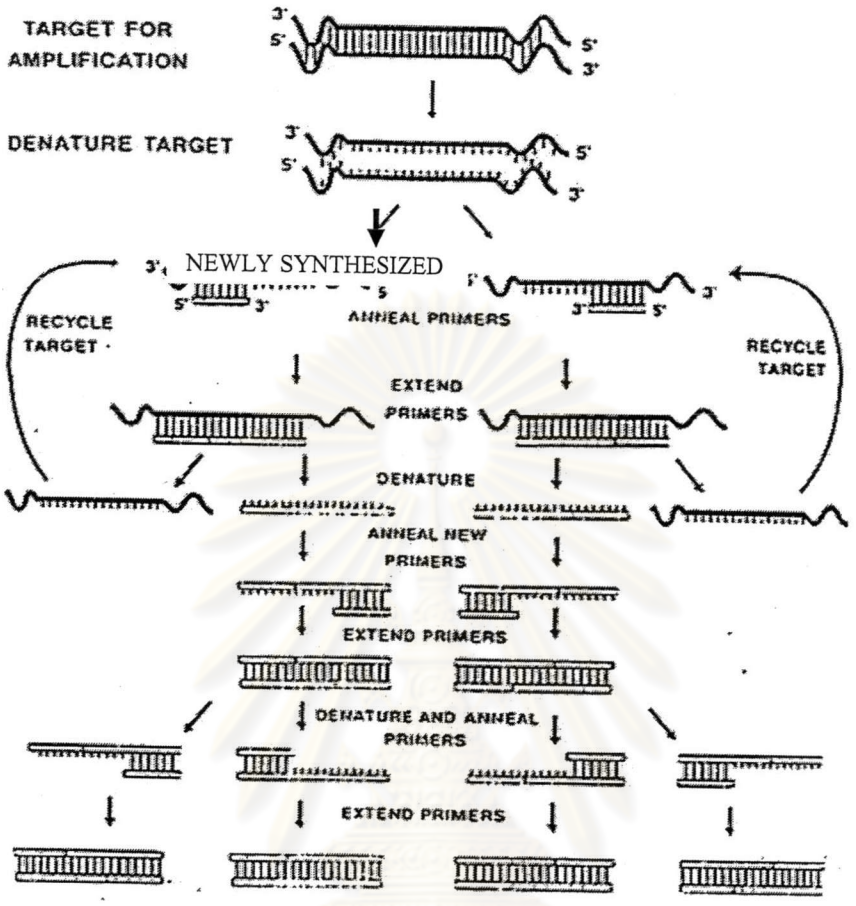


Figure 3. Schematic diagram of PCR [88]



5.2 DNA Sequencing (Dideoxy sequencing) [89]

The dideoxy enzymatic method as originally developed by Sanger F. [89] 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 [88] 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) [89].

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 [90]. 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 [89]

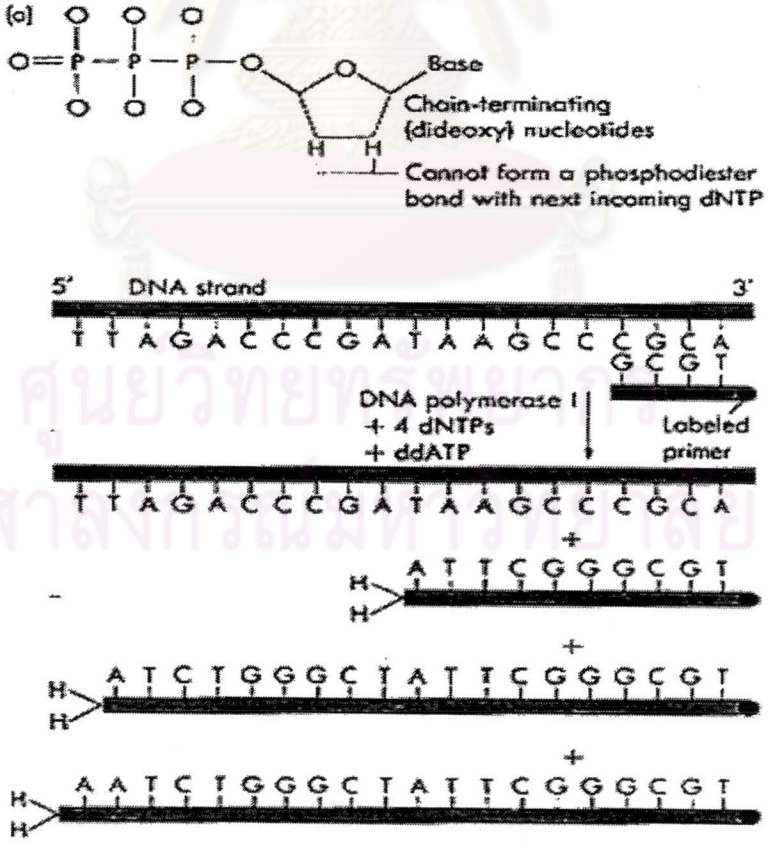
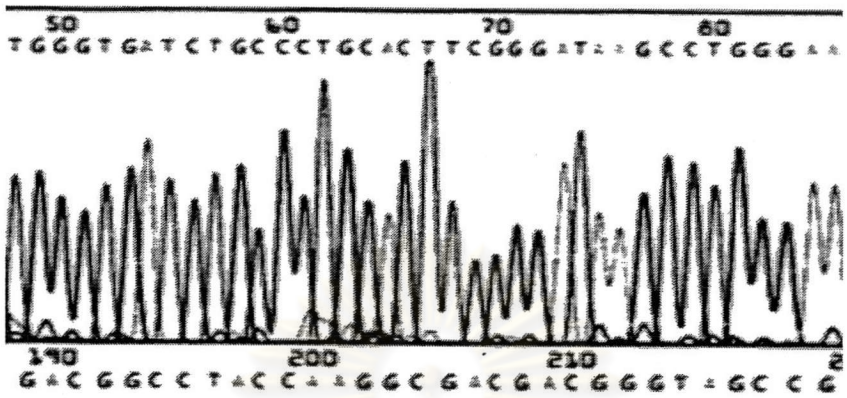


Figure 5. Chromatogram of sequencing by automate sequencer



5.3 Hybridization

Nucleic acid hybridization tests for the detection of specific DNA and RNA sequences are now extensively used in research and routine laboratories [26, 27, 91-93]. Labeled nucleic acid probes are utilized in a variety of assay formats including dot blots. Southern blots (DNA target), Northern blots (RNA target), in situ hybridization, plaque hybridization and colony hybridization. An important aspect of nucleic hybridization assays in the choice of the substance used to label a nucleic acid probe and the label detection method. The first assays used radioactive ^{32}phosphorus label. However, this label has the major disadvantage of a relatively short half-life (14.2 days). Many different substances have been tested as nonisotopic replacement for ^{32}phosphorus.

Choice of label and detection system

In choosing which particular label to use for a gene probe, it is again important to assess the sensitivity of detection required in a given experiment. For many applications, radiolabeled offer the twin advantages of excellent sensitivity and compatability with many labelling techniques. However, there are inherent disadvantages of radioisotope labelling such as the half-life of the radioisotope (therby limiting the storage time of the probe), potential health hazards, containment, and the need for relatively long detection periods (compared with, for example, chemiluminescent detetion). An increasing number of non-isotopic labelling and detection schemes are now available which are now used by

many researches for many applications where radiolabels were formerly the method of choice.

Non-isotopic methods of labelling and detection

Choice of label

Recently a number of non-isotopic labelling and detection systems has become available for applications where absolute sensitivity of detection is not required. The two main advantages of such systems are:

(1) their relative safety in comparison to radioisotopic labels;

(2) their storage stability (greater than one year has been claimed) which, unlike radiolabelled probes, is not limited by the half-life of the label.

However, although it is difficult to make direct comparisons, the sensitivity of non-isotopic labelling and detection appears to be at best about one-tenth that of the most sensitive of radioisotopic methods. Practically, this may obviate the use of non-isotopic detection in situations requiring very sensitive detection of target species, although successful use for these applications has been claimed by some manufactures. Non-isotopic labels, however, have found particularly successful application for more abundant targets suchas in library screening and the detection of PCR product, particularly in combination with chemiluminescent detection. non-isotopic labels are currently of three main types:

(a) Direct labels are deoxyribonucleotide triphosphates chemically tagged with a hapten reporter group which can be incorporated into DNA by eatablished techniques for uniform labelling. When used as a hybridization probe, detection is mediated either directly (if using fluorescent haptens) or indirectly, for instance via incubation with an anti-hapten antobody conjugated to an enzyme, or with a streptavidinenzyme conjugate if biotin is the hapten. The signal is then visualized using either a colorimetric or chemiluminescent substrate for the conjugated enzyme.

(b) The second method of labelling nucleic acid probes non-isotopically is to cross-link directly an enzymic activity to the DNA. Either horseradish peroxidase (HRP) or alkaline phosphatase (AP) can be cross-linked directly to nucleic acid whilst remaining their activity.

(c) The third main non-isotopic labelling method uses photoactivatable analogues of biotin or dioxygenin to label DNA probes directly. Upon brief irradiation with visible light of high intensity, stable linkages can be formed with both single- and double-stranded nucleic acids.

Choosing which is the best non-isotopic label for a particular application is often dependent on such factors as the level of sensitivity required , ease of use, and cost. The use of chemiluminescence for detection with most systems has allowed non-isotopic labelling to be used in many conventional molecular biology techniques, including Northern and Southern blotting, library screening, and DNA sequencing.

Detection of non-isotopic labels

The principle types of detection methods for non-isotopic labels are :

(a) Chemiluminescence and bioluminescence

Chemiluminescence is the emission of light that occurs in certain chemical reactions because of decay of chemiexcited molecules to the electronic ground state. Bioluminescence is the chemiluminescence of nature, which involves luciferin substrates and luciferase enzymes, or photoproteins. Both method are very sensitive, rapid (minutes to hours) and resolution (very sharp banding), and they provide X-ray film copies of the results.

(b) Colorimetry

Colorimetry assays produce soluble colored products and are relatively intensitive compared to luminescent assays (chemiluminescence, fluorescence, etc.). Most attention has focused on reactions to produce insoluble colored products for locating hybrids on solid phases. These have advantage of a simple visual read-out and a permanent record. Especially for mambrane-based assays.

(c) Electrochemilunescence

In this process, an electrochemical reaction produces excited-state species that decay to produce a ground state product and light. A disadvantage of this detection reaction is the need for specialized equipment that combines electrochemical-generation and light-detection capabilities.

(d) Fluorescence and Time-Resolved Fluorescence

Fluorescence measurement are capable of detecting single molecules of fluorescien[94]. In practice, however, fluorescence is plagued by background fluorescented to be short-lived, it can be avoided by using a long-lived fluorophore that is excited by a rapid pulse of excitation light. The fluorescence emission is then measured after the short-lived background has decayed, thus eliminating interference.

EPIDEMIOLOGY

Tuberculosis is the world's second commonest cause of death from infectious disease, after HIV/AIDs. There were an estimated 8-9 million new cases of tuberculosis in 2000, fewer than half of which were reported; 3-4 million cases were sputum-smear positive, the most infectious form of the disease [95]. Most cases (5-6 millions) are in people aged 15-49 years. Sub-Saharan Africa has the highest incidence rate (290 per 100,000 population), but the most of cases: India, China, Indonesia, Bangladesh, and Pakistan together account for more than half the global burden, 80% of new cases occur in 22 high-burden countries [96]

Before the advent of the AIDS epidemic, disease caused by nontuberculous mycobacteria was pulmonary, confined to cervical lymph nodes, limited to skin, or, in rare cases, disseminated [97]. The picture of nontuberculous mycobacterial disease has been radically changed by emergence of the AIDS epidemic throughout the world. Some 25 to 50% of patients with AIDS in the United States and Europe are infected with nontuberculous mycobacteria [98-102]. The rise in the incidence of nontuberculous mycobacterial disease has accelerated rapidly since the first reports of nontuberculous mycobacterial disease in AIDS patients in 1982 [103]. In the United States and Europe, where the incidence of tuberculosis is low, the incidence of nontuberculous infections is high in AIDS patients [98-102, 104]. In addition to the fact that mycobacterial disease in AIDS patients is usually disseminated, there has been a change in the distribution of mycobacteria causing disease. Disease in AIDS patients is caused principally by *M. avium*.

In King Chulalongkorn Memorial Hospital, there was an increased incidence of NTM infection. In 2002, a total of 777 (23.7%) mycobacteria were isolated from 3,278 clinical specimens. Out of these 777 isolates, 485 (62.42%) were *M. tuberculosis*, 292 (37.58%) were NTM. These NTM could be differentiated to be *M. avium* complex, *M. kansasii, M. gordonae, M. chelonae, M. flavescens, M. abscessus, M. scrofulaceum, M. fortuitum*, and unidentified NTM at the number of 189 (64.72%), 15 (5.14%), 11 (3.77%), 9 (3.08%), 9 (3.08%), 4 (1.37%), 4 (1.37%), 1 (0.34%), and 50 (17.12%), respectively.

When only hemocultures was considered, a total of 174 (14.73%) mycobacteria were isolated from 1,181 specimens. Out of these 174 isolates, 32 (18.39%) were *M. tuberculosis* and 142 (81.61%) were NTM. These NTM could be differentiated to be *M. avium* complex, *M. gordonae*, *M. flavescens*, and unidentified NTM at the number of 125 (88.03%), 3 (2.11%), 2 (1.41%), and 12 (8.45%), respectively.