CHAPTER II

LITERATURE REVIEWS

1. General characteristics of *Mycobacterium avium*

Mycobacterium avium was first described by Chester (1901). There are three subspecies including *M. avium* subsp. *avium, M. avium* subsp. *paratuberculosis,* and *M. avium* subsp. *silvaticum* (Chester, 1901; Thorel *et al*, 1990). *M. avium* is straight or slightly curved rod-shaped bacilli, 0.2-0.6 x 1.0 µm. It is non motile, non spore forming, without capsule, weakly gram positive, aerobic or microaerophillic, and no true branching (Wayne & Kubica, 1986). It can be stained with classical carbol fuchsin method. The staining techniques are based on the resistance of the mycobacteria to decolorization by acid or a mixture of acid and alcohol. Hence the term is called "acid-alcohol fast". The basis of the acid-alcohol fast staining is not clearly understood, but appears to be related to the presence of mycolic acids in the cell wall, to the integrity of the cell and to the viability of the cell (Kölbel, 1984).

1.1 The cell wall and envelope

Essentially, the cell envelope of mycobacteria can be divided into four major layers. The first or the innermost layer is the cytoplasmic or plasma membrane. Similar to those found in other bacteria, this layer consists of a permeable lipid bilayer with interacting proteins. The second layer is sometimes called the electron dense layer because of its staining properties when observed by transmitted electron microscopy. In this area, peptidoglycan alabinogalactan moieties that make up the basic structural component of the cell wall are found. The next layer is electron transparent layer. One of the primary components in this layer is mycolic acid. The outer layers of *M. avium*, the predominant superficial components are the serovar-specific glycopeptidolipid antigen (GPL) (Barrow *et al*, 1995). Also the alabinomannan extension of lipoalabinomannan (LAM) is found in this layer. The internal phosphatidylinosital portion of LAM is either anchored in the plasma membrane or positioned in the outer layer.

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1.2 The lipid activity

The total extractable lipid derived from *M. avium* can interfere with the capacity of human peripheral blood mononuclear cell (PBMC) to proliferate in response to concavalin A (ConA), purified protein derivative of tuberculin (PPD), and phytohemagglutinin (PHA) stimulation (Tsuyuguchi *et al*, 1990). In addition, the lipid fractions can induce prostaglandin E2 (PEG2) and tumor necrotic factor (TNF)-alpha that stimulated the production of interleukin (IL)-12 (Barrow *et al*, 1995). A recent study, using the Mono Mac 6 monocytic cell line, shows that the total extractable lipid and GPL can induce the inflammatory cytokines (i.e., TNF-alpha, IL-1, and IL-6), PEG_2, and TXB_2 (Davis *et al*, 1996).

Apparently, there is not any study describing the isolation and characterization of LAM from *M. avium*. It will be assumed that the location of LAM in *M. avium* is similar to that of other mycobacteria. LAM from *M. tuberculosis* and *M. leprae* have a variety of biological activities; including strong serologic activity, inhibition of gamma-interferon-mediated activation of macrophages (Sibley *et al*, 1988), induction of cytokine production and release by macrophages, suppression of T-cell proliferation (Kaplan *et al*, 1987), inhibition of mRNA synthesis encoding IL-2, IL-5, and GM-CSF in the human Jurket T-cell line (Chujor *et al*, 1992).

2. Molecular biology of *Mycobacterium avium*

The genome of mycobacteria has been found to consist of a single circular DNA molecule. It is not contained by a nuclear membrane. The molecular and recombinant DNA analyses of mycobacterial genomes reviewed that: (i) the genomes of most species of mycobacterium contain repetitive DNA elements, (ii) mycobacteria have only one or two copies of the genes encoding the ribosomal RNAs, and (iii) the genomic DNAs of *M. leprae* and *M. tuberculosis* display little variation in strains isolated from around the world. Most of mycobacteria have large genomes, in the range of 2.8×10^6 to 4.5×10^6 base pairs (Clark-Curtiss, 1990). The DNAs of most mycobacteria have between 64 and 70 mol% guanine (G) plus cytosine (C).

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Repetitive DNA elements

The repetitive DNA elements or insertion sequences (IS) are the simplest types of transposons. They vary in length from 780 bp to 2.5 kb. They encode only the functions required for their transposition, including a transposase, which catalyzes this reaction. In general, they are flanked by inverted repeat (IR) sequences 10 to 40 bp which bind the transposase and are required for transposition. The insertion sequences (IS) have been identified in *M. avium* such as IS*900*, IS*901*, IS*902*, IS*1110*, **IS*1245*** and IS*1311* (McAdam *et al*, 2000).

The IS*900*, IS*901*, IS*902* and IS*1110* belong to the IS*110* family of insertion sequence. The IS*901* insertion element has a nucleotide sequence of 1,422 bp with an open reading frame (ORF1), which encodes a protein of 401 amino acids. It was also determined that the terminal ends and target sites of IS*901* were similar to those of the IS*900* insertion element of *M. paratuberculosis*, while the DNA sequence of both elements exhibited only 60% homology (Green *et al*, 1989). IS*901* was found in pathogenic strains of *M. avium* but absent in *M. avium* isolated from patients with AIDS (Kunze *et al*, 1991).

The **IS*1245*** is a 1,414 bp long and contain 40-bp imperfect inverted repeat with an open reading frame (ORF) and encodes a protein of 410 amino acids. This insertion sequence exhibits a high degree of homology (64% amino acid similarity) with *M. bovis* IS*1081*. Both elements belong to the *Staphylococcus aureus* IS*256* family of insertion sequences (Guerrero *et al*, 1995). Also IS*1311*, a 1,317 bp long, showed 85% homology at DNA level with IS*1245* (Roiz *et al*, 1995).

These insertion sequences have been proposed as possible epidemiological tools to type and distinguish isolates of the different groupings within the *M. avium*. On the basis of **RFLP typing**, determination of the host range of IS*1245* is limited to *M. avium* (Bono *et al*, 1995; Guerrero *et al*, 1995). Devallois and Rastogi (1997) showed that the highly similar IS*1245* and IS*1311* possess a similar discriminatory potential for *M. avium* isolates.

Highly polymorphic multibanded IS*1245* RFLP patterns were almost invariably found among *M. avium* isolates from humans. A significant part of the IS*1245* DNA fingerprints of *M. avium* isolated from pigs shared a high degree of similarity with the human isolates. In contrast, isolates from a wide variety of bird species were found to possess identical three-band patterns (Bono *et al*, 1995; Ritacco *et al*, 1998). The three-band pattern found in birds was also found in

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small fraction of the pig isolates. As this pattern was only rarely encountered among human isolates so, birds were not to be an important source of *M. avium* infections in humans (Ritacco *et al*, 1998).

In vivo, the IS*1245* banding pattern exhibited by isolated from the same patient over time is quite stable (Pestel-Caron & Arbeit, 1998). Similarly, *in vitro*, only one- to two-band variations were observed in four out of six-*M. avium* strains which were subcultured in liquid media over a period of one-year (Bauer & Andersen, 1999).

3. Classification

3.1 Conventional Criteria

M. avium is classified as Runyon group III, non photochromogen, acid-fastness and slowly growing bacilli that sometime may produce a yellow pigment in the absence of light (Table 1) (Runyon, 1959; Berlin, 1990). Tuberculosis, caused by mycobacteria in the *M. tuberculosis* complex, has been traditionally viewed as the "typical" mycobacterial disease; thus, other species of mycobacteria (except for *M. leprae*) have been viewed by contrast as "atypical". Consequently, mycobacteria other than *M. tuberculosis* and *M. leprae* have been commonly referred to by the imprecise and taxonomically inappropriate term atypical mycobacteria. Other terms commonly applied to these mycobacteria are mycobacterium other than tuberculosis, or MOTT, nontuberculous mycobacteria (NTM), and potentially pathogenic mycobacteria (PPEM) (Wayne & Sramek, 1992).

3.2 Colony Variant Types

M. avium is one of *Mycobacterium avium* complex (MAC) that was classified as slowly growing mycobacteria (the colonies appear in more than 7 days). Colonies of MAC strains have been described: (i) a smooth, opaque, and dome type; (ii) a smooth, transparent, and flat type; and (iii) a rough type. The colonial morphology appear to be related to the chemical structure of their surface antigens, exopolysaccharides and cell wall (Belisle & Brennan, 1994), and the virulence of them may be associated with the presence of a surface glycopeptidolipid. Woodly and David (1976) showed that the rate of transparent-to-opaque transition was 1.6×10^{-4}, while the rate of opaque-to-transparent was about 10^{-6} per bacterium per generation. The colonies

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of MAC that were isolated from AIDS patients with disseminated disease are frequently exclusively of the smooth transparent type, but the opaque and the rough type develop in subcultures. The nonpigmented colonies were significantly more resistance to antimicrobial agents than the pigmented (Stormer & Falkinham, 1989).

3.3 Serotypes

The MAC is a serological complex of 28 serovars of two species including *M. avium* and *M. intracellulare*. MAC may be typed by using a seroagglutination assay described by Schaefer (1965). Currently the serovars 1 through 6, 8 through 11 and 21 are recognized as *M. avium*, 7, 12 through 20 and 23 through 25 as *M. intracellulare* while serovars 41 through 43 are designated *M. scrofulaceum*. The status of serovars 26 through 28 is not clear at present (Thierry *et al*, 1993). Serotyping is based on the presence of specific oligosaccharide haptens (Brennan & Goren 1979; Brennan *et al*, 1981; McNeil *et al*, 1987) that form the sugar moiety of glycopeptidolipids (GPL), located on the cell surface of smooth-colony-forming strains. Rough colony variants are not amenable for serotyping because they agglutinate spontaneously. However, their GPL can be extracted and analyzed by thin layer chromatography (TLC) procedures (Brennan & Goren, 1979).

3.4 Multilocus Enzymatic Electrophoresis Types

The multilocus enzyme electrophoretic typing of 35 strains of the MAC, using 20 different enzymes, showed 24 electrophoretic types (ETs). The 24 ETs can be classified into 2 clusters: *M. avium* cluster and *M. intracellulare* cluster. The clustering agree entirely with the species identity as determined by the GenProbe nucleic acid hybridization system. The electrophoretic type of *M. paratuberculosis* strain joined to the *M. avium* cluster and the electrophoretic type of *M. scrofulaceum* strain joined to the *M. intracellulare* cluster (Wasem *et al*, 1991). The characterization of *M. avium* serotype 4 and 8 from AIDS patients showed one major ET within each serotype and these two ETs were closely related (Yakrus *et al*, 1992). The ETs of *M. avium* isolates from soil were found to be similar to isolates recovered from AIDS patients (Yajko *et al*, 1995) but in animals only some porcine isolates belonged to the same ETs as certain human isolates (Feizabadi *et al*, 1996).

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3.5 Plasmid Types

At least three types of plasmids were isolated from *M. avium* strains including pLR7 (Crawford & Bates 1984), pLR20 (Hellyer *et al*, 1991) and pVT2 (Jucker & Falkinham 1990). Plasmid typing may be similarly limited for epidemiology studies. However, there is evidence that there may be an epidemiologically significant uneven distribution of MAC strains, both clinical and environmental, which carry plasmids.

Crawford and Bates (1986) showed that all strains of MAC, which isolated from AIDS patients, carried plasmid that hybridized to recombinant molecules carrying fragments of a small plasmid (pLR7) derived from serotype 4 strain of the MAC. However, the observation in Europe found that 5 of 16 MAC isolates from AIDS patients in Denmark carried plasmids (Jensen *et al*, 1989) and there was no difference in the rate of plasmid carriage from AIDS and non-AIDS patients in United Kingdom (Hellyer *et al*, 1991). The role of plasmids in the biology and pathogenicity of the MAC may be important because of the association of plasmids with virulent factors (Gangadharam *et al*, 1988; Pethel & Falkinham 1989) and with antibiotic resistance (Franzblau *et al*, 1986; Mizuguchi *et al*, 1981).

3.6 Phage types

Although phage typing has proven to be a useful tool for discriminating between strains of *M. tuberculosis* (Snider *et al*, 1984), there has been only a limited application of phage typing to the epidemiology of the MAC. Crawford *et al.* (1981) showed that only approximately one-third of *M. avium-M. intracellulare-M. scrofulaceum* in their study were susceptible to the mycobacteriophages tested. Later, Crawford and Bates (1985) pointed out that several factors can influence the susceptibility of mycobacteria to phage infection, including a requirement for accessible cell surface receptors, lysogenic immunity, the presence of a restriction-modification system, and plasmid interference.

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3.7 Polymerase chain reaction (PCR) types

The primers, PA (5'-CAGAGCCTCAGGCGA-3') and PB (5'-CAGAGCCTCACGC GGA-3'), were designed by Picardeau and Vincent (1996) to hybridize to an inverted repeat (IR) found in both IS*1245* and IS*1311*, and the PCR was designed to amplify DNA fragments between

copies of both of these insertion sequences (IS). The result of *M. avium* isolated from AIDS and non-AIDS patients showed that the banding patterns consisted of less than 10 bands. The unrelated strains showed different patterns and related strains displayed identical patterns, excepted the isolates from one patient. The authors suggest that the occurrence of subsequent or polyclonal infections.

By using the primers, the study of a PCR typing method for the comparison of clinical isolates and food isolates of *M. avium* establishes a possible relatedness between the isolates. Then, the study suggests that food is a potential source of *M. avium* (Yoder *et al*, 1999).

3.8 Pulsed-field gel electrophoresis (PFGE)

Pulsed-field gel electrophoresis (PFGE) is often considered as the "gold standard" of molecular typing methods. In which restriction enzymes that cut genomic DNA infrequently, are used to produce a small number of very-large-molecular-length DNA fragments ranging from 10 to 800 kb (Schwartz & Cantor, 1984). Many studies that define the banding patterns of *M. avium* have been conducted. The studies of genetic diversity among strains of *M. avium* in AIDS patients, using PFGE, showed each patient was infected by a unique strain (Arbeit *et al*, 1993). Although, PFGE analyses show a high discriminatory power (Picardeau *et al*, 1997; Pestel-Caron & Arbeit, 1998; Garriga *et al*, 2000; Legrand *et al*, 2000), it is time consuming and expensive apparatus.

3.9 Restriction fragment length polymorphism (RFLP) analysis

The RFLP analysis has been used extensively in epidemiological investigations of mycobacteria. This technique is based on digestion of whole chromosomal DNA with a restriction enzyme, and the fragments are separated by electrophoresis through an agarose gel. The separated DNA fragments are transferred from the agarose gel to either of a nitrocellulose or nylon membrane by Southern blotting (Southern, 1975). The membrane-bound nucleic acid is then hybridized to labeled probes homologous to the interested gene. Probes can be labeled with a number of detectable moieties, including enzyme-colorimetric substrates or enzyme-chemiluminescent substrates. This classical method has been adapted to differentiation of bacterial strains on the basis of the observation that the location of various restriction enzyme

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recognition sites within a particular genetic locus of interest can be polymorphic from strain to strain, resulting in gel bands that differ in size between unlike strains. Thus, the name restriction fragment length polymorphism (RFLP) refers to the polymorphic nature of the locations of restriction enzyme sites within defined genetic regions. Only the genomic DNA fragments that hybridize to the probe are visible in RFLP analysis, which simplifies the analysis greatly.

In 1995, Restriction fragment length polymorphism (RFLP) was used by Roiz *et al* to study 75 clinical isolates identified as *M. avium*. Two repetitive insertion sequences, IS*1311* and IS*900*, were used as DNA probes. The study reveals that less than 25% of isolates showed RFLP patterns with IS*900*, whereas, IS*1311* can totally differentiate. The study also shows that *M. avium* strains isolated from patients with AIDS exhibited marked polymorphism with both probes. In the same year, Guerrero et al. had described a novel insertion element, IS*1245*. The insertion element appears limited to *M. avium*, as this element was not identified in any other of mycobacteria species tested. The chromosome of *M. avium* strains isolated from human source show a high number of copies of IS*1245*, in range of 3 to 27(median, 16). The RFLP pattern of swine isolates shared the characteristics of human isolates; those from several avian sources exhibited a very low copy number of IS*1245*.

Picardeau and Vincent (1996) compared the PCR typing to RFLP of *M. avium* isolated from AIDS and non-AIDS patients. The RFLP study showed that the average IS*1245* copy number for the strains was 20, ranging from 1 to 27 copies. There is only one strain presented a single-band pattern. The strains that isolated from 18 patients produced different RFLP patterns. The study also showed the stability of IS*1245* distribution. On the other hand, the two distinct RFLP patterns were found in a patient that might be the occurrence of subsequent or polyclonal infections. The results of PCR typing were as discriminant as the results of RFLP analysis.

In 1997, Picardeau *et al* studied the characteristics of 93 M. *avium* strains isolated from blood of AIDS patients. The average IS*1245* copy number per strain was 17 and the range was 1 to 27 copies. The result is that IS*1245*-RFLP analysis showed 95.7% diversity. The diversity increased to 96.8% if the PFGE was also used.

In the year later, Ritacco *et al* investigated the IS*901*- and IS*1245*- RFLP typing of *M. avium* complex including; serovar reference strains, human and animal isolates. The 22 different patterns were observed among the 37 *M. avium* reference strains which were positive for IS*1245*,

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while three others lacked this element. In animal isolates, the IS*1245* DNA fingerprint of all 29 bird isolates invariably displayed the three-band pattern found among serovar 1, 2 and 3. The pattern is referred to as 'bird' type. A wide diversity of patterns was observed among 21 swine isolates. Only 2 isolates from pig lacked IS*1245*. Fifty-four isolates from patients displayed multibanded IS*1245* patterns. Most of these carried more than 20 IS*1245* copies, 8 isolates had 2 to 10 copies and only one clinical isolate that corresponded to the 'bird' type. The presence of IS*901* was found in only one isolate from patient. In the same year, Pestel-Caron and Arbeit differentiated the *M. avium* strains isolated from AIDS patients and their environment. By using IS*1245* as a probe, it was found that all 40 strains carried DNA homologous to IS*1245* and therefore were typeable. Twenty-five (63%) strains had ≥ 10 copies of the element, 6 (15%) had 4 to 9 copies, and 9 (23%) had only 1 to 3 copies.

Later, van Soolingen *et al* proposed the standard of IS*1245*-RFLP typing involved the following issues: the choice of restriction enzyme, the electrophoresis conditions, the preparation of the probe, the hybridization stringency and the use of molecular size of DNA. So that facilitates the establishment of databases of *M. avium* fingerprints and help to trace true sources of infection of this emerging potential pathogen. In the end of the year, the study of Lari *et al* showed that all but 2 of 63 *M. avium* isolates from Italian exhibited marked polymorph, multibanded IS*1245*-RFLP patterns; 2 isolates showed the low-number banding pattern typical of bird isolates. By computer analysis, 41 distinct IS*1245* patterns and 10 clusters of essentially identical strains were detected; 40% of the isolates showed genetic relatedness, suggesting the existence of a predominant AIDS-associated IS*1245*-RFLP pattern.

Bauer and Anderson (1999) studied the stability of IS*1245*. The result showed that the *M. avium* which was subcultured 33 times in liquid media exhibited identical or almost identical IS*1245* patterns. Only one- to two-band changes was observed in the patterns of 6 *M. avium* strains.

The comparison of IS*1245*-RFLP pattern of *M. avium* isolated from porcine and human showed no identical DNA fingerprints. Sixty percent of the isolates from both sources had a similarity of at least 75% among the IS*1245*-RFLP patterns (Komijn *et al*, 1999).

The RFLP analysis of *M. avium* isolates using probes derived from the insertion sequences IS*901*, IS*1245* and IS*1311* showed that 42 of the deer isolates and 2 of bovine isolates

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contained IS*901*, while this insertion sequence was absent from all of human and porcine isolates. All of the IS*901*-positive isolates had a characteristic three-band IS*1245* hybridization pattern and a characteristic single-band of IS*1311* hybridization pattern. The IS901-negative isolates exhibited highly polymorphic of IS*1245* and IS*1311* hybridization patterns, which differentiated the human and porcine isolates into a wide diversity of strain types (O'Grady *et al*, 2000).

4. Pathogenesis

M. avium is ubiquitous in nature. This microorganism is frequently isolated from water, plants and soil, and distributed throughout the world. In the pre-HIV era, *M. avium* complex was occasionally responsible for localized pulmonary infections. The infections are diagnosed in patients with predisposing lung condition such as pneumomococcosis, silicosis, cured tuberculosis and chronic obstructive lung diseases (Falkinham III, 1996; Indeelied *et al*, 1993). *M. avium* has been identified as causing disease in patients who uptake alcohol (Runyon, 1974). In additional, *M. avium* infection has been increased in middle aged women most of whom have structural changes in the chest (Iseman, 1989). Lymphadenitis in children is also frequently caused by *M. avium* complex (Wolinsky, 1979). Advent of AIDS, *M. avium* is associated with disseminated disease in 40-50% of the patients with less than 50 $CD4^+$ T cells/mm^3 of blood.

4.1 Attachment and invasion of mucosal surfaces

While in AIDS patient's current evidence suggests that the majority of infected patients acquired *M. avium* through the intestinal tract, on the other hand, in non-AIDS patients, the most likely route of infection is the respiratory tract. In both cases, *M. avium* comes in contact with the host's mucosa before establishing infection. Once in the alveolar space, *M. avium* can colonize and infect both alveolar macrophages and type II alveolar epithelial cells (Bermudez & Goodman, 1996) and potentially can use this route to translocate across the mucosal barrier. Nonetheless, the pathway used by *M. avium* to infect epithelial cells is presently unknown.

Colonization of the intestinal tract by *M. avium* is common finding in a large number of patients with AIDS. Colonization has been shown to precede bacteremia by several months (Toriani *et al*, 1995) and specifically in this group of patients, *M. avium* is found in the lamina

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propia infecting submucosal macrophages (Damsker & Bottone, 1985). *M. avium* interacts with the intestinal brush border by an uncharacterized manner and subsequently establishes contact with the epithelial cell membrane. In additional, host signal transduction mechanisms appear to be necessary for bacterial internalization. A recent study showed that the ability of *M. avium* to invade intestinal cells is controlled by environmental factors. A high osmolarity, low oxygen tension and temperature in the environment significantly increase the ability of *M. avium* to invade intestinal epithelial mucosal cells (Bermudez & Young, 1994; Bermudez *et al*, 1997). The release of chemokines by epithelial cells does not immediately follow inversion by *M. avium*. Its secretion appears to be only delayed from two to three days in oropharyngeal cells but is completely suppressed in HT-29 intestinal epithelial cells (Sangari *et al*, 1999). The manipulation of the host immune response by blocking chemokine production may be explain the lack of inflammatory response in the intestinal wall during the first day after oral ingestion of the organisms (Kim *et al*, 1998). The observation by electron microscopy suggests that the *M. avium* can enter the intestinal mucosa by crossing either enterocytes or M cells (Hsu *et al*, 1996).

4.2 Interaction with phagocytes

Mycobacteria are facultative intracellular pathogens that characteristically reside within mononuclear phagocytes. The interaction of *M. avium* with macrophages is a typical example for a bacterium subverting host defenses on several levels. This phenomenon is the interaction of *M. avium* with receptors on the macrophage membrane. *M. avium* appears to recognize a number of receptors on the surface of macrophages and *M. avium* that was uptaked by monocytes and macrophages has been associated with the presence of integrins, such as complement receptors (CR3, CR4 and CR1), and vitronectin receptor (Bermudez *et al*, 1991; Roecklein *et al*, 1992; Rao, 1992).

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4.3 Intracellular lifestyle

Following phagocytosis by macrophages, *M. avium* resides in a membrane-bound vacuole does not acidify and never expresses proton pump ATPase (Sturgill-Koszycki *et al*, 1994; Frehel *et al*, 1986). The pH of the *M. avium* endosome is approximately 6.5 to 6.8. The intracellular environment certainly has great influence on *M. avium* resistance to killing

mechanisms. The mechanisms by which activated macrophages kill *M. avium* are poorly understood. Although, all the evidence to date indicates that the *M. avium* phagosome never fuses with lysosomes. The *M. avium* strains vary in their susceptibility to oxygen radicals. While most of the AIDS isolates seem to be resistant to superoxide and hydrogen peroxide production, some isolates have shown to be at least partially susceptible to reactive oxygen intermediates (Bermudez & young, 1989; Sarmento & Appelberg, 1996). Because *M. avium* expresses a superoxide dismutase (Mn-SOD) encoded by *sod*A (Escuyer *et al*, 1996), *M. avium* can synthesize a superoxide dismutase of 23-25 KDa that can inactivate macrophage derived superoxide anion. Additionally, *M. avium* contains a functional *oxy*R gene which is a regulator responsible for inducing the expression of several genes involved in the response to oxidative stresses. Although, the majority strains of *M. tuberculosis* seem to be susceptible to nitric oxide produced by phagocytic cells, in case of *M. avium*, nitric oxide has no apparent role in a host defense against the bacteria (Doherty & Sher, 1997).

Bactericidal proteins produced by phagocytic cells have been investigated as a potential mechanism of *M. avium* killing. Results from the studies using rabbit defensins, small bactericidal proteins produced by rabbit neutrophils, support the concept that *M. avium* may be susceptible to those proteins (Ogata *et al*, 1992). However, no effect of human-derived bactericidal proteins has been demonstrated yet.

5. Clinical disease (presentation) and Diagnostic criteria

5.1 Patients without AIDS

5.1.1 Pulmonary disease

The first case of human disease due to *M. avium* was reported in 1943 in a miner from Minnesota in what became a classical description of pulmonary disease. The disease predominantly involves middle age white males with preexisting pulmonary disease (Etzkorn *et al*, 1986; Wallace *et al*, 1990) but there is tremendous variation in the sex, age, and race of these patients. Predisposing conditions such as chronic obstructive pulmonary disease (COPD), bronchiectasis, chronic aspiration or recurrent pneumonia, inactive or active tuberculosis, pneumoconiosis, and bronchogenic carcinoma are present in 54 to 77% of patients with pulmonary MAC disease (Etzkorn *et al*, 1986).

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The symptoms are varied and nonspecific, commonly including chronic productive cough, sputum production, dyspnia, sweats, malaise, fatigue, and less commonly hemoptysis. Fever and weight loss are not common but may occur. Approximately 75% of patients have evidence of cavitary infiltrate on chest roentgenograms, typically involving the apical and anterior segments of upper lobes, but dense unilobular or multilobular infiltrates, diffuse interstitial or reticulonodular infiltrates, or a solitary pulmonary nodule may occur (Christensen *et al*, 1979; Gribetz *et al*, 1981; Marchevsky *et al*, 1982; Prince *et al*, 1989).

MAC organisms may be isolated from the sputum in the absent of apparent disease, particularly in the patients with chronic respiratory disorders; such low-grade infection or colonization is more common than true disease. Guidelines have been suggested for distinguishing patients with NTM lung disease from patients who are colonized. NTM disease in patients with noncavitary infiltrates can be assumed to be present when (i) two or more sputum or bronchoalveolar wash specimens are smear positive and/or result in moderate to heavy growth in culture; (ii) sputum cultures fail to revert despite good pulmonary toilet or two weeks of antimycobacterial therapy; and (iii) reasonable attempts fail to identifying causes of disease (Wallace *et al*, 1990). It is important to note that these guidelines may not apply to immunodeficient patients.

5.1.2 Lymphadenitis

Infection of upper anterior cervical, submandibular, submaxillary, and pre-auricular lymph nodes in children between 1 and 5 years old is the most common presentation of NTM lymphadenitis (Lincoln & Gilbert, 1972; Schaad *et al*, 1979; Wolinsky, 1995). It is the most common disease manifestation of MAC in children and, in the absence of HIV infection, rarely affects adults. The disease occurs insidiously, with only rare associated systemic symptoms. The involved lymph nodes are generally unilateral (95%) and not tender. The nodes may enlarge rapidly, and even rupture, with formation of sinus tracts that result in prolonged local drainage. Other nodal groups outside of the head and neck may be involved occasionally (Wolinsky, 1995). There is typically no history of exposure to tuberculosis, screening PPD skin test of family members are usually negative, and the chest radiograph is normal.

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Most children with NTM lymphadenitis will react to skin test antigens prepared from *M. avium* complex, such as PPD-B (Schaad *et al*, 1979; Del Beccaro *et al*, 1989; Huebner *et al*, 1992). A study of NTM antigens from the CDC that used PPD-B, however, was terminated early, due to a blistering in several of the children (Huebner *et al*, 1992). More recent studies using a less potent, protein weight-standardized *M. avium* skin test material called "sensitin" and a dual skin test technique to determine *M. avium*-dominant versus PPD-dominant reactions have suggested to improve specificity with this antigen preparation in studied populations with known disease (von Reyn *et al*, 1994).

The utility of fine needle aspiration in obtaining diagnotic material is controversial (Baily *et al*, 1985; Lau *et al*, 1991; Gupta *et al*, 1993). However, granulomata or other compatible cytopathology such as a mixture of degenerating granulocytes, lymphocytes, and epithelioid histiocytes are seen in most cases. A positive culture may be obtained in up to 50% of HIV-seronegative patients.

A definite diagnosis of NTM lymphadenitis is made by recovery of causative organism from lymph node cultures. A simple diagnostic biopsy or incision and drainage of the involved lymph nodes should be avoided, since most of these procedures will be followed by fistulae formation with chronic drainage. However, even with excised nodes with compatible histopathology, only about 50% will yield positive cultures (Schaad *et al*, 1979), although the recovery rate may be as high as 82% (Wolinsky, 1995). Currently, approximately 80% of culture-proven cases of NTM lymphadenitis are due to *M. avium* complex (Lai *et al*, 1984). Now in the United States, only about 10% of the culture-proven mycobacterial cervical lymphadenitis in children is due to *M. tuberculosis* ; the remainder is due to *M. avium* complex and *M. scrofulaceum*. In contrast, in adults more than 90% of the culture-proven mycobacterial lymphadenitis is due to *M. tuberculosis*.

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5.1.3 Disseminated infection

Disseminated infection with the MAC was extremely unusual prior to the AIDS epidemic. Typically, the disease occurred in individuals with underlying malignancy or inherited or therapeutic immunodeficiency, especially children and young adults with hematogenous

malignancy or severe combined immunodeficiency syndrome, transplant recipients, and patients receiving cytotoxic chemotharapy or corticosteroids (Stone *et al*, 1992).

The most frequent presentation of disseminated infection in the immunocompromised host is fever of undetermined etiology. Dissemination may involve any organ system but, most commonly, the lungs and large airways, the mononuclear phagocyte system including the liver, spleen, and retroperitoneal nodes, the gastrointestinal tract, the skeletal system, and the skin. The isolation of organisms from sterile, closed sites such as bone marrow or blood or from skin biopsy (in the setting of multiple lesions) is diagnostic of the disease.

5.1.4 Unusual sites of infection

Chronic granulomatous infection caused by NTM may develop in tendon sheaths, bursae, joints, and bones after direct inoculation of the organisms through accidental traumas, surgical incisions, puncture wounds, or injections. *M. avium* complex are particularly prone to causing tenosynovitis of the hand (Hellinger *et al*, 1995). In only 15% of the cases can the diagnosis be made culture of joint aspirate and surgical biopsy, and culture of synovial material is necessary for diagnosis in most cases. With preservation of joint function, the majority of cases respond to a combination of surgical excision of infected material and antituberculous chemotherapy (Eggelmeijer *et al*, 1992).

5.2 Patients with AIDS

5.2.1 Focal disease

Some patients with AIDS may present with focal pulmonary infection due to *M. avium* without evidence of dissemination. The clinical presentation is similar to that of immunocompromised hosts but is generally minder than tuberculosis (Modilevsky *et al*, 1989). The pattern of radiographic involvement is varied. In contrast to non-AIDS patients with pulmonary MAC infection, cavitary disease is unusual (<5%). The thick pleural reaction often seen in normal hosts with chronic pulmonary disease is not seen, and pleural effusions are rare.

MAC pulmonary disease may be clinically and radiographically indistinguishable from bacterial pneumonia or any pulmonary disease. Determination of the etiologic agent may be difficult, and more than one pathogen may be present. Despite the isolation of *M. avium* from

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cultures of sputum or bronchoalveolar lavage fluid, a careful search for other potential pathogens should be made. In a patient who has a single sputum or bronchoalveolar lavage culture positive for MAC, radiographic evidence of pulmonary infiltrative disease more likely signals the presence of a pathogen other than MAC organisms. Transbronchial biopsy or percutaneous needle biopsy may be necessary, but open lung biopsy should be considered in those patients in whom other measures have failed to reveal the diagnosis and in whom assessment suggests that the benefits outweigh the risks.

5.2.2 Disseminated disease

Disseminated disease due to the MAC in patients with HIV infection usually occurs only in those with very advanced immunosuppression. Because these patients frequently have other complications, the diagnosis of mycobacterial infection may be confused or delayed. The diagnosis is exceedingly rare in person with > 100 $CD4^+$ T cells per mm^3, and it should usually be suspected only in person with < 50 $CD4^+$ T cells per mm^3 (Nightingale *et al*, 1992; Nightingale *et al*, 1993; Hoover *et al*, 1995). Most patients (>90%) have prolonged fevers, which may be as high as 103-104°F, frequently accompanied by night sweats. Weight loss is common, and some patients complain of abdominal pain and diarrhea. Physical findings may be only those of advanced HIV disease, although abdominal or retroperitoneal adenopathy and hepatosplenomegaly may be present. Anemia is the most the striking laboratory abnormality, with many patients having a hematocrit of < 25%. Alkaline phosphatase is elevated in approximately one-third of patients and may be indicative of hepatic disease due to *M. avium*. Thus, the diagnosis of disseminated *M. avium* should be aggressively pursued in any person with < 50 $CD4^+$ T cells per mm^3 who has a history of fever, weight loss, anemia, diarrhea, or elevated alkaline phosphatase, especially in one with a history of other opportunistic infections.

The diagnosis of disseminated *M. avium* is most commonly confirmed by isolation of *M. avium* in blood. The bacteremia, with the organism found almost exclusively in circulating monocytes, occurs in 86 to 98% of patients with disseminated disease. Most patients have colony counts in the range of 10^1 to 10^3 CFU/ml of whole blood (Havlir *et al*, 1993). The tissue load of infection may be 10^2 to 10^5 times greater than that in the blood. While a few of patients have continuous low levels of mycobacteremia in their bone marrow and bloodstream, suggesting that

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they have, to a limited degree, control of the infection, intracellular replication within macrophages is unchecked in many patients.

The bacteremia in *M. avium* is ongoing, and a single culture has a sensitivity of approximately 90%. It is recommended that a single culture be drawn, with repeat cultures only if the first is negative. Routine blood cultures of asymptomatic patients has a very low yield and is not recommended. In a prospective study of HIV-infected patients with < 50 $CD4^+$ T cells, approximately 67% of Patients with *M. avium* in sputum or stool had disseminated disease within 1 year, although most did not develop pulmonary disease. However, only one-third of all patients with disseminated disease had a prior positive stool or sputum. Therefore, routine screening of stool or sputum is not indicated, but a positive culture of one of these sites needs to raise concern about future dissemination. Sputums that are smear-positive for the AFB in the setting of HIV should always be regarded as tuberculosis until proven otherwise, since *M. tuberculosis* is a common cause of pulmonary disease in HIV-infected patients and is more likely than *M. avium* complex to produce positive AFB smears (Inderlied *ed al*, 1993).

6. Treatment

6.1 Treatment of pulmonary *M. avium* complex disease

6.1.1 Drug treatment

Drug therapy for *M. avium* complex disease involves multiple drugs; therefore, the risk of drug toxicity is relatively high. Additionally, the optimal therapeutic regimen has yet to be established. Therefore, the treatment of *M. avium* complex disease may best be served by physicians experienced in pulmonary or mycobacterial diseases.

The empiric combination regimens including clarithromycin, ethambutol, and rifamycin (rifampin or rifabutin) make sputum conversion rates for pulmonary *M. avium* complex disease in adults patients to be able to tolerate the medications up to 90% (Dautzenberg *et al*, 1995; Wallace *et al*, 1996). Rifabutin is the preferred rifamycin because it is more active *in vivo* than rifampin against *M. avium* complex, but it may also produce more problematic adverse affects (uveitis, leukopenia). All untreated strains of *M. avium* complex are macrolide susceptible (clarithromycin MICs of 0.25 to 4.0 μg/ml), while microbiologic relapses associated with

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symptom recurrence reveal isolates with MICs of > 32 μg/ml. The isolates of *M. avium* complex that resist to clarithromycin are also cross-resistant to azithromycin (Heifets *et al*, 1993).

The newer macrolides are the choice for disseminated *M. avium* complex disease. Initial therapy for adult HIV-negative patients with *M. avium* complex disease (needing should) consists of a minimum three drug regimen of clarithromycin (500 mg twice a day) or azithromycin (250 mg/d or 500 mg three times a week) rifabutin (300 mg/d) or rifampin (600 mg/d), and ethambutol (25 mg/kg per day for 2 months followed by 15 mg/kg per day for x months). For patients of small body mass and/or an age over 70, clarithromycin at 250 mg twice a day or azithromycin 250 mg three times a week may better tolerated.

For extensive disease, intermittent streptomycin for the first 2 to 3 months of therapy may be considered in addition to above regimen. The exact dose of streptomycin in this multiple drug regimen will depend on patient's age and weight (Table 1). The patient and physician should be alert to signs and symptoms of streptomycin toxicity.

TABLE 1 Suggested doses of streptomycin relative to age and weight in patients with normal serum creatinine

Wight and Age	Initial Therapy	Maintenance therapy
≥ 50 kg and ≤ 50 yr.	1 g 5x/wk	1 g 3x/wk
< 50 kg and ≤ 50 yr.	500 mg 5x/wk	750 mg 2x/wk
> 50 kg and 50-70 yr.	500 mg 5x/wk	750 mg 2x/wk
> 70 yr.	750 mg 2x/wk	750 mg 2x/wk

(American Thoracic Society, 1997)

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A study using 12 months of culture negatively as the treatment endpoint observed no pulmonary disease relapses with a mean follow-up of 18 months (Wallace *et al*, 1996), while the second study, which used 7 to 9 months of culture negatively, resulted in no early pulmonary disease relapses with a mean follow-up of 7 months (Dautzenberg *et al*, 1995). Early relapses

with less than 10 months of culture negatively were seen in the first study. These initial studies suggest that culture negatively of 10 to 12 months while on a clarithromycin-containing regimen is adequate for most patients.

Acid-fast bacilli smears and cultures of sputum should be obtained monthly for pulmonary *M. avium* complex disease to assess response, then periodically after completion of therapy to evaluate possible relapse. The desired endpoint is negative sputum cultures; patients who respond to therapy should develop negative AFB smears and cultures. One or more cultures containing small numbers of *M. avium* complex organisms (single colony on solid media or positive liquid media cultures only) may occur after sputum conversion and should not necessarily be interpreted as indicative of treatment failure or relapse. Rather, these culture results should be interpreted in light of the patient's overall clinical status.

The role of immune therapy in patients who failed in drug therapy has not been established. Interleukin and gamma interferon have been used in selected patients, and some investigation in this area continues.

6.1.2 Surgical treatment

Patients whose disease is localized on one lung and who can tolerate resectional surgery might also be consider for surgery, if there has been poor response to drug therapy or if the patient's isolate has become macrolide resistant. For some patients successfully treated by surgical resection, the prognosis has been better than for patients treated medically, although these results predated the use of macrolide-containing regimen (Corpe, 1981; Moran *et al*, 1983). However, the bilateral nature of *M. avium* complex lung disease, the advanced age of the patients, and the frequency of underlying chronic lung disease have limited the number of patients who are good candidates for surgery.

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6.2 Treatment of localized extrapulmonary *M. avium* complex disease

6.2.1 Lymphadenitis

Excisional surgery without chemotherapy is the recommended treatment for children with NTM cervical lymphadenitis, including those with disease caused by *M. avium* complex. The success rate with this procedure is about 95% (Schaad *et al*, 1979). Incisional biopsy or the

use of antituberculosis drugs alone (without a macrolide) has frequently been followed by persistent clinical disease, including sinus tract formation and chronic drainage, and should be avoided. For children with recurrent disease, a second surgical procedure is usually performed. An alternative for recurrent disease or for children in whom surgical risk is high (risk of nerve involvement) may be the use of a clarithromycin multidrug regimen such as that use for pulmonary disease (Green *et al*, 1993; Stewart *et al*, 1994).

6.2.2 Skin, Tissue, and Skeletal disease

For adult patients with extrapulmonary, localized *M. avium* complex disease involvement skin, soft tissue, tendons and joints, and occasionally bone, a combination of Excisional surgery (or surgical debridement) and chemotherapy is usually performed. Whether a three-drug regimen alone in this setting would be adequate is not known. The optimal duration of treatment is also unknown, but drug treatment usually lasts 6 to 12 months.

6.3 Treatment of disseminated *M. avium* disease

Disseminated *M. avium* is associated with an increased mortality in patients with AIDS. In one study, the median survival was 134 days after the first positive blood culture, and only 13% of patients were alive at 1 year (Nightingale *et al*, 1992). Based on the increased morbidity and mortality associated with disseminated *M. avium*, prophylaxis should be strongly considered in high-risk patients and therapy should be offered to all patients with established disease.

At least three drugs are advisedly used. One of which should be clarithromycin (500 mg twice daily) or azithromycin (250 mg or 500 mg daily). Most investigators would use ethambutol as the second agent at a dose of 15 mg/kg per day, although consideration should be given to an initial course of 25 mg/kg for the first 2 months. Rifabutin has the best potential as the third agent. Use of rifabutin will be problematic, however, in patients also on protease inhibitors, given its induction of the cytochrome P-450 system that metabolizes all currently approved members of this drug class. Clofazimine has also been used, as has a quinolone, but neither seems to contribute much to the regimen, and clofazimine has been associated with a higher mortality in two comparative treatment trials (Chaisson *et al*, 1996). Amikacin (Baron &

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Young, 1986) and streptomycin are both active, and one or other should be considered for use in patients with severe symptoms due to *M. avium* complex, especially as part of initial therapy.

6.4 Prophylaxis of disseminated disease in AIDS

The incidence of disseminated *M. avium* can be reduced by prophylactic antimicrobials. Some studies show that rifabutin was demonstrated to be effective. *M. avium* bacteremia developed in 8% of patients receiving 300 mg of rifabutin daily and 17% of patients on placebo (Nightingale *et al*, 1993). Because rifabutin is highly active against *M. tuberculosis*, it is probable that daily use of rifabutin would also provide prophylaxis against tuberculosis. Active tuberculosis must be ruled out before initiating rifabutin prophylaxis in order to prevent the development of drug-resistant tuberculosis. Clarithromycin in a dose of 500 mg twice daily was effective in a controlled trial of adult patients in reducing the incidence of *M. avium* complex bacteremia from 16% in the placebo group to 6% in the treatment group (Pierce *et al*, 1996; Benson *et al*, 1996), while in a related trial it was shown to be more effective than rifabutin (Benson *et al*, 1996). Azithromycin at a dose of 1,200 mg once weekly, either alone or in combination with rifabutin, has also been shown to be in a clinical trial study (Havlir *et al*, 1996). The final selection of agents may depend on cost, tolerability, and potential drug interactions of the agents. Rifabutin should generally be avoided in patients on protease inhibitors because it markedly enhances their metabolism and reduces serum levels of the protease inhibitors. The US Public Health Service has advocated use of indinavir but not other currently available protease inhibitors (retonavir, saquinquir) with reduced dose rifabutin if both drugs are deemed essential.

The development of drug resistance during prophylaxis is also concern, and it has already been noted to occur with the use of clarithromycin (Pierce *et al*, 1996; Benson *et al*, 1996) or azithromycin (Havlir *et al*, 1996) as monotherapy, but not the rifabutin monotherapy (Nightingale *et al*, 1993) or azithromycin when combined with rifabutin (Havlir *et al*, 1996). Because of very high risk of disseminated *M. avium* in persons with advanced HIV infection, prophylaxis should be offered to all patients with < 50 $CD4^+$ T cells per mm^3, especially in patients with a history of opportunistic infection.

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6.5 Toxicity monitoring

Monitoring of patients for toxicity, given the number of drugs and the older of these patients, is essential. Monitoring should include visual acuity (ethambutol and rifabutin), red-green color discrimination (ethambutol), liver enzymes (clarithromycin, azithromycin, rifabutin, rifampin, isoniazid, ethionamide) (Brown *et al*, 1995), renal function (streptomycin and amikacin), auditory and vestibular function (streptomycin, amikacin, clarithromycin, azithromycin), leukocyte and platelet counts (rifabutin) (Shafran *et al*, 1994; Griffith *et al*, 1995), and the nervous system (cycloserine). Patients who receive both macrolide and rifabutin must be monitored for the development of toxicity related to the interaction of these drugs (Shafran *et al*, 1994; Griffith *et al*, 1995). Clarithromycin enhances rifabutin toxicity (especially uveitis). Compared to rifabutin, clarithromycin serum drug levels will be lower reduced by rifampin.

7. Laboratory diagnosis

7.1 Isolation

Several methods can be used to culture *M. avium* from many kinds of specimens. For example, the principles and practices of culturing *M. tuberculosis* were update in 1993 by the CDC, with these methods having proved very effective for NTM species.

The most sensitive laboratory diagnosis requires the use of at least two culture systems including both solid and liquid media. Use of solid media as the primary or sole culture is no longer recommended by CDC because the greater recovery rate and more rapid recovery of all mycobacteria are given in rapid broth system. The methods used for digestion and decontamination of clinical samples in order to eleminate other bacteria and yeast prior to culture for recovery *M. tuberculosis* have also proved useful for the NTM. However, NTM are more susceptible to killing by NaOH, and for this reason, care must be taken not to exceed the recommended concentration of NaOH and time guidelines (Tenover *et al*, 1993).

The recommended methods for mycobacterial blood cultures single medium could be used with the BACTEC 13A broth or BACTEC 12B broth or BACTEC MYCO/F LYTIC broth (Becton Dickinson Diagnostic Instrument Systems, Sparks, Md.) or the lysis centrifugation method with plating on 7H10 or 7H11 (Isolator; Wampole Laboratories, Cranbury, New Jersey). In general, positive cultures are detected within 7 to 14 days. The cultures that are positive in less

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than 7 days have the mycobacteria > 400 CFU/ml, whereas those that are positive at $\geq$ 12 days have low level of bacteremia (< 9 CFU/ml). The cultures should be confirmed by performing an acid-fast stain on the broth culture or plate-isolated microorganism. Quantitation of growth on agar plates (generally 0 to 4+) is important to estimates of clinical significance and therapeutic response.

7.2 Identification

7.2.1 Staining

The direct examination of blood lymphocytes (buffy coat blood film) stained with acid-fast stain or stained with Wright's or Romanovsky stain may reveal mycobacteria. Also, auramine-rhodamine staining with fluorescent microscopy of bone marrow aspirations may be of value. However, the positive predictive value of these techniques is variable (35 to 86%), and one cannot exclude *M. tuberculosis* bacteremia on the basis of a smear alone (Inderlied *et al*, 1993).

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7.2.2 Biochemical test

Traditionally, mycobacteria have been definitively identified by using biochemical tests. Many of the biochemical tests are qualitative and based on color reactions. Good growth of the isolates is important for performing these tests.

A list of commonly used biochemical tests are as following:

- Niacin test
- Susceptibility to thiophene-2-carboxylic acid hydrazide (TCH) 5 µg/ml
- Nitrate reduction
- Semiquantitative catalase (> 45 mm)
- 68°C Catalase
- Tween hydrolysis (5 days)
- Tellurite reduction
- Tolerance to 5% NaCl
- Iron uptake
- Arylsulfatase (3 days)
- Growth on MacConkey agar
- Urease
- Pyrazinamidase (4 days) test

The rapidity of biochemical tests depend on the growth rate of the organisms, therefore, identification of mycobacteria to the species level can take 4 to 8 weeks from the visible growth of the organisms (Figure 1). The more efficient methods for laboratory identification and differentiation of mycobacterial species are still being improved.

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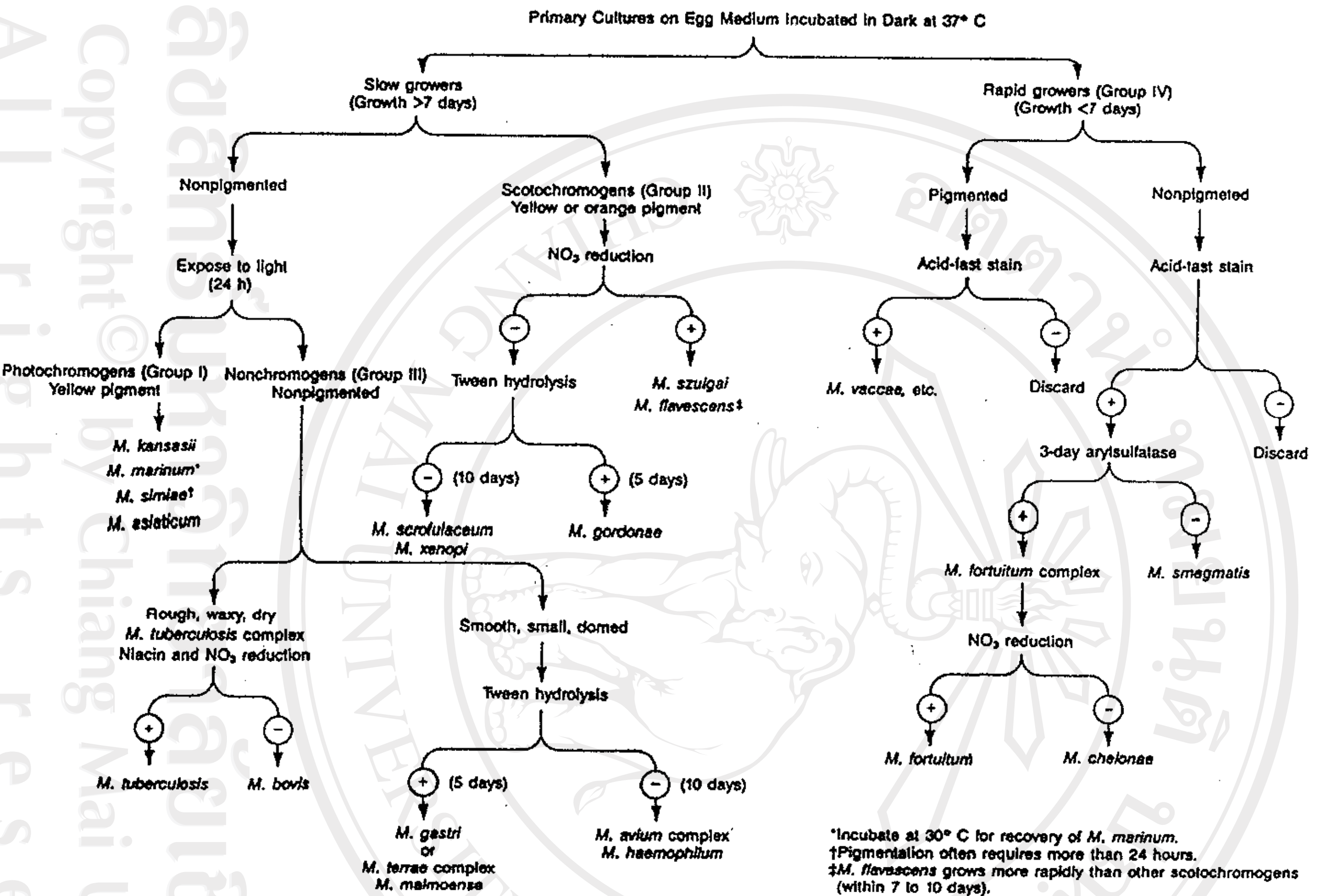

Figure 1 The identification of mycobacteria

From Ellen et at., 1994 (127)

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7.2.3 DNA probe method

The identification of mycobacteria can be achieved within a few hours, once sufficient growth is available, by using non-radioactively labeled DNA probes. The DNA probe tests are considered highly specific and sensitive. In one study, 114 MAC isolates were tested with the three available probes (GenProbe; San Diego, Calif.) and the results were compared with conventional identification. Initial results yielded a sensitivity of 93% and a specificity of 97%, but on repeat testing the sensitivity and specificity increased to 97% and 100%, respectively (Musial *et al*, 1988). The acridinium ester-labeled probes from GenProbe (San Diego, Calif.), was used to detected 40 MAC isolates and the result showed 100% specificity and 95.2% sensitivity (Lebrun *et al*, 1992). Later, the probe was redesigned, and in the primary study it had a sensitivity of 99.5% (Jonas *et al*, 1992).

In 1998, the digoxigenin-labeled MV222 oligonucleotide probe was used to identify *M. avium*. The probe could hybridize to the amplified products of 16S-23S rRNA gene spacer of *M. avium* isolates but not to those of any other species of bacteria including *M. intracellulare* and unclassified MAC isolates. The results completely agree with those obtained from the AccuProbe (Sansila *et al*, 1998).

7.2.4 High Performance Liquid Chromatography

Each species of mycobacteria appears to synthesize a unique set of mycolic acids (Minikin & Goodfellow, 1980; Butler & Kilburn, 1988), and this has been exploited for speciating mycobacteria (Figure 2). In this method, mycolic acids are extracted from saponified mycobacteria, converted to *p*-bromophenacyl esters and analyzed by high performance liquid chromatography (HPLC). The resulting pattern is compared to a library of reference patterns to identify the species. This rapid assay can provide definitive species identification for essentially any of more than 50 *Mycobacterium* spp. Within less than 4 hours, in contrast to the weeks required for conventional tests for speciation.

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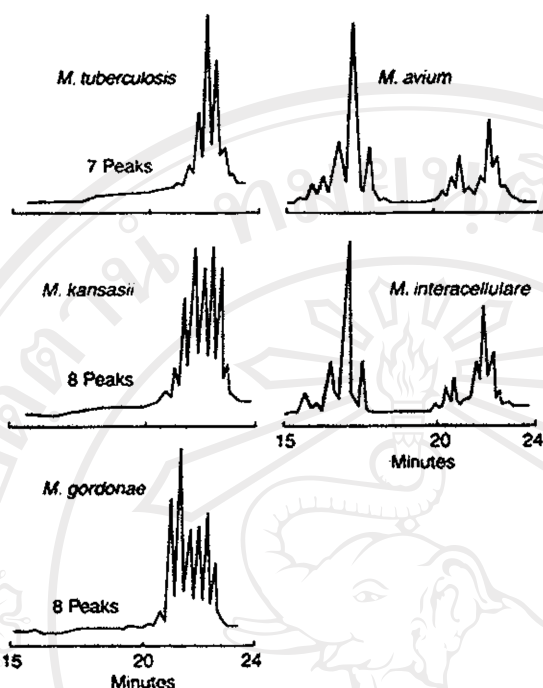

Figure 2 HPLC patterns of mycolic acids from 5 species of mycobacteria.

(Photograph from Mycobacterium, chapter 26, p. 559)

7.2.5 Polymerase Chain Reaction

There are several studies that using PCR analysis for detection and identification of *M. avium*. By using primers B (5'-CAGCCAGCCGAATGTCATCC-3') and (5'-CAACTCGCGAC ACGTTCACC-3') (Kunze *et al*, 1992) specific for the IS*901* insertion in MAC. Although, the result shows that the strains with 300- or 1,776-bp PCR products amplified by the primers were identified as *M. avium* and the strains with the 1,070-bp product were identified as *M. intracellulare* but none of the 110 strains of MAC isolated from swine possessed IS*901* (Nishimori *et al*, 1995).

Telenti *et al* (1993) used primers Tb11 (5'-ACCAACGATGGTGTGTCCA T-3') and Tb12 (5'-CTTGTCGAACCGCATACCCT-3') that amplified a 439-bp fragment between position 398 and 836 of the *hps65* gene of mycobacteria. The PCR-restriction enzyme pattern analysis (PRA) using the *Hae*III was done by computer software (Excel; Microsoft). The software converted the running distance on electrophoresis (in millimeters) to apparent molecular size. The software built a standard curve from migration distance of a molecular size standard (*Hae*III-

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digested ØX174 DNA). Thereafter, the distance values for each sample were automatically referred to the standard curve and expressed as the calculated molecular size. The calculated molecular sizes of *M. avium* were 140 and 105 base pairs. To avoid confusion with primer and primer-dimer bands, restriction fragments shorter than 60-bp were disregarded. Amplification products form Tb11- and Tb12-primers were also obtained from isolates of *Streptococcus albus* and *Rhodococcus equi.* However, the restriction patterns of these nonmycobacterial species did not resemble the pattern of *M. avium* (Taylor *et al*, 1997). Two isolates of *M. avium* subsp *avium* that lacked the IS*1245* element show the same PCR-restriction enzyme analysis (PCR-REA) pattern as described above. Although, the isolates were tested with AccuProbe™ and showed positive for MAC but negative *M. avium* and *M. intracellulare* (Garriga *et al*, 2000).

In 1993, the differentiation of *M. avium* and *M. intracellulare* could be performed by double PCR test. The AV6 (5'-ATGGCCGGGAGACGATCTATGCCGGCGTAC-3') and AV7 (5'-CGTTCGATCGCAGTTTGTGCAGCGCGTACA-3') primers directed the amplification of a 187-bp fragment within the DT6 sequence. Whereas, the IN38 (5'-GAACGCCCGTTGGCTG GCCATTCACGAAGGAG-3') and IN41 (5'-GCGCAACACGGTCGGACAGGCCTTCCTCG A-3') primers directed the amplification of a 666-bp fragment within the DT1 sequence. The study showed DT6-positive strains correspond to *M. avium*, DT1-positive strains correspond to *M. intracellulare* and strains positive with both pairs of primers can be identified as *M. avium* serotype 2 or 3 (Thierry *et al*, 1993; Sola *et al*, 1996)

The PCR-REA of the 16S-23S ribosomal deoxyribonucleic acid (rDNA) spacer of mycobacterial species was developed. A single pair of primers, *PL*1 (5'-GAAGTCGTAACAAG G-3') and *PL*2 (5'-CAAGGCATCCACCAT-3'), was used to amplify the spacer. A 370-bp product indicated the slow growing mycobacteria including *M. avium.* When digested with *Hae*III restriction enzyme, the *Hae*III -digested product of *M. avium* contained four fragments of 130-, 115-, 80- and 40-bp. However, the primers were not specific to mycobacteria and the number of *M. avium* isolates was small (Lappayawichit *et al*, 1996). Later, Sansila *et al* (1998) improved the method by using more specific primers. The primers, 16SC (5'-TCGAAGGTGGGATCGGC-3') and 23SG (5'-GCGCCCTTAGACACTTAC-3') was developed to amplify the *M. avium* spacer. The amplified products of 44 isolates of *M. avium* were about

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380-bp and the *Hae*III-digested amplified products of all *M. avium* contained four fragments of 155-, 115-, 65- and 40-bp. The PCR-REA pattern was unique to *M. avium* (Figure 3).

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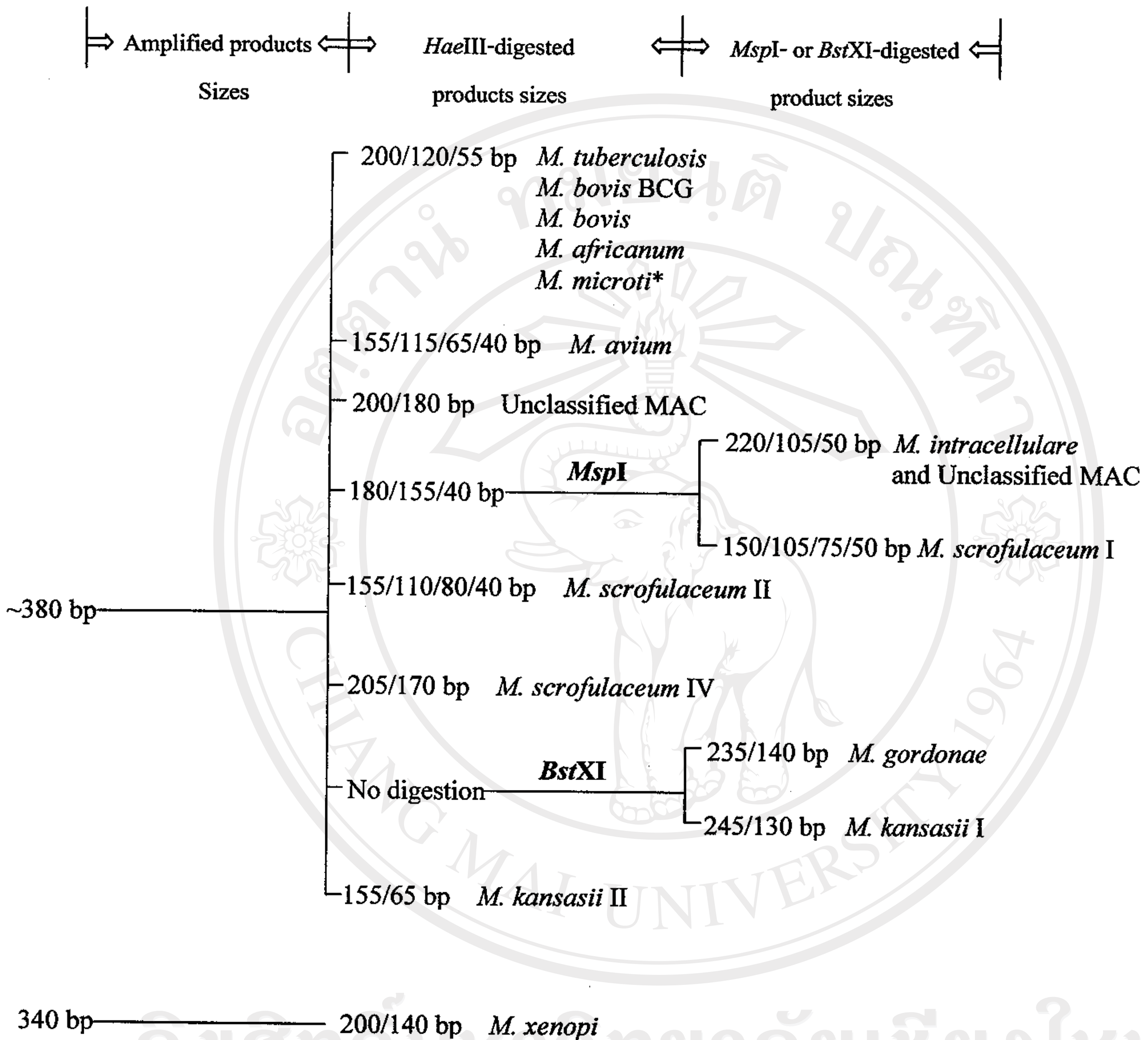

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Figure 3 The algorithm for the differentiation of slow-growing mycobacteria

(Sansila *et al*, 1998; *Panjaisee *et al*, 2002)