AIDS-Related Mycobacterium avium-intracellulare

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To the love, encouragement, understanding and selfless devotion of my wife Priscilla.
"La science favorise un esprit préparé"

Louis Pasteur
DECLARATION

No part of the work described in this thesis has been submitted in support of an application for another degree or qualification in this or any other institution of learning.

Robert-A. Ollar
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Summary
Summary

Recent literature indicates that between 40-53% of AIDS patients are infected with disseminated *M. avium-intracellulare* (MAI) complex.

In order to determine how TB and MAI have evolved at St. Vincent's Hospital during the current AIDS epidemic in which differing populations have entered into the scenario, a retrospective analysis was made between 1979-1989. The study demonstrated that the MAI:TB ratio which expresses changes in MAI and TB exposure patterns, can serve as a useful indicator of change in the composition of the AIDS population pool.

The paraffin wax slide culture baiting technique or Para SL/C for viewing *in situ* mycobacterial cording provided a useful means for making the distinction between the serpentine cording of non-tuberculous and tuberculous cording. Twenty percent of the St. Vincent's blood isolates displayed cording.

An adaptation of the Para SL/C method was developed for antimicrobial sensitivity testing. Clinical isolates of MAI were tested against ciprofloxacin, amikacin and azithromycin by Para SL/C. The procedure was reproducible with all agents tested.

The plasmid profile of St. Vincent's blood derived MAI isolates showed that pLR-7 type plasmids were found in 85% of the MAI isolates obtained from St. Vincent's Hospital and Memorial Sloan-Kettering Cancer Center. By contrast, only 20% of the aforementioned isolates showed the presence of pLR-20 type plasmids.

This investigation has observed that the A1 R.F.L.P. pattern occurred in 50% of the MAI isolates analyzed. R.F.L.P. patterns A/I, A2 and A3 occurred, respectively, at 12.5%, 25%, 12.5%.

HeLa Cells have epithelial cell characteristics and were used to indicate MAI invasiveness. The loss of MAI cording, presence or absence of pLR-7 or the presence of both the pLR-7 and pLR-20 types plasmids appeared not to be indicative of a heightened invasiveness. The sampling of R.F.L.P. types analyzed did not permit a direct correlation between specific R.F.L.P. type and a heightened index of invasiveness.
Section I
General Introduction
INTRODUCTION

1.0 Mycobacterium Avium-intracellulare Complex MAI

Rivolta was credited by Strauss and Gamaleia as being the first to express the idea that human and avian tuberculosis were the result of two different species (Strauss and Gameleia, 1891). Robert Koch also stated that the bacilli responsible for the human and avian forms of tuberculosis were two different organisms. In 1891, Strauss and Gameleia were the first to isolate the avian bacilli from birds. The organism was named *Mycobacterium avium* by F.D. Chester in 1901 (Chester, 1901).

*M. avium* can cause tuberculosis in domestic fowls and other forms of bird life (Runyon et al., 1974). *M. avium* has been seen less frequently in lesions or lymph nodes of cattle and swine and other animals. It has also been found to produce a localized disease in pigs. In experimental rabbits and mice, it is able to proliferate but, it did not produce macroscopic tubercles. *M. avium* serotypes have infrequently been implicated in human pulmonary disease. It has been found on rare occasions in soil or as an etiologic agent of human disease.

The avian bacillus is readily distinguishable from *M. tuberculosis* and *M. bovis* (Wolinsky, 1980). The individual cells are smaller; the colonies are smooth; it grows optimally at 41° C; it is resistant to most of the antituberculosis drugs; and it is pathogenic for chickens and rabbits but not for guinea pigs.

In 1949, Cuttino and McCabe isolated from both biopsy and necropsy a mycobacterial agent which grew in the cytoplasm of cells
but, did not produce necrosis (Cuttino and McCabe, 1949; Smith et al., 1968). Their organisms were strongly acid-fast and could grow on a variety of routine lab media (Smith, et al., 1968). This isolate was found to be non-pathogenic for mice, guinea pigs, rabbits or fowl. Because the organism displayed extreme branching in hanging drop slides, it was named \textit{Nocardia intracellularis}.

A peculiar mycobacterial isolate accounted for 1\% of all hospital admissions at Battey State Hospital for Tuberculosis during the 5 year interval from 1950-1955 (Smith et al., 1968). This organism was found to branch in the same manner as that described by Cuttino and McCabe. When Magnusson prepared sensitin derived from \textit{Nocardia intracellularis} and from the Battey Hospital strain, he found that both strains caused identical allergic reactions (Smith et al., 1968). In 1967, Runyon named the organism \textit{Mycobacterium intracellulare} (Runyon, 1967).

\textit{M. intracellulare} has been isolated from a fatal system disease in a child (Runyon et al., 1974). It is seen most often in the pulmonary secretions and surgical specimens of patients with a tuberculosis-like disease. When \textit{M. intracellulare} is isolated from human secretions, it is considered as an etiologic agent of pulmonary disease. However, it should be noted that this organism has been occasionally isolated as an apparent casual resident. The organism has been isolated in cattle and pigs. It has been occasionally found in soil.

\textit{M. intracellulare} is generally less thermophilic than \textit{M. avium} and the classical distinction between \textit{M. avium} and \textit{M. intracellulare} had been based upon pathogenicity tests in chickens (Barksdale and Kim, 1977; Wolinsky, 1980). \textit{M. avium} was virulent for chickens but, \textit{M. intracellulare} was not (Barksdale and Kim, 1977). However, virulence in chickens due to \textit{M. avium} can easily be lost (Cross and Goodfellow, 1973). There are forms that are intermediate between \textit{M. avium} and \textit{M. intracellulare} in terms of colonial morphology, cultural behavior and
chicken pathogenicity thus making the two strains indistinguishable (Runyon et al., 1974). Therefore, because both *M. avium* and *M. intracellulare* share many properties, it was proposed they should be placed under the common heading of *M. avium complex* (Cross and Goodfellow, 1973; Wolinsky, 1980; Sommers and Good, 1985).

1.1 Pre-AIDS-ERA *M. avium-intracellulare* Infection

Before the AIDS era, clinically important infection caused by *M. avium-intracellulare* were generally confined to older patients who suffered from chronic lung disease that was slowly progressive and often resembled those due to *M. tuberculosis*. (Ellner et al., 1991). It was also an uncommon cause of pneumonia in persons with chronic pneumonia (Horsburgh, 1991). In this pre-AIDS period, disseminated *M. avium-intracellulare* infection involving the blood and multiple organs was extremely rare with only 24 cases cited in the literature.

1.2 AIDS-ERA *M. avium-intracellulare* Infection

The MAI complex infection in AIDS patients has been shown to be widely disseminated in the host with involvement of the blood, bone, lungs, spleen, liver, spinal fluid, and intestinal tract (Reichert, et al., 1985). An infection of the gastrointestinal tract with *M. avium-intracellulare* usually in the presence of the disseminated form of the disease, is associated with diarrhea, abdominal pain, malabsorption, weight loss, and fever with or without night sweats (Smith et al., 1992). Recent literature indicates that between 40-53% of AIDS patients are infected with disseminated MAI complex (Guthertz et al., 1989; Yakrus and Good, 1990; Nassos, et al., 1991). Disseminated *M. avium* complex infection is the most common cause of bacillemia in patients with AIDS (Brown et al., 1991). It has been
called the most common systemic bacterial infection in patients suffering from AIDS (Nassos et al., 1991).

Genetic analysis of AIDS-related MAI strains indicated that 97%-98% of the isolates were *M. avium* (Guthertz et al., 1989; Hellyer, et al., 1991; Ellner, et al., 1991). The remaining 3% of MAI isolates in AIDS patients were *M. intracellulare*. In the case of MAI isolates derived from non-AIDS patients, only 60% were *M. avium* (Hellyer, Brown, Dale and Easmon, 1991).

Jacobson et al. (1991) found that of 137 consecutive patients who had a sterile body site cultured for mycobacteria within 3 months of their first *Pneumocystis carinii* infection, those with disseminated *M. avium* displayed significantly shorter (107 days) median survival when compared to those with negative cultures (275 days). In addition, these investigators found that among 34 patients with AIDS and respiratory MAI, 22 later developed the disseminated form of MAI. This investigation concluded that disseminated MAI infection was linked with a shorter survival in AIDS patients. They also found that when MAI was isolated from AIDS patient respiratory specimens, it carried a 65% predicative value for subsequent disseminated MAI infection.

When *M. avium-intracellulare* is definitively identified and it has been decided to offer therapy, the following drugs are among those that have been tried in combination: ansamycin, clofazimine, ethambutol,isoniazid, amikacin, ciprofloxacin, and imipenem (Powell, 1991). The optimal duration of therapy is undetermined. None of the aforementioned chemotherapeutic agents has thus far proved effective in eradicating the infection.

Thus, *Mycobacterium avium-intracellulare* complex once a rare causative mycobacterial agent of disseminated infection, is now
a devastating and frequent secondary infectious agent of disseminated infection in the AIDS patient population.

1.3 Serotype Analysis of M. avium-intracellulare

J. Firth in 1926 first studied the serological properties of Avian tubercle bacilli by agglutination and complement fixation with bacillar suspension as the antigen (Schaefer, 1965). Schaefer was able to serologically identify strains of nonphotochromogenic and scotochromogenic atypical mycobacteria which were derived from a variety of human and veterinary sources by their specific agglutination. These specific agglutinations were classified into 20 serotypes (Schaefer, 1965; Schaefer, 1968). The M. avium strains studied by Schaefer were found to belong in serotypes 1 and 2 (Schaefer, 1968). Barksdale and Kim (1977) noted that M. avium types 1 and 2 were found to be the cause of human disease. Type 2 occurred more frequently as the causative infectious agent in chickens, other birds, cattle, pigs and man (Barksdale and Kim, 1977).

The seroagglutination protocol as described by Schaefer for serotyping, however, suffers from inherent weaknesses such as a high frequency of cross-reactivity and the occasional presence of false-positive results or multiple reactions (Denner et al., 1992). In addition, agglutinating antibodies cannot distinguish between serovars with similar glycopeptidolipid compositions such as serovars 8 and 21. There is also the issue of semirough variant strains which autoagglutinate.

When the conventional Schaefer seroagglutination protocol was combined with the results of animal virulence studies, the recognition of serovars 1 to 3 of M. avium and 17 serovars (serovars 12-28) of M. intracellulare, as well as, 8 intermediate serovars (serovars 4-11) were realized. This classification was again altered on the basis of
genomic analysis and there is currently an ongoing debate as to which serovars are of the *M. avium* or the *M. intracellulare* species. The 28 well-documented serovars are termed the *M. avium* complex (Denner et al., 1992).

The serovar specific typing antigens of the *M. avium* complex have now been identified as glycolipids, or more specifically glycopeptidolipids (GPLs) which are composed of an invariant monoglycosylated lipopeptide core to which a variable oligosaccharide hapten is glycosidically linked (Denner et al., 1992). These have been called polar GPLs to distinguish them from the singly glycosylated, apolar GPLs which are nonspecific, being present in all serovars. This fundamental knowledge has allowed the development of simple chemical tools for the identification of *M. avium* complex isolates, notably thin-layer chromatography (TLC) of the alkali-stable, serovar-specific polar GPLs. The easy resolution by gas chromatography (GC) of the characteristic sugars that make up the haptenic oligosaccharide of the specific GPLs has provided yet another powerful tool for the identification and classification of mycobacteria. Enzyme-linked immunosorbent assay (ELISA) based on the GPL antigens and the original type-specific rabbit antisera has proved beneficial in this respect, and more recently, the generation of murine monoclonal antibodies (Mab) to specific epitopes within the GPLs of individual serovars has increased the ease and specificity of identifications. All of these methods have been applied by Tsang et al. in a recent investigation to distinguish *M. avium* serovars with great accuracy, and to help answer clinical and epidemiological questions such as the relevance of nontuberculous bacterial isolates from nonsterile body sites, the distribution patterns of individual serovars in the environment and within patient populations, and the consequent epidemiological implications (Tsang et al., 1992).
Saito et al. (1990) have applied DNA probes specific for either \textit{M. avium} complex or \textit{M. intracellulare} for the identification of a variety of serovar strains of \textit{M. avium} complex (MAC). When reference strains of \textit{M. avium} complex organisms belonging to serovars 1 to 28 were examined with DNA probes specific for either \textit{M. avium} or \textit{M. intracellulare}, the investigation found that earlier designations of MAC serovars, in which serovars 1 to 3 and 4 to 28 were regarded as \textit{M. avium} and \textit{M. intracellulare} respectively, should be revised. The study stated that \textit{M. avium} should include serovars 1 to 6, 8 to 11, and 21. In addition, the investigation stated that \textit{M. intracellulare} should include serovars 7, 12 to 20 and 25. These investigators also stated that other serovars, such as serovars 22 to 24 and 26 to 28, involve \textit{M. intracellulare}, \textit{M. scrofulaceum}, and MAC that were lacking the reactivity with either the \textit{M. avium} or \textit{M. intracellulare} probe. The absence of reactivity of serovars 22 to 24 and 26 to 28 to either the \textit{M. avium} or \textit{M. intracellulare} probe precluded a description related to taxonomic position in the \textit{M. avium} complex. Tsang et al. (1992) have stated that the DNA probes to date have not been able to identify different serovars within one species and that only the immunogenic markers involved in serotyping are able to discern differences and similarities among strains of the same species.

1.4 Serotype of \textit{M. avium-intracellulare} in AIDS Patients

Serotyping of the \textit{M. avium} complex provided important epidemiological information, especially in tracing the origins of infections (Denner et al., 1992). The seroagglutination protocol of Schaefer has been instrumental in the assembly of reference strains in the study of the geographic distribution of serovars in humans with underlying AIDS and in animals and in tracking sources of such isolates.
An analysis of the *M. avium* serovars from AIDS patients indicated that approximately 70% were either serovar 1, 4 or 8 (Morris et al., 1990). When a sample collection of AIDS-related *M. avium* isolates (50% of which came from medical centers in California and New York State) was serotyped using a modification of the basic Schaefer serotype protocol, regional variation in serotype was observed (Yakrus and Good, 1990). In the state of California, serotype 8 was found slightly more frequently than serotype 4. In western states other than California and New York, serotype 4 was found in slightly greater abundance than serotype 8. The serotypes other than 4 and 8, namely, 1, 9 and 10 had even distribution throughout the western and eastern United States (Yakrus and Good, 1990).

The frequency of isolation of specific serotypes for *M. avium* were determined for the cities of New York, Los Angeles and San Francisco (Yakrus and Good, 1990). In the city of Los Angeles, serotype 8 accounted for 36% of AIDS-related *M. avium* isolates. Serotype 8 was found in only 8% of New York City isolates and only in 16% of San Francisco isolates.

In Europe, a Swedish study of 25 AIDS-derived strains of *M. avium* found serotype 6 to be dominant followed by 4 and 9 (Hoffner et al., 1989; Tsang et al., 1992). A German study found that 90% of patient isolates were serotype 8/21 followed by serotype 8 and serotypes 6, 9, 12 and 4/8 (Ruf et al., 1989).

All of these investigations share two basic points: a) individuals acquire whatever serotype predominates in the immediate region, b) certain serotypes are found more frequently in one geographic location than another.
1.5 Gen Probe

The basic problem encountered with the conventional biochemical identification plans or schemes is that these protocols rely on metabolic processes that occur during the growth of the organisms under consideration (Tenover, 1989). There can occur random mutations in the genome of the organism that can alter the biochemical profiles and therefore make identification difficult. Assays which employ rapid monoclonal antibody-based tests rely on the production of specific antigens that may be produced only late in the life cycle of the specific organism. A DNA based probe protocol does not require the expression of a gene but, utilizes the unique sequence of the nucleic acids in an organism as a "fingerprint" for identification (Tenover, 1989).

In 1987, the Gen Probe Company of San Diego, U.S.A. introduced nucleic acid probes for the identification of *M. tuberculosis* complex (*M. tuberculosis*, *M. bovis*, *M. africanum* and *M. microti*), *M. avium*, and *M. intracellulare* (Roberts et al., 1991). The Gen Probe procedure utilized nucleic acid hybridization assays based upon the ability of complementary nucleic acid strands to bond and yield stable, double stranded complexes under appropriate assay conditions. The original Gen Probe employed an $^{125}$I-labeled single stranded DNA probe which was complementary to the rRNA from the target organism. The actual rRNA of the target organism was released from the test organism via the action of a lysing reagent, heat and sonication. The resulting hybridization of the $^{125}$I-labeled DNA probe and the rRNA of the target organism formed a stable DNA-RNA hybrid. A separation suspension separated the labeled complex (DNA-RNA hybrid) from the unhybridized DNA. The resulting radiolabelled DNA-RNA hybrids were counted and the assay results were expressed as a percentage of input probe.
hybridized. The cutoff value for positive hybridization with the probe is set at 10% hybridization (Gonzalez and Hanna, 1987).

Gonzalez and Hanna evaluated the efficacy of the Gen-Probe diagnostic protocol for *M. avium-intracellulare* complex (MAIC) and found that it correctly identified 63 of 64 MAIC clinical isolates and controls but, yielded 1 false negative value for a sensitivity of 98.43% (Gonzalez and Hanna, 1987). These investigators stated that whereas conventional techniques required subculture and are growth-dependent, the DNA probes can be utilized directly on the primary isolate as long as there is sufficient growth (3-6 x 10⁸ org/ml). The false negative result obtained by Gonzalez and Hanna was explained by the fact that there is a contaminant in their specimen. These investigators recommended Gen Probe not be utilized on heavily contaminated clinical specimens.

The Gen Probe system was able to demonstrate the presence of *M. avium* directly from BACTEC 12B hemoculture vials by centrifuging 3ml of the Bactec broth culture at 3,000 x g for 10 min if the Bactec radiometric system had a growth index of greater or equal to 999 (Kiehn and Edwards, 1987). Ellner et al. (1988) utilized Gen Probe DNA probes specific for *M. tuberculosis*, *M. avium* and *M. intracellulare* on Bactec 12B radiometric hemocultures (specifically utilized for the isolation of mycobacteria) for the purpose of speeding up the time to final report and elimination of the need for biochemical testing. When the Gen Probe system and the radiometric system (BACTEC 12B) were utilized in combination, there was a marked reduction in the time to final report (two-thirds of these isolates were detected and identified within 2 weeks of inoculation and the rest were detected by 4 weeks—a reduction of 5-7 weeks to final report) with the total elimination of biochemical testing. The utilization of the Gen Probe system for *M. avium* and *M. intracellulare*
permitted Conville et al. (1989) to identify for the first time in the clinical literature, the presence of a mixed *M. avium* and *M. intracellulare* from blood samples derived from an AIDS patient. Sherman et al. (1989) found that the results of their investigations utilizing the Gen Probe DNA probes for *M. tuberculosis*, *M. avium* and *M. intracellulare* were greatly improved by expressing percentage hybridization values of >15% as positive results, percentage hybridization values of <5% as negative values and values of 5%-15% as indeterminant values. These investigators noted that if they had utilized the manufacturers recommended cutoff value of 10%, they would have resulted in 27 misidentified cultures, 16 false-negative cultures and 11 false positive cultures.

Recent developments in Gen-Probe technology has made possible the use of non-radioactive nucleic acid probes. These probes utilize an acridinium ester-labeled single stranded DNA probe complementary to the rRNA of the target organism (Roberts et al., 1991). In this non-isotopic protocol, rRNA released from the target organism combines with labeled nucleic acid probe to form a stable DNA-RNA hybrid. The differentiation of hybridized and nonhybridized probe is achieved by the use of a differential hydrolysis reagent. Only the acridinium ester label that is bound to the probe in the hybrids is chemiluminescent and is detected by a luminometer. These nonisotopic nucleic acid probes have an extended shelf life of at least 6 months and are cheaper than the current isotopic probes. This latter feature makes these DNA probes very useful in the routine clinical microbiology lab.
1.6 **Restriction Fragment Length Polymorphism (RFLP) Analysis**

The restriction fragment length polymorphism (RFLP) analysis procedure was developed as a method of detecting small differences (i.e. single basepair changes) in the chromosomal DNA of closely related organisms as seen via conventional genomic analysis such as total chromosomal DNA-DNA hybridizations or in sequence determinations of highly conserved genes (Clark-Curtiss, 1990). In the RFLP protocol, restriction endonucleases are utilized to generate numerous fragments from the genomic DNA and these fragments are then separated via electrophoresis on the basis of their size. RFLP typing is useful as either a method of species identification, or as a method for subdividing species into a number of RFLP types (McFadden et al., 1990). Thus, among the closely related DNA's, the differences in the length of a specific restriction fragment could be due to genotypic variations that resulted in differences of one or more individual bases such that the resulting cleavage sites for a specified endonuclease could be lost or gained; alternatively, these differences could be the result of insertions or deletions of blocks of DNA within the fragment (Clark-Curtiss, 1990). RFLP studies have been used to estimate base-substitution between closely related strains and species (McFadden et al., 1990). The use of labelled restriction fragments as a DNA probe permits variation among restriction fragments in total genomic DNA to be detected by using Southern hybridization (Clark-Curtiss, 1990).

1.7 **Application of RFLP Analysis to M. Paratuberculosis and M. avium Complex Organisms**

Crohn's disease (CD) is a chronic granulomatous inflammation found chiefly in the large and small intestine and also found in other parts of the alimentary canal as well as in the skin, liver and joints
(McFadden et al., 1987). This disease, however, resembled intestinal tuberculosis and therefore, was initially suggestive of a mycobacterial infection. The idea of a mycobacterial causative agent was revived because of the isolation of slow growing, mycobactin dependent *Mycobacterium* species from the intestinal mucosa of patients (McFadden et al., 1987; Butcher et al., 1988; Hampson et al., 1989; Kunze et al., 1991). It was found that the biochemical and cultural characteristics of this *Mycobacterium* species were similar to those of *M. paratuberculosis*, the causative agent of an intestinal disease in ruminants called Johne's Disease (McFadden et al., 1987). *M. paratuberculosis* is similar to mycobacteria of the *M. avium-intracellular* complex (McFadden, et al., 1987). It was thus decided to utilize RFLP methodology in order to clearly be able to distinguish between these closely related mycobacterial species.

DNA was extracted and cloned from an unclassified *Mycobacterium* species that was isolated from CD (McFadden et al., 1987). McFadden et al., conducted RFLP analysis to distinguish between these mycobacteria. Upon RFLP analysis it was discovered that no RFLP's were detected between the DNA derived from the unclassified CD isolated *Mycobacterium* species and *M. paratuberculosis* but, there were several RFLP's detected and distinguished between *M. paratuberculosis* and both *M. avium* complex serovars 2 and 5 (McFadden et al., 1987). In addition, it was found that the frequency of DNA base substitution between *M. paratuberculosis* and *M. avium* complex serovar 2 was measured as 0.87. Of the probes examined by McFadden et al., one probe pMB22 (this probe contains the insertion sequence IS900) hybridized to give multiple banding patterns with *M. paratuberculosis* and the CD *Mycobacterium* species (Clark-Curtiss, 1990). However, pMB22 hybridized to far fewer bands when applied to *M. avium* and weakly to *M. kansasii* and *M. phlei*. Thus, from these RFLP studies,
it was determined that the degree of relationship of *M. paratuberculosis* to the other mycobacteria studied was therefore: *M. paratuberculosis* > *M. avium* > *M. kansasii* > *M. phlei*. The findings thus indicated that although *M. paratuberculosis* and *M. avium* were very closely related, *M. paratuberculosis* appeared to be a genetically distinct, highly conserved pathogen (Clark-Curtiss, 1990).

### 1.8 Application of RFLP to *M. avium* Complex Organisms

DNA probes were utilized to identify RFLP's in DNA samples of the *M. avium* complex and thus were able to divide it into *M. avium* and *M. intracellulare* strains (McFadden et al., 1987). There was less than 2% base substitution between *M. avium* strains but, the *M. intracellulare* strains had greater than 15% base substitution.

Hampson et al. (1989) looked at the RFLPs in strains of *M. avium-intracellulare* complex organisms derived from 45 AIDS patients, 18 Non-AIDS patients and 8 mycobacteria isolated from the stools of healthy patients. These investigators utilized DNA probes pMB16, pMB17, pMB20 and pMB22 (plasmid clones isolated from a genomic library of *M. paratuberculosis*) for RFLP analysis on the target mycobacterial genomic DNA that was previously digested with the PvuII restriction enzyme. The majority of the AIDS derived strains (33 isolates (73%)) exhibited Type A RFLP banding pattern and 2 AIDS strains from Africa exhibited a Type C banding pattern and the remainder showed heterogeneity.

The type A banding pattern included organisms classified as both serovars 4 and 8. Thirty one of the 33 AIDS derived isolates had been serotyped and of these, one each were serovars 3 and 9, 6 each were serovars 1 and 28, 10 were serovar 8 and 7 were serovar 4 (Hampson et al., 1989). All of the organisms serotyped as serovar 28 (theoretically an intracellular type) had displayed a type A banding
pattern. Of the non-AIDS derived \textit{M. avium-intracellularare} complex organisms, 39\% (7 isolates) belonged to the Type A RFLP banding pattern, with 4 isolates exhibiting RFLP banding pattern B, 1 exhibiting RFLP banding pattern C and the rest showing heterogeneity. The 8 isolates that were derived from healthy patients displayed an RFLP banding pattern that was distinct from that seen in any of the AIDS-related strains of \textit{M. avium-intracellularare} complex. The probe pMB22 was found to differentiate as many strains as the other four probes and thus their subsequent study utilized only pMB22. This investigation has supported the finding that the AIDS-derived mycobacterial serovars differ from those found in non-AIDS patients. However, serotyping correlated poorly with the genetic identity of these mycobacteria (Hampson et al., 1989).

Mcfadden et al. (1990) have reviewed the early work of Hampson et al. in an effort to better characterize the RFLP banding pattern. The banding pattern given to the MAI serovar 4, strain 1241 was originally designated type A (6 bands) by Hampson et al. but, it was changed to \textit{M. avium} complex RFLP type A6 (McFadden et al., 1990). The related banding patterns have been designated \textit{M. avium} complex RFLP type A4.2 (4 bands in common with A6, two unique). The more distantly related mycobacteria (greater than 13\% base substitution) such as \textit{M. intracellularare} isolates that have been considered part of the MAI complex give completely dissimilar patterns and have been designated as \textit{M. avium} complex RFLP type C1. Some of the closely related \textit{M. avium} strains and \textit{M. paratuberculosis} strains give a group of additional bands and the presence of these additional bands are due to the presence of repetitive elements related to IS900 present in these strains. These strains have been designated as \textit{M. avium} RFLP type A5.1/I6 (McFadden et al., 1990). Extensive studies by McFadden and co-workers have found that after an examination of hundreds of \textit{M.}
avium complex strains from a variety of sources most strains fall into a very limited number of highly conserved RFLP types.
Section II

Retrospective Analysis of *M. tuberculosis* and *M. avium-intracellulare* at St. Vincent's Hospital During the Decade of 1979-1989
INTRODUCTION

2.0 AIDS-Related Tuberculosis

In October of 1984, the Centers for Disease Control reported that six groups of adults were known to be at risk for AIDS (Goedert and Blattner, 1985). Homosexually active males made up the vast majority of cases (72.9%) followed by Nonhomosexual parenteral drug users (17.2%), non-homosexual non-drug-using immigrants from Haiti (3.6%), hemophiliacs (0.7%), heterosexual contacts of persons with or at risk for AIDS (0.7%), and recipients of blood transfusions (1.2%). Studies done on Haitian immigrants to the United States demonstrated that up to 10% of AIDS cases develop tuberculosis and over 45% of cases of active TB were HIV seropositive (Maayan et al., 1991). The children who have a parent who either has or is at risk for AIDS make up 64.8% of the pediatric cases (Goedert and Blattner, 1985). Nearly 4% of the cases cannot be attributed to the aforementioned risk groups. In 1989, Ma and Armstrong stated that 62% of the AIDS population was made up of homosexual and bisexual men (Ma and Armstrong, 1989).

In the period from 1963-1985, the incidence rate of Mycobacterium tuberculosis (TB) had declined in the United States on an annual percentage rate of 5.9% (McCray et al., 1990). However, the incidence of TB stabilized in 1984 and increased in 1986 (Fife et al., 1991). In 1986, the United States had its first increase in indigenous TB morbidity in 33 years (Onorato et al., 1992). The Centers for Disease Control (CDC) estimates that there were greater than 14,000 more tuberculosis cases than expected during 1984-1988. It was found that this increase occurred mainly in areas such as California and New York. The New York City Department of Health identified a high rate of TB cases in AIDS
patients in the period of 1981-1985 (Lin and Goodhart, 1991). Reported tuberculosis in New York City has been increasing since 1979 after decades of steady decline (Brudney and Dobkin, 1991). The growth of homelessness among urban drug abusers in the 1980's paralleled the spread of HIV infection, greatly complicating tuberculosis treatment and probably promoting further spread of this infection. There has occurred an increase in the incidence of TB in the total New York City metropolitan population since 1985 compared to 1981 (Lin and Goodhart, 1991). Many of these cases of TB have occurred in the disadvantaged areas of New York City which are known to have a high number of individuals who use intravenous drugs, are non-white and homeless. These regions of the city also have a high incidence of AIDS. Coté et al. (1990) have stated that in the most recent AIDS population surveys, patients who are intravenous drug users or Haitians appear to be at higher risk for TB than other AIDS patients. In the preceding year (1990), 3520 cases of tuberculosis were reported in the New York City area, a rise of 38% over the 2545 cases cited in 1989 (Charatan, 1991). The incidence in New York City rose from 1980-1990 by 132%. The incidence of tuberculosis was 46 cases per 100,000 which is 5 times the national U.S. average and accounting for 15% of the nation's reported cases. When the incidence of tuberculosis is defined by race and ethnic group with sexes combined, tuberculosis reveals itself to be the scourge of minorities. The incidence for white New Yorkers is just below 10 per 100,000 whereas the incidence for black, hispanic and Asian people are respectively 129, 72 and 62 per 100,000. The classification of cases in 1990 by area of birth showed that one in five occurred among immigrants, mainly those from the Caribbean and from Central and South America. The largest increases of tuberculosis occurred among blacks and Hispanics 25-44
years old (Onorato et al, 1992). The Centers for Disease Control data state that 13-46% of patients with tuberculosis are also infected with HIV and the highest rates of coinfection occur in New York City and are on par with those in parts of Central Africa (Charatan, 1991). *M. tuberculosis* has been seen as an important complication of AIDS in those countries where it is usually endemic (Gold and Armstrong, 1989).

In equatorial Africa, about half the adult population is infected with *M. tuberculosis* and 5 million new cases of tuberculosis are diagnosed each year (Maayan et al., 1991). In this population in which endemic tuberculosis is present, HIV infection was diagnosed in 17-55% of Tuberculosis cases. The World Health Organization data indicated that 30% of the AIDS cases in Zimbabwe and Ethiopia showed an initial presentation of tuberculosis.

The most striking clinical feature of tuberculosis in patients with AIDS infection is the extremely high frequency of extrapulmonary involvement, usually with concomitant pulmonary tuberculosis (Barnes et al., 1991). The most frequent forms of extrapulmonary involvement in persons with HIV infection are lymphadenitis and miliary disease. In addition, there is involvement of the bone marrow, genitourinary tract and central nervous system.

The number of cases of extrapulmonary tuberculosis increased by 20% from 1984-1989 as compared with 3% for pulmonary tuberculosis (Barnes et al., 1991). Extrapulmonary disease occurs in more than 70% of patients with tuberculosis and preexisting AIDS or AIDS diagnosed soon after the diagnosis of tuberculosis. However, the extrapulmonary disease is found in only 24-45% of patients with tuberculosis and less advanced HIV infection.
Proof of hematogenous dissemination of *M. tuberculosis* was initially reported in the 1900's and was found to be most frequent in patients with miliary TB (Clark et al., 1991). When Clough reviewed the literature in 1917, bacteremia was found in up to 66.6% of the patients with miliary tuberculosis but in only 6.7% of patients with other forms of *M. tuberculosis* disease. Later reports cited cases of patients with disseminated TB but, *M. tuberculosis* bacteremia was rare during the pre-AIDS Era. In the AIDS-Era, however, *M. tuberculosis* bacteremia has become a more frequent occurrence in HIV infected patients.

Because tuberculosis often appears before other opportunistic infections in persons infected with HIV, a large number of persons who have otherwise latent HIV infection may seek care at TB Clinics (Onorato et al., 1992).

2.1 AIDS-Related Disseminated MAI

In the Pre-AIDS era, disseminated *M. avium-intracellulare* infection was very rare; in 1980 only 24 cases had been cited in the literature (Horsburgh, 1991). However, in 1982, when the disseminated form of MAI infection was recognized in patients with AIDS, the number of cases increased dramatically. Disseminated MAI infection is concurrent with the current AIDS epidemic. In December of 1990, the CDC reported that there were 12,202 cases of disseminated nontuberculous disease out of an AIDS patient population of 161,073. The cumulative incidence was cited as 7.6%.

The studies from clinics have stated that 15-24% of AIDS patients have concomitant disseminated MAI infection (Horsburgh, 1991). The number of disseminated MAI cases in AIDS patients is most certainly underestimated because MAI infection occurs after the initial AIDS indicating diagnosis. The disseminated *M. avium-
intracellular infection is now the most common systemic bacterial infection of AIDS patients in the United States and has been found in as many as 50% of AIDS patients upon autopsy (Nassos et al., 1991).

In Europe and Australia, the percentages are similar to those in the United States (Horsburgh, 1991). In addition, MAI has been reported in Africa but, dissemination was not identified (Horsburgh, 1991; Okello et al., 1990).

2.2 AIDS-Related TB and MAI at St. Vincent's Hospital

The first AIDS-related TB case reported at St. Vincent's occurred in 1983, and the first MAI case was cited in 1984. In order to determine how TB and MAI have evolved at St. Vincent's Hospital during the current AIDS epidemic in which differing populations have entered into the scenario, a retrospective analysis was made over the decade between 1979-1989.
METHODS AND MATERIALS

2.3 Statistical Analysis

All statistical analysis of the data was determined with the aid of a STATISTIX II statistical software package (NH Analytical Software, Roseville, Minnesota U.S.A.).

A Chi-Square analysis was made for the purpose of determining if there occurred statistically significant changes in the annual distribution of mycobacterial disease caused by

*M. avium-intracellulare* and *M. tuberculosis* during the 1979-1989 decade.
RESULTS AND DISCUSSION

2.4 AIDS-Related MAI and TB 1979-1989

Data from the years 1979-1989 came from the files of the Mycobacterial Lab and the Department of Infection control of St. Vincent's Hospital and Medical Center of New York. This data can be seen in Table 1.

Table 1 reveals that prior to the AIDS Era, the incidence of M. avium-intracellulare complex at St. Vincent's Hospital (first AIDS-related TB case at St. Vincent's Hospital seen in 1983; first AIDS-related disseminated MAI case seen in 1984) was very rare (3 cases in 1979; 6 cases in 1980). The distribution of mycobacterial infection between MAI and TB (as reflected by the MAI: TB ratio) was 0.07 (See Table 1). In 1981 onward there occurred increases in the number of MAI infection as reflected by changes in the annual incidence of MAI and its distribution. This 1981 increase in the numerical value of the MAI:TB ratio could actually represent the true starting point of AIDS-related MAI at St. Vincent's hospital which represented undiagnosed cases of AIDS infection. By 1984, the number of MAI cases seen in St. Vincent's Mycobacteriology unit actually exceeded the TB cases (See Table 1). The highest MAI:TB ratio of 2.07 was reached in 1984 (See Table 1). In the subsequent years following 1984, however, the number of cases of TB again exceeded those with MAI (See Table 1). By 1989, the annual incidence of TB was two times that of MAI. A Chi-Square analysis of heterogeneity of the MAI/TB distribution per annum for the decade of 1979-1989 revealed that there did occur a statistically significant variation (P<0.0001).

In the early period of the AIDS Era (up to 1985), the vast majority of AIDS patients in the U.S. (72.9%) were homosexual males (Goedert and Blattner, 1985). The early AIDS patient population
seen at St. Vincent's Hospital (located in the Greenwich Village Section) in New York City was confined to a predominantly middle class male homosexual population in which *M. tuberculosis* was not endemic. The increases in the MAI:TB ratio in the years from 1981 through 1984 reflect an AIDS population consisting primarily of individuals who were not normally exposed to *M. tuberculosis*.

From 1983 onward, a change in the composition of the AIDS population occurred in the United States and Europe with an increase in the number of heterosexual individuals infected with the HIV virus (Holmes et al., 1989). The 1985 St. Vincent's Mycobacterial lab data also reflected the citywide increase in the annual incidence of TB. Thus, despite the fact that there occurred increases in the per annum incidence of MAI in 1985 and thereafter, there also occurred an even greater corresponding increase in the annual incidence of TB. These increases in the annual incidence of TB surpassed those of MAI by the end of the decade (See Table 1).

In 1986, the United States experienced its first increase in indigenous tuberculosis morbidity (Onorato, McCray et al., 1992). The change in the composition of the AIDS patient population reflected a decline in the number of homosexual and bisexual males to 62% (Ma and Armstrong, 1989). This new patient population consisted of intravenous drug users, minorities (blacks and Hispanics), homeless, Carribean immigrants (Haitians and Cubans) and immigrants from Central and South America (Onorato et al. 1992). This new patient pool consisted of individuals normally exposed to high levels of endemic TB (Brudney and Dobkin, 1991; Onorato et al., 1992, Lin and Goodhart, 1991). The literature has stated that 1985 marked the first increase in TB cases in many years for the City of New York (Lin and Goodhart, 1991). The decreases in the MAI:TB ratio in the years from 1985
through 1989 reflect a differing AIDS patient population consisting primarily of individuals who had a high exposure to *M. tuberculosis*. The study did not go beyond 1989 because the new stricter patient confidentiality regulations introduced in 1990 allowed only physicians and health care professionals directly involved with the patients to have access to file data.

The study demonstrated that the MAI:TB ratio which expresses changes in MAI and TB exposure patterns, can serve as a useful indicator of change in the composition of the AIDS patient population pool.
Table 1: Annual Incidence of MAI and TB at St. Vincent's Hospital 1979-1989

<table>
<thead>
<tr>
<th>Year</th>
<th>MAI</th>
<th>TB</th>
<th>MAI:TB Ratio</th>
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<tr>
<td>1979</td>
<td>3</td>
<td>41</td>
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<tr>
<td>1980</td>
<td>6</td>
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Section III
Paraffin Wax Slide Culture for Isolation and Speciation of *Mycobacterium avium-intracellulare*
INTRODUCTION

3.0 Conventional Mycobacteriology

The isolation of mycobacterial agents has been achieved conventionally on egg medium (Lowenstein Jensen) and clear agar medium (7H11) (Runyon et al., 1974). In addition, prior to isolation, highly contaminated and viscous samples such as sputum were treated to a variety of homogenization and decontamination procedures prior to cultivation. To decrease the possibility of cross-contamination of the mycobacterial culture, the media included a variety of agents (penicillin, nalidixic acid and polymixin). The conventional system worked but, required from 7-67 days for M. avium detection and additional time for identification (a minimum of two weeks) (Roberts et al., 1987). In 1975, a new system of detection for mycobacteria was developed called Radiometric (Ellner et al., 1988). An automated BACTEC Radiometric apparatus for detecting the presence of mycobacteria in sputa was developed in 1977. The BACTEC system used broth with $^{14}$C-labelled palmitic acid. The apparatus measured the release of $^{14}$CO$_2$ from the metabolism of the $^{[14]}$C-labelled palmitic acid to detect the presence of mycobacteria. A growth index or GI of 10 or more showed the release of $^{14}$CO$_2$. The assay was measured until a GI of 10 was reached. With the onset of the AIDS era, disseminated infection due to MAI and its associated mycobacteremia have become common (Motyl et al., 1989). The automated radiometric system was then easily adapted for the analysis of blood culture specimens using 7H12 broth containing $^{14}$C-labelled palmitic acid to analyze blood for the presence of mycobacteria in AIDS patients. The automated Radiometric BACTEC (Johnston Laboratories, Inc., Towson, Maryland, U.S.A.) provided a more rapid MAI detection time of from 3-23 days but, it did not provide a more rapid identification of
any of the Mycobacteria Other Than Tuberculosis (MOTT) (Roberts et al., 1987).

Most recently, it has been reported by several investigators (Ellner et al., 1988; Gonzalez and Hanna, 1987) that a DNA probe called the "Gen-Probe Rapid Diagnostic System" was able to rapidly speciate (within 2 hours) *M. avium* organisms from a variety of noncontaminated mycobacterial isolates. Ellner et al. stated that with the use of the DNA probe all their isolates were identified within 4 days of their detection by BACTEC or on conventional media (Ellner et al., 1988). The DNA probe assay, however, costs about $12.00 per assay (Gonzalez and Hanna, 1987).

A limitation of the radiometric assays and DNA probe is that they are dependent upon skilled personnel and the support of a well equipped clinical laboratory including expensive detecting devices (Ollar et al., 1991). A simpler and cheaper method for the detection, isolation and speciation of MAI complex organisms would be quite beneficial in those circumstances where expensive radiometric systems, highly skilled personnel and or financial resources are limited.

3.1 Paraffin Wax Baiting

Sohngen in 1913 first developed the use of the paraffin wax baiting technique as a means of isolating certain bacteria including the Mycobacteria and Nocardia species solely on their ability to utilize paraffin wax as a carbon source (Sohngen, 1913). Gordon and Hagan isolated acid-fast saprophytic mycobacteria from soil solely based upon the ability of the latter group of organisms to utilize paraffin wax as a source of carbon (Gordon and Hagan, 1936). This was accomplished by introducing paraffin wax coated rods into media that lacked all other sources of carbon. This
technique has been called Paraffin Baiting. Waksman has often referred to this technique in the isolation of nocardias from soil (Waksman, 1967).

In the realm of clinical microbiology, Mishra and Randhawa, for the first time applied the paraffin baiting technique for the isolation of *Nocardia asteroides* from sputum, bronchial aspirates and gastric lavage (Mishra and Randhawa, 1969). Kurup, Randhawa and Mishra further studied the use of this procedure for the isolation of *Nocardia asteroides* from sputum (Kurup et al., 1970). Later, Mishra, et al. (1973) conducted a more detailed study on the paraffin baiting procedure and its application as a laboratory diagnostic procedure in Nocardial infection.

Mishra et al. found that the property of paraffin wax metabolism was very useful since it is a property not commonly found among human pathogens and permitted heavily contaminated sites to be examined for the presence of nocardioform organisms without the need to incorporate antibiotics into the media. The inoculated media that contained these paraffin utilizing organisms showed colonial growth on the paraffin coated rods (Ollar, 1976). Palmer et al. further cited reasons for the utilization of the paraffin baiting procedure, "Finally paraffin techniques using the ability of Nocardia to utilize paraffin as a sole source of carbon has proven to be helpful as a simple method in detection of low concentrations of the organisms in respiratory secretions." (Ollar, 1976).

### 3.2 Slide Culture

Traditionally mycologists and microbiologists studying the filamentous bacteria (actinomycetes) have been very interested in observing the nondisturbed or "in situ" microscopic growth of their
respective fungal and filamentous bacterial elements in order to identify morphologic cultural changes (Moss and McQuown, 1969). The method utilized to study this "in situ" growth is known as Slide culture. The technique of slide culture has conventionally utilized either agar coated glass slides or agar blocks mounted upon glass slides (Vanbreuseghem, 1966; Riddell, 1950). The resulting "in situ" growth was then subsequently stained and mounted with a combination of lactophenol and cotton blue within a saturated aqueous solution of potassium acetate (Emmons et al., 1970).

3.3 Paraffin Wax Slide Culture

The Nocardial mycelium fragments easily, and therefore the growth taken from solid media often reveals only the bacillary or coccoid forms. Thus, in order to see the undisturbed or "in situ" growth, slide culture of Nocardia must be used. Ollar has shown that paraffin wax coated slides could be utilized for baiting (Ollar, 1976). The application of paraffin wax coated slides for baiting was a great advance over the conventional baiting achieved with rods because it united the factors of "in situ" growth on paraffin wax, nocardial microscopic morphology and acid-fastness (via Kinyoun cold acid-fastness) in presumptive identification of nocardioform organisms.

3.4 Paraffin Slide Culture and MAI

Blood has become the most common source of isolation in AIDS related MAI (Horn et al., 1989). The basic procedure currently used for the detection of MAI in blood relies on the Bactec Radiometric system (Kirihara et al., 1985). For sites other than the blood, Lowenstein-Jensen, Middlebrook 7H9 and related
Middlebrook media are currently used (Ollar et al., 1990). The speciation of MAI is based upon several important conventional criteria; namely, tellurite reduction after 3 days, negative urease reaction, negative niacin production and negative Tween 80 hydrolysis assay.

Fuhs in 1961 noted that many mycobacteria including M. avium and M. intracellulare but not Mycobacterium tuberculosis were able to make use of paraffin wax as a sole source of carbon in basal salt media devoid of all other carbon sources (Fuhs, 1961).

Therefore, because the MAI complex is also able to make use of paraffin wax, a slide culture protocol could be adapted for use with conventional biochemical assays such as tellurite reduction, nitrate reduction, urease utilization and Tween 80 assay in simplified and economical systems for isolation and speciation of MAI complex organisms.
3.5 Paraffin Wax Slide Culture Slides

Standard microscope slides were cut longitudinally so that they could fit within test tubes and could be easily withdrawn (Ollar, 1976; Ollar et al., 1990). The test tubes were plugged and sterilised by autoclaving. Hollow glass tubing was bent into U-shaped or V-shaped rods (to act as slide supports) and added to standard size glass petri dishes which were then wrapped and autoclaved.

For preparation of paraffin wax coated slides, several tubes of histological grade paraffin embedding wax (previously sterilised by autoclaving) were melted in a boiling water bath, while a glass petri dish containing a slide support was heated on an electric hot plate to a temperature sufficient to keep the paraffin wax molten. Molten paraffin wax was then poured into the heated petri dish to a level sufficient to cover a slide on the support.

Ethanol-flame sterilised forceps were used to transfer a previously autoclaved microscope slide onto the supporting glass rod in the petri dish which contained molten paraffin wax. The slide was kept there for only a few seconds so that it was covered by a thin coat of paraffin wax. The coated slide was quickly returned to the tube from which it was initially withdrawn. A tube of molten paraffin wax was added to the petri dish after 6-10 slides had been processed to ensure that there was always sufficient wax to cover the supported slides.
3.6 Czapek Broth for Slide Culture Isolation

Czapek broth (See Appendix IV; Media) to 1.0 litre in distilled water, and adjusted to pH 7.5 (Ollar, 1976). The broth was dispensed in 4.5 ml amounts and sterilized by autoclaving for 15 min at 1.0 kg/cm².

3.7 Blood Culture Isolates

Aliquots of 3-5 ml of blood from AIDS patients were first added to Bactec 13A Blood Culture Medium (30 ml Bactec 13A (Becton Dickenson) per vial). Aliquots of 0.5 ml. subcultured from the inoculated Bactec 13A vial were added initially to six tubes containing 4.5 ml Czapek broth. Paraffin wax coated slides were then added to each of the six tubes. At intervals of 7 days additional tubes were inoculated as cited above. A total of 3-4 seven day samples were usually taken. These paraffin slide cultures were then incubated at 37°C for a period of 30 days before declaring the subculture to be negative (Ollar et al., 1990). When growth was observed on a series of paraffin slide cultures, it was scraped off the surface of a paraffin slide culture with a flame-sterilized inoculating needle and subcultured aseptically onto two Lowenstein-Jensen slopes. The five remaining paraffin slide cultures of the series were utilized for the speciation assays previously described (see Speciation Assays cited below).

3.8 Stool Culture Isolates

Fresh fecal suspensions were prepared from stools of AIDS patients by inoculating a 4mm loop of sample into 5.0 ml of sterile saline. Aliquots of 500 μl were added to 4.5 ml of sterile Czapek broth. Paraffin wax coated slides were then added. Cultures were incubated and periodically checked. In addition, when broth level
became low or growth on paraffin slide was observed, slides were transferred to new tubes containing sterile Czapek broth. The growth from paraffin slide culture surface was subcultured and speciated in the same manner as cited above for Blood Culture Isolates.

A modification of the standard fecal isolation protocol as cited above was also used. In the modification, the Czapek broth which was to be inoculated with fecal suspension contained a 1:100 final dilution of antibiotic cocktail called Bactec Panta Plus (manufactured by Becton and Dickenson: stock solution contained: Polymyxin B 10,000 units/ml; Amphotericin B 1,000 μg/ml; Nalidixic Acid 4,000 μg/ml; Trimethoprim 1,000 μg/ml; Azlocillin 2,000 μg/ml). The paraffin wax coated slides were transferred as cited above but, in this protocol the new tubes also contained antibiotic cocktail. The subculturing and speciation procedures the same as those cited below.

3.9 Sputum Culture Isolates

Suspensions of sputum were prepared from sputum derived from AIDS patients by inoculating 500 μl of sputum into 4.5 ml of sterile Czapek broth which also contained 1:100 final dilution of Bactec Panta Plus antibiotic cocktail. The subculturing speciation procedures were the same as previously cited.

3.10 Speciated MAI Isolates

The strains speciated as MAI complex organisms were: 953, 1516, 1762, 4861, 5097, 6475, 8515, 8997, 15113, 10,000, 10,001, 10,002, 10,008, 10,010, 10,011, 10,012, 10,013, 10,014, 10,015 and 10,016. The aforementioned strains were initially isolated from the blood of the AIDS-patient population of St. Vincent's Hospital.
and Medical Center of New York via the Paraffin Wax Slide culture protocol. These were speciated by the following conventional biochemical criteria: reduction of tellurite in 3 days, absence of nitrate reduction, negative urea hydrolysis and absence of Tween 80 hydrolysis reaction. A parallel species conformation of the MAI complex organisms was performed via the GEN-PROBE hybridization protocol.

3.11 Stock Cultures

The stock cultures of *Mycobacterium fortuitum* NI and *Mycobacterium kansasii* NII were kindly supplied to us by Mr. William Millett, Assistant Director, Department of Microbiology, St. Vincent's Hospital and Medical Center of New York.

3.12 Stock Culture Media

The stock cultures were grown in Trypton broth (See Appendix IV; Media), made up to 1.0 litre in distilled water, and adjusted to pH 7.5 (Ollar, 1976). The trypton broth was dispensed in 4.5 ml amounts and sterilized by autoclaving for 15 mins at 1.0 kg/cm².

3.13 Speciation Media

The media utilized for speciation were dispensed in 4.5 ml amounts and sterilized by autoclaving for 15 min at 1.0kg/cm². Urea broth and nitrate broths were prepared according to the method cited in the Ninth Edition of the Difco Manual (Difco Laboratories Inc., 1974). Tween 80 broth and tellurite reduction indicator were prepared according to the method cited in the Second Edition of the Manual of Clinical Microbiology (Runyon et al., 1974).
3.14 Preparation of Stock Culture for Speciation Assays

A loopful (10 μl) of stock culture was added to 4.5ml amounts of trypton broth and incubated at 37° C. The cultures were grown for 6-14 days. Thereafter, 0.5ml of stock culture broth culture were pipetted into 6 tubes containing sterile Czapek broth, and the paraffin wax coated slides were added (Ollar, 1976), incubated at 37° C and checked daily for growth on the slide (paraffin slide culture).

3.15 Speciation Assays

3.15a Paraffin utilization and "in situ" acid alcohol fastness assay

When visible growth was observed on the paraffin wax-coated culture slides, one of the slides was removed and stained for acid-alcohol fastness by placing it into a tube containing Kinyoun carbolfuchsin (See Appendix V; Stains) for 15 min, immersing it several times in distilled water, placing it in a tube containing acid-alcohol (See Appendix V; Stains) for 5 min, immersing it in a tube containing distilled water and then immersing it in a tube containing 1.0% (v/v) aqueous solution of methylene blue (See Appendix V; Stains) counterstain for 1 minute. The slide cultures were given a final rinse in a tube containing distilled water, removed and gently blotted (with care not to remove the stained growth from the paraffin wax surface) with a clean absorbent paper tissue. It is important to note that during each step of the staining the entire slide should be totally submerged. The slides were then viewed at 250x, 450x and 1000x oil immersion (Ollar et al. 1990).
3.15b Tellurite reduction assay

Slide cultures showing heavy growth were tested for the ability to reduce tellurite in 72 hours by transferring them to sterile tubes containing sterile Czapek broth to which 200 µl of potassium tellurite reagent had been previously added (Runyon et al., 1974; Ollar et al., 1990). The slide cultures were then incubated at 37° C and checked daily for tellurite reduction for a period of 72 hours. A blank sterile paraffin wax coated slide was added to sterile media containing tellurite as a reagent control. The strains of *M. avium-intracellulare* and *M. fortuitum* cited above were used as positive controls and the known strain of *M. kansasii* cited above was utilised as a negative control.

3.15c Nitrate reduction assay

Slide cultures showing heavy growth were assayed for the ability to reduce nitrates by adding them to tubes containing sterile nitrate broth (Runyon et al., 1974; Ollar et al., 1990). After a period of 12-24 hours incubation at 37° C, the slides were removed from the broth and 5 drops of sulfanilic acid reagent solution followed by 5 drops of alpha naphthylamine reagent solution were added to the tubes. The same procedure was carried out with sterile media exposed to sterile paraffin slides as reagent controls. The strains of *M. fortuitum* and *M. kansasii* cited above were used as positive controls and the strains of *M. avium-intracellulare* cited above served as negative controls.

3.15d Urea Hydrolysis Assay

Slide cultures were added to tubes containing 4.5ml amounts of sterile urea broth (Runyon et al., 1974; Ollar et al., 1990). The cultures were incubated at 37° C and checked for a
period of 3 days. Sterile paraffin slides, added to sterile urea broth, served as reagent controls. The strains of *M. fortuitum* and *M. kansasii* served as positive controls and the strains of *M. avium-intracellularare* served as negative controls. A positive reaction involved a colour change to pink or red after a period of 3 days.

3.15e **Tween 80 Hydrolysis Assay**

Slide cultures were added to sterile tubes containing Tween 80 media and incubated at 37° C (Runyon et al., 1974; Ollar et al., 1990). The reagent controls were sterile Tween 80 media and sterile paraffin slides. A slide culture of the strain *M. kansasii*, added to sterile Tween 80 media, served as a positive control. Positive reaction involved the appearance of pink colour within 5 days. A slide culture of the strain *M. avium-intracellularare* served as a negative control.

3.15f **Gen Probe Hybridization**

The GEN-PROBE hybridization analysis of the Paraffin slide culture isolates was performed by the Dept of Clinical Microbiology of St. Vincent's Hospital.
RESULTS AND DISCUSSION

3.16 Paraffin Wax Slide Culture isolation of MAI

The use of slide culture for the mycobacteria has largely been overlooked, namely, because of the fact that in some species of the genus Mycobacterium hyphae are never seen, whereas other species have incipient hyphae only in young cultures (Gottlieb, 1973).

Prior to this investigation, the concepts of Paraffin Slide Culture "in situ" growth isolation linked with a slide culture based biochemical assay of "in situ" growth were unknown concepts in the realm of Mycobacteriology. At the onset of this investigation, the Paraffin Slide Culture was a device which I had developed 17 years earlier for isolation of Nocardia asteroides and therefore did not have any Mycobacterial focus (Ollar, 1976). In its original form, the Paraffin Wax Slide Culture merely examined "in situ" morphology and the acid-fastness of the latter.

The literature at the beginning of this project, however, noted that several early investigations had determined there were species of mycobacteria capable of utilizing paraffin wax as a sole source of carbon in basal media devoid of all other carbon sources (Sohngen, 1913; Gordon and Hagen, 1936; Fuhs, 1961). In addition, one investigator had found that M. tuberculosis was incapable of utilizing paraffin wax (Fuhs, 1961). However, these citings were related to growth upon paraffin wax coated rods so that Paraffin Wax Slide Culture or its application to the examination of "in situ" mycobacterial growth was ignored.

In the scenario of AIDS-related mycobacterial infection, M. avium complex organisms are often found growing concomitantly with M. tuberculosis. A Paraffin Slide therefore could serve as a means of distinguishing between "atypical" mycobacteria and M.
tuberculosis because the latter cannot utilize paraffin wax as a sole source of carbon. The Paraffin Wax Slide Culture System has the advantage of being a highly selective milieu, and would reduce the risk of contamination because very few human pathogens and commensals are able to grow on paraffin wax. In addition, the acid-alcohol fast staining procedure allows for additional screening to determine whether the organisms are nocardioforms or atypical mycobacteria. The nocardioforms such as Nocardia asteroides are able to utilize paraffin but are not acid-alcohol fast (by the staining method used) and are filamentous upon slide culture. Thus they would be ruled out by the Paraffin utilization and "in situ" acid-alcohol fastness assay (cited below) in the paraffin-wax based system.

The role of the Paraffin Wax Slide Culture was further enhanced by the inclusion of a series of key conventional biochemical assays performed upon the "in situ" growth (Tellurite reduction, Nitrate reduction, Urea hydrolysis and Tween 80 hydrolysis) which are indicative of MAI complex organisms. The concept of performing biochemical speciation upon "in situ" slide culture growth especially Paraffin Wax Slide Culture is a completely new approach. These "in situ" biochemical assays will be discussed in greater detail below.

3.17 Blood

The median time needed to isolate acid-alcohol fast organisms from blood culture via the Paraffin Wax Slide Culture system (Para SL/C) was 22 days (See Tables 2&3). The range of time needed for detection of MAI via the Para SL/C was 13 to 41 days. The literature has revealed that the range of time needed for detection with the Bactec Radiometric system was 3 to 23 days.
(Roberts et al., 1987). A Chi-square analysis of the two time distributions revealed that there did not occur a statistically significant \( P=0.1892 \) difference in the time intervals needed for MAI detection by these two diverse systems.

This is the first time that a paraffin-wax system has been successfully used in AIDS patients to isolate paraffinophilic acid-alcohol fast organisms \( (953, 1516, 1762, 4861, 5097, 6475, 8515, 8997, 15113, 10,000, 10,001, 10,002, 10,003, 10,004, 10,008, 10,009, 10,010, 10,011, 10,012, 10,013, 10,014, 10,015 \) and \( 10,016 \) ) from blood. Unfortunately, due to incubator malfunction, 3 blood isolates \( (10,003, 10,004, 10,009) \) were destroyed before speciation could be carried out.

\[ 3.18 \text{ Stool} \]

The experiments with stool culture marked the first time that Paraffin Wax Slide Culture had been applied to isolate paraffinophilic acid-alcohol fast organisms in stool specimens of AIDS-patients. Stool isolates are quite important since the gastrointestinal tract has been cited as a focus of MAI infection (Hellyer et al., 1991) and as a possible causative agent of diarrhea (Smith et al., 1992). The initial experimental work in this highly contaminated environment proved that the Para SL/C linked with acid-alcohol fast staining could discern the presence of acid-alcohol fast elements and non-acid-alcohol fast but paraffinophilic elements as seen in figure 1.

The isolation of uniquely acid-alcohol fast elements by the Paraffin Wax Slide Culture System, however, was only achieved by the addition of 1:100 dilution of an Anti-bacterial and Anti-fungal antibiotic Cocktail called Panta Plus (Becton and Dickenson) as seen in figure 2. This Panta Plus additive killed all possible
paraffinophilic non-acid alcohol fast growth as is demonstrated in figure 2. Stool sample analysis was possible without the need for vigorous pre-inoculation decontamination procedures as are required for conventional Lowenstein & Jensen preps.

The time needed to isolate acid-alcohol fast organisms from a stool culture via the Paraffin Slide Culture system varied from 7 to 24 days. The mean isolation time was 15.5 days.

When the Paraffin Slide Culture incorporated 1:100 dilution of the Bactec Panta Plus antibacterial and antifungal antibiotic cocktail in specimen 10,006, the time needed for isolation was 7 days. By contrast, in specimen SK7B where the Bactec Panta Plus antibacterial and antifungal was not utilized, it required 24 days for the isolation of acid-alcohol fast organisms.

Despite the encouraging initial experiments involving AIDS-related stool specimens, these experiments with stool culture were incomplete due to the destruction of these stool isolates (10,005, 10,006, SK7B) in an incubator thermostat malfunction. Further experimentation with fecal isolation proved difficult due to the official reluctance on the part of Medical Staff to permit the taking of fecal samplings. It should be noted that the official line among the Hospital Staff at St. Vincent's Hospital is that blood is the only relevant MAI specimen.

3.19 Sputum

During the course of this investigation one sputum sample was obtained from an AIDS patient. The time needed to isolate acid-alcohol fast organisms from a sputum culture was 7 days. Tragically, this specimen was among those lost due to incubator malfunction.
Figure 1: Stool isolate from AIDS patient. Atypical Mycobacteria SK7B (acid-alcohol fast staining) and yeasts (non-acid-alcohol fast staining) 24 days in situ growth on Para SL/C at 37° C (1000x Oil Immersion, Microscopic Magnification).
Figure 2: Stool isolate from AIDS patient. Atypical Mycobacteria 10,006 (acid-alcohol fast staining) 7 days in situ growth on Para SL/C at 37° C. The Czapek broth also contained 1:100 dilution of Bactec Panta Plus (antibacterial and antifungal antibiotic) cocktail. (1000x Oil Immersion, Microscopic Magnification).
3.20 Paraffin utilisation assay and "in situ" acid-alcohol fastness.

A macroscopic view of a Paraffin Wax Slide Culture where growth occurred, displayed the presence of a heavy pellicle that was either non-pigmented or pigmented at the level of the menicus. The macroscopic view of the same Paraffin Wax Slide below the menicus revealed small white punctiform colonies. The kinyoun acid alcohol fast staining of the slide cultures revealed, under the light microscope at 450x and 1000x, bacillary forms, branching filaments, and thick colonial mass (See Figures 3 and 4).

The growth of M. fortuitum usually appeared after a minimal growth period of 48-72 hours at 37° C and the M. kansasii growth usually appeared after a minimal growth period of more than 148 hours at 37° C. In both these organisms there were again observed a variety of acid alcohol fast staining forms.

A useful finding obtained from these studies with the Paraffin Wax Side Culture was that we were able to observe the presence of cording in some of our isolates (10,002, 10,010, 10,011 and 10,015) without having to perform any additional assays. The subject of MAI cording is examined in greater detail in Section IV.

3.21 Tellurite reduction assay

A heavy growth on the paraffin wax slide culture was necessary in order to perform this assay. After a period of 24-72 hours exposure of the tellurite to paraffin slide culture, the positive control mycobacterial species (M. fortuitum NI) displayed a heavy black precipitate at the level of the meniscus pellicle indicative of the classifical tellurite reduction (See Figure 5). By contrast, the reagent controls which were processed in the same manner failed to show a positive tellurite reduction. The negative
control mycobacterial species (\textit{M. kansasii} NII) failed to show any reduction of the tellurite (See Figure 5). \textit{M. avium-intracellulare} complex organisms are able to reduce tellurite (Runyon et al., 1974; Ollar et al., 1990). The reliability and reproducibility of this assay was confirmed by the fact that in 20 isolates where it was run (See Table 2) a tellurite reduction was achieved. These isolates were also confirmed as being \textit{M. avium} by a subsequent GEN-PROBE analysis.

The system even permitted us to view microscopically the "in situ" microcolonies that had reduced tellurite since they were in effect stained by the tellurite reaction.

3.22 Nitrate reduction assay

Nitrate broth that had been exposed to slide cultures that contained heavy growth of \textit{M. fortuitum} and \textit{M. kansasii} for a period of 12-24 hours showed a typical red colour when treated with the sulfanilic acid and alpha naphthylamine reagents revealing a reduction of the nitrate to nitrite. By contrast, the reagent controls which were processed in the same manner failed to show a positive nitrate reaction. \textit{M. avium-intracellulare} complex fail to reduce nitrate (Runyon et al., 1974; Ollar et al., 1990). The reliability and reproducibility of this assay was confirmed by the fact that in 20 isolates where it was run (See Table 2) a nitrate reduction was not achieved. A GEN-PROBE analysis confirmed the species as being \textit{M. avium}.

This investigation found that because the Czapek broth control contained a nitrate source, namely, \textit{NaNO}_3, it also could be used as an additional medium to assay for nitrate reduction. In fact, the nitrate reduction assay worked equally well with either Czapek or nitrate broth. This finding added to the utility of the
Paraffin Wax Slide Culture System by reducing the number of special speciation media required and thereby cutting down costs incurred by the purchase of these additional assay broth media.
Figure 3: Microcolonial growth of MAI. Paraffin slide culture stained with Kinyoun acid alcohol fast staining (200x, Microscopic Magnification)
Figure 4: **Bacillary and rudimentary filamentous forms.** Paraffin slide culture stained with Kinyoun acid alcohol fast staining (2000X Oil immersion, Microscopic Magnification)
Figure 5: Comparison of paraffin slide cultures. A positive tellurite (black) assay and a routine paraffin slide culture growth.
3.23 **Urea hydrolysis Assay**

When slide cultures that contained heavy growth of *M. fortuitum* were added to sterile urea broth, a pink or rose colour developed 3 days at 37° C, indicative of urea hydrolysis. By contrast, the reagent controls which were processed in the same manner failed to show a positive Urea hydrolysis reaction. *M. avium-intracellulare* complex do not hydrolyze Urea (Runyon et al., 1974; Ollar et al., 1990). The reliability and reproducibility of this assay was confirmed by the fact that in 20 isolates where it was run (See Table 2) a Urea hydrolysis was not achieved. These isolates were also tested by GEN-PROBE analysis and found to be *M. avium*.

3.24 **Tween 80 Hydrolysis Assay**

Slide cultures containing heavy growth of the strain *M. kansasii* added to sterile tubes containing Tween 80 media produced positive reactions within 5 days incubation at 37° C, seen as the appearance of red colouration of the colonial growth at the meniscus surface of the slide culture. By contrast, the reagent controls which were processed in the same manner failed to show a positive reaction. *M. avium-intracellulare* complex do not hydrolyze Tween 80 (Runyon et al., 1974; Ollar et al., 1990). The reliability and reproducibility of this assay was confirmed by the fact that in 20 isolates where it was run (See Table 2) a Tween 80 hydrolysis was not achieved. These isolates were also speciated by GEN-PROBE analysis and found to be *M. avium*.
3.25 Pigmentation of MAI Isolates

The presence of colonial pigmentation was seen to be variable. Several MAI speciated isolates (1516, 6475, 10,000, 10,013 and 10,014) from the total collection of speciated MAI isolates were pigmented whereas the remaining MAI speciated isolates were not (See Table 2). The presence of pigmentation of the paraffin slide culture was correlated by its presence on subsequent subcultures made on conventional L&J slopes.
<table>
<thead>
<tr>
<th>Spec. number</th>
<th>ParSL/C and Alcohol acid fast.</th>
<th>Tellurite Reduct.</th>
<th>Nitrate Reduct.</th>
<th>Urea Hydro</th>
<th>Tween 80 Hydro</th>
<th>Gen Probe Hybrid with (M. avium) probe</th>
<th>Pigment</th>
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<td>953 (MAI)</td>
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<td>-</td>
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<td>+</td>
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<td>1762 (MAI)</td>
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<tr>
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<td>-</td>
<td>+</td>
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<tr>
<td>(M. kansas) II NII</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>(M. fort) NI</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>nd*</td>
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*nd=not done
Table 3: Total Time Needed For Isolation and Speciation of MAI from Blood by Paraffin Wax Slide Culture System

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Time in Bactec 13A SL/C (in days)</th>
<th>Time in vial (in days)</th>
<th>Total time for (+) in Par SL/C growth before seeding in Para SL/C</th>
<th>Final time</th>
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<tr>
<td>10,000</td>
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<td>5</td>
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<td>25</td>
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<tr>
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<td>18</td>
<td>21</td>
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<td>7</td>
<td>28</td>
<td>31</td>
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<td>3</td>
<td>13</td>
<td>16</td>
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<tr>
<td>10,016</td>
<td>25</td>
<td>6</td>
<td>31</td>
<td>34</td>
</tr>
</tbody>
</table>
3.26 The Duration of Speciation

The literature revealed that the range of time needed for MAI speciation was 7 to 27 days via the Bactec Radiometric/GENE-Probe (Roberts, McMillan and Coyle, 1987; Ellner, Kiehn Cammarata and Hosmer, 1988). The range of time needed for speciation with the Para SL/C system was 16 to 44 days. The Chi-square analysis of the time distributions for speciations also failed to reveal statistically significant differences (P=0.5101). The median time needed to confirm the presence of MAI organisms on Para SL/C (i.e. speciation of MAI complex organisms) was 25 days (See Table 3).

The combination of Bactec 13A media and the Paraffin Wax Slide Culture System was successfully able to isolate and speciate MAI organisms from AIDS blood without the need to purchase a sophisticated radiometric detector. In addition, these experiments have also revealed that the Para SL/C system could indeed isolate and speciate MAI at time intervals comparable with the more sophisticated Bactec Radiometric and GENE-Probe assays.

3.27 General Comment On Paraffin Wax Slide Culture

As the AIDS epidemic spreads through the developed and developing areas of the world, this world-wide problem will oblige many bacteriologists who have not hitherto worked with mycobacteria to focus on the MAI organisms. This investigation has shown that a Para SL/C can successfully isolate and speciate MAI complex organisms. The simplicity, low cost, and reduced risk of contamination make the system especially suitable for small rural laboratories and field stations where the small AIDS population would not warrant the extra expenditure of expensive equipment and extra personnel. The procedure is specially useful for
laboratories in developing countries where manpower and financial resources are both in short supply (Ollar et al., 1990).
Section IV
Cording in *Mycobacterium avium-intracellularare*
Complex Organisms
INTRODUCTION

4.0 Serpentine Cording

The mycobacteria are inducers of granuloma formation (Youmans et al., 1975). The component of the mycobacterial cell that appears to be primarily responsible for inducing the granulomatous response is the cord factor. This substance, found in the mycobacterial cell wall surface, was thought to be responsible for the manner in which virulent mycobacteria adhere to one another and grow as intertwining serpentine cords in which the bacilli aggregate with the long axes parallel. Virulent strains of M. tuberculosis complex organisms have been observed to grow as characteristic ropes, bundles or serpentine cords of acid-fast organisms, however, most avirulent strains grow in a more disordered manner (Yagupsky et al. 1990). The correlation between cording and virulence has proven to be imperfect because the presence of serpentine cords has been shown to be also found in saprophytic strains such as M. phlei (Runyon, 1965).

Lorian found he could achieve enhanced cording of M. tuberculosis by culturing it upon 7H10 Agar supplemented by Triton WR 1339 and OADC (Lorian, 1969). Non ionic detergents (i.e. Tween 80) reduce cord formation by coating the cell surface (Wolinsky, 1980). Therefore, Buttiaux et al. have recommended that all media utilized for examining cording should exclude Tween 80 (Buttiaux et al., 1974). Thus the ability of M. tuberculosis to grow in vitro as serpentine cords was indeed influenced by the composition of the culture milieu (Yagupsky et al., 1990).
4.1 Cording Factor

The cord factor which may be responsible for both virulence and serpentine cording has been identified as being the mycoside 6,6'-dimycolate trehalose (Wolinsky, 1980). The toxic glycolipids that contain trehalose occur with distinctive modifications in members of the genera Corynebacterium, Mycobacterium and Nocardia (Barksdale and Kim, 1977). It was theorized that the trehalose esters such as the cord factor behaved as detergents and this coupled with the fact that they are suggestive of a role in facilitating the inward movement of certain molecules important to the growth of the cell (Barksdale and Kim, 1977).

Mycobacterium tuberculosis H₃₇Rv elaborates the cord factor 6,6' dimycoly1-alpha, alpha prime -D-trehalose which, in microgram amounts, kills mice (Barksdale and Kim, 1977). This killing is associated with the destruction of mitochondrial membranes and phosphorylation. In addition, it has been found that a side effect of the cord factor has been the intoxication upon the microsomal enzymes pyrazinamide deasamidase and aminopyrine demethylase. In contrast to its toxic effect in mice, cord factor is relatively nontoxic for the rat.

There are several factors which are thought to link cord factor to virulence, namely, a) its extraction renders cells phenotypically nonvirulent, b) it inhibits migration of normal polymorphonuclear leukocytes in vitro (as in the case of virulent tubercle bacilli), and when 10 μg of cord factor are given subcutaneously to a mouse it will die, c) cord factor is more abundant in virulent strains of tuberculosis, d) M. tuberculosis bacilli which have been recovered from animals or from young cultures are more virulent, and exhibit a greater content of cord
factors than do mycobacterial cells of the same strain from older cultures, e) mice have been protected against *M. tuberculosis* infection by being actively immunized with a complex of cord factor and methylated bovine serum albumin or via passive transfer of rabbit anti-cord-factor serum (Wolinsky, 1980).

Recently, it has been proposed that the trehalose glycolipids that have been found on the surface of the mycobacteria may be important in the inflammatory response caused by virulent mycobacterial strains (Behling et al., 1991). Injections of trehalose 6,6'-dimycolate into susceptible mice was found to be lethal in much lower amounts than other mycolic acid-disaccharide esters. Trehalose 6,6'-dimycolate (TDM) injections also produced pulmonary granulomas in mice. In those mice treated with TDM, pulmonary granulomas were larger and accompanied by alveolar wall thickening and hyperplasia not seen in other treatments. This investigation supported earlier investigators that the glycolipid structure of TDM is important for its biological activity and suggest that TDM contributes to the granulomatous response in mycobacterial infections (Behling et al., 1991).

### 4.2 Corded *M. avium*-intracellulare Colonial Morphology

The essence of mycobacterial roughness is the cohesion of the bacteria, usually in cords or strands; these cords or strands are commonly serpentine (Sommers and Good, 1985). Moehring and Solotorovsky have found that rough isolates of *M. avium* have been seen less frequently than smooth isolates (Moehring and Solotorovsky, 1965). These rough isolates were hydrophobic and not easily suspended in liquid and were found in low proportions (1 to 10 per cent) in several of the cultures studied. The virulence of the different colonial morphological types was performed by chick
virulence assay. The smooth, transparent colony type was present in all the virulent M. avium strains and comprised 80-100 per cent of the total colonies observed. Rough granular variants were variable in virulence. Pattyn investigated the colonial morphology of M. avium correlated with in vitro characteristics and fowl virulence (Pattyn, 1967). He found that rough or freshly isolated variants were capable of bringing about morbidity in chicks (Pattyn, 1967). Rastogi et al. (1989) have also noted that a rough mutant of M. avium appeared to be as virulent for rabbits and chickens as their parental smooth strain. In addition, Rastogi et al. have noted that attempts to correlate the colonial morphology of M. avium with virulents has given conflicting data. Rastogi et al. have stated that certain reports have given greater virulence to the rough strains while other reports have stated the opposite. It has been found that a small proportion of M. intracellulare colonies may be partially or completely rough and that in the case of M. avium the proportion of rough may be often greater (Sommers and Good, 1985). There have been strains of M. avium complex organisms that have colonies so rough that these isolates have been confused with M. tuberculosis.

In a recent study of 126 isolates of M. avium complex from specimens other than blood, only 0.8% (1/126) of these isolates was seen to exhibit cording (Yagupsky et al., 1990).

The majority of the strains of AIDS-related M. avium that have been isolated in my study have been derived from blood. Horn and associates have said that most of the isolates of AIDS-related M. avium complex organisms are derived from blood isolates (Horn et al., 1989). The current literature does not mention anything about the presence, occurrence and significance of
hypercorded strains of M. avium complex organisms derived from blood isolates of AIDS patients.
METHODS AND RESULTS

4.3 Strains

The 20 strains of MAI (953, 1516, 1762, 4861, 5097, 6475, 8515, 8997, 15113, 10,000, 10,001, 10,002, 10,008, 10,010, 10,011, 10,012, 10,013, 10,014, 10,015 and 10,016) which had been isolated and speciated in Section III were utilized in this section.

4.4 Detection of in situ Cording

The 20 alcohol-acid fast stained paraffin wax slide culture slides derived from the strains cited above (originally obtained during the process of Speciation in Section III) were all further scrutinized via light microscope at 450X for the presence of Serpentine Cording.

Of the 20 M. avium complex strains examined, the 4 isolates previously cited in section III (10,002, 10,010, 10,011 and 10,015) as being hypercorded were the only isolates that displayed Serpentine Cording (See Figures 6, 7, 8; See Table 4). Hypercorded isolates accounted for 20% (4/20) of the speciated St. Vincent's M. avium complex strains.

4.5 Stability of Cording

The isolates of M. avium complex originally derived from AIDS blood cultures which displayed hypercording (10,002, 10,010, 10,011, 10,015) had been previously subcultured on Lowenstein-Jensen slopes and stored at 4° C (See Section III).

A 4 mm loopful of original hypercorded isolate was aseptically removed from the Lowenstein-Jensen slope and added to 2.0 ml of sterile saline. Aliquots of 0.5ml were added to 2 tubes of Czapek liquid Para SL/C (Para SL/C was utilized to determine non-disturbed in situ colonial morphology) and 2 tubes of 7H9 broth.
supplemented with 2% glycerol and 4% OADC (See Appendix IV; Media). The tubes were then incubated at 37° C for 10-13 days. The Para SL/C's were stained via the Kinyoun acid-alcohol procedure (as cited in Section III) to determine the presence or loss of hypercording. The 7H9 tubes were utilized to inoculate a new series of cultures consisting of 2 tubes of Czapek Para SL/C and 2 tubes of 7H9. The process of subculturing and Para SL/C in situ analysis was carried for 4 successive subcultures.

The Para SL/C's of the original patient isolates of all four strains examined showed hypercording. In strain 10,002, diminished hypercording in the first subculture with a total loss of hypercording at the third subculture. In strain 10,010, the first subculture showed strong hypercording, however, subcultures 2-3 showed diminished cording and at subculture four there was a complete absence of hypercording. The loss of hypercording was seen in strain 10,011 at subculture 2. In strain 10,015, however, the loss of hypercording was detected by subculture 3. (See Figures 9-11; Table 5).
Figure 6: **Corded in situ growth on Para SL/C.** Para SL/C growth of MAI isolate 10,010 derived from an AIDS patient blood sample (100x, Microscopic Magnification)
Figure 7: Serpentine cording of in situ growth on Para SL/C. MAI isolate 10,010 on Para SL/C derived from an AIDS patient blood sample (450x, Microscopic Magnification).
Figure 8: **Corded in situ growth on Para SL/C.** Growth on Para SL/C of MAI isolate 10,011 on Para SL/C derived from an AIDS patient blood sample (450x, Microscopic Magnification).
Figure 9: MAI 10.011-Initial isolate. Initial AIDS patient blood isolate showing hypercording (100x, Microscopic Magnification).
Figure 10: MAI 10.011-First Subculture. First subculture (10 days) showing diminished hypercording (100x, Microscopic Magnification).
Figure 11: MAI 10.011-Second Subculture. Second subculture (10 days) showing loss of hypercording (1000x Oil Immersion, Microscopic Magnification)
<table>
<thead>
<tr>
<th>AIDS-Related MAI Isolate</th>
<th>Presence of Cording</th>
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<tbody>
<tr>
<td>953</td>
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<tr>
<td>1516</td>
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<td>1762</td>
<td>-</td>
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<td>4861</td>
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<td>5097</td>
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<td>8515</td>
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<td>10,016</td>
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### Table 5: Effects of Subculturing on Hypercorded MAI Isolates

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<th>Subculture II</th>
<th>Subculture III</th>
<th>Subculture IV</th>
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<tr>
<td>10,002 +/- +/ -</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>10,010 + +/ -</td>
<td></td>
<td>+/ -</td>
<td>+/-</td>
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</tr>
<tr>
<td>10,011 +/ -</td>
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<td></td>
</tr>
<tr>
<td>10,015 +/- +/ -</td>
<td>+/-</td>
<td>+/ -</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

+ = presence of cording
+/- = presence of diminished cording
- = complete absence of cording
DISCUSSION

4.6 Significance of Corded MAI

Virulent strains of *M tuberculosis* have been shown to often grow as characteristic ropes, bundles or serpentine cords of acid-fast bacilli in liquid culture (Yagupsky et al., 1990). By contrast, the avirulent strains of *M. tuberculosis* have been found to grow in liquid media in a nonoriented, dispersed manner. The serpentine cording that occurs in *M. tuberculosis* also occurs in the saprophyte *M. phlei*, and other mycobacterial species such as the *M. avium* complex and *M. triviale* (Runyon, 1965; Sommers and Good, 1985). In fact when serpentine cording does occur in *M. avium* and *M. triviale*, these strains have on occasion been confused with tubercle bacilli and it was only after subsequent biochemical assays that speciation as non-tuberculous mycobacteria was possible (Gilkerson et al., 1966, Sommers and Good, 1985). The Para SL/C for viewing in situ mycobacterial cording provided a useful means for making the distinction between the serpentine cording of non-tuberculous and tuberculous cording. The ability to make this distinction of cording is based on the fact that only atypical corded or non-corded mycobacteria can utilize a paraffin wax substrate whereas, *M. tuberculosis* cannot (Fuhs, 1961; Ollar et al., 1990). This in situ protocol fulfills the basic notions for the observation of cording cited by Buttiaux and his co-workers, namely, 1) that the culture be seeded in liquid milieu, 2) does not contain Tween 80. Nonionic detergents such as Tween 80 reduce the formation of cording by coating the cell surface (Wolinsky, 1980). The third notion for observing cording requires that the mycobacterial growth not be agitated during its growth upon the surface of the slide (Buttiaux et al., 1974). Buttiaux et al. have in reality described a slide culture system. The finding of corded
in situ growth on slide culture would indeed make slide culture a relevant protocol in mycobacteriology.

The relationship of serpentine cording to virulence is demonstrable with the species M. tuberculosis and M. bovis but, cording and virulence is not known to be so correlated in other mycobacterial species (Runyon, 1965). Pattyn has demonstrated that a rough strain of M. avium was as virulent for chicks as the more commonly occurring virulent smooth strains of M. avium (Pattyn, 1967). Runyon has stated that achieving equal inocula of rough and smooth strains in terms of numbers and activity is difficult and perhaps rarely obtainable and, thus, caution is needed in crediting differences in virulence due to smooth and rough colonial morphology. Studies with human non-peripheral blood isolates has shown that the occurrence of corded strains was extremely rare and occurred in 0.8% or 1/126 isolates (Yagupsky et al., 1990). The studies conducted at St. Vincent's Hospital on AIDS patient peripheral blood isolates of MAI found that 20% or 4/20 isolates displayed cording. This increased incidence of rough or hypercorded strains is all the more curious given the fact that rough or hypercorded strains are seldom seen in clinical laboratories (Rastogi et al., 1989). These hypercorded isolates lost their hypercording after 4 successive subcultures. Thus, the significance of more frequent occurrence of the hypercorded or serpentine corded colonial morphological forms of MAI complex organisms in AIDS-patient peripheral blood isolates and any possible differences in virulence when compared with the noncorded AIDS-patient peripheral blood isolates still remain unclear. The relationship between hypercording and its loss upon MAI virulence will be discussed in further detail in Section VIII.
Section V

Antibiotic Sensitivity Methodology

for Mycobacterium avium-intracellulare Complex Organisms
INTRODUCTION

5.0 A Therapeutic Dilemma

The role of *Mycobacterium avium* as a pathogen in the HIV infected patients has been confusing and controversial to clinical practitioners (Ellner et al., 1991). Laughon et al. (1991) have stated, "Uncertainty about the extent of the morbidity and mortality caused by *Mycobacterium avium* complex infections in AIDS patients and the resistance of *Mycobacterium avium* complex to most therapeutic interventions has created controversy and frustration among researchers and clinicians regarding the relative merits of specific therapy". Clinical isolates have been often resistant to conventional antimycobacterial agents and a lack of correlation between in vitro sensitivities, and clinical response in uncontrolled series has been observed (Hawkins et al., 1986; Young, 1988). At one time in the current AIDS story, it was suggested that patients with disseminated MAI complex should not be treated because the therapy have proven to be of no value and also was potentially toxic (Bach, 1989). Linked to this early leaning to therapeutic nihilism was the debate that was concerned with whether disseminated infection caused symptoms and contributed to patient mortality (Ellner et al., 1991).

5.1 Antibiotic Sensitivity Testing for MAI

Conventional disk and well diffusion protocols are not suitable for antimicrobial sensitivity testing of slowly growing mycobacteria (Runyon et al., 1974). Most studies on susceptibility testing have traditionally employed agar dilution methods (Hawkins, Wallace and Brown, 1991; Ollar et al., 1991). Heifets stated that an important drawback in the agar dilution assay was that the drug concentrations incorporated in solid media are not always the same
as those actually interacting with the mycobacterial inoculum during the period of incubation. For this reason, Heifets preferred a mycobacterial sensitivity assay that could be performed via a radiometric BACTEC (See Section I) system which used radiolabelled 7H12 broth. This radiometric assay was able to be completed in 8 days and was able to avoid using excessive inoculum which would be necessary to obtain comparable results if a conventional tube dilution turbidimetric protocol had been utilized. Heifets and his co-workers noted that broth dilution methods for the determination of antibiotic susceptibility are preferable to agar dilution methods because they have a shorter incubation period which reduces the risk of drug degradation and because the concentration of antibiotic dissolved in broth is directly responsible for producing the antibacterial effect (Heifets, 1988; Heifets, Lindholm-Levy, 1989). The radiometric method is now commonly applied in both clinical and research settings.

The conventional broth dilution protocol employs turbidimetric determination in either microdilution wells or test tubes. The microdilution assay works well but must be cautiously interpreted with those antimicrobials that produce a "tail without a clear borderline between growth and inhibition (Heifets, 1988; Heifets and Lindholm-Levy, 1989). Concern for the production of potentially infectious aerosols may restrict the use of microtiter testing to special containment facilities. Macrodilution tubes assays with MAI are possible but MIC's determined by this method may be inaccurate because of the heavy inoculum required to produce detectable growth within 8 days (Heifets, 1988; Heifets and Lindholm-Levy, 1989; Heifets et al., 1990). A practical limitation of the radiometric procedure described by Heifets is its dependence
upon highly skilled personnel and the support of a well equipped clinical laboratory, including an expensive radiometric detector.

All current methods require the use of perishable media which must be prepared and maintained under sterile conditions. Furthermore, no consensus has been reached as to which of the currently employed methods for antibiotic sensitivity testing should be accepted as a standard for sensitivity determinations in atypical mycobacteria (Ollar et al., 1991).

Paraffin baiting (Ollar et al., 1990) may be particularly useful for the isolation of organisms from heavily contaminated specimens and where the use of sophisticated microbiological techniques are impractical. With the growing appreciation of the pathogenicity of MAI in patients with AIDS as well as in the normal host, this tool may be of particular importance in locations where modern techniques are unavailable. The capacity to determine antimycobacterial sensitivities under these circumstances would extend the usefulness of this Para SL/C tool.
5.2 Strains

Strains of MAI used in this study were originally isolated from AIDS patients at St. Vincent's Hospital and Medical Center of New York and at Memorial Sloan-Kettering Cancer Center. The strains from both institutions were identified by routine morphologic and microbiological procedures with confirmation by DNA hybridization (Gene Probe; Biogen) for *M. avium* and *M. intracellulare*. Strains isolated at St. Vincent's Hospital were: 10,000, 1762, 1516, 15113, 8515, 6475, 5097, 8197 and 4861. Those strains from Memorial Sloan-Kettering Cancer Center were: SK015, SK016, SK095, SK069, SK037, SK060, SK024. All strains were isolated from patients with AIDS and identified as *M. avium* with the exception of SK069 which was an *M. intracellulare* isolated from an immunocompromised patient with cavitary pulmonary disease.

5.3 Sensitivity Testing

Stock solutions of antimicrobial agents were prepared. Amikacin (Bristol-Myers) was dissolved in distilled water and filter sterilized; azithromycin (Pfizer) was dissolved in 95% ethanol and ciprofloxacin-HCl (Miles Laboratories) was dissolved in distilled water and filter sterilized.

5.4 Microtiter broth dilution assay

A modification of previously described methods for MIC determination for MAI by microtiter broth dilution was employed (Fenlon and Cynamon, 1986).

To make working solutions, stock solutions of amikacin and ciprofloxacin-HCl were diluted in sterile distilled water. Azithromycin was diluted in sterile phosphate buffer adjusted to a
pH of 6.5 (Retsema et al., 1987). 100μl amounts of the working solutions were distributed in serial 2-fold dilutions in sterile 96-well round bottomed microtiter trays at 2 times the final concentration to be tested. Trays were stored frozen at -70° C until used. Trays not used within 1 month of preparation were discarded.

Strains previously maintained on Lowenstein-Jensen slants were cultured on Middlebrook 7H9 media with OADC (Becton-Dickenson) enriched agar. Strains maintained as frozen stocks in 7H9 with 19% DMSO were thawed and plated as confluent lawns on enriched 7H11 agar. Lawns were harvested after 3-5 days and suspended in normal saline (NS) containing 0.5% Tween 80. Single cell suspensions were obtained by centrifugation 50xg for 3 min. The supernate was then spun at 1000xg for 10 min. The sediment from this was re-suspended in double strength 7H9 broth with OADC and the number of organisms per ml estimated by optical density. Actual inoculum size was determined by plating serial 10-fold dilutions on the initial inoculum on OADC enriched 7H11 agar and determining colony counts after 7-10 days.

The bacterial suspension was diluted in double strength 7H9 broth and distributed in 100 μl amounts to each well of the thawed plates to yield a final concentration of 1-5 x 10⁴ organisms per well. Final dilutions were amikacin 128-0.125μg/ml, azithromycin 512-0.5μg/ml, and ciprofloxacin 128-0.125μg/ml.

Plates were read after 4 and 7 days incubation in a humidified chamber at 37° C. The MIC was defined as the lowest concentration of drug showing no visible growth. All studies were performed in triplicate.
5.5 Paraffin Slide Culture Assay

The paraffin slide culture (Para SL/C) system for selective growth of MAI species as previously cited (Ollar et al., 1990).

Stock solutions of antibiotics which were 11 times the final concentration were diluted in sterile distilled water to obtain initial working concentrations.

From stock cultures which were maintained on Lowenstein and Jensen slants, a 4 mm loopful was added to 4.5 ml of tryptone broth and incubated at 37° C for 6-7 days. Cultures were diluted in normal saline to \(10^5\) bacilli/ml or 10 bacilli per 100x oil immersion field (Buttiaux et al., 1974).

For each antimicrobial concentration tested, 0.5 ml each of infectious inoculum and the initial working antimicrobial solution, and paraffin wax coated slides (Para SL/C) were added to 4.5 ml of Czapek medium. This resulted in a final concentration of organisms to just under \(10^4\)/ml. The antibiotic concentrations tested were amikacin 16, 12.8, 9.6, 6.4, 3.2 µg/ml; azithromycin 13.2, 10.6, 7.9, 5.3, 2.6 µg/ml and ciprofloxacin 18.2, 14.5, 10.9, 7.3, 3.6 µg/ml. A control tube for azithromycin containing 0.5 ml of 95% ethanol in 5.0 ml Czapek broth was included to ensure that any inhibition observed in the higher concentrations of antibiotic was not the result of ethanol. Stock solutions of azithromycin (1.452 mg/ml) were prepared in 95% ethanol. Controls for amikacin and ciprofloxacin contained 0.5 ml of normal saline added to 5.0 ml Czapek broth.

There were three experimental series consisting of two experiments each. The first experiment in each experimental series consisted of strains 10,000, 1762, 1516, 15113, 8515, 6475, 5097,
8197 and 4861. The second experiment of each experimental series consisted of strains SK015, SK016, SK069, SK037, SK060 and SK024.

The paraffin slide cultures were usually read after 5-10 days incubation at 37° C. The MIC was defined as the lowest concentration of drug allowing no visible growth on the paraffin slide culture when compared with the control tube which was devoid of antibiotic and exhibited confluent growth on the paraffin slide culture.

5.6 Statistical Analysis

All statistical analyses were performed on an Amstrad Model PC 1512 microcomputer utilizing a Statistix II statistical software programme which was produced by the NH Software Company of Roseville, Minnesota, U.S.A. A non-parametric Wilcoxon Matched Pairs Signed Rank statistical assay was applied to the ciprofloxacin studies to determine if statistically significant interseries variation occurred in the MIC values between the two related or paired experimental series (II and III). The non-parametric Friedman statistical assay was applied to the MIC values of three related experimental series to determine if statistically significant variation had occurred between the two antibiotic sensitivity methods. Where absolute MIC values were not available, the nearest absolute value was utilized for purposes of statistical calculation.

5.7 The Effect of pH On MIC Values for Azithromycin

5.7a Paraffin Slide Culture Assay

There were two batches of Czapek broth utilized in this procedure. One batch of Czapek broth was adjusted to an initial pH
of 7.53 and the other batch adjusted to pH 6.52. The pH values of both batches of Czapek broth were determined by pH meter. The concentrations of azithromycin initially analyzed at pH 7.53 and pH 6.52 were the same as that previously cited for azithromycin. Two additional azithromycin concentration series were assayed at pH 6.52, in order to determine the specific MIC at this pH. One pH 6.52 concentration series was 66, 53, 40, 26 and 13 μg/ml. The other pH 6.52 concentration series was 136, 106, 82, 55 and 27μg/ml.

The Para SL/C assay followed the same methodology as previously cited for azithromycin but, was read, and MIC values determined after 8 days following the criteria set forth by Heifets (Heifets, 1988).
5.7b **Microtiter Assay**

There were two batches of double strength 7H9 broth utilized in this procedure. One batch of 7H9 broth was adjusted to an initial pH of 7.5 and the other batch adjusted to pH 6.5. The pH values of both batches of 7H9 broth were determined by pH meter. The preparation of the azithromycin working solution at pH 6.5 was the same as that previously cited for azithromycin but, the final range concentration range was 128-2μg/ml. The azithromycin working solution at pH 7.5 was diluted in phosphate buffer adjusted to pH 7.5. The final range concentration of azithromycin at pH 7.5 was 64-2μg/ml.
RESULTS

5.8 Ciprofloxacin-HCl

The basic methodology for the Para SL/C antibiotic sensitivity assay was derived from experimental series I (see Table 6) with ciprofloxacin-HCl. As expected, the control tube showed the greatest amount of growth on the paraffin wax surface. When visible confluent growth on the paraffin slide culture in the control tube occurred, the Para SL/C tubes containing varying dilutions of ciprofloxacin-HCl were examined. The effect of increased concentration of ciprofloxacin-HCl (See Figure 12) on MAI growth were clearly visible on the Para SL/C. The concentration of ciprofloxacin in the tube containing the lowest antimicrobial concentration in which there were no visible colonies on the paraffin wax coated slide was defined as the minimal inhibitory concentration (MIC), for this system. Lack of growth at this concentration was confirmed by microscopic examination of slides stained by the Kinyoun acid-fast staining method (Ollar et al., 1990). This method was used to determine the MIC in all subsequent series of Para SL/C antimicrobial sensitivity tests.

In all of the experimental series with ciprofloxacin-HCl, the median time to development of confluent growth in control tubes was 8 days, range 7-9 days.

There was no statistically significant variation (p=0.68) of series II and III for ciprofloxacin-HCl (See Tables 6 and 7). This confirmed the reproducibility of the Para SL/C method between experimental series.

The overall sensitivity pattern was found to be similar with Para SL/C and microtiter procedures, with the MIC$_{50}$ and MIC$_{90}$ values of 7µg/ml and 18.2µg/ml for the Para SL/C and 2µg and
16μg/ml for the microtiter assays. This comparison is considered in the discussion.
Figure 12: Paraffin slide culture system for antibiotic sensitivity scanning. A view of a 7 day culture of M. intracellulare SK069 (non-AIDS) showing: (A) Control tube containing 7-day-old culture in Czapek broth showing confluent growth on Para SL/C. (B) Assay tube containing 7-day-old culture in Czapek broth plus 3.6 μg/ml ciprofloxacin-HCl showing heavy growth on Para SL/C. (C) Assay tube containing 7-day-old culture in Czapek broth plus 14.5 μg/ml ciprofloxacin-HCl showing sparse growth on Para SL/C.
5.9 **Amikacin**

In the amikacin study, the median time to confluency of the control tubes was 6.5 days, range 6-7 days.

The MIC values for the experimental series I-III making the Para SL/C assay did not vary to a statistically (p=0.39) significant level (See Table 8).

There was close agreement between the MIC$_{50}$ and MIC$_{90}$ values obtained by the Para SL/C and microtiter assays—6.4μg/ml and 12μg/ml for the Para SL/C assay and 8μg/ml and 16μg/ml for the microtiter assay.

5.10 **Azithromycin**

In the azithromycin experimental series I-III, the median time to confluency of the control tubes was 6.5 days, range 6-7 days. The MIC values for the experimental series I-III making the Para SL/C assay did not vary to a statistically (p=0.14) significant level (See Table 9).

By contrast to the overall sensitivity patterns obtained for ciprofloxacin-HCl and amikacin, the MIC$_{50}$ and MIC$_{90}$ values obtained by the two procedures for azithromycin were quite different: 2.6μg/ml and 5.3μg/ml for the Para SL/C and 64μg/ml and 128μg/ml for the microtiter assay. The Azithromycin effect will be considered further in the next paragraph, as well as in the discussion section.
5.11 Effect of pH On MIC Values for Azithromycin

5.11a Para SL/C ASSAY

The cumulative MIC$ _{50} $ and MIC$ _{90} $ values for experimental series I and II at pH 7.53 were 5.3$ \mu $g/ml and 10.6$ \mu $g/ml respectively. By contrast, the cumulative MIC$ _{50} $ and MIC$ _{90} $ values for experimental series III and IV at pH 6.53 were 82$ \mu $g/ml and >136$ \mu $g/ml. The shift downward in pH to 6.5, caused a corresponding upward shift in the cumulative MIC$ _{50} $ and MIC$ _{90} $ values such that their respective values were 15.5 and >12.8 times that noted at pH 7.53 (See Table 10).

5.11b Microtiter Assay

The MIC$ _{50} $ and MIC$ _{90} $ values for experimental series I pH 7.5 were 32$ \mu $g/ml and >64$ \mu $g/ml respectively (See Table 16). The MIC$ _{50} $ and MIC$ _{90} $ values for experimental series II pH 6.5 were 128$ \mu $g/ml and >128$ \mu $g/ml respectively (See Table 10). The lower pH brought about a corresponding increase in the MIC$ _{50} $ and MIC$ _{90} $ and their respective values were 4 and >2 times that noted at pH 7.5.
Table 6: **MIC values (µg/ml) of ciprofloxacin-HCl in paraffin slide culture assay**

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Table 7: MIC values (µg/ml) of ciprofloxacin-HCl in paraffin slide culture and microtiter assays

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*NG= No growth
Table 8: MIC values (µg/ml) of amikacin in paraffin slide culture and microtiter assays

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*NG = No growth
Table 10: **Effect of pH on MIC values (µg/ml) of Azithromycin**

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<th>MIC Para SL/C pH 6.5</th>
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<td>7.9, -</td>
<td>&gt;13.2, &gt;13.2, &gt;66, 136</td>
<td>&gt;64</td>
<td>&gt;128</td>
</tr>
<tr>
<td>SK024</td>
<td>5.3, -</td>
<td>&gt;13.2, &gt;13.2, 66, 82</td>
<td>&gt;64</td>
<td>&gt;128</td>
</tr>
</tbody>
</table>

*-= absence of growth in control and assay tubes.
DISCUSSION

5.12 Paraffin Slide Culture in MAI Antibiotic Assay

The overall results obtained from this investigation suggested that the determination of the MIC values in the Para SL/C assay could be further refined. Confluent growth in the control slides is generally achieved in 8 days. A standard reading of the MIC on the eighth day of incubation would comply with the time limit found by Heifets and his co-investigators to be most reliable for accurate MAI sensitivity testing (Heifets, 1988; Heifets and Lindholm-Levy, 1989; Heifets et al., 1990). Those assays not achieving confluent growth in the control tube by the eighth day would be reassayed.

Further modifications of this methodology may make it possible for the Para SL/C to be utilized for the determination of Minimal Bactericidal Concentration (MBC) level. This could be achieved by simply removing slides on which no growth was observed at the eighth day post-inoculation and transferring them to antibiotic-free Czapek Media for a period of 5-8 days at 37° C. Those Para SL/C's that were at the MBC would remain free of growth on the surface of the paraffin coated slide after the additional 5-8 days incubation period in antibiotic-free Czapek Media.

It should be feasible to employ simultaneously the basic Para SL/C assay for primary MAI isolation with its role as an antibiotic sensitivity test. When there is a high suspicion of non-tuberculous mycobacterial disease, for example with AFB seen in a smear of clinical specimen, simultaneous testing would be rapid and economical. During the time when isolation is being attempted, additional paraffin slides would be placed into a supplementary series containing specimen, Czapek media and antibiotic at
predetermined "break point" concentrations. This may also have application in large scale surveys on environmental isolates.

The MIC$_{50}$ and MIC$_{90}$ values obtained via the Para SL/C for ciprofloxacin-HCl and amikacin were quite close to those obtained by other investigators (Heifets and Lindholm-Levy, 1989; Inderleid et al., 1987; Leysen et al., 1989; Saito et al., 1989; Tomoika et al., 1989) and to those obtained by us using the microtiter method. However, the MIC$_{50}$ and MIC$_{90}$ values obtained by the Para SL/C for azithromycin were divergent from values obtained by microtiter method, and also from values obtained by other investigators (Inderleid et al., 1989, Naik and Ruck, 1989).

Azithromycin is an azalide antibiotic that is structurally related to the macrolide erythromycin. An azalide is a dibasic molecule which contains an N-methylgroup in the lactone ring. Experiments with S. aureus strains showed that the MIC varied from 50µg at pH 6.0 to 0.025µg/ml at pH 8.0 (Retsema et al., 1991). The effect of pH variation of in vitro potency (MIC values) was explained by the fact that the pka values of the basic groups on azithromycin and erythromycin are 8.4-8.8. Thus, at pH 8.0 the unionized compounds should cross the cytoplasmic membrane more readily and bind to the bacterial ribosome. Since the pH inside the cell is more neutral (pH 7.2), greater ionization will occur, causing the intracellular concentration to become greater than the exterior concentration.

When the milieu is at pH 6.0 azithromycin is completely ionized and will therefore have difficulty in crossing the cytoplasmic membrane. Therefore, the relevant activity of azithromycin in the clinical situation is probably its activity at approximately pH 7.2. Retsema and co-workers thus advised that when evaluating the susceptibility of organisms to azithromycin or
basic macrolides, attempts should be made to keep the pH within the physiological range of pH 7.2-7.4 (Retsema et al., 1991).

When another macrolide Clarithromycin was tested against M. avium complex organisms by Heifets and co-workers, it was noted that differences in pH were particularly important (Heifets, et al., 1992). Clarithromycin was found to be more active at pH 7.4 and less active at pH 5.0, with intermediate activity at pH 6.8. The MIC values for 90% (25 strains) were 0.85μg/ml at pH 7.4, 6.0μg/ml at pH 6.8 and >16μg/ml at pH 5.0.

Thus, when reviewing the MIC₅₀ (5.3μg/ml) and MIC₉₀ (10.6μg/ml) for the Para SL/C originally performed at pH 7.5, and the MIC₅₀ (64μg/ml) and MIC₉₀ (128μg/ml) of the Microtiter Assay originally performed at pH 6.5, it is not surprising that the respective MIC values are so divergent given the differences of pH of the media in the two assays.

When the pH of Para SL/C was reduced to 6.5 there occurred a corresponding increase in MIC (reduced sensitivity) comparable to the MIC obtained with the microtiter assay but, when the microtiter assay was run at pH 7.5 it did not give a comparable change in MIC. Heifets has stated that the composition of the medium, specifically the metallic cations, phosphates, and some other salts can affect the in vitro activity of some antimicrobial agents (Heifets, 1988). Thus, when reviewing the data for the Para SL/C and the microtiter assay, the divergent MIC values for azithromycin are not surprising when the following points are taken into consideration: a) the differences in the composition of the respective media and b) the presence of phosphate only in the microtiter broth. Other explanations for the divergent results obtained with azithromycin are plausible, such as alterations in mycobacterial cell wall permeability to drug.
However, the MIC values obtained by the Para SL/C at pH 7.5 gave results more in tune with in vivo sensitivity levels obtained with experimental lab animals than did the microtiter assay (Dr. James Retsema, Pfizer Labs, Groton, Connecticut, U.S.A.—Personal Communication).

These experiments with the Para SL/C have shown that it is possible to perform a modified broth assay utilizing basal salt media and paraffin coated slides in a sensitive and reproducible antibiotic protocol for susceptibility testing of MAI.
Section VI

The Plasmids of *Mycobacterium Avium* Complex Organisms
6.0 General Description of Plasmids

A large number of important bacterial genes are not found on the chromosomal DNA but, rather on plasmids. The molecular weights of plasmids can range from about $1 \times 10^6$ to $200 \times 10^6$ (Hardy, 1981). Most of the plasmid DNA inside bacteria occurs in the form of a covalently-closed circle. If one of the two polynucleotide strands in a closed-circle plasmid is broken, or nicked an open circle is formed. Some very large plasmids are extremely difficult to maintain in the covalently-closed circle state during plasmid isolation and purification.

Plasmids can be stably inherited without being linked to the chromosome. It should be noted that some bacteriophages can be classified as plasmids according to the aforementioned definition. Plasmids have great importance in medicine and agriculture due to their ability to confer antibiotic resistance on human and veterinary pathogens and for their ability to code for such virulence factors as toxins and adhesions (Hardy, 1981). Plasmids can also confer other properties such as lactose fermentation, hydrogen sulphide production, urea breakdown. Plasmids encode genes that enable bacteria to degrade unusual substrates such as xylene, toluene, 2,4-D(2,4-diphenoxacycetic acid) and several components of mineral oils (Hardy, 1981).

6.1 Mycobacterium Avium-intracellulare Plasmids

The investigation of Crawford and Bates first demonstrated the presence of mycobacterial plasmids in Mycobacterium avium complex organisms (Crawford and Bates, 1979). The presence of plasmids has also been demonstrated in strains of Mycobacterium fortuitum, Mycobacterium intracellulare and
Mycobacterium scrofulaceum (Crawford and Falkinham III, 1990; Rauzier et al., 1988; Meissner and Falkinham III, 1984).

Crawford and Bates found that a strain of M. intracellulare, namely, LR113 carried a small unique plasmid of 15.3 kb which they called pLR7 (Crawford and Bates, 1984). This plasmid was extensively studied and cloned into E. coli. The pLR7 plasmid has been mapped and it possessed unique sites for BamHI, Hind III and Xba I. In addition, further digests of the pLR7 plasmid revealed that there occurred a unique site for Xho I and a second site for Xho II (Crawford and Bates 1984). A clone of the pLR7, pJC20 consisted of the entire pLR7 sequence joined to an E. coli pBR322 plasmid via the unique Hind III site (Crawford and Bates, 1984; Crawford and Falkinham III, 1990).

Jucker and Falkinham III have found a 12.9 kb plasmid, pVT2, from a clinical isolate of M. avium, MD1 (Jucker and Falkinham III, 1990). The pVT2 plasmid was found to hybridize to both clinical (13 of 13 strains) and environmental (5 of 5 strains) plasmids of 13.5 kb or smaller. However, there was no hybridization between the pVT2 and pLR7 plasmids. Crawford found that the pVT2 plasmid cited by Jucker and Falkinham III was similar to his pLR20 plasmid which he found in M. avium strain LR541. The pLR20 plasmid was cloned into pUC 18 as an Eco RI fragment (Crawford and Falkinham III, 1990).

Crawford has stated that the large plasmids (>150kb) have to date not been characterized other than by their size (Crawford and Falkinham III, 1990). He notes that many strains of MAI carried similar-sized large plasmids but, studies have not been performed to determine if these are similar or related. The problem with the large plasmids has been that work with these
plasmids has been inhibited by the modest yields that were obtained in most of the preparations.

6.2 MAI Plasmids in Clinical and Environmental Isolates

Crawford and Bates examined 26 strains of MAI isolated from AIDS patients and examined their plasmid content (Crawford and Bates, 1986). It was found that all 26 strains carried small plasmids (9-15 megadaltons), 11 of the strains carried one small plasmid and 15 strains carried 2 small plasmids. In addition, 10 strains carried large plasmids (>100 megadaltons). All of these AIDS-related strains of MAI were probed with a nick-translated and radiolabelled probe derived from pLR7:pBR322. The Southern blot analysis revealed that each of the 26 AIDS-related MAI isolates carried plasmids related to the pLR7 plasmid. The pLR7 related plasmid, however, in the AIDS-related strains of MAI showed considerable heterogeneity of size. Bates and Crawford proposed that the pLR7 clone could serve as a useful marker for epidemiology and might also be useful for the detection of MAI in clinical material.

It has been found that 56% of clinical isolates of MAI and 75% of aerosol isolates of MAI from endemic areas have plasmids but, only 5-6% of soil isolates have plasmids (Gangadharam et al., 1988). Gangadharam et al. reported that all of their 26 AIDS-related strains of MAI that possessed plasmids were of serotypes 4 or 8. These strains all carried a small plasmid that was closely related to the pLR7 plasmid. In a study performed in Denmark, a plasmid profile analysis was performed on isolates of MAI isolates derived from AIDS patients, children with cervical lymphadenitis and from environmental sources (Jensen et al., 1989). The Danish study reported that plasmid presence was found in 31% of AIDS-
related MAI isolates, 27% of pediatric cervical lymphadenitis-related MAI and 7% in environmental isolates of MAI. These findings showed that plasmid presence occurs more frequently in clinical isolates of MAI than in environmental isolates.

Morris et al. (1990) studied the plasmid profiles of 12 *M. avium* strains from 12 different AIDS patients. Nine isolates including all those of serotypes 4 and 8 carried multiple plasmids of less than 100 kb, while two serotype 3b strains and one untypeable strain had no detectable plasmids. The predominant band found in six of the serotype 4 and 8 strains was 16kb. In addition, in 4 of the aforementioned 6 strains there also occurred a high copy 13 kb plasmid band. High concentrations of an approximately 40 kb plasmid were observed in extracts from 2 isolates. An untypeable isolate carried a plasmid profile which was unique in that it carried three large plasmids of >20 kb. All 7 serotype 4 and serotype 8 isolates displayed similar hybridization patterns with a pLR7 probe. These aforementioned strains showed hybridizing bands of approximately 16, 25 and 40 kb.

Hellyer et al.(1991), studied the plasmid content of 147 MAI isolates which were derived from 88 British AIDS-related MAI isolates, 9 U.S. AIDS-related MAI isolates; 36 British non-AIDS related MAI isolates and 14 British veterinary isolates. The pLR7 and pLR20 type plasmids were detected with the pLR7 and pLR20 probes of Crawford. The plasmids observed on electrophoretic analysis displayed two major groupings, namely, the small variety that consisted of principally 14 and 30 kb and the large variety which were> 150 kb. These investigators noted that there was no significant difference in the rate of plasmid presence in MAI isolates from AIDS and non-AIDS patients in Great Britain. By
contrast, the American AIDS-related strains displayed a higher rate of plasmid carriage. The American strains which were derived from blood and isolated at St. Vincent's Hospital in New York, possessed at least one plasmid. The British veterinary isolates were found not to carry large plasmids. In addition, not one of the 147 isolates surveyed was found to possess more than three plasmids. Hybridization studies in which small-sized plasmids were probed with the pLR20 probe revealed that this type of plasmid was less common. Both the pLR7 and pLR20 probes did not hybridize to either the large sized plasmid or chromosomal DNA. None of the small plasmids were found to have homology with both probes. Hellyer and co-workers found that the pLR7 related plasmids showed considerable variation in size (15-30 kb) and by contrast, the pLR20 related plasmids were more consistent in size (14-16 kb). The plasmids which showed homology with the pLR20 probe were always seen in the presence of another small plasmid and were always the smallest plasmid present.
6.3 Specific Plasmid Associated Functions

6.3a Plasmid Coded Mercury Resistance

Meissner and Falkinham III have isolated an environmental strain of *M. scrofulaceum* (closely related to *M. avium* and *M. intracellulare*) from Chesapeake Bay which carried a plasmid (pVT1) of 177 kb in size (Meissner and Falkinham III, 1984). This plasmid bearing strain was able to grow in the presence of 100 μM of HgCl$_2$ and was able to convert $^{203}$Hg$^{2+}$ to volatile mercury at a rate of 50 pmol/10$^8$ cells per min. The extracts from these *M. scrofulaceum* cells were found to contain a soluble mercuric reductase whose activity was not dependant on exogenously supplied thiol compounds. A derivative of the parent strain was found to be mercury sensitive and lacked both the pVT1 plasmid and mercuric reductase activity. It is this latter finding that linked mercury resistance to a plasmid.

6.3b Plasmid Coded Copper Resistance

Subsequent studies with the isolate of *M. scrofulaceum* described by Meissner and Falkinham III above, found that those strains lacking the pVT1 plasmid also were more susceptible to copper than parental strains (carrying the pVT1 plasmid) (Crawford and Falkinham III, 1990). The parental copper resistant strains formed a black precipitate (copper sulphide) when grown in the presence of copper sulphate.

6.3c Plasmid Presence and Colonial Variation

The investigation of Mizuguchi and his co-investigators found that a wild type strain (strain 103) of *M. intracellulare* carried two plasmids of 2 Mdal and 50 Mdal plasmid and displayed a
transparent colonial morphology (Mizuguchi et al., 1981). When the wild type strain was treated with mitomycin C and other antibacterial agents, the 2 Mdal plasmid was lost and the colonial morphology changed from translucent to opaque. This finding of the lost 2 Mdal plasmid and the transformation colonial morphologies from translucent to opaque was suggestive of a plasmid role in determining colonial morphology.

6.3d Plasmid Role In Catalase Activity

Pethel and Falkinham III observed that the ability of Crawford Strain LR25 to grow at 43° C was the result of the strain's resistance to elevated levels of oxygenation in broth (Pethel and Falkinham III, 1989). These investigators measured the catalase activity in these aforementioned strains because of the probable role of hydrogen peroxide in oxygen dependent killing (Pethel and Falkinham III, 1989; Crawford and Falkinham III, 1990). The LR25 strain at log phase was observed to have a catalase activity that was three times higher than those LR25 strains which had undergone a loss of plasmid due to curing. These results have, however, been invalidated by the subsequent finding which has shown that the "so-called" cured strains derived from LR25 have been shown to be a contaminant (Dr. J.W. Dale, Personal Communication).

6.3e Antibiotic Resistance and Plasmids

Franzblau et al. (1986) found that the MIC levels for 16 plasmid bearing strains (8 strains carried a plasmid >100 Mdal and produced translucent colonies) of MAI showed consistent levels of sensitivity \(\geq 100\mu g/ml\) for kanamycin, streptomycin and rifampin. Non-plasmid bearing strains, however, showed considerable variation in MIC levels.
6.3f Plasmid Content and Virulence

Crawford and his co-workers found that all of their 26 AIDS-related isolates of *M. avium* possessed pLR7 related plasmids. In addition, 56% of clinical isolates of MAI and 75% of aerosol isolates of MAI from endemic areas have plasmids but, only 5-6% of soil, dust, and environmental isolates possess plasmids (Gangadharam et al., 1988). These findings thus were suggestive of a possible link between the presence of plasmids and virulence (Crawford and Bates, 1986).

Gangadharam and associates demonstrated that a Crawford isolate LR25 which carried 3 plasmids exhibited a high degree of virulence in the beige mouse model when virulence was displayed by high mortality and a progressive increase in MAI organisms in the spleen and lungs (number of organisms were expressed as colony forming units or c.f.u.). When a cured variant (plasmid lost) of LR25 called LR163 was judged by the criteria cited above, the virulence was lower (Gangadharam et al., 1988). However, this study was invalidated by the finding that "LR163 is a contaminant not a cured derivative of LR25" (Dr. J.W. Dale, Personal Communication). Morris et al., however, stated that the increased virulence noted in plasmid bearing strains could be a consequence of the presence of minor amounts of a unique plasmid-encoded product within cell (Morris et al., 1990).

The investigation of Hellyer et al. (1991) at St. Mary's Hospital in London found increasing evidence to support the notion that the portal of entry in MAI infection is the gastrointestinal tract. This notion of G.I. tract involvement was based upon the finding that individuals without HIV infection do not carry MAI in feces. In intestinal infections, it is important for organisms to
penetrate the epithelium and thus survive within the gut associated lymphoid tissues; mycobacterial cell-wall agents of adhesion could perhaps be involved in this process. Plasmids have been known to be linked to code for adhesins in other organisms. Thus, perhaps MAI virulence could be the result of heightened G.I. tract adhesion which could be plasmid encoded. However, because of the complex nature of MAI related plasmids the relationship between plasmids and virulence is still unclear.

This section will focus upon the pLR7 and pLR20 plasmid content of M. avium complex strains isolated at two New York City hospitals, namely, St. Vincent's Hospital and Memorial Sloan-Kettering Cancer Center.
6.4 pJC-20 Plasmid Prep

The pJC-20 recombinant plasmid (pLR7: pBR322) contains an MAI pLR7 plasmid which had been inserted into a pBR322 plasmid at the Hind III site. A 4mm loopful of *E. coli* containing the pJC-20 recombinant plasmid was inoculated into 4 ml of LB broth (See Appendix IV) containing 25 μg/ml of ampicillin to select for plasmid bearing strains and incubated overnight at 37° C. This overnight growth served as inocula for a 150 ml (in a 500 ml erlenmeyer flask) LB broth batch culture (containing 25 μg/ml ampicillin) which was placed into a 37° C shaker bath. The $E_{650nm}$ reading was taken at 30 min intervals. When the $E_{650nm}$ reading reached 0.9 O.D. units, spectinomycin was added to a final concentration of 150μg/ml, and the culture was returned to the shaker bath and incubated overnight at 37° C. The cells were harvested and plasmids isolated via the basic Qiagen protocol (see Qiagen protocol).

6.5 pJC-70 Plasmid Prep

Recombinant plasmid pJC-70 (pLR20 :pUC18) contains an MAI pLR20 plasmid which had been inserted into a pUC18 plasmid at the Eco RI site. A 4mm loopful of *E. coli* containing the pJC70 recombinant plasmid was inoculated into 4 ml of LB broth which contained 100 μg/ml ampicillin to select for plasmid containing strains and incubated overnight at 37° C. The overnight culture served as inocula for a 150 ml broth batch culture (containing 100 μg/ml of ampicillin) which was placed into a 37° C shaker bath. The remainder of the protocol was the same as that cited for pJC-20.
6.6 Qiagen Plasmid Isolation Protocol

6.6a Qiagen Midi (30-150ml) Plasmid Protocol for E. coli pJC-20

The cells (150 ml.) were pelleted by centrifugation at 4,320 x g for 15 min at 4° C in a Sorvall Model RC-2B Refrigerated High Speed using a Sorvall GSA rotor (Qiagen INC., Chatsworth, California, U.S.A.). The bacterial pellet was resuspended in 4.0 ml of P1 buffer (See Appendix III). Four ml of P2 buffer (See Appendix III) was added to the bacterial cell suspension, was gently mixed and allowed to incubate at room temperature for 5 min. An Aliquot of 4.0 ml of P3 buffer (See Appendix III) was added to the bacterial cell suspension which was gently mixed and placed in a Sorvall RC-2B centrifuge using a Sorvall SS-34 rotor and spun at a speed of 23,420 x g for 30 minutes at a temperature of 4° C. The supernate was removed to a clean sterile tube and it was centrifuged two additional times at 23,420 x g for 15 minutes at a temperature of 4° C. After each spin the supernate was transferred to a clean sterile tube. The final supernate was then filtered on a 0.45 micron pore size Nalge filter unit to remove any remaining particulate matter. The resulting filtrate was added to a Qiagen-pack 100 syringe pressure operated column which had been equilibrated with 2.0 ml of QB buffer (See Appendix III). The Qiagen-pack 100 column was washed with 2 x 4.0 ml of QC buffer (See Appendix III), and the column was eluted with 2.0 ml of QF buffer (See Appendix III). The resulting eluate (plasmid DNA) was aliquoted (500 µl per eppendorf tube) into eppendorf tubes. The plasmid DNA per aliquot was precipitated with 450 µl of isopropanol (equilibrated to room temperature) and was centrifuged at 23,430 x g at 4° C for 30 min to pellet the plasmid DNA. The supernates per aliquot were carefully removed and the plasmid DNA therein was
washed with 70% ethanol and spun for 30 minutes at 23,420 x g at 4°C. The supernate per aliquot was again removed carefully and the pellet was dried briefly (5 min) under vacuum. A single volume of 200 µl of TE buffer (See Appendix I) was pipetted through all the eppendorf tubes that contained the dried pellet. The resulting pooled re-suspended plasmid DNA was stored at -20°C.

6.6b *Qiagen Maxi (150 - 500 ml) Plasmid Protocol for E. coli pJC-70*

The cells (150 ml) were pelleted in the same manner as that cited for the Midi prep (Qiagen INC. Chatsworth, California, U.S.A.). For the Maxi prep the bacterial pellet was resuspended in 10 ml of P1 buffer (See Appendix III). A 10 ml aliquot of P2 buffer was added to the bacterial suspension, was gently mixed and allowed to incubate at room temperature for 5 min. A 10 ml aliquot of P3 buffer was added to the suspension, gently mixed and placed in a centrifuge (Sorvall RC-2B centrifuge using SS-34 rotor) and spun at a speed of 23,420 x g for 30 min at a temperature of 4°C. The supernate was removed to a clean sterile tube and was centrifuged two additional times (each time the supernate was transferred to a clean sterile tube) for 15 minutes at 4°C. The supernate was then filtered on a 0.45 micron pore size Nalge filter unit to remove any remaining particulate matter. The resulting filtrate was added to a Qiagen Gravity operated tip-500 column that had been equilibrated with 10 ml of QBT buffer (See Appendix III). The Qiagen tip-500 column was washed with 30 ml of QC buffer (See Appendix III) and eluted with 15 ml of QF buffer (See Appendix III). The resulting eluate was aliquoted (1000µl per eppendorf tube) into eppendorf tubes. The plasmid DNA eluate per aliquot was precipitated with 500 µl of isopropanol (equilibrated to room
temperature) and centrifuged at 23,420 x g at 4° C for 30 minutes to pellet plasmid DNA. The supernates per aliquot were removed carefully and the plasmid DNA therein contained were washed with 70% ethanol and spun for 30 min at 23,420 x g at 4° C. The supernate per aliquot was again removed carefully and the pellet was dried briefly (5 min) under vacuum. A single volume of 200 µl of TE buffer (See Appendix I) was pipetted through all the eppendorf tubes that contained dried plasmid pellet. The resuspended pooled plasmid DNA pellet was stored at -20° C.

6.6c Modified Qiagen Midi and Maxi Preps for MAI

MAI strains were first grown in 10ml of 7H9 broth (BBL) which was supplemented with 4% OADC (BBL) and 1% glycerol for a period of 7 days at 37° C. The 10 ml 7H9 broth culture served as inoculum for a 150 ml batch culture contained within a 500 ml dimpled erlenmeyer flask which also contained the aforementioned supplements of OADC and glycerol. These cultures were grown at 37° C in a shaker bath for a period of 8 weeks. The flasks containing the MAI cells were exposed to sterile glycine (stock concentration 20% ; final concentration per flask 2%) for a period of 48 hours prior to harvest (in shaker water bath at 37° C in order to make the MAI cell walls leaky thus rendering them more susceptible to the action of alkaline lysis. Cultures were spun down at 11,950 x g in a Sorvall GSA rotor at 4° C. The protocol then followed the basic Qiagen Midi Prep protocol as cited above for E. coli.

The modifications to the Qiagen protocol did enable the visualization of plasmid bearing strains of MAI via ethidium bromide staining of the agarose, as well as, the identification of specific plasmid types by Southern Blotting. Early in these studies, it became quite clear that the Qiagen Midi Column which
the manufacturer cited as having the capacity to utilize 30-150 ml aliquots of crude *E. coli* lysate as the source of plasmid DNA, was inadequate for use in 8 week old 150ml MAI culture (this volume yielded the necessary biomass needed in order to view the low copy MAI plasmid via ethidium bromide staining and carry out successful nonradiometric colorimeter detection). The Midi column which had to be loaded to its upper capacity (150ml) with crude MAI lysate, often became clogged. Therefore, the higher capacity Maxi Qiagen Column (150ml-500ml capacity) replaced the Midi Column in MAI preps. The Qiagen system, however, failed to totally remove chromosomal DNA from the MAI prep as demonstrated on the electrophoretic and southern blots.

6.7 Electrophoretic Analysis of MAI Plasmid DNA

The samples analyzed were: a) MAI plasmid preps consisting of 60μl of plasmid prep and 20μl of loading buffer (See Appendix I) and b) BRL Supercoiled DNA ladder consisted of 12μl BRL Supercoiled DNA (94ng/ml), 48μl of TE buffer (See Appendix I) and 20μl of loading buffer. Sixty microliter aliquots of MAI plasmid sample and BRL supercoiled DNA were loaded into wells (2.0mm x 7.5mm) in 0.6% agarose. The agarose was 9.68mm thick, contained 0.5μg/ml ethidium bromide, and was 11 x 14 cm. The electrophoretic run was carried out in a BRL Model H5 Electrophoretic Horizontal Submarine Gel Unit tank using 1X TAE as the electrophoretic buffer (See Appendix I). The electrophoresis was run at 104 Volts generated from a BRL Model 250 Power Pack for a period of 2 hours and 40 min. The resulting electrophoretic run was viewed on a 254nm UV transilluminator. A photographic record was made by the use of a Polaroid Quick Shooter Camera using Polaroid 667 film.
6.8 The Preparation of Probe DNA for detecting pLR-7 and pLR-20 Type Plasmids in AIDS-related MAI

6.8a pLR7 Plasmid Prep from PJC-20

Fifteen microliters of Qiagen Midi Prep eluate PJC-20 plasmid digested with 2 µl of Hind III (Boehringer Mannheim; 11 units/ml) restriction enzyme in a total volume of 20 µl for 60 minutes at 37°C. Five microliters of loading buffer were added to the 20 µl of digest. A 25µl aliquot of the PJC-20 digest and several additional samples were subsequently analyzed electrophoretically. The additional samples analyzed were: a) PJC-20 uncut plasmid sample which consisted of 15µl of PJC-20 plasmid, 2µl 10x react buffer, 3µl of ddH₂O and 5µl of loading buffer; b) BRL Supercoiled DNA sample which consisted of 4µl of supercoiled DNA molecular weight ladder (94ng/µl), 2µl 10X react buffer, 14 µl T.E. buffer, 1µl ddH₂O and 5 µl loading buffer; c) Digoxigenin Labelled Lambda Hind III Fragments Sample which consisted of 15µl Digoxigenin Labelled Hind III Fragments (10ng/µl), 2µl TE buffer, 5µl loading buffer. The samples were loaded into wells (0.8mm x 5.3cm) in 0.8% agarose gel. The agarose gel was 4mm thick, contained 0.5µg/ml ethidium bromide, and was 5.7 x 8.3 cm. The electrophoretic run was performed in a BRL Horizon 58 baby gel electrophoretic tank using 1X TAE buffer as electrophoretic buffer. The electrophoresis was run at 100 volts for a period of 43 min using a BRL Model 200 power supply. The resulting electrophoretic run was viewed on a 254nm UV transilluminator. A photographic record was made by the use of a Polaroid Quick Shooter Camera using Polaroid 667 film.
6.8b **pLR-20 Plasmid Prep from PJC-70**

Fifteen microliters of Qiagen Maxi Prep eluate PJC-70 plasmid digested with 2 μl Eco R1 (Boehringer Mannheim; 12 units/ml) restriction enzyme in a total volume of 20 μl for 60 minutes at 37° C. Five microliters of loading buffer was added to the 20 μl of digest and the resulting 25 μl were loaded onto the same agarose gel configuration as cited for pLR-7. The electrophoresis was run at 100 volts for a period of 45 min.

6.8c **Gene Clean Protocol**

Either a Hind III(pLR-7) or EcoR1 (pLR-20) fragment obtained from the preceding protocols was excised from an ethidium bromide stained gel. The excised gel slice was added to a (Pre­weighed) eppendorf tube(Bio 101). This tube which contained the gel slice was weighed in order to determine the approximate volume of the gel slice by weight (1gm agarose gel equals approximately 1ml (Bio 101)). To the eppendorf tube containing the agarose slice was added two to three volumes of NaI stock solution (Bio 101 Gene Clean kit stock solution). This tube was then placed into a 55° C waterbath for a period of 5 min until all the gel had melted. To a 5μl aliquot (5μl of GLASSMILK solution added to 5μg or less of DNA) of GLASSMILK solution( silica matrix: Bio 101 Gene Clean kit stock solution) was added to the DNA solution, which was then mixed by pipetting and incubated for 5 min in an ice bath (at 2 min intervals the GLASSMILK suspension was mixed to ensure that it remained in suspension). The GLASSMILK was pelleted in an eppendorf centrifuge for 5 secs at 23,420 xg. The NaI stock solution supernate was carefully removed so as not to disturb the GLASSMILK pellet. The GLASSMILK was resuspended in an additional wash with NaI (400 μl) and incubated for 3-5 min in a waterbath at
55°C. The GLASSMILK was pelleted for 5 secs at 23,420 x g in an eppendorf microcentrifuge. The NaI supernate was again removed from the GLASSMILK pellet. An aliquot of 700μl of ice cold NEW solution (Bio 101 Gene Clean kit stock preparation) was added to the pellet which was resuspended by pipetting back and forth. The GLASSMILK pellet was pelleted by centrifugation at 23,420 x g in an eppendorf microcentrifuge for 5 sec. The supernate was then discarded. The wash procedure with NEW solution was repeated two more times. After the supernate from the third and final NEW wash was removed, the eppendorf tube was spun for a few additional seconds to the last bit of New supernate. The GLASSMILK pellet was resuspended in 10 μl of TE buffer and incubated for 3 minutes in a 55°C waterbath. The suspension was pelleted at 23,420 x g in an eppendorf microcentrifuge. The supernate was transferred to a sterile 1.5 ml eppendorf tube. The GLASSMILK pellet was resuspended for a second time in 10μl of TE buffer and incubated for 3 minutes in a 55°C waterbath. The suspension was pelleted again at 23,420 x g in an eppendorf microcentrifuge. The supernate was pooled with the supernate obtained in the preceding step. The resulting 20ul aliquot of either the pLR-7 or pLR-20 plasmid was then ready to be labelled and utilized as a probe.

6.8d Random Primer Labeling of DNA with digoxigenin-11-dUTP

The GENE CLEAN plasmid prep(s) derived in the preceding section served as the purified DNA for digoxigenin labeling in a scaled up labeling reaction(Boehringer Mannheim). The amount of DNA utilized in the labeling reaction varied from 200 ng to 500 ng (DNA concentration was estimated based upon the intensity of ethidium bromide staining of a molecular weight standard on known concentration as seen on a 254nm UV Transilluminator). The 20 µl
aliquot of DNA was added to a sterile eppendorf tube and boiled in a waterbath for a period of 10 min to achieve denaturation. The denatured DNA was then placed in an ice bath for 3 min. To the DNA aliquot were added 5μl of Hexanucleotide mixture (Boehringer Mannheim Genius Digoxigenin Labeling and Detection Kit), 5μl dNTP (Boehringer Mannheim Genius Digoxigenin Labeling and Detection Kit), 17μl of dd H₂O and 3μl of Klenow enzyme (DNA Polymerase, Large Fragment, 2 Units/μl). The total volume of the reaction was 50μl. The labeling aliquot was briefly spun down in a microcentrifuge and then incubated in a 37°C incubator overnight. The labeling reaction was stopped by adding 4μl of a 0.2M EDTA solution (pH 8.0) and it was precipitated with 5μl of 4M LiCl and 150 μl of prechilled (-20°C) ethanol. The eppendorf tube which contained the labelling mixture was mixed by inversion of the tube. The eppendorf tube was then placed in a -70°C freezer for 30 minutes. The tube was then centrifuged at 12,000 x g for 30 min. The resulting supernate was removed gently so as not to disturb the pellet. The pellet was washed with 40 μl of cold (-20°C) ethanol (70% v/v) and centrifuged again for 10 min at 12,000 x g for 30 min. The supernate was again carefully removed so as not to disturb the pellet. The pellet was dried under vacuum for 1-2 min. The pellet was re-suspended in 50 μl of TE/SDS buffer (See Appendix II). The labelled probe was stored at -20°C until needed.

6.8e Checking the Specificity of the Labelled Probe VIA Southern Blotting

The original pBR322 plasmid vector which contained the pLR-7 plasmid inserted at the Hind III site or the pUC-18 plasmid which contained the pLR-20 plasmid inserted at the Eco RI site were first digested with the appropriate restriction enzyme. The
restriction digest was the same as previously cited for pLR7 or pLR20 Plasmid Preps. The plasmid digests were electrophoretically analyzed against the appropriate uncut plasmid vector, BRL Supercoiled DNA and Digoxigenin Labelled Lambda Hind III Fragments under the same conditions as previously cited for pLR-7 or pLR-20 Plasmid Preps. The gel was further exposed to Ultraviolet light (254nm) for a period of 5 min to induce photonicking thus facilitating the transfer of plasmid or supercoiled DNA (Crawford and Bates, 1986).
6.8f Transfer of DNA from Agarose Gels to Nylon Membranes

The DNA within the agarose gel was denatured by exposure to several volumes of 1.5 M NaCl and 0.5M NaOH for 1 hr at room temperature with constant shaking and was followed by a subsequent neutralization step. During neutralization, the gel was exposed to 1.0 M Tris-HCl buffer (pH 8.0) + 1.5M NaCl for a period of 1 hr at room temperature under constant shaking. The gel was then inverted. A piece of Whatman 3MM paper was then wrapped around a BRL plexiglass gel support and placed in a BRL plexiglass reservoir which was filled with 10 X SSC (See Appendices I and II). This Whatman 3MM paper served as a wick. The wick and supporting BRL plexiglass were then set up with the wick resting upon the plexiglass and its ends immersed in the reservoir. Two additional pieces of Whatman 3mm paper were cut to the same size as the gel (6 x 8 cm) and soaked in 2x SSC (See Appendix II) for 2-3 min. A nylon membrane (Biodyne A, Pall Corp. U.S.A.) was cut to the same dimensions as the gel (6 x 8 cm) and immersed in 2xSSC for 2-3 min. One of the pieces of Whatman 3MM paper was placed atop the wick. The inverted gel was placed upon the Whatman 3MM paper and it was smoothed down with a glass rod to remove air bubbles. A Pall Biodyne A nylon transfer membrane was placed upon the upper surface of the inverted gel and was also smoothed down to remove air bubbles. The second Whatman 3MM paper was placed upon the nylon transfer membrane and was then smoothed down to remove air bubbles. Finally, atop the Whatman 3MM paper a stack of 7 BRL blotting pads were added. A second plexiglass plate was placed atop the blotting pads. On this plexiglass plate was placed a 200 gm weight. The weighted blotting stack or transfer assembly was not disturbed for 12-24 hours in order to facilitate the transfer of DNA from the agarose gel to the nylon transfer membrane by capillary action.
The transfer assembly was subsequently taken apart and the side of the nylon membrane in contact with the gel surface was marked with soft pencil. The nylon membrane (marked surface up) was placed into a baking dish containing 6xSSC and placed upon a rotatory shaker for 5 min at room temperature. The nylon membrane was placed upon a dry piece of Whatman 3MM paper and allowed to air dry. The air dried nylon membrane was then placed between two sheets of Whatman 3MM paper and baked for 2 hours at 80°C in order to bind the DNA to the nylon membrane. The membrane was then ready for Prehybridization and hybridization.

6.8g Hybridizing Digoxigenin-11-dUTP Labelled pLR-7 or pLR-20 Probes to Immobilized target DNA

The nylon membrane was then placed within a BRL heat sealable hybridization bag (7 X 10 cm), which was then filled with 25ml of hybridization fluid (See Appendix II) which had been prewarmed (70°C) for 1 hr. prior to its use in prehybridization. The hybridization bag was then heat sealed and placed upon a rotator in a 68°C incubator for 3-4 hrs. The microtube which contained the labelled probe DNA (pLR-7 or pLR-20) was boiled for 10 min to denature the probe DNA. The denatured labelled probe DNA was cooled for 3 min in an ice bath. The heat sealed bag was opened and the Pre-hybridization fluid was removed and replaced with 3.99 ml of prewarmed hybridization fluid (prewarmed for 1 hour at 70°C) plus 10μl of denatured labelled probe DNA (pLR-7 or pLR-20). The bag was again heat sealed and was again placed upon a rotating platform in a 68°C incubator. After overnight incubation, the bag was opened and the hybridization fluid was discarded. The nylon membrane was washed twice for 5 min at room temperature in 50 ml solution of 2x SSC, 0.1% (w/v) SDS. The washings were carried
out on a shaking platform. The nylon membrane was then washed an additional two times, 15 min each in 50 ml of 0.1x SSC, 0.1% SDS on a shaking platform at 68° C. The membrane was now ready for the detection of probe DNA-target DNA hybrids.

6.8h Detecting Probe DNA - Target DNA Hybrids.

The nylon membrane was washed in Buffer #1 (See Appendix II) for 1 min under constant shaking at room temperature. This washing was followed by a 30 min incubation in 100 ml of prewarmed (prewarmed for 1 hour at 70° C) Buffer #2 (See Appendix II) under constant shaking at room temperature. The nylon membrane was then briefly washed on a shaking platform for 1 min. in Buffer #1 at room temperature. The nylon membrane was subsequently exposed to a diluted anti-digoxigenin antibody conjugated with alkaline phosphatase diluted 1:5000 in Buffer #1 thus yielding a final activity of 150mU/ml for a period of 60 min at room temperature on a shaking platform. Enhanced sensitivity of detection was achieved with a 1:500 dilution of anti-digoxigenin antibody (final activity of 1500 mU/ml). The unbound antibody-conjugate was removed by two 15 min washings at room temperature in 100 ml of Buffer #1 (under constant shaking). The nylon membrane was equilibrated with Buffer #3 (See Appendix II) for 2 min at room temperature on a shaking platform. The nylon membrane was placed into a BRL heat sealable bag, and a freshly prepared Colorimetric detecting solution consisting of 9.920 ml of Buffer #3 plus 45μl of NBT solution (Boehringer Mannheim) and 35μl of X-phosphate-solution was added. The bag was heat sealed and placed in a darkened desk drawer for a period of 2-3 hours at room temperature. The nylon membrane was removed from the bag and the color reaction was stopped by washing the nylon membrane for 5 min in 50 ml of Buffer #4 (See Appendix
II) under constant shaking (at room temperature). The detected bands were documented by either photocopying or photographing the wet nylon membrane.

6.9 Southern Transfer of MAI Plasmid DNA

Aliquots of MAI plasmid sample and BRL Supercoiled DNA ladder were loaded onto an 11 x 14 cm agarose gel in a BRL H5 Electrophoretic Tank and run under the same conditions as cited for electrophoresis of MAI plasmid DNA. The gel was prepared for southern blotting in the same manner as cited above but, in the actual southern blotting procedure for MAI plasmids, I utilized a larger nylon membrane (11 x 14 cm) and a blotting stack of 12 BRL pads (11 x 14 cm) and a heavier weight 500 gm weight. The Southern blot protocol, hybridization procedures and non-radioactive detection procedures utilized for the MAI plasmid DNA preps were identical to that cited above.
RESULTS AND DISCUSSION

6.10 Qiagen Midi (30-150ml Plasmid Prep and Electrophoresis for the isolation of):

6.10a E. coli plasmid pJC-20

The use of 150 ml Qiagen Midi preps did indeed yield (pJC-20= pBR322 : pLR-7 Hind III insert) plasmid DNA devoid of contaminating chromosomal DNA. Figure 13a showed that the intact plasmid was >16kb, and was composed of two Hind III fragments: pBR322 (4.3kb) and pLR7 (15.7kb) (See Figure 13a).

6.10b E. coli pJC-70

The Qiagen syringe pressure operated Midi column was replaced by the newer Qiagen gravity operated Maxi column which had a higher DNA capacity (150-500ml) than the midi column. The Qiagen Maxi prep (150ml) gave a (pJC-70=pUC-18 : pLR20 Eco RI insert) plasmid DNA devoid of contaminating genomic DNA (See Figure 13b). The uncut pJC-70 plasmid was >16 kb when measured against a supercoiled DNA (See Figure 13b). The Eco RI digest of pJC-70 produced two fragments of 13 kb (pLR-20 Eco RI insert) and 2.7 kb (pUC-18 plasmid vector)(See Figure 13b).

6.10c Checking the Specificity of Labelled Probes Via Southern Blotting

When the previously purified (via Gene Clean) and digoxigenin labelled Hind III fragment (pLR-7) probe was tested for specificity, it revealed a strong homology with pLR-7 at a 1:500 conjugate (alkaline phosphate conjugated to anti-digoxigenin Fab fragments) dilution (See Figure 14). This check of specificity also revealed some hybridization with the parent pBR322 plasmid
vector segment of pJC-20. This result indicates some degree of carryover of the parent plasmid vector (pBR322) (Figure 14).

Similarly the probe pLR-20 (Eco RI fragment of pJC-70) showed very strong homology with the heavier fragment (pLR-20) at 1:500 conjugate dilution (alkaline phosphate conjugated to anti-digoxigenin Fab fragments) (See Figure 15). This specificity check also displayed a very faint band of hybridization with the parent pUC-18 plasmid vector segment of the Eco RI digest of pJC-70. This second faint band of homology would, therefore, be indicative of a slight degree of carryover of the parent plasmid vector (pUC-18) (See Figure 15).
Figure 13a: Electrophoresis of plasmid pJC-20. Agarose Gel Electrophoresis

Lane 1: Lambda Hind III digest, Lane 2: BRL Supercoiled DNA Ladder, Lane 3: pJC-20 Hind III Cut Plasmid, Lane 4: pJC-20 Uncut Plasmid
Lane 1: Lambda Hind III digest, Lane 2: Supercoiled DNA Ladder, Lane 3: pJC-70 Eco RI Cut Plasmid, Lane 4: pJC-70 Uncut Plasmid
Figure 14: **Specificity Check of probe pLR-7.**

L: Agarose Gel Electrophoresis

R: Southern Blot of Agarose Gel Electrophoresis

Probe Conditions: pLR-7 (10 μl pLR-7 probe/ 4.0 ml hybrid. fluid)

Detection: 1:500 dilution Anti-digoxigenin Fab

Colorimetric Visualization Interval: 2 hrs

Lane 1(L&R): Lambda Hind III fragments, Lane 2(L&R): pJC-20 Hind III Cut Plasmid
Figure 15: Specificity check of probe pLR-20.
L: Agarose Gel Electrophoresis
R: Southern Blot of Agarose Gel Electrophoresis
- Probe Conditions: pLR-20 (10 μl pLR-20 probe/4.0 ml hybrid. fluid)
- Detection: 1:500 dilution Anti-digoxigenin Fab
- Colorimetric Visualization Interval: 2 hrs

Lane 1 (L&R): Lambda Hind III fragments, Lane 2 (L&R): pJC-70 Eco RI Cut Plasmid
6.11 **Electrophoresis of MAI Plasmid DNA for the Presence of PLR-7 and PLR-20 Type Plasmids**

6.11a **PLR-7 Type Plasmids**

The majority (85% or 17/20) of MAI strains isolated from AIDS patients at St. Vincent's Hospital and Memorial Sloan-Kettering Cancer Center, and analyzed via Southern Transfer for homology with Digoxigenin-Labelled pLR-7 probe, were shown to possess pLR-7 type plasmids (See Figure 16; Table 11). The AIDS related strains in this study that showed homology with the probe (pLR-7=15.7kb) displayed pLR-7 type plasmid bands varying from 14-18kb when measured against BRL supercoiled molecular weight markers.
Figure 16: Detecting the presence of pLR-7 type plasmids.

L: Agarose Gel Electrophoresis
Gel Specifics: (11 x 14 cm gel, 0.6% Agarose, 9.68mm in Thickness)
Voltage: 104 volts
Duration of run: 2hrs 35 min

R: Southern Blot of Agarose Gel Electrophoresis
Probe Conditions: pLR-7 (10 µl pLR-7 probe/ 4.0 ml hybrid. fluid)
Detection: 1:5000 dilution Anti-digoxigenin Fab
Colorimetric Visualization Interval: 2 hrs 35 min

Lane 1 (L&R): BRL Supercoiled DNA Ladder, Lane 2 (L&R): LR25,
Lane 3 (L&R): 10,000, Lane 4 (L&R): 10,001, Lane 5 (L&R): 10,002,
Lane 6 (L&R): 10,011
6.11b **PLR-20 Type Plasmids**

By contrast to the results obtained with the pLR-7 probe, those for the pLR-20 were quite different. When the same 20 strains derived from St. Vincent's Hospital and Memorial Sloan-Kettering Cancer Center were analyzed via Southern Transfer for homology with Digoxigenin-Labelled pLR-20 probes, only 20% (4/20) of the isolates (See Table 11) showed homology with the pLR-20 probe (See Figure 17). The plasmid bands which displayed homology with the pLR-20 probe were 13 kb (pLR-20)=± 13 kb). The pLR-20 probe was derived from a pUC-18 parent plasmid vector. This pUC-18 plasmid vector contained a pBR322 ampicillin resistance gene sequence (Davis et al., 1986). The presence of this pBR322 ampicillin resistance gene sequence in the pUC-18 parent plasmid vector enabled pLR-20 probe homology with the BRL supercoiled DNA ladder to occur, therefore permitting visualization of the supercoiled DNA ladder on the nylon transfer membrane. This visualization of the BRL supercoiled DNA molecular weight marker permitted a direct molecular weight determination of the plasmid DNA. A summary of the pLR-20 type plasmid bearing strains can be seen in Table 11.
Figure 17: Detecting the presence of pLR-20 type plasmids.

L: Agarose Gel Electrophoresis
   Gel Specifics: (11 x 14 cm gel, 0.6% Agarose, 9.68mm in Thickness)
   Voltage: 106 volts
   Duration of run: 2hrs 35 min

R: Southern Blot of Agarose Gel Electrophoresis
   Duration of run: 2hrs 35 min
   Probe Conditions: pLR-20 (10 μl pLR-20 probe/4.0 ml hybrid. fluid)
   Detection: 1:500 dilution Anti-digoxigenin Fab
   Colorimetric Visualization Interval: 2 hrs 53min

Lane 1 (L&R): BRL Supercoiled DNA Ladder, Lane 2 (L&R): MD,
Lane 3 (L&R): 10,002, Lane 4 (L&R): 10,0010
Lane 5 (L&R): 10,010 IV, Lane 6 (L&R): 10,011,
Lane 7 (L&R): 10,011 IV, Lane 8 (L&R): 10,015
6.12 Cross Hybridization of pLR-7 and pLR-20 probes with chromosomal DNA

When we sought to increase the sensitivity of the non-radioactive digoxigenin colorimetric detection by utilizing a more concentrated anti-digoxigenin conjugate (1:500) rather than the dilution recommended by the manufacturer (1:5000), there occurred cross hybridization of the pLR-7 probe with chromosomal DNA. Cross hybridization of the pLR-20 probe with chromosomal DNA, however, was not encountered at the more concentrated 1:500 dilution of the anti-digoxigenin conjugate.

6.13 Conservation of Plasmids

Two of the four corded MAI isolates derived from patient blood revealed the presence of plasmids (See Table 11). Two of these corded MAI isolates (10,010, 10,011) carried pLR-7 type plasmids, while the two remaining corded strains (10,002, 10,015) carried neither the pLR-7 nor the pLR-20 type plasmids. The pLR-20 type plasmids were not seen in any of the original four corded isolates. When corded strains 10,010 and 10,011 were passed through 4 subculturings, cording was lost by the fourth subculture but, the presence of the pLR-7 plasmid still remained (See Table 12). Crawford and Falkinham have stated that plasmid carriage in many plasmid bearing strains of MAI remained despite the fact that these strains had been maintained in laboratory culture media for many years (Crawford and Falkinham, 1990). This continued conservation of MAI plasmids, was found also by J.J. McFadden and his Co-workers (J.J. McFadden, Personal Communication).
6.14 Presence of Mixed Cultures

Initially corded strains 10,002, 10,010 and 10,015 were isolated and subcultured with the Para SL/C cited in Section IV. The results as concerns the plasmid profile for strains 10,002, 10,010 and 10,015 and their respective fourth subcultured strains (10,002-IV, 10,010-IV, 10,015-IV) show that these strains are basically the same (See Tables 11 & 12).

When corded strain 10,011 was subcultured 4 times, it lost cording but, its fourth subcultured strain 10,011-IV, had present both pLR7 and pLR20 type plasmids (See Table 12). The plasmid profile seen on the ethidium bromide stained agarose gel in Figure 17 (See lanes 6L, 7L) shows plasmids of differing sizes in the fourth subcultured strain 10,011-IV when run alongside the original corded strain 10,011 (See Figure 17: lanes 6L&7L). This result clearly shows these two strains are different.

The original corded strain 10,011, and the fourth subcultured strain 10,011-IV, were subcultured in the same manner as that cited above for the other corded strains (10,002, 10,010, 10,015). This manipulation with Para SL/C would rule out the presence of a non-acid-alcohol fast, non-paraffinophilic and nonmycobacterial contaminant. However, the application of Para SL/C does not rule out the possibility of the existence of mixed cultures of MAI within the initial AIDS patient blood isolate. Arbeit and his co-investigators have noted that it is not uncommon for AIDS patients to suffer from bacteremia due to more than one strain of MAI occurring simultaneously (Arbeit et al., 1992).

The initial presence of two distinct strains of MAI isolate 10,011 does provide a plausible explanation for the differing plasmid profiles obtained for 10,011 and 10,011-IV in
Section VI and the differing R.F.L.P profiles obtained for these organisms in Section VII.
<table>
<thead>
<tr>
<th>MAI Strain</th>
<th>Hospital Where Isolated</th>
<th>PLR-7 Type Plasmid Presence</th>
<th>PLR-20 Type Plasmid Presence</th>
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*ND=not done
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6.15 General Plasmid Carriage

The plasmid profile of St. Vincent's blood derived MAI isolates showed that the predominant plasmid type was the pLR-7. This finding is in complete agreement with the findings of several other groups of investigators (Crawford and Bates, 1986; Jucker and Falkinham, 1990; Hellyer et al., 1991). Hellyer et al. examined a small collection of our St. Vincent's blood isolates (953, 1516, 1762, 4861, 6475, 8197, 8515, 15113, 10,000) and found that the pLR-7 type plasmid was present in 89% of these isolates whereas, the pLR-20 type plasmid was present in 33%. In addition, this investigation again substantiated the earlier findings of Hellyer et al. that reported pLR-20 related plasmids were almost always found in those strains that also carried a plasmid related to pLR-7. The pLR-20 plasmid was only seen in the presence of the pLR-7 plasmid in our investigation.

The findings of Hellyer et al. served as a check on our plasmid isolation protocols since when we repeated their analysis of the above strains, we obtained the same results (Hellyer et al., 1991). PLR-7 type plasmids were found in 85% of the MAI isolates obtained from St. Vincent's Hospital and Memorial Sloan-Kettering Cancer Center. By contrast, only 20% of the aforementioned isolates showed the presence of pLR-20 type plasmids. The study of pLR-7 and pLR-20 type plasmid carriage in MAI performed by Hellyer and co-workers included British AIDS-related isolates, U.S. AIDS-related isolates, British non-AIDS isolates and British veterinary isolates. Hellyer found that 62% of his isolates carried pLR-7 type plasmids but only 17% carried pLR-20 type isolates. A chi-square analysis of the distribution of plasmids among pLR-7 and pLR-20 types which compared the data from Hellyer's investigation with this investigation failed to show any statistically
significant difference in (P>0.05) plasmid carriage. The study of Hellyer and co-workers noted the presence of plasmids of >150 kb in St. Vincent's strains 953, 1516, 1762, 4861, 6475, 8515, 10,000 and 15113 but, I was never able to find these plasmids of >150 kb in the aforementioned strains.

6.16 Hybridization and Variation in pLR-7 and pLR-20 Plasmid Size

The pLR-7 and pLR-20 plasmid types were found not to cross hybridize with each other as had been noted by Hellyer et al. (1991). It was noted in our investigation that those pLR-7 type plasmids which showed homology with the pLR-7 probe varied from 14 kb to >16.2kb. An accurate measurement of supercoiled DNA was only possible up to the upper limit of the BRL Supercoiled DNA Ladder (16.2 kb) which served as the Supercoiled molecular weight standard. The study of Hellyer et al. found a greater variation in size in those plasmids showing homology with pLR-7 probe (15-30 kb). The pLR-20 type plasmids which showed homology with the pLR-20 probe were found to be 13 kb which was a bit smaller than the 14-16 kb ranged noted by Hellyer et al. Our pLR-20 plasmid, however, did fit within the range of 12.8 kb -15.3 kb cited for pVT2 type plasmids by Jucker and Falkinham (Hellyer et al., 1991; Jucker and Falkinham, 1990). The pLR-20 plasmid of Crawford and the pVT2 plasmid of Jucker and Falkinham may be closely related to each other (Crawford and Falkinham, 1990; Crawford, Personal Communication).

There did occur major differences in the percentage of AIDS-derived MAI carrying pLR-7 type plasmids between the British blood isolates (29%) and our total St. Vincent's blood-derived isolates (79%) (Hellyer et al., 1991). In the British alimentary tract and respiratory tract, the percentage of isolates carrying pLR-7 type plasmids were 25% and 28% respectively. The higher carriage of pLR-7 type plasmids in the American isolates was also noted by Crawford et al. (100%) and Morris et al. (75%) (Crawford and Bates, 1986; Morris et al., 1990).

6.18 AIDS-related Plasmid Presence and Virulence

The literature has shown that members of the M. avium, M. intracellulare, and M. scrofulaceum group or MAC organisms have been shown to encode functions that are involved in restriction and modification and mercury and copper resistance (Jucker and Falkinham, 1990). For the most part, the studies that have focused upon the functions of plasmids such as the pLR-7 and pLR-20 type plasmids isolated from M. avium still are inconclusive at best. The current state of knowledge concerning the presence of plasmids in most MAC complex organisms and their relationship to MAC virulence is still not clearly understood.
Section VII

The Application of Restriction Fragment Length Polymorphism (R.F.L.P)

To

AIDS-related Mycobacterium avium Isolates
INTRODUCTION

7.0 Restriction Fragment Length Polymorphism Analysis

The restriction fragment length polymorphism (RFLP) analysis and its application to MAI complex organisms has been previously discussed in Section I (See paragraphs 1.6-1.8).

This section will focus upon the application of restriction fragment length polymorphism analysis to AIDS-related M. avium complex strains isolated at St. Vincent's Hospital and Memorial Sloan-Kettering Cancer Center.
7.1 Isolation of Genomic DNA

A 7 day old 5ml starter culture (7H9 broth supplemented with 1% glycerol and 4% O.A.D.C) inoculated with *M. avium* cells grown at 37°C served as inoculum for an erlenmeyer flask containing 20 ml of the 7H9 broth. The 20 ml aliquot of *M. avium* cells were incubated at 37°C for a period of 2-3 weeks. The cells were exposed to 2% (w/v) glycine for a period of 48 hours prior to their being harvested. The cells contained within the erlenmeyer were pelleted by centrifugation at 11,950 x g in a Sorvall SS-34 rotor at 4°C for 20 min. The pellet was washed in TEN buffer (See Appendix I) and re-suspended into 5.0 ml TEN (McFadden et al., 1990; Van Soolingen et al., 1991). The 5.0ml of re-suspended pellet was then digested with 10mg/ml of subtilisin (Sigma) for 3 hours at 37°C which was then followed by exposure to lysozyme (1mg/ml; Boehringer Mannheim) for an additional 3 hours at 50°C. SDS was then added to 1% and pronase (Calbiochem) added to 3mg/ml and incubated at 37°C for 24 hours, with a further addition of pronase (to a total of 6mg/ml) after 18 hours. The DNA was then extracted by multiple phenol/chloroform extraction, Ribonuclease A digestion and ethanol precipitation. The sample was spun down at 15,000 x g for 30 min at 4°C in a Sorvall SS-34 rotor and the supernate was gently removed and discarded. The resulting pellet was washed in 70% (v/v) ethanol and was spun in a Sorvall SS-34 rotor at 15,000 xg for 15 min at 4°C. The supernate was gently removed and discarded. The remaining pellet was air dried at room temperature for 10 minutes. The pellet was re-suspended in 300 μl of TE buffer and stored at -20°C.
7.2 **Boehringer Mannheim Column Chromatography Protocol for MAI Genomic DNA Isolation**

The *M. avium* cells were grown and harvested in the same manner as cited above. The pellet was gently re-suspended in 2ml of the Boehringer Mannheim Solution #1 (lysis buffer) (Boehringer Mannheim Biochemicals, Indianapolis, Indiana, U.S.A.). An aliquot of 60 μl heat treated (boiled in 100° C water bath for 15 minutes) RNase A (See Appendix I) solution and 160 μl of a Lysozyme (See Appendix I) solution was added to the tube. The tube was mixed by gentle inversion and incubated at 37° C for a period of 30 min. An aliquot of 100 μl of Proteinase K solution (See Appendix I) was then added to the tube which was gently mixed by inversion and incubated for a period of 30 min at 55° C. During the proteinase K incubation, a Boehringer Mannheim Column was equilibrated with 6 ml of Boehringer Mannheim Kit Solution #2 (Column Wash Buffer). The column eluate port was capped when the level of the Solution #2 or Column Wash Buffer was 0.5 cm above the surface of the column gel matrix. The Proteinase K digest or sample was then prepared. Column was further equilibrated with 4 ml of Boehringer Mannheim kit Solution #2.

The 6 ml sample was added to the column with eluate port still capped. The sample was then pipetted back and forth through the column so as to cause a re-suspension of the column matrix and sample. The column bed was then allowed to settle for a period of 10 min at room temperature. The column eluate port cap was then removed and the column flow was allowed to proceed via gravity flow. When the wash buffer level was 0.5 cm above the gel matrix, an additional aliquot of 3 ml of wash buffer was carefully layered onto the column so as not to disturb the gel matrix. A 10ml syringe was added to the top of the column and slight pressure was
applied. When the residual of the wash buffer reach 0.5 cm above the gel matrix, the syringe was removed and the column was then primed with 0.5ml of Boehringer Mannheim Solution #3 (Column Elution Buffer) under gravity flow. When the primer aliquot of elution buffer reached 0.5 cm above the gel matrix, 2 additional milliliters of elution buffer were added and a 10 ml syringe was connected to the column. A slight pressure was applied to the syringe. The eluate resulting from the treatment with the elution buffer contained the purified Genomic DNA. The column was then discarded.

The column eluate (2ml) was treated with 0.5 volumes of isopropanol, then placed in a Sorvall SS-34 rotor and spun at 17,200 x g for 20 min at 4° C. The supernate was carefully removed with a sterile pipette so as to not disturb the DNA pellet. The DNA pellet was then washed by adding 1.0 volume of 70% (v/v) ethanol and further centrifuged at 17,200 x g for a period of 20 min at 4° C. The supernate was carefully removed so as not to disturb the DNA pellet. The Genomic DNA pellet was air dried for 10 min. The Genomic DNA pellet was resuspended in 300 µl of TE buffer and stored at -20° C.
7.3 Modified Qiagen Prep For The Isolation of Genomic DNA From MAI

7.3a Mini Prep Protocol

The basic inoculum was the same as that cited in the preceding genomic protocols but an aliquot of 40ml aliquot of M. avium cells were utilized rather than the 20ml previously cited for genomic DNA preps. The cells were then grown and harvested in the same manner as cited above for genomic DNA (Qiagen Inc., Chatsworth, California, U.S.A.).

The cells were then ready for the Qiagen procedure for genomic DNA isolation which differs from that utilized for plasmid DNA isolation. The pellet was resuspended in 1ml of Qiagen P1 buffer (See Appendix III; Qiagen Genomic DNA Prep). The composition of this P1 buffer utilized for genomic DNA preps differed from that used for plasmid DNA preps. A 1ml aliquot of aqueous solution of lysozyme (20mg/ml) was then added to the solution to give a total volume of 2 ml and a final lysozyme concentration of 10mg/ml. The solution was then vortexed and incubated at 37° C for 20 minutes. One ml of Qiagen P2 buffer (See Appendix III; Qiagen Genomic DNA Prep) was added to the solution which was then incubated for 20 min in an ice bath. The P2 buffer utilized in Qiagen Genomic DNA preps also differed from that utilized in Qiagen plasmid DNA preps. A 1ml aliquot of an aqueous solution of Proteinase K (40mg/ml) was added to the solution to give a total volume of 4ml and a final Proteinase K concentration of 10mg/ml. The solution was then vortexed and incubated at 50° C for 2 hrs. The incubation was followed by a centrifugation at 20,000 x g in a Sorvall SS-34 rotor for 15 min at 4° C. The supernate (lysate) was then carefully removed and saved. The buffers utilized for the remainder of the Qiagen Genomic DNA prep
were the standard Qiagen buffers which were routinely utilized for plasmid DNA preps. A Qiagen Maxi Column was equilibrated with 10 ml of QBT buffer (See Appendix III; Qiagen Plasmid DNA Prep). The lysate was loaded onto the Qiagen Maxi Column and when the lysate was almost absorbed into the gel matrix (0.5 cm above column gel matrix) the column was washed with 2 x 30ml of QC buffer (See Appendix III; Qiagen Plasmid DNA Prep). The column was eluted with 15ml of QF buffer (See Appendix III; Qiagen Plasmid DNA Prep). The eluate was aliquoted in 1000μl amounts into microcentrifuge tubes and 500μl of isopropanol was added per 1000μl aliquot of eluate. The microcentrifuge tubes were placed into an eppendorf microcentrifuge (microcentrifuge held within a 4°C refrigerated chamber) and spun at 16,000 x g for 30 min. The supernate derived from the centrifugation was carefully removed and discarded. The genomic DNA pellets were washed in 1000μl of 70% (v/v) ethanol by being spun for 30 min at 16,000 x g in an eppendorf microcentrifuge kept at 4°C. A single volume of 300 μl of TE buffer was pipetted through all the microcentrifuge tubes that contained dried Genomic DNA pellet. The resuspended pooled Genomic DNA pellet was stored at -20°C.

7.3b Maxi Prep Protocol

The basic inoculum was the same as that cited in the preceding genomic protocols but an erlenmeyer flask containing 150 ml of the 7H9 broth rather than the smaller volumes previously cited. In addition, the M. avium cells were incubated at 37°C for a period of 4-6 weeks under constant shaking conditions.

The cells were then grown and harvested in the same manner as cited previously for genomic DNA. The pellet was
resuspended in 3ml of P1 which was 1/50 volume (manufacturer's recommendation) of the initial 150ml culture. Lysozyme was added dry to the solution rather than as an aqueous concentrate. The final concentration of lysozyme, however, was the same as that cited for the Mini Prep (10mg/ml). The solution was then treated in the same manner as cited for the Mini prep. Proteinase K was added dry to the solution rather than as an aqueous concentrate. The final concentration of Proteinase K, however, was the same as that cited for the Mini Prep (10mg/ml). The protocol from this point on was the same as that cited for the Mini prep.

7.4 Check of Quality of DNA

Samples of 650-1500 ng of genomic DNA preparation were applied to 11 x 14 cm 0.8% agarose gels which contained 0.5μg/ml of ethidium bromide and were 9.68 cm in thickness. Samples of 1320ng (66ng/μl) Lambda Hind III fragments served as both molecular weight markers and an ethidium bromide intensity standard to be utilized to estimate the DNA concentrations of the genomic DNA samples in the gel. The gels were placed within a BRL Model H5 Horizontal Electrophoretic Tank containing 1X TAE buffer and 1.0ug/ml ethidium bromide. Electrophoretic runs were carried out using a BRL Model 250 Power Unit set at 50-60 Volts and run for 3-4 hours. The gel was viewed on a 254nm transilluminator and the electrophoretic run photographed. Those aliquots of genomic DNA found displaying a band of >20 kb in size were utilized in the subsequent restriction digests and RFLP analyses.

7.5 pMB-22 Plasmid Prep

The pMB22 recombinant plasmid is composed of a pGEM-1 plasmid vector which is 3.0 kb and has a 5.4kb insert at the
Bam HI site. The insert in pMB22 contains IS900 + flanking DNA (M. paratuberculosis). A 4mm loopful of E. coli containing the pMB22 recombinant was inoculated into 4 ml of LB broth (See Appendix IV) containing 25 μg/ml of ampicillin to select for plasmid bearing strains and incubated overnight at 37° C. This overnight growth served as inocula for a 150 ml (in a 500 ml erlenmyer flask) LB broth batch culture (containing 25 μg/ml ampicillin) which was placed into a 37° C shaker bath. The A650nm reading was taken at 30 min intervals. When the A650nm reading reached 0.9 O.D. units, spectinomycin was added to a final concentration of 150μg/ml, and the culture was returned to the shaker bath and incubated overnight at 37° C. The cells were harvested in the same manner as that previously cited (See Section VI; 6.4). The Qiagen Maxi Plasmid protocol utilized for the isolation of pMB-22 remained the same as that cited for pJC-20 (See Section VI; 6.6, 6.6a, 6.6b).

Fifteen microliters of Qiagen Maxi Prep eluate pMB22 plasmid clone was digested with 2 μl of BamHI (Boehringer Mannheim) and was subjected to agarose gel electrophoresis. The 5.4 kb fragment was excised from the gel and recovered by the Gene Clean Protocol (Section VI; 6.8c). The 5.4kb fragment was labelled by digoxigenin (See Section VI; 6.8d).

7.6 Specificity of the Labelled PMB-22 Probe

Fifteen microliters of Qiagen Maxi Prep eluate pMB22 plasmid was digested with 2 μl of BamHI (Boehringer Mannheim; 11 units/ml) restriction enzyme in a total volume of 20 μl for 60 minutes at 37° C. Five microliters of loading buffer were added to the 20 μl of digest. A 25μl aliquot of the pMB22 digest and several additional samples were subsequently analyzed electrophoretically. The additional samples analyzed were:
a) pMB22 uncut plasmid sample which consisted of 15μl of pMB22 plasmid, 2μl 10x react buffer, 3μl of ddH₂O and 5μl of loading buffer; b) BRL Supercoiled DNA sample which consisted of 4μl of supercoiled DNA molecular weight ladder (94ng/μl), 2μl 10X react buffer, 14 μl T.E. buffer, 1μl ddH₂O and 5μl loading buffer; c) Digoxigenin Labelled Lambda Hind III Fragments Sample which consisted of 15μl Digoxigenin Labelled Hind III Fragments (10ng/μl), 2μl TE buffer, 5μl loading buffer. The samples were electrophoresed and photographed in the same manner as that cited for pLR-7 (See Section VI; 6.8a). Southern blotting and hybridization were carried out using the same methodologies cited above (See Section VI; 6.8f, 6.8g). Non-radioactive colorimetric detection was carried at the enhanced detection (1:500 dilution) level previously cited (See Section VI; 6.8h).

7.7 Restriction Digests Of Genomic DNA

Genomic DNA (650-1500ng/130μl) were totally digested with 72 Units Pvu II (Boehringer Mannheim 12 Units/μl) restriction endonuclease and 15μl of 10X React Buffer in a total volume of 151μl for a period of 3 hours at 37° C. The digests were stored overnight at -20° C.

7.8 Agarose Gel Electrophoresis of Genomic DNA Digests

An aliquot of 37μl of loading buffer was added to the restriction digests of the Genomic DNA samples cited above. These samples of genomic digest were loaded onto a 1% agarose gel which was 11 x 14 cm, 9.68mm in thickness and contained 0.5μg/ml ethidium bromide. A sample of 1250 ng/lane of digoxigenin labelled Lambda Hind III (10ng/μl) served as a linear molecular weight standard.
The gel was placed in a BRL H5 electrophoretic tank containing 1X TAE Buffer and run at 60 Volts for 4 hours using a BRL Model 250 Power Unit. The electrophoretic gel was viewed on a 254nm Transilluminator to determine the completeness of the Pvu II restriction digestion per genomic prep. Adequately digested genomic preps were seen as even smears down the gel. A photographic record of the agarose gel was then made.

7.9 Southern Transfer of Genomic DNA Digest

The gel was prepared for southern blotting in the same manner as cited above but, in the actual southern blotting procedure for cited MAI plasmids, I utilized a larger nylon membrane (11 x 14 cm) and a blotting stack of 12 BRL pads (11 x 14 cm) and a heavier weight 500 gm weight. The Southern blot protocol and hybridization procedures utilized for the pMB-22 probe specificity check MAI Genomic DNA preps were identical to that cited above. The duration of the period required for non-radioactive/colorimetric detection of R.F.L.P. bands was slightly longer (3-3.5 hrs.) than that cited for the pMB-22 specificity check.

7.10 Restriction Fragment Length Polymorphisms R.F.L.P. Analysis

The R.F.L.P banding patterns obtained in the present investigation were classified according to R.F.L.P. designations established by McFadden and co-workers for AIDS-related M. avium complex organisms (McFadden et al., 1990).
RESULTS AND DISCUSSION

7.11 Isolation of Genomic DNA

The basic protocol as cited by McFadden et al. and Van Soolingen et al. did yield sufficient quantities of MAI genomic DNA for R.F.L.P. assays but, necessitated the use of toxic reagents such as phenol and chloroform as well as, generated toxic organic wastes that posed a problem as concerns compliance with the new safety guidelines for proper handling and disposal of these reagents (McFadden et al., 1990; Van Soolingen et al., 1991). The red tape created by these new regulations, forced us to abandon these Chloroform/phenol based extractions in favor of genomic DNA isolation alternatives that did not require an organic phase (chloroform/phenol). These non-organic phase genomic DNA isolations alternatives were based upon column chromatographic protocols.

7.12 Boehringer Mannheim Column for Purification of Genomic DNA

The Boehringer Mannheim Column chromatographic protocol which was adapted to MAI complex organisms did yield pure restriction endonuclease grade samples of genomic DNA that were able to be analyzed in R.F.L.P. analyses and did show that it was possible to eliminate the need to utilize organic phase (chloroform/phenol extraction) for genomic DNA isolation. The column, however, suffered from an important drawback as concerns its application in MAI genomic DNA isolations, namely, with the crude sample lysate derived from 15-20ml of 7-14 day old cultures of MAI, the column (syringe pressure operated) often became clogged producing an eluate that either showed lower yields of genomic DNA or a complete absence of genomic DNA. When increased operating pressure was applied to the syringe to overcome the effects of
column clogging, a blowback in the column occurred. The blowback produced a corresponding destruction of the column gel bed.

The column clogging and the associated reductions in genomic DNA yields limited the usefulness of the Boehringer Mannheim column protocol for MAI genomic DNA isolation. The larger amounts of MAI genomic DNA that were needed (1250-2000ng/lane) for successful R.F.L.P. analysis via Southern Transfer and non-radiometric digoxigenin/colorimetric detection could not always be obtained via the Boehringer Mannheim protocol. Therefore, the Boehringer Mannheim column system was abandoned.
7.13 Qiagen Column Protocol For Genomic DNA Isolation

7.13a Mini Prep Protocol

The Mini prep protocol was only utilized on a trial basis to determine the feasibility of Qiagen Maxi Columns for isolation of M. avium genomic DNA (See Figure 18). The presence of intact genomic DNA >20 KB seen on gel electrophoresis proved that Qiagen Maxi columns offered an additional column based method capable of isolating M. Avium genomic DNA without the need for an organic phase (chloroform/phenol). In addition, the Qiagen Maxi column which was gravity operated, did not become clogged and had a very large capacity for crude DNA lysates (150-500 ml). From the initial success of the pilot mini prep series, it was decided that the Qiagen Maxi Column be utilized in all subsequent MAI genomic DNA isolations. It was also decided that all future Batch Culture Preps be 150ml aliquots (Maxi Preps) of MAI strain grown up at 4-6 week intervals to take advantage of the greater DNA binding capacity of the Qiagen Maxi Column.
Figure 18: Qiagen Genomic DNA Preparation.
Agarose Gel Electrophoresis (Qiagen Mini Prep)
Gel Specifics: (11 x 14 cm gel, 1.0% Agarose, 9.68mm in Thickness)
Voltage: 60 volts
Duration of run: 4 hrs.

Lanes 2-5 each contained 100µl of DNA preparation.
Lane 1: Lambda DNA Hind III markers, Lane 2: 10,002,
Lane 3: 10,010, Lane 4: 10,011, Lane 5: 10,015,
The Mini prep protocol demonstrated that the large capacity of the Qiagen Maxi Column could be utilized for MAI genomic DNA isolation derived from a larger biomass generated during the longer period of growth (4-6 weeks) thus providing isolations yielding higher concentrations of genomic DNA. The larger yields of MAI genomic DNA are extremely important especially when performing R.F.L.P. with the non-radioactive Digoxigenin/colorimetric detection which required slightly larger sample sizes (650-1500ng sample mycobacterial genomic DNA /lane) to be added to the gel than do radioactive based detection systems (300-1000ng sample mycobacterial genomic DNA/lane; Hampson et al., 1989).
7.14 Quality of MAI Genomic DNA Preps

M. avium genomic DNA preparations were run electrophoretically on agarose gel. Those preparations found to be intact and >20 kb as determined against Lambda Hind III markers, were utilized in the subsequent restriction digests and RFLP analyses.

7.15 Check of the Labelled PMB-22 Probe

Specificity of the labelled probe Via Southern Blotting revealed a strong homology with the heavier fragment (5.4kb) at an anti-digoxigenin conjugate dilution of 1:500 (See Figure 19). This check of specificity also revealed that there did occur lesser homology with the parent pGEM-1 plasmid vector segment (3.0kb) of the Bam HI digest of plasmid clone pMB-22. This result therefore indicates that there did occur some degree of carryover of the parent plasmid vector (See Figure 19).
Figure 19: Specificity of Labelled PMB-22 Probe.

L: Agarose Gel Electrophoresis
   Gel Specifics: (5.7 x 8.3 cm gel, 0.6% Agarose, 4.0mm in Thickness)
   Voltage: 100 volts
   Duration of run: 45 min.

R: Southern Blot
   Probe Conditions: pMB-22 (10 μl probe/ 4.0 ml hybrid.
   Detection: 1:500 dilution Anti-digoxigenin Fab
   Colorimetric Visualization Interval: 2hr

Lane 1 (L&R): Digoxigenin-labelled Lambda DNA Hind III markers,
Lane 2 (L&R): pMB-22 plasmid clone Bam HI digest
Figure 20: R.F.L.P. Banding Patterns

L: Agarose Gel Electrophoresis (Qiagen Columns)
   Gel Specifics (11 x 14 cm gel, 1.0% Agarose, 9.68 mm in thickness)
   Voltage: 60 volts.
   Duration of run: 4 hrs.

R: Southern Blot
   Probe Conditions: pMB-22 (10 \mu l probe / 4.0 ml hybrid. fluid)
   Detection: 1:500 dilution Anti-digoxigenin Fab
   Colorimetric Visualization Interval: 3 hr.

Lanes 2–7 contain 130 \mu l of Pvu II digested genomic DNA.
Lane 1 (L&R): Digoxigenin-labelled Lambda DNA Hind III markers,
Lane 2 (L&R): 10,002-IV, Lane 3 (L&R): 10,011,
Lane 4 (L&R): 10,011-IV, Lane 5 (L&R): 10,010,
Lane 6 (L&R): 10,010-IV, Lane 7 (L&R): 10,015-IV
7.16 Non-radioactive Detection System For R.F.L.P.

The non-radioactive digoxigenin-labelled probed linked to a colorimetric detecting system was sensitive enough to yield discernible R.F.L.P. banding patterns (See Figure 20). It was however necessary to add slightly larger amounts of sample genomic DNA digest (650-1500ng/lane) to achieve results comparable to that obtained by Hampson and co-workers via a $^{32}$P radiolabelled probe (300-1000ng/lane) linked to an autoradiographic detection system (Hampson et al., 1989). Other investigators have also found that the non-radioactive-digoxigenin labelled probe could serve as an adequate substitute of radiolabelled probes in R.F.L.P analyses (McFadden et al., 1990). Recent refinements in the non-radioactive detecting systems such as chemiluminescent labelling and autoradiographic detection have now improved these systems to levels of sensitivity that are comparable to that obtained via radiolabelled systems.

7.17 R.F.L.P. Patterns

The data cited in Table 13 reveals that the AIDS-related MAI isolates from patients at St. Vincent's Hospital and Memorial-Sloan Kettering Cancer Center consisted of the currently known M. avium R.F.L.P. banding patterns.

This investigation has observed that the R.F.L.P. pattern most closely resembling A1 occurred in 50% of the MAI isolates successfully analyzed. R.F.L.P. patterns A2, A3 and A/I occurred respectively at 25%, 12.5% and 12.5% (See Table 14).

The A/I banding pattern has been previously reported to occur particularly in animal derived MAI isolates and not occur in AIDS related MAI isolates (Kunze et al., 1991). Therefore, the presence of an AIDS blood isolate of MAI (SK060) having an A/I
bANDING PATTERN IS A RARE AND SIGNIFICANT FINDING (DR. SHELDON BROWN, PERSONAL COMMUNICATION).


<table>
<thead>
<tr>
<th>M. avium Strain</th>
<th>Hospital where isolated</th>
<th>R.F.L.P. Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>10,002</td>
<td>St. Vincent's Hospital</td>
<td>NS*</td>
</tr>
<tr>
<td>10,010</td>
<td>St. Vincent's Hospital</td>
<td>A1</td>
</tr>
<tr>
<td>10,011</td>
<td>St. Vincent's Hospital</td>
<td>MC*</td>
</tr>
<tr>
<td>10,015</td>
<td>St. Vincent's Hospital</td>
<td>A3</td>
</tr>
<tr>
<td>SK015</td>
<td>Memorial Sloan-Kettering Cancer Center</td>
<td>A1</td>
</tr>
<tr>
<td>SK016</td>
<td>Memorial Sloan-Kettering Cancer Center</td>
<td>A1</td>
</tr>
<tr>
<td>SK024</td>
<td>Memorial Sloan-Kettering Cancer Center</td>
<td>A2</td>
</tr>
<tr>
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<td>A1</td>
</tr>
<tr>
<td>SK060</td>
<td>Memorial Sloan-Kettering Cancer Center</td>
<td>A/I</td>
</tr>
<tr>
<td>SK069</td>
<td>Memorial Sloan-Kettering Cancer Center</td>
<td>ND*</td>
</tr>
<tr>
<td>SK095</td>
<td>Memorial Sloan-Kettering Cancer Center</td>
<td>A2</td>
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*NS= Not successful, DNA degradation after several attempts
+ND= not done
'MC= mixed culture
<table>
<thead>
<tr>
<th>R.F.L.P. Type</th>
<th>Strains displaying Specific R.F.L.P. Type</th>
<th>Frequency of Occurrence of Specific R.F.L.P. Type (%) Among MAI Strains Successfully Analyzed</th>
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<tbody>
<tr>
<td>A6</td>
<td>SK015, SK016, SK037, 10,010</td>
<td>50%</td>
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<tr>
<td>A/1</td>
<td>SK060</td>
<td>12.5%</td>
</tr>
<tr>
<td>A2</td>
<td>SK024, SK095</td>
<td>25%</td>
</tr>
<tr>
<td>A3</td>
<td>10,015</td>
<td>12.5%</td>
</tr>
</tbody>
</table>
7.18 R.F.L.P. Types Among Corded and Non-Corded AIDS-related MAI Strains

The R.F.L.P. analyses of corded strains 10,010 and 10,015 from AIDS patients at St. Vincent's Hospital, as well as, their variants that had lost cording revealed that there was conservation of R.F.L.P. type despite a loss in cording (See Table 15; See Figure 20).

The plasmid analysis of corded strain 10,011 and its lost cording subculture 10,011-IV had revealed differing plasmid profiles when reviewed in Section VI. This finding was further substantiated by the distinctly differing R.F.L.P. banding patterns for these strains seen in Figure 20. The parental strain 10,011 contained bands both similar and also distinct from that of the 10,011-IV subculture. In addition, there were more bands seen in the initial isolate and fewer in the 4x subcultured variant (See Figure 20). This isolate was originally derived from an AIDS bacteremia, isolated on Para SL/C, and speciated biochemically and via GEN PROBE as M. avium. The presence of distinctly different R.F.L.P. banding patterns would support the notion of a polyclonal M. avium bacteremia as has been cited in the literature by Arbeit et al.(1992).

The experimental results obtained in this section would therefore indicate that the loss of MAI cording is a complex multifactorial mechanism which cannot be easily explained in terms of a simple alteration of the MAI Genomic R.F.L.P. pattern generated via the pMB-22 probe in a Southern DNA Fingerprinting motif. The significance, however, of a particular R.F.L.P. pattern type with respect to corded and related lost corded derivatives still remain unclear.
<table>
<thead>
<tr>
<th>M. avium Strain</th>
<th>Hospital where isolated</th>
<th>R.F.L.P. Type</th>
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<tr>
<td>10,002, 10,002-IV</td>
<td>St. Vincent's</td>
<td>NS</td>
</tr>
<tr>
<td>10,010, 10,010-IV</td>
<td>St. Vincent's</td>
<td>A1</td>
</tr>
<tr>
<td>10,011, 10,011-IV</td>
<td>St. Vincent's</td>
<td>MC</td>
</tr>
<tr>
<td>10,015, 10,015-IV</td>
<td>St. Vincent's</td>
<td>A3</td>
</tr>
</tbody>
</table>

*NS = Not successful, DNA degradation after several attempts  
+MC = mixed culture
Section VIII

Invasion

of HeLa Cells By

Strains of MAI
8.0 Bacterial Adhesion

Pathogenicity has been defined as the ability of microorganisms to cause disease (Arbuthnott and Smyth, 1979). With few exceptions, pathogenicity is rarely the property of a single determinant of the pathogen but rather, it is multifactorial. In flowing systems, such as the gastrointestinal tract, a successful pathogen must be able to colonise and/or penetrate the body surfaces, namely, the skin or mucosal epithelia of the respiratory tract, oropharynx, urogenital tract or conjunctiva and it must be capable of multiplying on or in the tissues of the host (Arbuthnott and Smyth, 1979; Marshall and Bitton, 1980). Thus, the outcome of the interaction between host and the invading microbe depends both on a combination of virulence factors produced by the pathogen and the susceptibility of the host (Arbuthnott and Smyth, 1979).

8.1 The Colonization Of The Gastrointestinal Tract By Atypical Mycobacteria And AIDS-related MAI

In mouse tissue, most environmental mycobacteria are of low pathogenicity requiring the introduction of very large numbers of organisms (Collins, 1988). Of the 31 serovars making up the MAI complex group of organisms, less than 12 serovars are capable of causing progressive disease following an intragastric challenge. A large number of the serovars making up the MAI complex, however, are able to colonize both nasopharyngeal and intestinal mucosa. In normal patient populations, as many as 30% of the patients fecal samples have atypical mycobacteria. In geographic areas where soil and water are contaminated with atypical mycobacteria, individuals may be repeatedly colonized by many different MAI serovars. Those MAI strains which do colonize the
gastrointestinal tract could in theory be expected to infect the submucosal tissues and its draining lymph nodes when the normal host cellular defenses are depressed.

The clinical picture of AIDS-related MAI infection is one associated with anorexia, abdominal pain, diarrhea and malabsorption (Hoyt et al., 1992; Hellyer et al., 1991: Stacey, 1986). These clinical findings are thus suggestive of a disease in which the infection occurs by way of the gastrointestinal tract (Hellyer et al., 1991; Hoyt et al., 1992). In the AIDS-patient, colonization by a food or water borne virulent serovar MAI complex organisms would initiate the chain of events leading to the terminal form of MAI infection (Collins, 1988; Okello et al., 1990). The main evidence for this hypothesis is that there occurs extensive infection of the lower gastrointestinal tract in some patients with AIDS and the concurrent isolation of *M. avium* complex organisms from stool and blood in many other AIDS patients (Okello et al., 1990). The role of *M. avium-intracellulare* complex organisms in causing diarrhea has still not been established but, a causative or contributory role for producing the diarrhea has been suspected because of the presence in some AIDS patients, of large numbers of mycobacteria in stool and mucosal samples from the small intestine (Smith et al, 1992). Positive MAI isolates from stool are thought to reflect either MAI colonization or gastrointestinal disease (Hoyt et al., 1992). Stacey noted that in 3 of 5 AIDS patients, the presence of disseminated MAI was first seen by the presence of acid-alcohol fast bacteria in fecal smears (Stacey, 1986). In 6 additional AIDS patients, however, Stacey noted that no clinical or pathological evidence of dissemination was found and he thus proposed this as evidence in support of the theory that the
gastrointestinal tract is a portal of entry for MAI (Stacey, 1986).

8.2 In Vitro Models for Determining MAI Uptake

8.2a Macrophage and Monocyte Models

Crowle and co-workers cultured normal human macrophages in the presence of *M. avium* and found that the uptake and replication of a variety of *M. avium* strains could be measured by colony forming unit (C.F.U.) counts of the mycobacteria resulting from lysis of the *M. avium* infected macrophages exposed to sonication (Crowle et al., 1986). In addition, Crowle and associates noted that the microscopic counts of acid-alcohol fast bacilli were not useful because the organisms which multiplied inside the macrophages were usually not acid-alcohol fast. When Crowle et al. utilized electron microscopy, however, the intracellular bacilli were observed to be undergoing transverse fission and were surrounded in individual vacuoles by a broad electronlucent zone and possessed cell walls which were thinner than extracellular cultivated *M. avium*. In the Crowle investigation, 15 strains of *M. avium* (serovars: 1, 2, 3, 4, 8 and 9) examined were derived from patients with nontuberculous granulomatous infection, HIV, unrelated problems, or laboratory reference isolates. Phagocytosis of the mycobacteria by the macrophages did not differ between lab strains and patient strains but, there did occur a difference in the ability of strains to replicate within the macrophage. The laboratory derived strains of MAI were termed virulent because they were able to replicate within the macrophage. The laboratory strains of MAI were termed avirulent because they did not replicate within the macrophage.
The virulent patient isolates could be compared with other patient isolates by intracellular replication rates.

The intracellular replication of MAI was further investigated by Stokes and Collins using mouse macrophages (Stokes and Collins, 1988). Macrophages were derived from MAI susceptible but, BCG infected mouse strain C57BL/6, and inbred MAI resistant mouse strain A/J which was not BCG infected. The BCG infected MAI susceptible macrophage strain C57BL/6 strains was better able to control the intracellular MAI replication than was the MAI resistant BCG uninfected inbred strain A/J. This investigation revealed that resistance to intracellular growth of MAI could be acquired.

An in vitro model for MAI pathogenicity which utilized normal human peripheral blood monocytes was employed by Toba et al. (1989). In Toba's investigation twelve strains of MAI complex organisms (7 non-AIDS isolates and 5 AIDS-related isolates) were used to obtain an in vitro infection of the monocytes. The monocyte model measured mycobacterial uptake and intracellular replication via a microscopic count of the alcohol-acid fast bacilli and the C.F.U. of mycobacteria, respectively, in lysed monocytes. The CFU count obtained from the 5 AIDS-related MAI strains revealed that only in one of the AIDS-associated strains of MAI had intracellular mycobacterial replication occurred. Among the non-AIDS-derived MAI strains, intracellular replication had occurred in 2 of the 7 strains. Further investigations with the monocyte model by Toba and co-workers revealed that pre-treatment of the monocytes with gamma interferon prior to infection with MAI (AIDS and Non-AIDS strains) brought about a decrease in phagocytosis but, had no effect on intracellular replication of Mycobacterium avium organisms. From this investigation with gamma interferon
pre-treatment, it was concluded that most strains of MAI do not multiply within monocytes from healthy individuals and that gamma interferon pretreatment does not have macrophage activating factor activity for MAI infection in human monocytes.

Shiratsuchi et al. (1990) carried out in vitro studies with normal human peripheral blood monocytes which were precultured for 2 days before MAI infection. The 13 MAI strains which were utilized to infect the monocytes were derived from AIDS and Non-AIDS strains (six AIDS-related MAI strains, 3 Non-AIDS-related MAI strains and 4 MAI strains selected on the basis of colonial morphology). The MAI uptake by the monocytes detected by counting intracellular acid-fast bacilli was found to differ according to colonial morphology. The MAI strains displaying round and opaque colonies were found to be phagocytosed more readily than those MAI strains with flat colonies. Shiratsuchi et al. noted that virulence defined as intracellular growth was also partly associated with colonial morphology. There did, however, occur exceptions in which some strains with flat colonial morphology did possess the capacity to multiply within normal human monocytes but, some round opaque strain did not possess the capacity to multiply within normal human monocytes.

The effects of gamma interferon and alpha/2A interferon upon monocytes and macrophages were further investigated by Blanchard and co-workers (Blanchard et al., 1991). This new investigation utilized a rapid radiolabel assay ([2-3H] glycerol) with monocytes obtained from normal human peripheral blood, 4-day cultured derived and matched monocyte cultures. The application of the radiolabel assay to normal fresh human peripheral blood monocytes resulted in the finding that these cells were able to kill 40-92% of inoculated MAI (laboratory strains; serovar 8). The
bactericidal activity was however, found to be significantly lower in 4-day old culture-derived macrophages which were compared with matched monocyte cultures. When gamma interferon was added to the fresh monocytes, there was a corresponding inhibition of bactericidal activity. The experiments with culture-derived macrophages found that pretreatment of culture-derived macrophages with gamma-interferon did not enhance either MAI intracellular survival or growth. Results similar to that obtained with gamma-interferon for monocytes and cultured macrophages were obtained with alpha/A2 interferon. From these studies, Blanchard and co-workers concluded that the innate efficiency of mycobacterial killing by monocytes can be down-regulated by interferon but, macrophages are not significantly affected.

Meylan and co-workers tested whether in vitro infection of macrophages with either HIV or mycobacteria would influence the replication of the other pathogen (Meylan et al., 1992). In this protocol, the macrophages were infected sequentially with the macrophage-tropic isolate HTLV-III_{Be-L/85} and _M. tuberculosis_ H37Rv or _M. avium_. The intracellular growth of mycobacteria was measured by colony counting and radiometric assay of macrophage lysates and the replication of HIV by the release of p24 antigen into the culture supernates. The phagocytosis and intracellular growth of the mycobacteria was similar in HIV-infected macrophages and controls. Mycobacteria were found not to affect the replication of HIV in macrophages. The ultimate result of these studies was the failure to find any direct intracellular interaction between HIV and mycobacteria in cultured macrophages that would explain the increased rate of mycobacterial diseases in patients infected with HIV or that would support a hypothesis that mycobacterial infection
of macrophages can intrinsically enhance HIV replication in these cells.

In an effort to improve the efficacy of the macrophage model systems vis à vis their application to macrophage virulent strains of MAI, Straub and co-workers have developed a miniaturization of the conventional macrophage system (Straub et al., 1990). The standard conventional macrophage assay required 10^6-10^7 cells/well (each well required 1-1.5ml of media) and needed numerous 35 or 100ml petri dishes which in turn required 60-70 mice. The miniaturized system by contrast, utilized microtiter plates into which were placed 10^5 macrophages/well (each well contained 200 µl volume of media) and permitted an entire assay to be performed within a 96 well plate and in turn required only 10 mice. This has proved to be a useful means of selecting human derived isolates of MAI in patients with pulmonary MAI disease based upon the ability of the MAI to infect and multiply within cultured mouse pulmonary alveolar macrophages.

8.2b Human Intestinal Epithelial Model

When the focus of AIDS-related MAI occurs within the gastrointestinal tract, there is colonization of the columnar epithelial layer with subsequent invasion of the layer and underlying tissue (Arbuthnott and Smyth, 1979; Marshall and Bitton, 1980). To study the invasion of gastrointestinal epithelial cells, models relying on epithelial or epithelial-like tissue culture systems rather than macrophage or monocyte tissue culture systems would provide a more relevant model.

Mapother and Songer (1984) examined the interaction of the pathogen *M. avium* and the saprophytes *M. phlei* and *M. smegmatis* with human intestinal epithelial cells *in vitro* to determine the
invasiveness of these mycobacteria. The human intestinal epithelial cell monolayers were inoculated with cultures of *M. avium* (serovars 2, 8, 10) that were viable, autoclaved, formalin killed, exposed to UV light or suspended in anti-*M. avium* serovar 2 serum. A parallel series of human intestinal cell monolayers were exposed to the aforementioned mycobacterial saprophytes. In addition, the effects of four reagents known to block phagocytosis or endocytosis (cytochalasin B, dibutyryl cyclic adenosine monophosphate, iodoacetate, and 2,4-dinitrophenol) on the mycobacterial–intestinal epithelial interaction were also studied. A maximal uptake of *M. avium* pathogenic mycobacteria by the human intestinal epithelial cell monolayer was achieved after 2–3 hours of incubation at 37° C. The *M. avium* cells belonging to serovar 2 were found to be taken up in greater quantity than either serovar 8 or 10. Neither of the saprophytes examined (i.e. *M. phlei*, *M. smegmatis*) were found to attach or penetrate the human intestinal epithelial cell monolayers. Only viable *M. avium* mycobacterial cells were ingested by the intestinal monolayer whereas, non-viable *M. avium* cells were not. The pre-treatment of the human intestinal epithelial monolayer by the phagocytotic or endocytotic reagents cited above did indeed bring about a diminished uptake of the *M. avium* cells thus suggesting that the apparatus of uptake is an endocytic process which is induced by virulent mycobacteria.

### 8.2c HeLa Cell Model

The use of macrophage, monocyte and human intestinal epithelial monolayer tissue culture systems all suffer from three basic shortcomings, namely, availability, cost and viability of the cell line in *vitro*. Therefore, a cell culture system which is less costly, more readily obtainable (i.e. commercially available) and
Riley has successfully utilized a HeLa Cell Model to study patterns of attachment of *E. coli* (Riley, 1988). The Riley model for *E. coli* attachment required HeLa Cells to be suspended and diluted to 1-2 x 10^5 cells/ml in growth medium, and 1.5ml of the suspension be added per well of a 24 polystyrene culture plate. The cells were incubated at 37° C in an atmosphere of CO₂ until the HeLa Cells covered 50% of the bottom of each well. The media per well was then replaced with 0.5% mannose (without antibiotics) as means to prevent nonspecific binding by *E. coli*. Finally, each of the wells was exposed to 0.1ml of overnight growth of *E. coli* and incubated for 3 hr at 37° C. The media was then removed and the infected cells of the monolayer were washed 6X with P.B.S. (pH 7.4) and fixed with methanol for 5 min. The monolayer was subsequently stained with Giemsa stain, washed with distilled H₂O and dried. The wells containing the infected monolayers were then viewed under an inverted light microscope for patterns of bacterial attachment.

The application of a HeLa Cell model for mycobacterial virulence was cited in the investigations conducted by Clark and Forrest in the 1950's (Clark and Forrest, 1959). Cultivation of tuberculous and non-tuberculous strains *in vitro* using the HeLa Cell model permitted Clark and Forrest to observe differences vis à vis the ability of tuberculous and non-tuberculous strains to grow within the HeLa cells. The non-tuberculous or atypical strains grew rapidly enough within the HeLa cells to permit cultures to be read in three days.

Given the fact that AIDS-related *M. avium* infection is found in a diversity of organs, it is reasonable to state that these organisms first associate and subsequently penetrate an
epithelial layer of cells before reaching the internal regions of the target organ. HeLa Cells have epithelial cell characteristics and could be used to indicate virulence as a function of invasiveness.
MATERIALS AND METHODS

8.3 MAI Strains

The MAI strains utilized for the HeLa Cell Invasion study were noncorded strains (SK015, SK016, SK024, SK037, SK060, SK069, SK090, SK095), corded strains (10,002, 10,010, 10,011, 10,015) and the lost cording strains (10,002-IV, 10,010-IV, 10,011-IV, 10,015-IV).

8.4 MAI Cultivation

A 4mm loopful of MAI strain was inoculated into a 5.0 ml tube of Middlebrook 7H9 broth supplemented with 2% glycerol and 4% O.A.D.C and incubated for 7 days at 37° C. This growth served as inoculum for infected HeLa Cells (cited below).

8.5 HeLa Model for Determining M. avium complex Invasion

8.5a Light Microscopy

Sterile tissue culture media (Eagles Minimum Essential Medium (MEM); without antibiotics) and 10% Fetal Calf Serum (F.C.S.) was added to tissue culture wells (24 well polystyrene culture plate) in aliquots of 1.0 ml. To these wells were added HeLa Cells (final concentration: 1-2 x 10^5 cells/ml) followed by cover slips. One control well contained MEM plus F.C.S. without HeLa Cells. The cells were incubated at 37° C in an atmosphere of CO2 until the HeLa Cells covered 50% of the bottom of each well. The MEM media per well was then replaced with fresh sterile MEM media (without antibiotics). Finally, each of the wells were exposed to 0.1ml of 7 day old Middlebrook 7H9 broth culture which contained MAI growth (cited above) of M. avium (a ratio of 100 MAI Colony forming units/ HeLa Cell) for 24, 48, 72 and 96 hours at
37° C. The media was then removed, and the infected cells of the monolayers were washed 3X with sterile P.B.S. (pH 7.4). The cover slips containing the infected monolayers were removed and fixed in concentrated methanol for 20 min. The cover slips containing the infected monolayers were subsequently stained via the Kinyoun Acid Alcohol Fast stain procedure (Modified from; Riley, 1988). The aforementioned cover slips were then viewed via light microscopy at 400X and 1000X Oil Immersion. The degree of invasion or association of MAI to the HeLa Cell was quantified by a number ranging from 0-3 (0= 0 organisms/1000 cells; 1= <1 organism/oil immersion field (5-10 cells) (non-invasive)): 2= 1 or more organism/ oil immersion field (5-10 cells) (invasive); 3= 1 or more organism/cell (invasive)).

8.5b Electron Microscopy

Sterile tissue culture media (Eagles Minimum Essential Medium (MEM); without antibiotics) and 10% Fetal Calf Serum (F.C.S.) was added to tissue culture wells (24 well polystyrene culture plate) in aliquots of 1.0 ml. To these wells were added HeLa Cells (final concentration: 1-2 x 10^5 cells/ml/). One control well contained MEM plus F.C.S. without HeLa Cells. The cells were incubated at 37° C in an atmosphere of CO₂ until the HeLa Cells covered 50% of the bottom of each well. The MEM media per well was then replaced with fresh sterile MEM media (without antibiotics). Finally, each of the wells were exposed to 0.1ml of 7 day old Middlebrook 7H9 broth culture which contained MAI growth (cited above) of M. avium (a ratio of 100 MAI Colony forming units/ HeLa Cell) for 48 hours at 37° C. The media was then removed, and the infected cells of the monolayers were washed 3X with sterile P.B.S. (pH 7.4). The MAI infected monolayers from each were aseptically
pipetted into separate microcentrifuge tubes. The microcentrifuge tubes were spun at 11,950 xg at a temperature of 4° C in a Sorvall RC-2B High Speed Centrifuge using an SS-34 Sorvall rotor fitted with microtube adaptors for 15 min. The resulting supernates generated from centrifugation were carefully removed and discarded. The resulting pellet was fixed 2x in 2.5% (v/v) buffered (in 0.2M Phosphate Buffer pH=7.3) glutaraldehyde (2 hours required for each fixation). The cells were again spun at 11,950 xg for 10 min and the resulting supernates carefully removed and discarded. The monolayer pellet was then washed 1X in PBS and spun at 11,950 xg for 10 min and the supernates were carefully aspirated off and discarded. The monolayer pellet was then treated with 4% buffered (0.2M Phosphate Buffer pH=7.3) Osmium tetroxide for 30 min at room temperature. The monolayer was again spun at 11,950 xg for 10 min and supernates carefully withdrawn and discarded. The pellets were passed through increasing concentrations of ethanol (30%, 50%, 75%, 95%, 95%, 100%, 100%) at intervals of 10 mins per specified concentration of ethanol to achieve dehydration of the pellet. The final supernates of 100% ethanol were carefully aspirated off, and the pellets were first transferred to electron microscopic embedding capsules. The pellets were then exposed to concentrated propylene oxide (at room temperature) for a period of 30 min. The propylene oxide was carefully aspirated off and the pellets were then infiltrated with a 60% : 40% ratio of propylene oxide : spur (embedding plastic) for 30 min. The infiltrating fluid (60:40) was aspirated off and replaced with a second infiltrating fluid consisting of 30% (propylene oxide) : 70% (spur) for an additional time interval of 30 mins. Finally, the latter infiltrating fluid was aspirated and replaced by 100% spur. The 100% spur was placed within a 60-70° C oven overnight to achieve polymerization of the
spur embedding plastic. The polymerized plastics were then sectioned on an LKB ultromicrotome at a thickness of 700-900 Angstroms (silver/gold interface). The sections were mounted on copper grids and first stained for 10 mins. in uranyl acetate followed by 3 mins lead nitrate. The sections were viewed on a Zeiss Model E.M. 9S2 Electron Microscope at a magnification of 4,500x (Modified from; Mokotoff, 1978).
RESULTS AND DISCUSSION

8.6 HeLa Model for Determining M. avium complex Invasion

8.6a Light Microscopy

Figure 21 shows a light microscopic view of a HeLa Cell monolayer displaying non-invasion by M. avium SK037 as seen by the absence of intracellular acid-alcohol fast bacilli. By contrast, figure 22 shows a light microscopic view of a HeLa monolayer showing invasion by M. intracellulare SK069 as seen by the abundance of intracellular acid-alcohol fast bacilli. The presence of intracellular forms in figure 22 was confirmed by electron microscopy (See Figure 23). Other strains of MAI were also analyzed via light microscopy using the HeLa Cell protocol. These subsequent analyses yielded results that indicated a variety of indices of invasiveness (See Table 16). Initial studies indicated that an incubation period of 48 hours provided an adequate incubation time for the invasion of HeLa Cells by M. avium bacilli.
Figure 21: *HeLa cell monolayer (light microscopy).*

A Light Microscopic View of a HeLa cell Monolayer showing Non-Invasion by *M. avium* strain SK037 as seen by an absence of intracellular acid-alcohol fast mycobacteria. Magnification: 400x (Microscopic Magnification)
Figure 22: HeLa cell monolayer (light microscopy).
A Light Microscopic View of a HeLa Cell Monolayer showing invasion by *M. intracellulare* strain SK069 as seen by an abundance of intracellular acid-alcohol fast mycobacteria. Magnification: 400x (Microscopic Magnification).
<table>
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<th>Strain</th>
<th>Invasion Index</th>
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<th>pLR-20 plasmid Present</th>
<th>R.F.L.P. Type</th>
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<td>-</td>
<td>-</td>
<td>NS*</td>
</tr>
<tr>
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<td>3</td>
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<td>-</td>
<td>NS</td>
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<td>-</td>
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<td>+</td>
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<td>A1</td>
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<td>+</td>
<td>-</td>
<td>+MC</td>
</tr>
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</tbody>
</table>

*(c)* = corded; *(lc)* = lost cording; *(nc)* = non-corded
NS* = Not successful, DNA degradation after several attempts;
ND** = not done; MC+ = Mixed culture

Invasion index number: 0 = 0 organisms/1000 cells; 1 = <1 organism/oil immersion field (5-10 cells) (non-invasive);
2 = 1 or more organisms/oil immersion field (5-10 cells) (invasive); 3 = 1 or more organisms/cell (invasive).

8.6b Electron Microscopy
8.6b Electron Microscopy

Electron microscopy of these HeLa Cell infected by *M. intracellulare* strain SK069 revealed that these bacterial cells were intracellular and were surrounded by an electron-transparent zone (See Figure: 23). The presence of this electron-transparent zone surrounding a mycobacteria located inside a phagocytic vacuole has been observed by other investigators (Draper and Rees, 1970). Draper and Rees suggested that the production of this inert and mechanically protective zone could perhaps explain why many mycobacterial pathogens are resistant to killing by phagocytic cells.
Figure 23: *Hela cell monolayer (electron microscopy).* An Electron Microscopic View of HeLa Cell showing invasion of cell by *M. intracellularare* strain SK069. Magnification: 4,500x (Microscopic Magnification).
8.7 HeLa Invasiveness Vis a Vis Cording, Loss of Cording, Non-Cording, Plasmids and R F L P Type

The loss of MAI cording did not correspond with a diminished index of invasiveness. In one case the parental corded strain (10,002) and subcultured derivative(10,002-IV) had the same index of invasiveness. In another case the corded parental strains (10,010, 10,015) had lower indices of invasiveness than the subcultured derivatives (10,010-IV, 10,015-IV). The total absence (strain 10,015), unique presence of pLR-7 (strain SK090) or the presence of both the pLR-7 and pLR-20 types of plasmids (strain SK037) appeared not to be indicative of a heightened index or invasiveness as can be seen in Table 16. The small sampling of R.F.L.P. types analyzed and cited in Table 16 does not permit a direct correlation between R.F.L.P. type and a heightened index of invasiveness. There were numerous examples, of strains differing in one or more of the variables cited above which were either hyperinvasive, hypoinvasive or noninvasive.

From the results obtained it would appear that virulence as measured by the ability of a particular strain of MAI to invade HeLa Cells involved much more intricate genetic machinery than variations with respect to cording, plasmids and R.F.L.P type.

8.8 Invasion Of The Gastrointestinal Epithelium by MAI And The HeLa Cell Model

The incidence of disseminated MAI infection among AIDS patient is high with antemortem rates varying between 17%-30% (Hopewell et al., 1992). Due to the wide variety of manifestations of the HIV viral infection, the simultaneous presence of opportunistic infections, neoplasms, and the toxicities and adverse reactions from numerous medications utilized for treatment, it is
difficult to assess the impact of MAI disease on the clinical status, survival or longevity of patients suffering from the AIDS Syndrome. What is, however, quite clear from a 1989 Centers for Disease Control study is that those patients with MAI appeared to have a significantly shortened life expectancy. The present situation as concerns AIDS-related MAI is made even gloomier by the fact that no large-scale studies have yet proven that chemotherapy for MAI infection reduces morbidity or mortality (Hopewell et al., 1992). Recent clinical data cited by several groups of investigators (Hoyt et al., 1992; Hellyer, et al., 1991; Okello et al., 1990; Collins, 1988; Stacey, 1986) would support the theory that the gastrointestinal tract is the portal of entry of MAI. Thus, when speaking of an infection of the gastrointestinal tract wherein M. avium complex infection involves the colonisation and subsequent invasion of columnar epithelial tissue, an in vitro model system focusing upon an association and invasion of an intestinal epithelial monolayer or epithelial-like monolayer would be a relevant model as relates to gastrointestinal tract virulence.

The application of HeLa cells which are well characterized, have epithelial characteristics and are readily available, to study the invasive capacity of MAI strains derived from AIDS patient isolates of M. avium complex has demonstrated that all strains are not equal with respect to their invasive capacity (See Table: 23). The finding of inequality as it relates to AIDS-related M. avium complex invasiveness of HeLa Cells in this investigation with AIDS-related M. avium complex organisms echoes those results obtained by Clark and Forrest 33 years ago with Non-AIDS-related tuberculous and nontuberculous strains. Thus, the HeLa Cell Model is a useful model which can be applied to study
invasiveness and related virulence factors (Clark and Forrest, 1959).
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APPENDICES
APPENDIX I: General Stock Solutions
General Stock Solutions:

0.5M EDTA (pH 8.0)
Add 93.05 g of disodium ethylene tetraacetate 2H2O to 400 ml of ddH2O. Stir on a magnetic stirrer. Adjust the pH to 8.0 with NaOH. Autoclave for 15min at 1.0kg/cm².

Ethidium bromide (50μg/ml)
Add 5mg of ethidium bromide to 100 ml of ddH2O. Store within a dark bottle at 4° C.

Glycine 20% (w/v)
Add 20 g of glycine to 50 ml of ddH2O and bring volume up to 100ml. Autoclave for 15min at 1.0kg/cm².

10% (w/v) N-Lauroylsarcosine
Add 20 g of N-Lauroylsarcosine to a final volume of 200ml of ddH2O. Autoclave for 15min at 1.0kg/cm².

Lysozyme
Add 50mg/ml of ddH2O. Filter sterilize on a 0.22μm Corning filter. Dispense into aliquots and store at -20° C.

1 M MgCl₂
Add 20.3g of MgCl₂.6H₂O to a final volume of 100ml ddH₂O. Autoclave for 15min at 1.0kg/cm².

5 N NaCl
Add 292.2g of NaCl in a final volume of 1.0 liter of ddH₂O.

10 N NaOH
Add 200 g of NaOH in 450ml of ddH₂O. Adjust the volume to 500ml with ddH₂O.

Proteinase K
Add 20mg/ml of ddH₂O. Filter sterilize on a 0.22μm Corning filter. Dispense into aliquots and store at -20° C.

RNase Free of DNase
Dissolve pancreatic RNase (RNase A) at a concentration of 10mg/ml in TE buffer and heating for 10 min at 70° C inactive DNase, followed by slow cooling to room temperature. Dispense in aliquots and store at -20° C.

Sample loading buffer
Glycerol 5ml
Na₂EDTA.2H₂O 0.37g
SDS 0.1g
Bromophenol blue
Adjust the volume to 10ml with ddH₂O.

10% (w/v) SDS (Sodium dodecyl sulphate).
Dissolve 100g of SDS in 900ml of ddH₂O. Heat to 68° C to facilitate dissolution. Adjust the pH to 7.2 by adding a few drops of conc. HCl. Adjust the volume to a liter.
20X SSC
Dissolve 87.65g of NaCl and 44.1g sodium citrate in 400ml of ddH2O. Adjust pH to 7.0 with a few drops of 10N NaOH. Adjust the volume to 500 ml. Autoclave for 15min at 1.0kg/cm².

50X TAE
To make 500ml:
Tris base 242g
glacial 57.1ml
acetic acid
0.5M EDTA 100ml
(pH 8.)
Autoclave for 15 min at 1.0kg/cm².

Tris 1M, pH 7.5:
To make 500ml:
dd H2O 400ml
Tris base 60.55g
HCl(conc) 32.5ml
Adjust the pH with additional concentrated HCl until pH 7.5 is reached. Bring the volume to 500ml with ddH2O. Autoclave for 15 min at 1.0 kg/cm².

Tris 1M, pH 8.0:
To make 500ml:
dd H2O 400ml
Tris base 60.55g
HCl(conc) 21ml
Adjust the pH with additional concentrated HCl until pH 7.5 is obtained. Bring the volume to 500ml with ddH2O. Autoclave for 15 min at 1.0 kg/cm².

Tris 2M, pH 8.0:
To make 500ml:
ddH2O 400ml
Tris base 121.1g
HCl(conc) 21ml
Adjust the pH with additional concentrated HCl until pH 8.0 is obtained. Bring the volume to 500ml with ddH2O. Autoclave for 15 min at 1.0 kg/cm².
Tris 1M, pH 9.5:
To make 500ml:
ddH₂O 400ml
Tris base 60.55g
HCl(conc) 10ml

Adjust the pH with additional concentrated HCl until pH 9.5 is obtained. Bring the volume to 50ml with ddH₂O. Autoclave for 15 min at 1.0 kg/cm².

TE (pH 8.0)
To make 250ml:
10mM Tris-HCl (pH 8.0) 2.5ml of 1.0M Tris-HCl (pH8)
1mM EDTA (pH 8.0) 0.5ml of 0.5M EDTA (pH8)

Bring volume up to 250ml with ddH₂O. Sterilize by filtration through a Corning 0.22 μm pore size filter membrane.

TEN (pH 8.0)
To make 250ml:
50mM Tris-HCl (pH 8.0) 12.5ml of 1.0M Tris-HCl (pH8)
150mM NaCl 7.5ml of 5.0M NaCl
100mM EDTA (pH 8.0) 50.0ml of 0.5M EDTA (pH8)

Bring volume up to 250ml with ddH₂O. Sterilize by filtration through a Corning 0.22 μm pore size filter membrane.
APPENDIX II: Solutions for Boehringer Mannheim Genius Kit
Solutions for Boehringer Mannheim Genius Kit

Labeling solutions

0.2M EDTA (pH 8.0)
To make 250ml:
0.2M EDTA (pH8.0)  100 ml of 0.5M EDTA Stock
ddH₂O  150ml
Sterilize on a Corning 0.22 μm pore size filter membrane.

4M LiCl
Add 84.78 g of LiCl to a final volume of 500ml of ddH₂O. Autoclave for 15 min at 1.0kg/cm².

10mM Tris-HCl, 1mM EDTA, 0.1% SDS
To make 250ml:
10mM Tris-HCl (pH 8.0)  2.5ml of 1.0M Tris-HCl(pH8)
1mM EDTA (pH 8.0)  0.5ml of 0.5M EDTA (pH8)
0.1% SDS  2.5ml of 10% SDS
Bring volume up to 250ml with ddH₂O.
Sterilize on a Corning 0.22μm pore size filter membrane.

Denaturation Solution
1.5M NaCl and 0.5M NaOH
To make 200ml:
1.5M NaCl  60ml of 5.0N NaCl
0.5M NaOH  10ml of 10N NaOH
Bring volume up to 200ml with ddH₂O.
Sterilize on a Corning 0.22μm pore size filter membrane.

Neutralization buffer
1M Tris-HCl (pH8.0) and 1.5MNaCl.
To make 200ml:
1.0M Tris-HCl (pH8.0)  100ml of 2M Tris-HCl (pH8.0)
1.5M NaCl  60ml of 5.0N NaCl.
Bring volume up to 200ml with ddH₂O.
Sterilize on a Corning 0.22μm pore size filter membrane.

DNA Transfer Solutions
2X SSC
To make 200ml:
2X SSC  20ml of 20X SSC
Bring up volume to 200ml with ddH₂O.
Sterilize on a Corning 0.22μm pore size filter membrane.

6X SSC
To make 200ml
6X SSC  60ml of 20X SSC
Bring up volume to 200ml with ddH₂O.
Sterilize on a Corning 0.22μm pore size filter membrane.

10X SSC
To make 200ml:
10X SSC  100ml of 20X SSC
Bring up volume to 200ml with ddH₂O.
Sterilize on a Corning 0.22\(\mu\)m pore size filter membrane.

Hybridization Solution:
To make 100ml:
5X SSC  25ml of 20X SSC
0.1% N-lauroylsarcosine  1ml of 10% N-lauroylsarcosine
0.02% SDS  0.2ml 10% SDS
Bring up volume to 100ml with ddH2O.
Sterilize on a Corning 0.22\(\mu\)m pore size filter membrane.
Heat to 50-70°C for 1 hr prior to use and add 1%(w/v) of Boehringer Mannheim Blocking Reagent (Vial 11).

Solutions for Stringency Washes:
2X SSC, 0.1% SDS
To make 100ml
2X SSC  10ml of 20X SSC
0.1% SDS  1ml of 10% SDS
Bring volume up to 100ml with ddH2O.
Sterilize on a Corning 0.22\(\mu\)m pore size filter membrane.

Solutions for probe DNA Detection:
(Buffer #1)
100mM Tris-HCl; 150mM NaCl; pH 7.5
To make 250ml
100mM Tris-HCl (pH7.5)  25ml of 1.0M Tris-HCl (pH7.5)
150mM NaCl  7.5ml of 5.0M NaCl
Bring volume up to 250ml with ddH2O.
Sterilize on a Corning 0.22\(\mu\)m pore size filter membrane.

(Buffer #2)
100mM Tris-HCl; 150mM NaCl; pH 7.5; 0.5% (w/v) Blocking Reagent.
To make 250ml
100mM Tris-HCl (pH7.5)  25ml of 1.0M Tris-HCl (pH7.5)
150mM NaCl  7.5ml of 5.0M NaCl
Bring volume up to 250ml with ddH2O.
Sterilize on a Corning 0.22\(\mu\)m pore size filter membrane.
Heat to 50-70°C for 1 hr prior to use and add 0.5%(w/v) of Boehringer Mannheim Blocking Reagent (Vial 11).

(Buffer #3)
100mM Tris-HCl; 100mM NaCl; 50mM MgCl2; pH 9.5.
To make 250ml:
100mM Tris-HCl(pH9.5)  25ml of 1.0M Tris-HCl pH 9.5
100mM NaCl  5ml of of 5.0M NaCl
50mM MgCl2  12.5ml of MgCl2
Bring volume up to 250ml with ddH2O.
Sterilize on a Corning 0.22\(\mu\)m pore size filter membrane.

Detect Color Solution (freshly prepared)
45 \(\mu\)l Boehringer Mannheim NBT solution (vial 9)
35 \(\mu\)l Boehringer Mannheim X-phosphate solution (vial 10)
10ml Buffer #3

(Buffer #4)
10mM Tris-HCl; 1mM EDTA; pH 8.0
To make 250ml:
10mM Tris-HCl (pH 8.0) 2.5ml of 1.0M Tris-HCl (pH 8.0)
1mM EDTA 0.5ml of 0.5M EDTA
Bring volume up to 250ml with ddH₂O.
Sterilize on a Corning 0.22μm pore size filter membrane.
APPENDIX III: Solutions for Qiagen Column Kits
Solutions for Qiagen Column Kits

Solutions for Qiagen Genomic DNA Prep

P1 Buffer
10mM Tris-HCl
100mM NaCl
5mMEDTA
pH 7.0

P2 Buffer
10mM Tris-HCl
250mM NaCl
1.2% Triton X-100
100ug/ml RNase A
12mM EDTA
0.5M Guanidine-HCl
pH 8.0

QBT Buffer (Equilibration Buffer)
750mM NaCl
50mM MOPS
15% Ethanol
0.15% Triton X-100
pH 7.0

QC Buffer (Wash Buffer)
1000mM NaCl
50mM MOPS
15% Ethanol
pH 7.0

QF Buffer (Elution Buffer)
1250mM NaCl
50mM Tris-HCl
15% Ethanol
pH 8.5

Solutions for Qiagen Plasmid DNA Prep

P1 Buffer
50mM Tris-HCl
10mM EDTA
100ug/ml RNase A
pH 8.0 (store at 4° C).

P2 Buffer
0.2 M NaOH
1% SDS

P3 Buffer
3.0 M Potassium Acetate
pH 5.5
QBT Buffer (Equilibration Buffer)
750mM NaCl
50mM MOPS
15% Ethanol
0.15% Triton X-100
pH 7.0

QC Buffer (Wash Buffer)
1000mM NaCl
50mM MOPS
15% Ethanol
pH 7.0

QF Buffer (Elution Buffer)
1250mM NaCl
50mM Tris-HCl
15% Ethanol
pH 8.5
APPENDIX IV: Media
Media:

Czapek Broth

\[
\begin{align*}
\text{NaNO}_3 & : 3.0 \text{ g} \\
\text{K}_2\text{HPO}_4 & : 1.0 \text{ g} \\
\text{MgSO}_4\cdot\text{7H}_2\text{O} & : 0.5 \text{ g} \\
\text{KCl} & : 0.5 \text{ g} \\
\text{FeSO}_4 & : 0.01 \text{ g}
\end{align*}
\]

Bring volume up to 900 ml with dd\text{H}_2\text{O}, and adjust the pH to 7.5 with 1N HCL and 1N NaOH. The volume is then brought to 1 liter by the further addition of dd\text{H}_2\text{O}. The broth is autoclaved for 15 min at 1kg/cm².

LB Broth

\[
\begin{align*}
\text{Tryptone} & : 10 \text{ g} \\
\text{Yeast Extract} & : 5 \text{ g} \\
\text{NaCl} & : 10 \text{ g}
\end{align*}
\]

Bring volume to 900ml with dd\text{H}_2\text{O}, and adjust the pH to 7.2 with 1N NaOH. The volume is then brought to 1 liter by the further addition of dd\text{H}_2\text{O}. The broth is autoclaved for 15 min at 1kg/cm².

L Agar

\[
\begin{align*}
\text{Tryptone} & : 10 \text{ g} \\
\text{Yeast Extract} & : 5 \text{ g} \\
\text{NaCl} & : 10 \text{ g} \\
\text{Difco Agar} & : 20 \text{ g}
\end{align*}
\]

Bring volume to 900ml with dd\text{H}_2\text{O}, and adjust the pH to 7.2 with 1N NaOH. The volume is then brought to 1 liter by the further addition of dd\text{H}_2\text{O}. The broth is autoclaved for 15 min at 1kg/cm².

Middlebrook 7H9 Broth

\[
\begin{align*}
\text{Monopotassium Phosphate} & : 1.0 \text{ g} \\
\text{Disodium Phosphate} & : 2.5 \text{ g} \\
\text{Monosodium Glutamate} & : 0.5 \text{ g} \\
\text{Sodium Citrate} & : 0.1 \text{ g} \\
\text{Ammonium Sulphate} & : 0.5 \text{ g} \\
\text{Pyridoxine} & : 0.001 \text{ g} \\
\text{Ferric Ammonium Citrate} & : 0.04 \text{ g} \\
\text{Magnesium Sulphate} & : 0.05 \text{ g} \\
\text{Zinc Sulphate} & : 0.001 \text{ g} \\
\text{Copper Sulphate} & : 0.001 \text{ g} \\
\text{Biotin} & : 0.5 \text{ mg} \\
\text{Calcium Chloride} & : 0.5 \text{ g} \\
\text{Glycerol} & : 10 \text{ ml}
\end{align*}
\]

Bring volume to 900ml with dd\text{H}_2\text{O}. The volume is then brought to 1 liter by the further addition of dd\text{H}_2\text{O}. The broth is autoclaved for 15 min at 1kg/cm². A supplement of BBL O.A.D.C. (final concentration 4% (v/v)) is added to the 7H9 broth just prior to inoculation with MAI complex organisms.
Tissue Culture Media
Eagle's Minimum Essential Medium (MEM)

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<th>L-Amino Acids</th>
<th>mg/liter</th>
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<tr>
<td>Phenol Red</td>
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<tr>
<td>Fetal Calf Serum</td>
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</tbody>
</table>

Filter sterilize with 0.22μm pore size filter membrane.
Tryptone Broth
Bacto Yeast Extract 3 g
Bacto Tryptone 5 g
Bacto Dextrose 1 g

Bring volume to 900 ml with ddH₂O. The volume is then brought to 1 liter by the further addition of ddH₂O. The broth is autoclaved for 15 min at 1 kg/cm².
APPENDIX V: Stains
Stains:
Kinyoun Acid-Fast Stain (Luna, 1968)

Kinyoun Carbol Fuchsin Solution

Basic fuchsin 4.0 g
Phenol crystals 8.0 ml
(heated)
Alcohol, 95% 20.0 ml
Distilled water 100.0 ml

Acid-Alcohol
Absolute Ethanol 97.0 ml
HCl (conc.) 3.0 ml

Methylene Blue Solution (Stock)
Methylene Blue 1.4 g
Alcohol, 95% 100.0 ml

Methylene Blue Solution (Working)
Methylene blue solution (stock) 10.0 ml
Distilled water 90.0 ml
MATERIAL REDACTED AT REQUEST OF UNIVERSITY