Non-tuberculous Mycobacteria Isolated from New Zealand Soil Environments

A thesis presented in partial fulfilment of the requirements for the degree of

Masters of Philosophy

in

Microbiology

at Massey University, Albany,

New Zealand.

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2006

Abstract

There is little information on the diversity of non-tuberculous mycobacteria (NTM) in New Zealand. This project has shown that diverse mycobacteria can be isolated from forest, pastoral and urban environments through the combined use of specialised decontamination techniques and selective media. *Mycobacterium avium-intracellulare* complex (MAIC) was the most commonly isolated mycobacteria (40%) followed by *M. montefiorense/M. triplex* (20%). This is the first known isolation of *M. montefiorense/M. triplex* from soils in New Zealand. The greatest numbers of mycobacteria were isolated from peat-rich pastoral soils, followed by urban dust/organic matter and native forest soils. The majority of mycobacteria isolated were slow-growing. The greatest numbers of isolates that were unable to be speciated further than *Mycobacterium* species using 16S rDNA sequencing (i.e. likely to be new species) were isolated from native forest soils.

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Chapter 1 Introduction

Worldwide, the incidence of environmental mycobacterial infections is increasing but there are limited data on the diversity of mycobacteria isolated from the environment. Often the studies available have focussed on a particular species associated with human disease rather than on overall diversity. Without a baseline knowledge of species diversity it is difficult to assess changes within the mycobacterial population and whether a previously uncommon isolate may now cause disease due to a change in habitat. Unlike tuberculosis, infection due to non-tuberculous mycobacteria is not a notifiable disease. Therefore, limited data on the number, type and epidemiology of non-tuberculous mycobacterial infections in New Zealand are available. It is believed that *Mycobacterium avium* complex (MAC) is the most common cause of non-tuberculous mycobacterial infections in New Zealand and Australia. It is a well known fact that the environment is the primary source of MAC infection (Falkinham 1996).

Exposure of MAC isolates to macrolides, antibiotics commonly used to treat MAC infections, has been identified as a major factor in the development of antimicrobial resistance (Stormer *et al.* 1989; Yago *et al.* 1995). Exposure to sub-inhibitory levels of these antibiotics found in the environment may also contribute to this resistance (Kummerer 2004). There are no local data available on the antimicrobial susceptibility of MAC isolates from the environment. Without available data it is difficult to assess if human impact (i.e., agriculture or urbanisation) has had any effect on the susceptibility of wild type MAC to macrolide antibiotics and if drug resistant MAC strains exist in the New Zealand environment.

The aim of this project was to develop improved techniques to isolate environmental mycobacteria and to compare the diversity of mycobacterial species from different New Zealand soil environments using genotypic methods for identification. The susceptibility of MAC isolates to macrolides was also assessed to investigate whether specific environments harbour isolates with increased resistance to macrolide antibiotics.

Knowledge of mycobacterial diversity and antimicrobial susceptibility may provide insights into the indigenous mycobacterial populations in New Zealand soil environments. Data on existing mycobacterial populations, their environmental niche and susceptibility to antibiotics can be used to enhance our ability to predict and prevent conditions that lead to infections. These data may be used to further understand the effect of human impact on the susceptibility and epidemiology of non-tuberculous mycobacteria.

Chapter 2 Literature Review

2.1 The Genus Mycobacterium

The Genus *Mycobacterium* belongs to the Phylum *Actinobacteria*, Class *Actinobacteria*, Subclass *Actinobacteridae*, Order *Actinomycetales*, Suborder *Corynebacterineae*, Family *Mycobacteriaceae* (www.ncbi.nlm.nih.gov/Taxonomy).

Mycobacteria are aerobic, slightly curved or straight bacilli, 0.2–0.6 x1.0–10 μm in size. The cell walls of mycobacteria are lipid-rich and contain characteristic mycolic acids with long, branched chains. The lipid-rich cell wall of mycobacteria enables them to resist staining with aniline dyes normally used to stain bacteria. Special staining techniques such as the Ziehl-Neelson hot stain are used to promote the uptake of dye. Once stained, mycobacteria are not easily decolourised with acid-alcohol solutions. Their resistance to decolourisation is termed acid-fastness, hence the name acid-fast bacilli (AFB).

Historically, mycobacteria taxonomic division was linked to growth rate. Mycobacteria were defined as being either slow- or rapid-growers. The relationships among the mycobacteria are most clearly displayed in the phylogenetic tree (Fig. 1). There is a natural coherence among the slow-growing mycobacteria suggesting a distinct line of evolutionary descent (Stahl and Urbance 1990). This supports the traditional separation of slow-growing from rapid-growing species.

The division between the fast- and slow-growing mycobacteria is marked by length and sequence variation within the helix formed by positions 451-482 in *E. coli* 16S rRNA numbering (Fig. 2). An extended helix is found only among members of the slow-growing mycobacteria (with the exception of *M. trivale*). The natural division between the fast- and slow-growing species is also reported to be reflected by the rRNA gene copy number: one genomic copy among slow-growing species and two copies among rapid-growing species (Suzuki *et al.* 1987).

In the 1950's Dr Earnest Runyoun classified mycobacteria based on growth rate and pigmentation properties. Although these broad groupings are no longer used to

speciate mycobacteria, many clinical laboratories continue to group mycobacteria according to the Runyoun groups. These groups are:

I photochromogens (yellow pigment formed in the light)

II scotochromogens (yellow pigment formed in the dark)

III non-chromogens

IV rapid growers

Stahl and Urbance (1990) demonstrated that phylogenetic relationships based on comparative 16S rRNA sequencing reflected traditional classification, with major branches of the phylogenetic tree in general correspondence to the four Runyoun's groups.

Figure 1: Phylogeny of 16S rRNA of characterised mycobacteria and related organisms.

MAIS, *Mycobacterium avium-intracellulare-scrofulaceum* complex. This tree was created by Stahl and Urbance (1990).

Today molecular methods predominate in mycobacterial identification. In 1980, 41 species of mycobacteria were included in the Approved List of Bacterial Names. Today there are 110 species of mycobacteria and the number continues to grow due to the use of molecular techniques such as 16S rDNA sequencing (Hall and Roberts 2006). Recent reports document that 30% of mycobacterial isolates from water, soil, air and patients do not belong to any of the identified species (Tortoli *et al.* 2001) indicating a potentially great number of novel mycobacteria yet to be assigned a species.

The *Mycobacterium tuberculosis* complex (MTC) contains *M. tuberculosis*, *M. bovis* (including BCG strain), *M. microti*, and *M. africanum*. Newly recognized additions to the MTC include *Mycobacterium caprae* and *Mycobacterium pinnipedii* (Huard *et al.* 2006). Although it is not presently an officially described organism, "*Mycobacterium canettii*" is another widely accepted member of the MTC. Lastly, there exist rare MTC variants, the so-called dassie and oryx bacilli, whose standing within the MTC remains to be defined (Huard *et al.* 2006). The remainder of the genus has been referred to by a variety of terms, such as environmental mycobacteria, mycobacteria other than tuberculosis (MOTT), atypical mycobacteria (ATM), and non-tuberculous mycobacteria (NTM). The term ATM was first used because these organisms were mycobacteria species but not typical of *M. tuberculosis*. These mycobacteria are not in fact atypical and are characteristic of their own species. Therefore the term NTM is now preferred and will be used when referring to these species as a group.

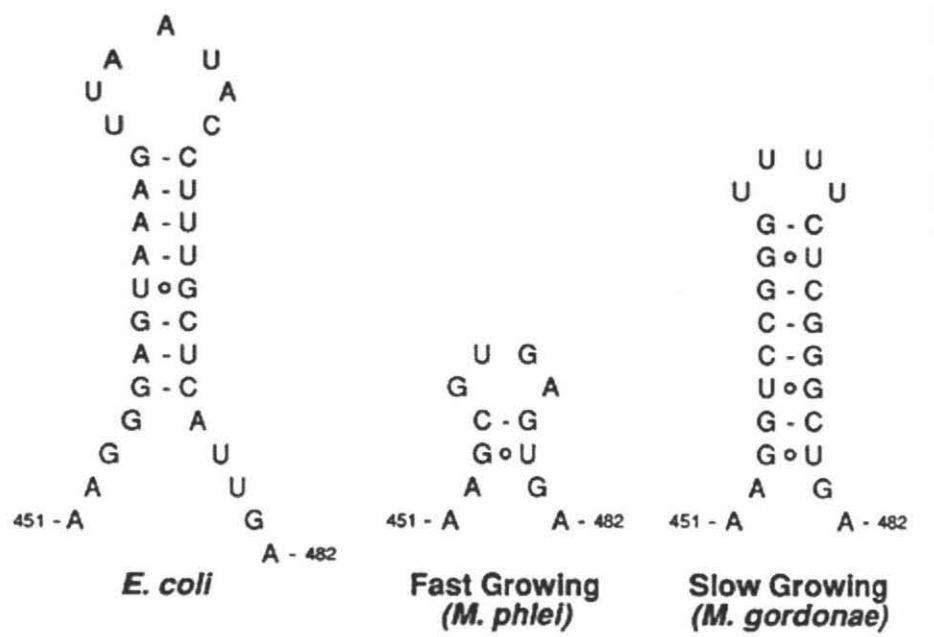


Figure 2: Signature for slow-growing mycobacteria.

Length variation in mycobacterial helices homologous to the helix in *E. coli* bounded by positions 455-477 (*E. coli* numbering). The extended helix is only present among mycobacteria composing the slow-growing line of descent. The structure for a representative fast-growing mycobacterium is *M. phlei*. *M. gordonae* is displayed as a representative slow-growing species. This figure was created by Stahl and Urbance (1990).

2.2 Clinical Significance

2.2.1 Primary Infections

Mycobacteria are responsible for considerable human morbidity and mortality worldwide. *Mycobacterium tuberculosis* is the causative organism of tuberculosis. Tuberculosis (TB) is primarily an illness of the respiratory system, and is spread by coughing and sneezing. Each year about 2 million people die from this curable disease (World Health Organisation Notification Data 2004).

The current TB notification rate in New Zealand is 10 per 100, 000 (EpiSurv Notification Data 2003). Incidence has increased slightly in recent years due to HIV/AIDS, immigration and deterioration of control programmes (Heffernan *et al.* 2000).

In addition to humans, tuberculosis infection also occurs in a wide range of domesticated and wild animals. *Mycobacterium bovis*, which causes tuberculosis in animals and rarely in humans, has one of the widest animal host ranges of all pathogens. Animal hosts include cattle, possums, deer, buffalo, numbats, horses, cats, pigs and seals.

Mycobacterium avium subspecies *paratuberculosis* is the causative agent of Johne's disease in ruminants. Johne's disease is a contagious, chronic and usually fatal infection that affects primarily the small intestine of ruminants. *M. paratuberculosis* shares some characteristics with *M. tuberculosis*: it is slow to develop, resistant to antibiotics and is contagious. *M. paratuberculosis* can replicate only within the host animal; it cannot replicate in the environment. However, this organism can remain infectious in soil or water for up to one year. The hypothesis that this organism might also be involved as a cause of the chronic inflammation of the intestines of patients with Crohn's disease was first reported in 1913, but due to the extreme difficulty of culturing this organism by standard techniques, the initial hypothesis was never clearly confirmed. Recently, several authors have reported the detection of *M. paratuberculosis* in 40% of diseased tissues from patients with Crohn's disease by an adapted *in situ* hybridization technique (El-Zaatari *et al.* 2001; Hulten *et al.* 2001). *M. paratuberculosis* remains difficult to culture, requiring the addition of mycobactin J (iron-chelated substance) to culture media. *M. paratuberculosis* is considered incapable of synthesizing mycobactin and hence is dependent on the presence of mycobactin in culture media for growth.

Leprosy is primarily a human disease caused by *Mycobacterium leprae,* although natural infections of armadillos and monkeys have also been recorded. Leprosy remains a major global health problem, especially in the developing world. *M. leprae* is non-cultivable and to this day remains poorly understood compared to other major bacterial diseases. Intriguing experiments performed in the 1980's have shown that sphagnum moss contains mycobacteria that could be propagated by serial passage in the footpads of mice, but bacilli from the footpads could not be cultured in vitro. Although it cannot be assumed these organisms are *M. leprae*, it indicates non-cultivable mycobacteria exist in the environment, possibly in symbiosis with plants or other living organisms (Kazda *et al.* 1990).

Two species of non-tuberculous mycobacteria that cause clinically distinct human skin infections are *Mycobacterium marinum* and *Mycobacterium ulcerans*. *M. marinum* skin infection is commonly referred to as "fish tank granuloma". Most often, infection from this species occurs following an exposure of a cut or abraded skin to organisms present in aquariums, pools and natural waters. The typical outcome of infection is the development of a localized skin lesion on the fingers, arms or legs (Grange 1996).

M. ulcerans causes distinctive, often severe skin lesions in tropical and sub-tropical areas of the world including Australia. These skin lesions are also known as Buruli ulcer. Infection is thought to occur through exposure to organisms from cuts made by vegetation (e.g. grass). Lesions develop as small, palpable, painless, subcutaneous swellings approximately four to ten weeks after infection. The growing nodule, which is firm, extends laterally involving fat and fascia around muscles. The skin overlying the lesion loses pigmentation, becomes filled with fluid and necrotic and often ulcerates. The ulceration often enlarges over many months leaving the patient debilitated and disabled (Grange 1996).

2.2.2 Opportunistic Infections

M. tuberculosis can successfully infect and cause disease in a healthy host in whom all defences are intact. Other organisms have a more limited pathogenicity and cause disease almost exclusively in hosts with defence defects. Such organisms are termed "opportunistic". The remainder of the non-tuberculous mycobacteria are opportunistic pathogens. They are capable of causing lymphadenitis and infections of the lungs, skin, soft tissues, bursa, joints, tendon sheath and bones.

Mycobacterium avium complex (MAC) is ubiquitous in the environment (Falkinham 1996) and infections due to this organism are more commonly observed in immunocompromised persons such as those with HIV/AIDS. Although rare, MAC pulmonary disease may occur in immunocompetent persons. A number of reports have demonstrated an association between lung disorders in immunocompetent humans and the occurrence of *M. avium* complex in spa pools (Embil *et al.* 1997;

Kahana *et al.* 1997). However, most researchers did not study the relationship between the clinical and the environmental isolates except for three studies that used restriction fragment length polymorphism (RFLP) and multilocus enzyme electrophoresis to demonstrate a genotypic link between MAC isolates from the patient and the spa pool (Lumb *et al.* 2004). Pulmonary MAC infections are typically thought to occur following exposure to contaminated aerosols from tap water i.e., inhalation of bacilli when showering. However, a study on the local environment of HIV patients living in San Francisco showed that MAC organisms were rarely recovered from tap water or food samples but were recovered from 55% of potted soils (Yago *et al.*1995).

2.3 The Ecology of Mycobacteria

The impact of non-tuberculous mycobacteria on morbidity and mortality of AIDS patients has stimulated the initiation of studies on the epidemiology, ecology, genetics, molecular biology and physiology of NTM (Primm *et al.* 2004). Studies of the physiology of human NTM infections have provided some understanding of the geographic distribution of mycobacterial species and identified some factors influencing their numbers. The term "physiologic ecology" was coined by Bruce Barker to describe mycobacterial studies of this nature (Falkinham 1996).

Soil organic matter is the predominant habitat of most mycobacteria, reflecting their close phylogenetic relationship with the *Actinomycetes*. Almost all species of mycobacteria have been isolated from various soil environments with the exception of the *Mycobacterium tuberculosis* complex, *M. leprae,* and *M. ulcerans*. Mycobacteria have also been recovered in large numbers from sphagnum vegetation and in waters with high levels of humic and fulvic acids (Katila *et al.* 1987; Kirschner *et a*l.1999). Some environmental mycobacteria have been shown to grow in amoebae and it is suggested that this habitat may provide a haven when environmental conditions deteriorate (Falkinham1996). *M. ulcerans* has been isolated from the salivary glands of aquatic insects and *M. paratuberculosis* has been recovered from trichostrongylid nematode larvae and earthworms. These findings suggest that the ecology of NTM

may be considerably more complicated than currently thought (Falkinham *et al.* 2004).

NTM grow over a wide range of temperature, pH, salinity, and oxygen tension. Thus, NTM can be isolated from a wide variety of environmental samples. A specific example of the relationship between mycobacterial physiology and ecology is provided by MAC. MAC grows best between pH 5-5.5, micro-aerobically and approximately 2-5mm below the surface of semi-solid mycobacterial media. It also grows well in waters with or without salt. Therefore, it is not surprising that MAC organisms can be found in large numbers in brackish swamps and estuaries (Falkinham 1996).

Physiologic traits (e.g. hydrophobicity) that directly influence host-to-host transmissibility (e.g. aerosolization) are becoming increasingly recognised as important. Mycobacteria are hydrophobic due to their thick lipid-rich cell wall and they collect at air-water interfaces where they can obtain oxygen from the atmosphere and nutrients from the water. Concentration at air-water interfaces also allows them to be easily aerosolized thereby facilitating infection.

The impermeability of the hydrophobic cell wall also permits resistance to hydrophilic antibiotics and disinfectants (Falkinham *et al.* 2004). NTM are less affected by the disinfection process than other bacteria. Resistance to disinfection coupled with their ability to persist in low nutrient environments leads to a predominance of mycobacteria in drinking water (Falkinham *et al.* 2004).

Mycobacteria are slow growing organisms. Slow growth provides an advantage as it allows greater time for adaptation to stressful conditions such as nutrient deprivation (Falkinham *et al.* 2004). It has been reported that *Mycobacterium intracellulare* persisted in sterile water for 1.4 years with only one log loss of viability (Primm *et al.* 2004). The slow-growth of mycobacteria may be due to the possession of a single rRNA cistron. There is a relationship between the number of ribosomes in cells and the rate of protein synthesis. It is possible that the small number of rRNA genes in mycobacteria limits the rate of protein synthesis and hence slows the rate of growth (Falkinham *et al.* 2004).

Much of the knowledge of NTM ecology and distribution has been inferred from records of human-NTM interactions (i.e. their epidemiology). From the pattern of the geographic distribution of NTM infections it can be inferred that their distribution in nature is not uniform. *Mycobacterium kansasii* infections are more common in Texas and the Midwest of America while rapidly growing NTM species such as *M. chelonae* and *M. fortuitum* are predominant in areas of the South-eastern USA (Anon. 2004). *M. kansasii* infections are common in some areas of the world but are rare in Australia and New Zealand (personal communication, Ross Vaughan, New Zealand TB Reference Laboratory). However, an increase in laboratory isolation of *M. kansasii* has been recently noted in Queensland, Australia (personal communication Chris Gilpin, Queensland Mycobacterium Reference Laboratory). *M. kansasii is* commonly isolated from water but rarely isolated from environmental samples. Its ability to survive long-term in soil is questionable (Alcaide *et al.* 1997; Picardeau *et al.* 1997), and this raises the question of whether the natural ecology of this organism or the nature of its interaction with human populations has changed, leading to an increase in disease and laboratory isolation.

The possibility that the natural population of MAC and *Mycobacterium scrofulaceum* in the environment is changing in its distribution and/or numbers is reflected in evidence that there has been a shift in the frequency of isolation of NTM from children suffering from cervical lymphadenitis. Up to 1979, *M. scrofulaceum* was the most frequent cause of cervical lymphadenitis in children. In contrast, recent reports have established that MAC is now the most common cause of cervical lymphadenitis in children in several countries including New Zealand (Primm *et al.* 2004). There are also reports from surveys over the period 1990-1993 that demonstrate *M. scrofulaceum* is no longer as prevalent in natural waters as before (i.e.1970-1980) and that *M. scrofulaceum* is absent from drinking water systems, unlike MAC (Falkinham *et al.*1980, 1990). These data highlight the significant effects that shifts in ecology and resultant selective pressures can have on NTM epidemiology.

2.4 Isolation Media for Mycobacteria

Various methods have been described for isolating mycobacteria from soil samples. However, none of these methods are universally applicable due to the physiological diversity of mycobacterial species.

The recovery of mycobacteria using agar culture medium was difficult and of low efficacy when the first attempts were made late in the 19^{th} century. A medium containing whole eggs, potato flour, glycerol and salts was found to be effective in isolating *M. tuberculosis*. Today this is the basis of Lowenstein Jensen (LJ) medium (Difco Manual 2003).

Mycobacteria have simple nutritional requirements despite their relatively slow growth. Most species are able to utilize a diverse range of nitrogen and carbon sources. In general, NTM show greater metabolic diversity than do the tubercle bacilli (Edson 1951).

A typical simple medium that contains sufficient nutrients is Sauton's medium, containing asparagine, glucose, glycerol, Na_2HPO_4, K_2HPO_4, $MgSO_4$ and ferric ammonium citrate (Parish and Stoker 1998).

All mycobacteria are aerophilic. Carbon dioxide stimulates growth and may be derived from the atmosphere or from carbonates or bicarbonates in the media. The vitamin biotin is a cofactor involved in CO_2 fixation and is incorporated into many media (Difco Manual, 2003).

Carbon may be obtained from sugars or organic acids. Glycerol is particularly well utilized and is metabolized to pyruvate. Nitrogen may be derived from ammonia, amides, amino acids, nitrate or nitrite. Asparagine is a particularly well utilized nitrogen source and is included in LJ and many other media (Difco Manual, 2003).

The metabolism of mycobacteria is similar to that of bacteria in other genera. The reason for the preference for asparagine and glycerol as nitrogen and carbon sources is unknown (Parish and Stoker 1998). Similarly, the biochemical basis for differences

in growth rates between rapid-growing and slow-growing mycobacteria is unknown. Possible explanations include differences in the rate of DNA replication, diffusion of nutrients, cell wall synthesis, and the absence or presence of key energy-generating pathways (Koneman *et al.* 1998).

The media which have been most widely used for isolation of mycobacteria are Lowenstein Jensen slopes, Middlebrook Enrichment agar and Sauton's medium.

2.4.1 Lowenstein Jensen (LJ) Slopes

LJ Medium is an egg-based medium developed from Jensen's modification of Lowenstein's formula (Table 1). LJ contains no agar; it is solidified by heating to 90°C for 45 minutes. The coagulation of the egg albumin during sterilization provides a solid medium for inoculation purposes. The process of solidifying protein-containing medium by heat is known as inspissation. The glycerol and egg mixture provides fatty acids and protein required for the growth of mycobacteria. The addition of defined salts is essential for growth and the relatively high concentration of malachite green selectively inhibits contaminants (Difco Manual, 2003).

Advantages of LJ medium include that it is inexpensive and readily available (Table 2). It has a reasonable shelf life which can be extended by refrigeration, and as a sloped medium it resists dehydration more effectively than plated media. It also allows for a luxuriant growth of mycobacterial species while being moderately selective due to the presence of malachite green. Another desirable feature is that chromogenic studies and biochemical tests are most accurate when performed from LJ media and it does not require strict capneic conditions for incubation (Koneman *et al.* 1998).

Disadvantages are that it is less sensitive than Middlebrook agar and is opaque; therefore microscopic analysis of microcolonies is very difficult. Another disadvantage is that an inspissated culture medium is more prone to liquefaction by proteolytic enzymes produced by contaminating bacteria than is a medium solidified by the addition of agar (Koneman *et al.* 1998).

2.4.2 Middlebrook 7H10 OADC Enrichment

Middlebrook 7H10 Agar, when supplemented with oleic acid, albumin, dextrose and catalase enrichment (OADC), is used for the isolation and cultivation of mycobacteria (Table 1).

Glycerol is the source of carbon and energy but supplementation of the agar base with OADC is required to obtain mycobacterial growth. In the enriched medium, sodium chloride maintains osmotic equilibrium while oleic acid and other long chain fatty acids can be utilized for growth. The primary effect of albumin is protection of the mycobacteria against toxic agents, which then enhances recovery on primary isolation. Albumin is not metabolized by the bacteria. Glucose is an energy source; and catalase destroys toxic peroxides that may be present in the medium. Partial inhibition of contaminating bacteria is achieved by the presence of the malachite green dye (Difco Manual, 2003).

Prepared plates of the complete medium are deep-filled to reduce the effects of drying during prolonged incubation.

An advantage of this medium is that it is transparent; thereby presumptive identification of microcolonies can be made after ten days incubation. Also, agar-based media have less protein content than egg-based media and therefore cause less non-specific protein binding of antibiotics in drug susceptibility testing (Koneman *et al.* 1998).

Disadvantages of this medium are that it is expensive and not readily available commercially. The rate of contamination on Middlebrook is higher than on egg-based medium due to the lower concentration of malachite green. Contamination can also occur after the addition of OADC, which must be added after autoclaving when the medium has cooled to 50°C. This medium has a shorter shelf life than LJ and storage of Middlebrook for more than 4 weeks may result in the release of formaldehyde, a chemical very inhibitory to mycobacteria (Koneman *et al.* 1998). The absolute requirement for capneic incubation for proper performance is also a disadvantage. Plates tend to dehydrate much more rapidly than slopes and prolonged incubation

results in loss of "free" water and reduction of colony size or total inhibition of organism growth (Table 2).

2.4.3 Modified Sauton's Medium (In-house)

While the above media were primarily designed to isolate the tubercle bacilli, Sauton's medium is a simple broth medium that adequately supports the growth of many organisms as well as mycobacteria. Sauton's is often used to cultivate large masses of *M. tuberculosis* for research work. The ingredients found in Sauton's medium are potassium phosphate, magnesium sulphate, ferric ammonium citrate, citric acid, glycerol and asparagine (Grange 1996).

Originally a liquid medium (Parish and Stoker 1998), this medium has been modified by the addition of agar which has solidified the medium allowing spatial separation of colonies and microscopic analysis (Table 1). It contains ammonium phosphate and glutamine as nitrogen sources and glycerol as a carbon source. Glutamine is the preferred nitrogen source over asparagine for two reasons, the first being technical. Glutamine is easily dissolved in water, unlike asparagine which requires strong acid or alkali to dissolve thereby affecting the final pH. The second reason for using glutamine rather than asparagine is based on the work of Edson, who demonstrated an increase in oxygen consumption of the tubercle bacilli and NTM in the presence of glutamate and glycerol (Edson 1951). A variety of inorganic salts and vitamins are also present for optimal growth.

Advantages of this medium are that it is translucent, enabling microscopic evaluation of microcolonies and it is low in inhibitory substances, allowing for diversity of species isolated. Also, due to the low nutrient level, this medium inhibits the growth of a wide variety of fungi and soil bacteria that can grow on Middlebrook 7H10. This makes it an exceptional medium for isolating the very slow growing mycobacteria.

Disadvantages of using the in-house medium are batch-to-batch variation and poor growth of *M. kansasii* (Table 2).

Table 1: Formulae of mycobacterial isolation media per litre

Lowenstein-Jensen		**Middlebrook 7H10**		**In-house medium**	
Asparagine	6.0g	Ammonium Sulfate	0.5g	Ammonium Phosphate	0.5g
Monopotassium Phosphate	4.0g	Monopotassium Phosphate	1.5g	Glutamine	4.0g
Magnesium Sulfate	40.0mg	Disodium Phosphate	1.5g	Glycerol	20.0mL
Magnesium Citrate	1.0g	Sodium Citrate	0.4g	B Vitamins	1.0mL
Potato Flour	50.0g	Magnesium Sulfate	25.0mg	CCY salts	1.0mL
Malachite green	0.7g	Calcium chloride	0.5mg	Agar	15.0g
Glycerol	20.0mL	Zinc Sulfate	1.0mg		
Whole egg mix	1.0L	Copper Sulfate	1.0mg	CCY salts:	
		L-Glutamic Acid	0.5g	$MgCl_2.6H_2O$	1.0g
		Ferric Ammonium Citrate	0.04g	$MnCl_2.4H_2O$	9.5mg
		Pyridoxine Hydrochloride	1.0mg	$FeCl_3$	135mg
		Biotin	0.5mg	$CaCl_2$	29.0mg
		Malachite Green	250µg	$ZnCl_2$	68.0mg
		Agar	15.0g		
		Glcerol	5.0mL		
		*OADC enrichment:			
		Sodium Chloride	8.5g		
		Dextrose	20.0g		
		Bovine Albumin	50.0g		
		Catalase	0.03g		
		Oleic acid	0.6mL		

* OADC: oleic acid, albumin, dextrose and catalase. Use 100mL of enrichment per litre of media.

Table 2: Comparison of media used for the isolation of non-tuberculous mycobacteria

Lowenstein Jensen	Middlebrook 7H10	In-house medium
Commercially available	Commercially available*	"In-house" medium
Sloped medium	Plate medium	Plate medium
Inexpensive	Expensive	Inexpensive
Long shelf life	Short shelf life	Moderate shelf life
Selective	Moderately selective	Non- selective
Moderate levels of contamination	High levels of contamination	Low levels of contamination
Microscopic analysis not permitted	Microscopic analysis enabled	Microscopic analysis enabled
Aerobic or capneic incubation	Strict capneic incubation	Aerobic or capneic incubation

* Middlebrook 7H10 is not readily available in New Zealand.

2.5 Sample Decontamination

Mycobacteria are slow-growing organisms, which makes them difficult to recover from samples containing rapidly growing bacteria. A feature of the mycobacteria that can be exploited for their selective isolation is resistance to chemical decontamination procedures. Chemical decontamination involves subjecting samples to harsh chemicals such as sodium hydroxide or sulphuric acid for set periods of time. No one method of decontamination is ideal for all types of samples. Whichever method is chosen, the microbiologist must be aware of the inherent limitations of that procedure. Sodium hydroxide is the most commonly used decontamination compound. However, with increasing concentrations and treatment time it is harmful to mycobacteria (Isenberg 2004).

A pre-incubation of the soil samples is necessary to allow any spore-formers (i.e. *Bacillus* species) to germinate, thus making them susceptible to decontaminating

agents. Splitting the decontamination procedure into two parts (A and B) is necessary because rapid-growers are more susceptible to sodium hydroxide (NaOH) than are the slow-growers (Parashar *et al.* 2004). Sodium dodecyl sulphate (SDS) is a non-ionic detergent used in combination with the NaOH to help concentrate the hydrophobic mycobacteria during centrifugation. The combination of SDS and NaOH is the most effective decontamination solution to minimize contaminants such as *Bacillus* and *Streptomyces* species, two of the most common soil organisms (Salfinger *et al.* 1988).

Contaminating bacteria are a nuisance when prolonged incubation of plates is required for the isolation of the slow-growers. In addition to the above, Cetrimide is used to reduce the extent of contamination. Cetrimide is a cationic detergent, which is a powerful disinfectant and has been proven through experiments to effectively reduce the level of contamination (Parashar *et al.* 2004).

2.6 Identification of Mycobacteria

Differentiation of species and subgroups of the genus *Mycobacterium* has traditionally relied on labour-intensive biochemical methods. Often these tests have poor sensitivity and are time consuming, with some tests requiring up to five weeks incubation. More recent approaches to rapid identification include analysis of mycolic acids using high performance liquid chromatography (HPLC), DNA probes, restriction fragment length polymorphism (RFLP), internal transcribed spacer regions (ITS) and rRNA gene sequencing.

Identification of microorganisms based on their genetic code began in the 1980's. Woese and others showed phylogenetic relationships of bacteria by comparing a stable part of their genetic code, the 16S gene (Clarridge 2004). Other genes that can also be used are the 5S, 23S and the spaces between these genes.

The 16S rRNA gene is highly conserved among bacteria (Clarridge 2004). This high degree of conservation is assumed to result from the importance of 16S rRNA as a critical part of cell function as opposed to genes used to make enzymes. Mutations to

genes encoding enzymes are tolerated more frequently as they do not affect essential structures like ribosomes. For example, if a bacterium does not have the gene to make enzymes needed to utilize lactose, it can use an alternative sugar as an energy source.

The 16S rRNA gene sequence is approximately 1500 base pairs (bp) long and is composed of variable and conserved regions. Universal primers are usually chosen as complementary to the conserved regions at the beginning of the gene and at either the 540 bp region or at the end of the whole sequence (1550 bp region), and the sequence of the variable region in between is used for comparative taxonomy (Clarridge 2004).

The whole 1500bp length or, more commonly 500bp can be sequenced. Sequencing the entire 1500bp length is required to distinguish between certain taxa or strains and is necessary for describing a new species. Generally a 500bp sequence provides adequate differentiation for identification (Clarridge 2004).

There have been several studies comparing the identification of mycobacteria by 16S rRNA gene sequencing and phenotypic methods (Odell *et al.* 2005; Ringuet *et al.* 1999). In all these studies 16S rRNA gene sequencing was judged to be superior to phenotypic methods. Since the introduction of bacterial 16S rRNA sequencing in 1990, three new species of NTM have been described per year (Tortoli 2004).

There are a few exceptions in which 16S rRNA gene sequencing cannot differentiate mycobacteria. Certain species are identical throughout this region making differentiations impossible (Odell *et al.* 2005; Ringuet *et al.* 1999). The species known to be highly conserved are *M avium* and *M. paratuberculosis*, *M. chelonae* and *M. abscessus*, *M. kansasii* and *M. gastrii*, *M. malmoense* and *M. szulgai* and the *M. tuberculosis* group.

The gene encoding the 65-kDa heat shock protein is highly conserved among mycobacteria and it has also been used to define taxonomic relationships (Odell *et al.* 2005; Ringuet *et al.* 1999). Heat shock proteins (hsp) are present in all living cells and are structurally highly conserved (Henderson *et al.* 2000). The hsp65 gene is conserved but shows significant sequence variation between species making it an ideal method to differentiate mycobacteria unable to be distinguished by 16S rRNA

sequencing (Odell *et al.* 2005; Ringuet *et al.* 1999). PCR of the hsp65 gene coupled with endonuclease analysis of the PCR products is an additional tool for differentiating mycobacteria (Steingrube *et al.* 1995).

Other areas of the rRNA gene have also been used for studying phylogenetic relationships among mycobacteria. Roth *et al.* (1998) used the 16S-23S rRNA gene internal transcribed spacer (ITS) sequences and found the technique useful for differentiating organisms that were unable to be distinguished by 16S rRNA gene sequences (Clarridge 2004). Several medical laboratories in Australia prefer to use ITS for identifying non-tuberculous mycobacteria (personal communication, Chris Gilpin, Queensland Mycobacterial Reference Laboratory).

Odell *et al.* (2005) used high resolution melting analysis of hsp65 PCR products and found it to be considerably faster than other methods and, like sequencing, can detect unexpected base changes.

2.7 Susceptibility of MAIC to Clarithromycin

There is increasing concern about the growing resistance of pathogenic bacteria to antibacterial compounds in the hospital environment. However, little is known about the effects of sub-inhibitory concentrations of antibiotics and disinfectants (antibacterials) on environmental bacteria (Kummerer 2004).

Resistance is a description of the relative insusceptibility of a microorganism to a particular treatment under set conditions. For antibacterials, resistance is quantified as the minimum inhibitory concentration (MIC) required to inhibit the growth of a population of cells. When an agent is ineffective (i.e. the MIC is elevated beyond a defined concentration) against a certain organism, this organism is known as "resistant". Many organisms are naturally (intrinsically) resistant to a particular agent by nature of their physiology or biochemistry, e.g. rapidly growing mycobacteria are typically resistant to standard anti-mycobacterial therapy, therefore, susceptibility testing is essential for guiding therapy (Jogi and Tyring 2004). Conversely, resistance

can be encoded in genes that are not expressed often, e.g. wild type *M. kansasii* isolates are resistant to critical concentrations of isoniazid *in vitro.* Despite this result isoniazid appears to be clinically active.

Chemotherapy of NTM infections is complicated because of the inherent relative resistance of mycobacteria to a wide range of antibiotics. Consequently, there has been a search for new and more effective anti-mycobacterial antibiotics and new targets for anti-mycobacterial therapy.

The major determinant of antibiotic resistance in NTM is the presence of a cell wall-associated permeability barrier (Falkinham 1996). To overcome that barrier synergistic combination therapy has been used. For example, antibiotic agents whose target is the cell wall (e.g. ethambutol) have been used in combination with others whose target is different (e.g. streptomycin, ciprofloxacin, or rifampin). The need to identify other effective, synergistic combinations of antimycobacterial drugs has been emphasized in the literature (Falkinham 1996).

Pang *et al.* (1994) demonstrated the potential for *Streptomyces* species to transfer tetracycline resistance genes to NTM. The transfer of resistance is usually greatest when both the donor and recipient are the same or related species. Some *Streptomyces* species share similarities with the genus *Mycobacterium* and they both share the same habitat. In addition, rRNA methylase determinants, which confer resistance to the macrolides, are often associated with tetracycline resistance determinants. The prophylactic use of the macrolides to prevent disseminated MAC infection in AIDS patients could provide the pressure to introduce a mobile methylase determinant in mycobacteria in the hospital and urban environment.

Pigmentation of MAC isolates is thought to be linked to antimicrobial susceptibility (Kiehn *et al.*1995). MAC colonies range from transparent, buff, or deep yellow-orange pigments. Pigmented strains tend to grow more rapidly and are less drug resistant than non-pigmented strains of MAC. Non-pigmented strains of MAC have been shown to be more virulent and more drug resistant than pigmented strains (Dunbar *et al.* 1968; Kajoika and Hui 1978).

Chapter 3 Materials & Methods

3.1 Media

Lowenstein Jensen Slopes with Glycerol were obtained from Fort Richard Laboratories, Otahuhu, Auckland.

Middlebrook 7H10 and 7H9 with OADC enrichments were obtained from BBL Difco (available from Fort Richard Laboratories, Otahuhu, Auckland).

In-house medium "Modified Sauton's" was made and autoclaved on site at BioDiscovery New Zealand Ltd (121°C for 15 minutes).

To ensure all media were able to isolate a wide variety of mycobacteria, plates from each batch were routinely tested with the following control cultures (kindly supplied by the New Zealand TB Reference Laboratory, Auckland).

- *M. fortuitum* ATCC 6841
- *M. chelonae* NZRM 4281
- *M. avium* wild type
- *M. intracellalare* ATCC 13955
- *M. kansasii** ATCC 12478
- *M. peregrinum* NZRM 4078
- *M. scrofulaceum* ATCC 19981

*Note: *M. kansasii* grew poorly on the in-house medium

3.2 Chemicals

All chemicals were obtained from Sigma-Aldrich, Auckland, New Zealand unless otherwise stated.

Sodium hydroxide pellets (NaOH)

Sodium dodecyl sulphate crystals (SDS)

Resazurin dye tablets

Zeihl-Neelsen (ZN) and Gram staining reagents were obtained from Becton and Dickinson (available from Fort Richard Laboratories, Otahuhu, Auckland).

Cetrimide (40%) was obtained from the Amcal Chemist, Meadowbank, Auckland

Klacid (Clarithromycin) was kindly gifted by the New Zealand TB Reference Laboratory, Auckland.

3.3 Sample Selection

To enhance the diversity and number of mycobacteria isolated, a range of soil types were sampled (Table 3). The environments tested included areas of high and low human impact.

Approximately 5g (wet weight) of soil was provided for testing by BioDiscovery New Zealand Ltd. Samples consisted of top soil approximately 2-5 cm below the surface with the exception of the urban samples. The urban samples were collected from concrete crevices and consisted of dust, leaf litter, and hair. Samples were stored at room temperature and tested within 48 hours of collection.

Table 3: Description of the soil environments tested and level of human impact.

Environment	Description	Human Impact
Native Forest	Central North Island native forest soil	Low
Artificial	Commercially available potting mix (Thrive©)	High*
Agricultural	Soil from a peat farm (maize and sheep) in the Waikato region	Moderate
Urban	Dust and organic matter collected from the corners of concrete steps – central Auckland City	High

* Although potting mix contains natural ingredients such as peat moss it also contains chemical fertilizers. Due to the artificiality of this medium the level of human impact is considered to be high.

3.4 Decontamination

Five mL of sterile nutrient broth was added to 1g of soil sample. The sample was mixed thoroughly for 1 min and then incubated for 4-6h at 30°C to allow bacterial spores to germinate. Settled soil supernatant (0.5mL) was transferred into two microfuge tubes labelled (A) and (B). A solution of 3%SDS - 4% NaOH solution was added to both microfuge tubes (0.5mL). Tube (A) was left to decontaminate at room temperature for 10 min, Tube (B) for 20 min. The differences in decontamination times was based on the work of Parashar *et al.* (2004) who demonstrated a difference in the susceptibility of rapid- and slow-growers to NaOH. The solutions were then neutralised by filling the microfuge tube with sterile water. After centrifuging the sample for 15 min at 8000g the pellets were washed with sterile water and re-centrifuged. Cetrimide solution (0.5mL, 4%) was added to the pellets. Part (A) was left for 5 min and part (B) for 10 min. The cetrimide was neutralised by filling the microfuge tubes with sterile water. The samples were then centrifuged for 15 min at 8000g and resuspended in 1mL sterile water. An aliqout (50µL) from each tube (A and B) was then plated onto each agar (Middlebrook 7H10 and the in-house medium). The sample was spread across the plates to obtain a lawn culture. The plates were incubated for up to 12 weeks at 25-30°C in air. Positive controls which included spiked soil suspensions of *M. fortuitum* and *M. avium* complex were included with every run. Un-inoculated plates were incubated with test samples to check the sterility of the media and acted as negative controls.

Records were kept on the percentage of contaminated plates. The acceptable range is 3-5%. A contamination rate of less than 3% suggests overly harsh decontamination. A rate significantly greater than 5% suggests the decontamination is too weak (Isenberg 2004). The level of contamination observed during this project was between 3-5%.

3.5 Colony Isolation

Due to the slow growth rate of many NTM and the risk of bacterial and fungal contamination on primary isolation plates, a technique was developed to detect early

growth. Agar plates were screened for growth at 4, 8 and 12 weeks of incubation using a Leica dissecting microscope allowing the detection of NTM micro-colonies at an early stage of growth. Colonial morphology of the control cultures (Section 3.1) was studied to better understand colonial variation of NTM. Familiarity with the morphological variability of NTM micro-colonies allowed for diverse isolations rather than multiple isolates of the same species. Any typical micro-colonies were purity-plated onto Middlebrook 7H10 and re-incubated for a further 4 weeks. The Middlebrook 7H10 purity-plates were then screened for purity. Presumptive mycobacteria cultures were then stained using the Ziehl-Neelsen hot stain. Once purity and acid-fastness was established the isolate was sub-cultured onto a LJ slope for long term storage. If no typical mycobacterial colonies were observed after 12 weeks of incubation the plates were discarded.

3.6 Antibiotic Susceptibility Testing

The Alamar Blue assay was used to test *M. avium-intracellulare* complex (MAIC) isolates against clarithromycin. Clarithromycin is a type of macrolide antibiotic and is a common "first line" drug used to treat MAIC infections. Routine testing of MAIC organisms against other antibiotics is not recommended (National Committee for Clinical Laboratory Standards, 2003).

The Alamar Blue Assay is a micro-broth dilution method performed in 96 well microtitre plates. It is simple, inexpensive and can be used for high throughput screening (Martin *et al.* 2003; Collins and Franzblau 1997; Palomino *et al.* 2002; Franzblau *et al.*1998). It involves the use of an oxidation-reduction dye (resazurin) to visually determine the MIC of an antimicrobial compound. The resazurin dye is a general indicator of cellular growth and/or viability; the blue oxidized form becomes pink upon reduction (Ahmed *et al.* 1994).

The change from blue resazurin to pink resofurin is not reversible by atmospheric oxygen. The second stage of reduction to the colourless state is reversible by atmospheric oxygen. It can be measured spectrophotometrically at 610nm, but visual

observation of a colour change is sufficient. Lack of any colour (i.e. clear) can be included as a positive result (Guerin *et al.* 2001).

Assays were performed in Middlebrook 7H9 broth medium supplemented with 2% glycerol as described by Franzblau *et al.* (1998).

The clarithromycin (50µg/mL) was dissolved in Middlebrook 7H9 broth according to the manufacturer's instructions. Serial two-fold dilutions of clarithromycin were then prepared in the 96 well microtitre plates. Bacterial inoculum (100µL) was added to the dilution series. The inoculum was prepared from fresh LJ medium. The broth was adjusted to McFarland 1 turbidity and then further diluted 1:10.

Plates were covered with Parafilm, sealed in plastic bags and incubated at 30°C in air. Growth controls containing no antibiotic and sterility controls without inoculum were included. Guard wells around the perimeter of the plate were also included to prevent dehydration. After 7 days of incubation, 30 µL of resazurin solution was added to the growth control well. Resazurin was prepared at 0.01% (wt/vol) in distilled water and filter sterilised. This was equivalent to 1 resazurin tablet (0.25g) with a dye content of 0.84% in 20mL of distilled water. Wells were incubated overnight at 30°C and assessed for colour development. A change from blue to pink (or clear) indicated a reduction of resazurin and therefore bacterial growth. Resazurin was then added to all wells in the assay. The MIC was defined as the lowest drug concentration that prevented a colour change.

MIC results were interpreted according to the National Committee for Clinical Laboratories Standards* (NCCLS) for testing *M. avium* and clarithromycin (Table 4). Any results indicating resistance were repeated.

* As of January 1st 2005 the NCCLS changed its name to Clinical Laboratory Standards Institute (CLSI).

Table 4: Interpretative criteria for clarithromycin when testing *M. avium* complex

(Woods 2005)

Antibiotic	Method/pH	MIC (ug/mL) for category:		
		Susceptible	Intermediate	Resistant
Clarithromycin	Broth microdilution pH 7.3-7.4	<8	16	>32

3.7 DNA Extraction and the PCR

A loopful of solid *Mycobacterium* mass was emulsified in 0.2mL of TE buffer (10mM Tris-HCl; 1mM EDTA; pH 8.0). Bacterial genomic DNA was then harvested using the commercially available kit Genomix Kit (Bioneer) in accordance to the manufacturer's instructions.

This project used an in-house method of the PCR. Approximately 800bp of the 16S rDNA gene were routinely sequenced. Primers were designed according to the 16S rDNA gene of *M. tuberculosis* complex (Appendix 1). The gene is 1549 bp long and primers were designed to cover both conserved and variable regions of the 16S rDNA sequence.

PCR was performed by adding 5µL of high quality genomic DNA to a 45µL PCR mixture containing 1 X Bioneer PCR Buffer, 1.5 mM $MgCl_2$, 200µM each deoxynucleoside triphosphate (dNTP's), 0.5µM each primer 16S2 (5'GGATTAGATACCC 3') and PB36F (5'AGGGGTTTGATCATCGCTCA 3'), and 1.25U Bioneer Taq polymerase. Initial DNA denaturation was carried out for 5 min at 94°C. Amplification consisted of 25 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, followed by a final extension step at 72°C for 5 min. Known mycobacteria were included with every run to test the reliability of the DNA extraction and the PCR procedures. The PCR product was detected by running 5µL of reaction on 0.8% agarose gel at 80V for 30 mins and staining with ethidium bromide. The PCR product

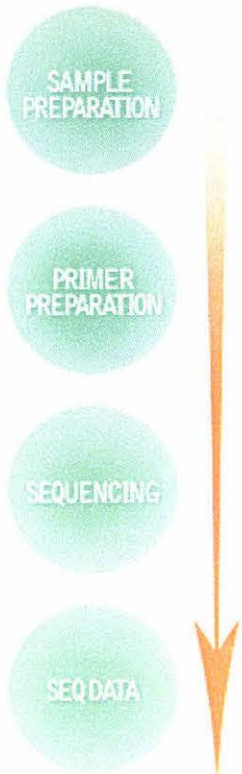
was then purified using the BioNeer PCR Purification Kit and quantified using a DNA quantification ladder standard.

PCR product (15µL of 80ng/µL) was then sent to Macrogen World Meridian Centre, Korea, for sequencing (see Fig. 3). The forward primer (PB36F) was used to sequence the isolates. Macrogen emailed the sequences to BioDiscovery New Zealand Ltd. The sequences were then edited for reading mistakes using the analysis software Sequencher 4.6. Edited sequences were next compared to nucleotide sequences in Genbank, (www.ncbi.nlm.nih.gov), using the BLAST (Basic Local Alignment Search Tool) algorithm.

The results of the BLAST- nucleotide search were then recorded into a spreadsheet containing information about the isolate (Appendix 2).

The Clustal X software was used to align derived 16S rDNA sequences to compare homology.

Figure 3: Macrogen's sequencing methodology (available at www.macrogen.com)



- 10~15μl with concentration of 50ng/μl based on purified PCR product (20-30μl in case of unpurified PCR product)
- 1.5mL microcentrifuge tube recommended

- 10μl with concentration of 5 pmole/μl (5μM) based on 5 samples
- Sufficient quantity required (increase of 10 pmole each time the reactions are increased)
- Vessel separated from samples at the time of provision of the client's primer
- Primer Tm suitable for Macrogen service: 50~58

- Sequencing conducted under BigDyeTM terminator cycling conditions
- The reacted products purified using Ethanol Precipitation and run using Automatic Sequencer 3730xl

- Phred score of 16 or higher and read length of 500bp or more guaranteed based on the normal plasmid template
- Abi file (electropherogram, raw data) created after 3730xl completes analysis
- Further detailed analysis conducted using the Macrogen analysis module; the results together with the raw data are uploaded to the client's account
- Notification mail automatically sent upon the completion of analysis; client may then access LIMS* and download the results

* LIMS: Laboratory information management system.

3.8 Molecular Reagents

All molecular reagents were purchased from Pacific Lab Products, Ellerslie, New Zealand unless otherwise stated.

Bioneer Genomix DNA Extraction Kit

Bioneer PCR Purification Kit

Bioneer 10X PCR Buffer

Bioneer Taq Polymerase 5U/μL

Bioneer Proteinase K

Bioneer MgCl2 20mM

Bioneer dNTP's (each 2.5mM)

DNA quantification ladder (Bioline Hyperladder) from BioLab, Albany, Auckland, NZ.

DNA ladders 25 and 100 bp from Invitrogen, Penrose, Auckland, NZ.

Restriction enzymes from Invitrogen, Penrose, Auckland, NZ.

Primers (custom made) from Invitrogen, Penrose, NZ.

6% TBE gel (1.0mm x 15 wells) from Invitrogen, Penrose, NZ.

3.9 Miscellaneous Equipment

Table top centrifuge. Eppendorf 5403.

Electrophoresis tanks. Horizontal Gel Electrophoresis Apparatus from Gibco BRL and Xcell SureLock Vertical Novex Mini-Cell from Invitrogen.

Pipettes. Biohit calibrated used with Neptune barrier tips, from Invitrogen.

Thermal Cycler. GeneAmp PCR System. 9700. Applied Biosystems.

Standard Heat Block. WWR Scientific Products.

Leica Dissecting Microscope CLS 150X

Olympus BX40 1000X Oil Immersion Microscope

3.10 Flowchart of Mycobacterial Isolation from Soil

The entire process of decontamination, isolation and identification is summarised in Figure 4.

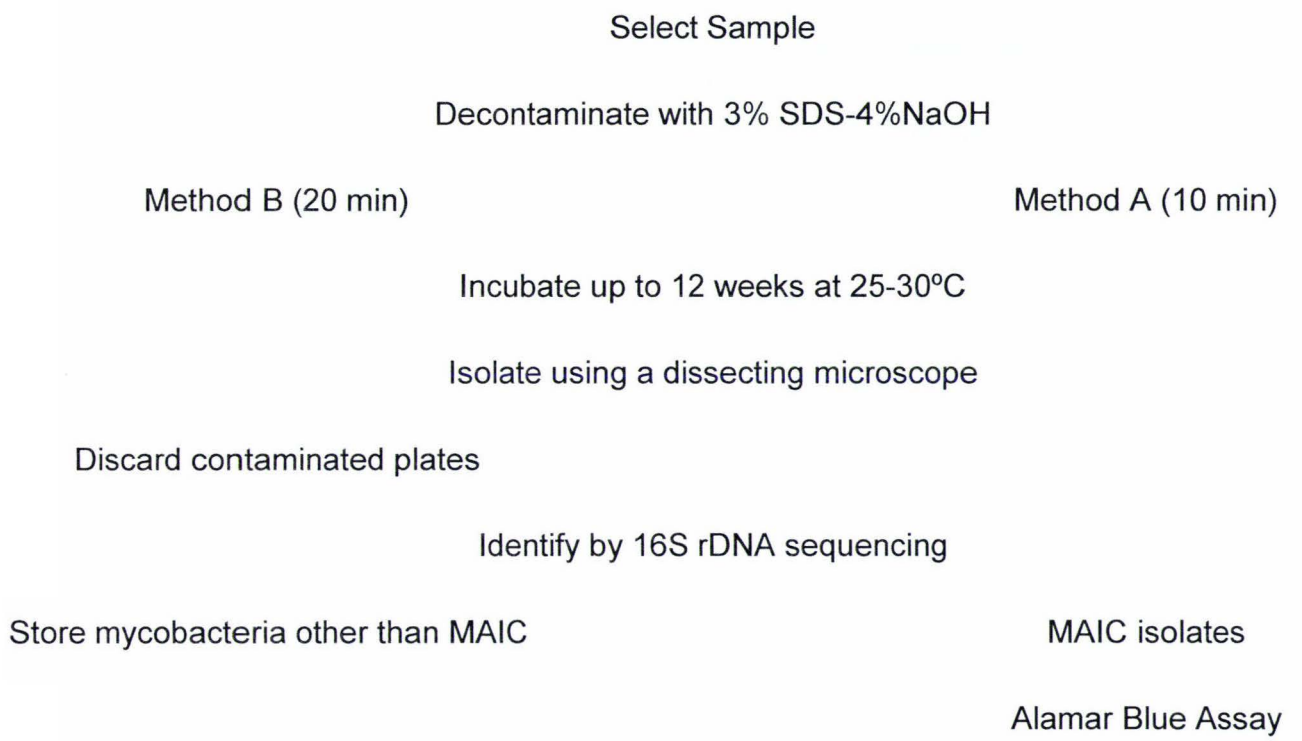


Figure 4: Procedure for isolating mycobacteria from soil.

Chapter 4 Results

4.1 Mycobacteria Isolation

A total of 122 mycobacterial colonies were isolated from the two media (Table 5). 72% of mycobacteria were isolated from Middlebrook 7H10 and 28% from the in-house medium.

Table 5: Comparison of the total number of mycobacteria isolated per medium

Medium	Number of NTM (%)
Middlebrook 7H10	87 (72)
In-house	35 (28)
Total	**122**

NTM, non-tuberculous mycobacteria.

The degree of contamination was noticeably less on the in-house medium than on Middlebrook agar which was prone to heavy bacterial and fungal contamination after prolonged incubation (Fig. 5).

Organisms belonging to the MAIC were the most frequently isolated followed by the *M. montefiorense/M. triplex* group (Table 6). The Middlebrook agar isolated more mycobacterial species than the in-house medium, especially MAIC organisms. However, the media were able to isolate similar numbers of *M. montefiorense/M. triplex*.

Single isolated colonies could be seen on the in-house medium after only two weeks of incubation as shown in Figure 6. The typical umbonate colony morphology of *M. fortuitum* on Middlebrook agar (Fig. 7) is clearly distinguishable from the rough bundle of rods formed in the early growth stages of *M. kansasii* (Fig. 8), or the

translucent undulate-edged colonies of *M. intracellulare* (Fig. 9). These early colony variations were of great value in recognising mycobacterial diversity.

The majority of mycobacteria were isolated after 4-5 weeks of incubation and again after 10 weeks of incubation as shown in Figure 10. Very few mycobacteria were isolated before two weeks or after 10 weeks of incubation.

There was a higher recovery rate of *M. monteflorense/M. triplex* using decontamination procedure (B), as shown in Table 7. However, the overall recovery rates were similar for both methods with procedure (A) accounting for 60 isolates, and procedure (B), 62 isolates.

The greatest numbers of mycobacteria were isolated from peat (Table 8). The peat isolates accounted for 52% of the total mycobacteria recovered.

M. avium-intracellulare complex (MAIC) were the most frequently isolated mycobacteria (Fig. 11). MAIC were the only species of mycobacteria to be recovered from every environment tested. MAIC were the most commonly recovered species of mycobacteria from peat and urban environments. Certain mycobacteria were limited to particular environments (Fig. 11). *M. asiaticum* was isolated only from urban environments while several species of mycobacteria were isolated only from peat (*M. cookie*, *M. florentinum*, *M. parmense*, M. *scrofulaceum* and *M. septicum*) or potting mix (*M. arupense*, *M. nonchromogenicum*, and *M. terrae*).

Few mycobacteria were isolated from the native forest samples, and the majority that were recovered were unable to be speciated further than *Mycobacterium* species (Fig. 11). This result indicates a high probability of novel species being isolated from this environment. Interestingly, the greatest numbers of mycobacteria were isolated from peat. However, mycobacteria unable to be speciated further than *Mycobacterium* species were isolated from every environment except peat.

Table 6: Number of *Mycobacterium* species isolated per medium

Organism	Total Number	MB7H10	In-house
M. arupense	1	-	1
M. asiaticum	3	3	-
M. cookii	2	2	-
M. florentinum	3	2	1
M. lentiflavum	9	7	2
M. montefiorense/triplex	24	14	10
M. nonchromigenicum	1	-	1
M. parmense	1	-	1
M. scrofulaceum	2	2	-
M. septicum	1	1	-
M. simiae	9	6	3
M. terrae	1	1	-
MAIC	49	37	12
Mycobacterium spp.	16	12	4
Total	**122**	**87**	**35**

MB7H10: Middlebrook 7H10 agar, MAIC: *Mycobacterium avium-intracellulare* complex.

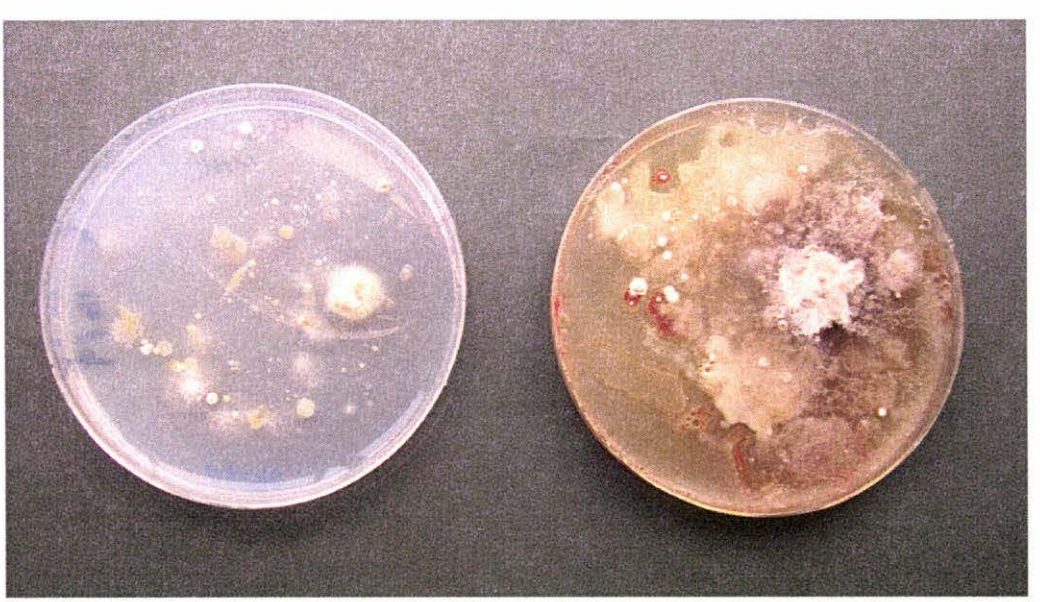


Figure 5: Comparison of contaminants on different media.

Middlebrook 7H10 (right) and the in-house medium (left) for primary isolation of mycobacteria from soil after 6 weeks incubation at 25°C.

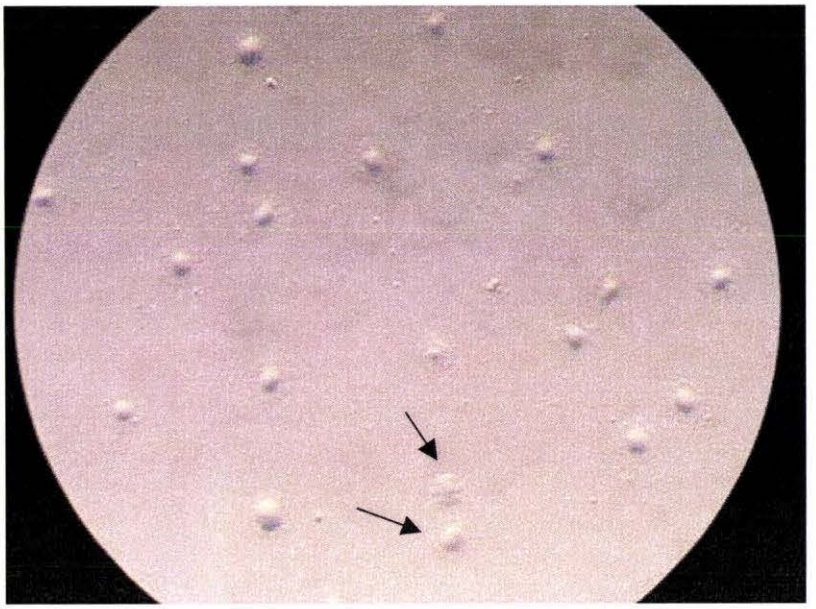


Figure 6: An example of colonial variation.

Single isolated colonies and colonial variation on the in-house medium indicating possible diversity (X50). This plate had been incubated for two weeks.

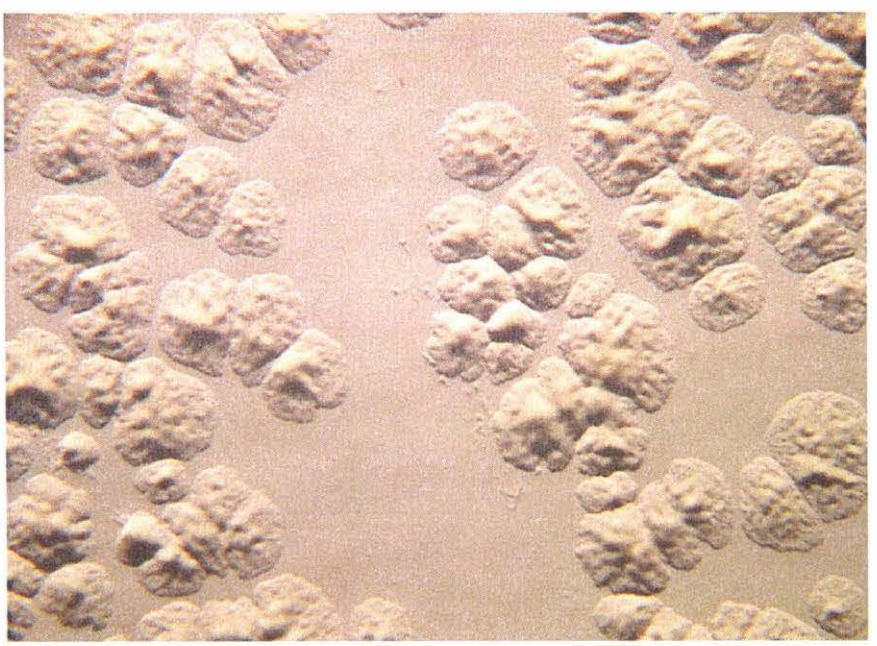


Figure 7: *M. fortuitum* colony morphology on Middlebrook agar.

This plate had been incubated for two weeks (X40).

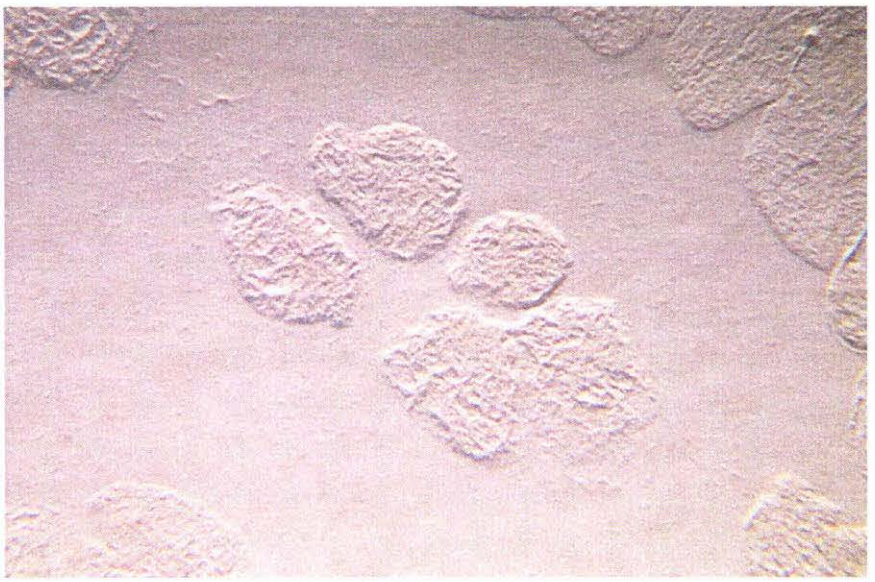


Figure 8: *M. kansasii* colony morphology on Middlebrook agar.
This plate had been incubated for two weeks (X60).

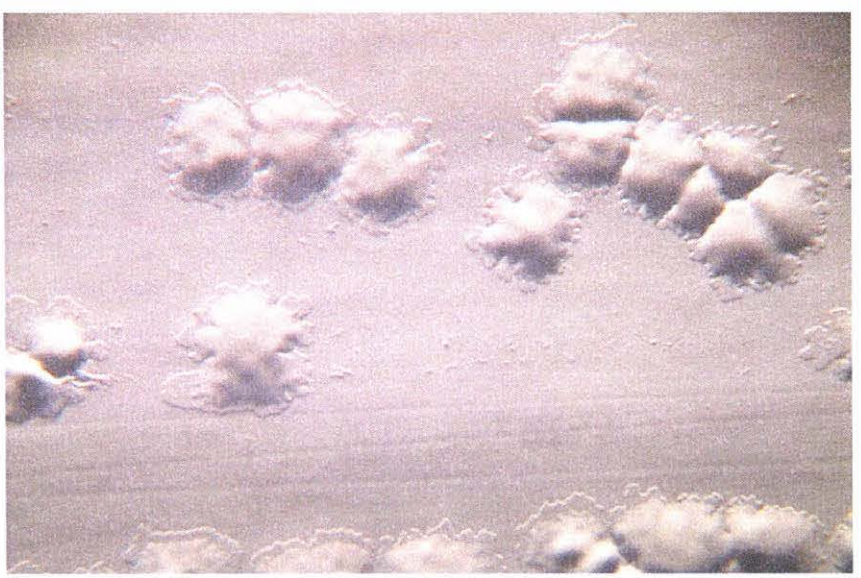


Figure 9: *M. intracellulare* colony morphology on the in-house medium.
This plate had been incubated for two weeks (X60).

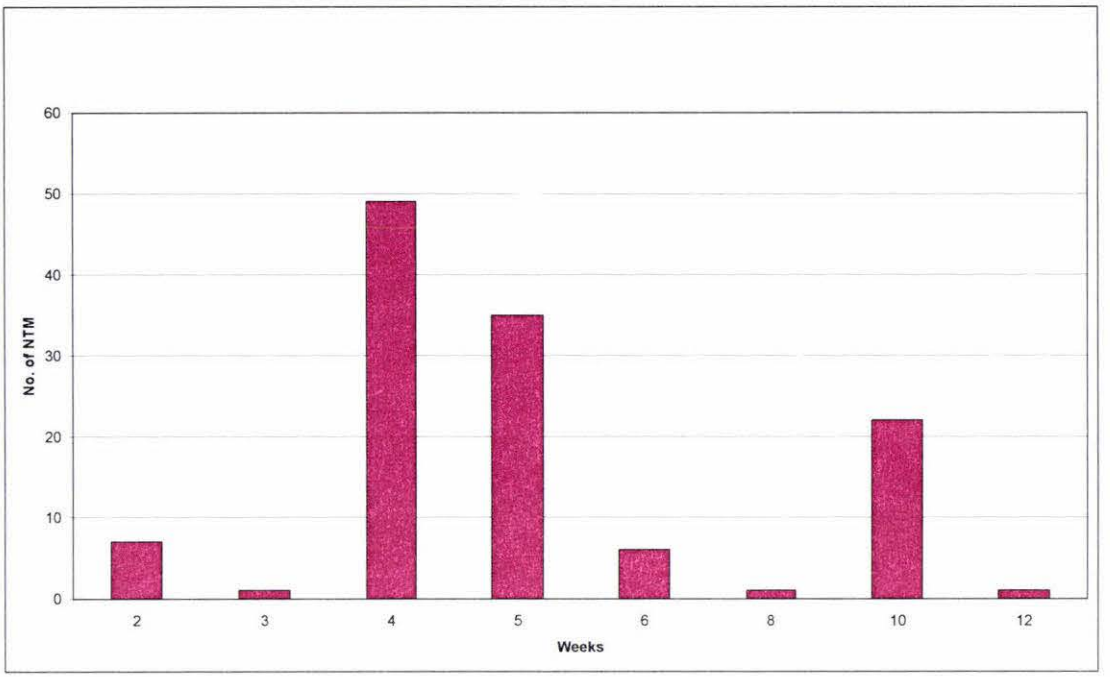


Figure 10: Incubation time in weeks for initial mycobacterial isolations.

Table 7: Number of mycobacteria isolated per decontamination technique.

Organism	A (10 min)	B (20 min)
M. arupense	-	1
M. asiaticum	2	1
M. cookii	1	1
M. florentinum	0	3
M. lentiflavum	6	3
M. montefiorense/triplex	7	17
M. nonchromigenicum	1	-
M. parmense	-	1
M. scrofulaceum	2	-
M. septicum	-	1
M. simiae	4	5
M. terrae	-	1
MAIC	28	21
Mycobacterium spp.	9	7
Total	**60**	**62**

Table 8: Total number of mycobacteria isolated per soil environment

Type of environment	Number of samples tested	Number of NTM isolated
Peat	10	64
Potting mix	10	27
Urban	10	22
Native Forest	10	9
Total	**40**	**122**

NTM, non-tuberculous mycobacteria.

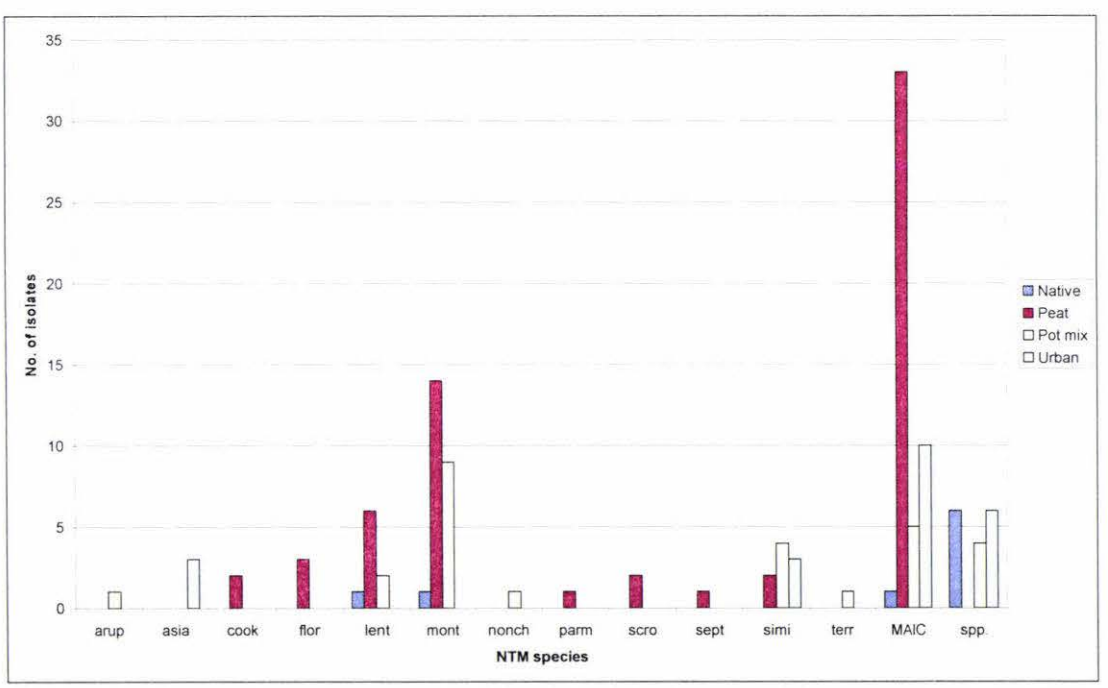


Figure 11: Species of mycobacteria isolated per soil environment.

Abbrevaited species: arup, *M. arupense*; asia, *M. asiaticum*; cook, *M. cookie*; flor, *M. florentinum*; mont, *M. montefiorense*; nonch, *M. nonchromigenicum*; parm, *M. parmense*; scro, *M. scrofulaceum*; sept, *M. septicum*; simi, *M. simiae*; terr, *M. terrae*; MAIC, *M. avium-intracellulare* complex; spp, M. species.
NTM, non-tuberculous mycobacteria.

4.1.1 Discussion

The decision to use an in-house medium as well as commercial media was based on the need to optimize the isolation procedure. The aim was to create a simple, inexpensive medium able to isolate a diverse range of environmental mycobacteria. Historically, complex nutrient-rich media have been used to isolate *M. tuberculosis* from clinical material. However, NTM are abundant in low nutrient environments like soil and water and a medium was designed with this in mind. Although the majority of NTM were isolated from the commercial medium (80%), there were certain species recovered only from the in-house medium such as *M. arupense*, *M. nonchromigenicum*, and *M. parmense* (Table 6). This finding validated the use of more than one medium to increase isolation diversity.

Traditional methods of decontamination use relatively large volumes of sample and decontaminating agents. Miniaturisation of the decontamination technique using microfuge tubes and smaller sample volumes allowed for the high throughput screening of environmental samples. This was important due to limited incubator and laboratory space and the high number of samples screened. In addition, spreading of the decontaminated samples across the agar surface was important. Spatial separation was critical for early microscopic detection of morphologically distinctive colonies before contamination occurred (Fig. 6). Single isolated colonies could then be harvested using a dissecting microscope thereby ensuring pure stocks for further genetic work.

The lack of rapid-growing mycobacteria isolated may be explained by the decontamination procedure. Rapid-growers have been shown to be more susceptible than slow-growers to the harsh chemicals involved in decontamination (Parashar *et al.* 2004). However, these chemicals are needed to control the levels of bacterial and fungal contaminants. Maintaining a balance between acceptable levels of contaminants and diverse isolations was difficult. Although two different time periods were used to decontaminate samples the results show that an equal number of mycobacteria were isolated using each method (Table 7). It is also possible that the low isolation rate is due to small numbers of rapid-growers in the environments

tested. NTM are not uniform in their distribution and geography as often demonstrated by the differences in epidemiology of NTM infections from one country to another. Human infection control data for the period 2003-2006 show that a total of 123 NTM were referred to the New Zealand TB Reference Laboratory for identification and antimicrobial susceptibility testing (Appendix 3). Of these isolates only 29 (24%) were rapid-growers.

Of the total mycobacteria isolated, 49 (40%) belonged to the MAIC (Table 6). The high isolation rate of MAIC and very low isolation rate of *M. scrofulaceum* correlates with previous studies suggesting that the distribution and number of *M. scrofulaceum* in the environment may be falling. The number of clinical isolates of *M. scrofulaceum* compared to MAIC (Appendix 3) is low providing further evidence to suggest a shift in the ecology of *M. scrofulaceum*, or in human/ *M. scrofulaceum* interactions.

The next most commonly isolated mycobacteria were *M. montefiorense/M. triplex*. These two species are very closely related and unable to be accurately differentiated over 800bp of the 16S rRNA gene. *M. montefiorense* has been recently been reported as the causative organism of chronic granulomatous disease in eels (Levi *et al.* 2003). Levi and others hypothesize the natural ecology of *M. montefiorense* may be marine environments. However, the results of this study suggest that *M. montefiorense* is also found in soil. To our knowledge, this is the first report of *M. montefiorense* isolation in New Zealand. To date no human infections have been reported by *M. montefiorense/M. triplex* in New Zealand (Appendix 3).

Peat is high in fulvic and humic acids and is known to be a rich source of mycobacteria (Kirshner *et al.* 1999). It is often used as bedding material in piggeries and is usually heat-treated to kill MAIC and prevent infections in pigs. When peat is used for sale as potting soil it is usually not heat-treated and contains a high mycobacterial load (Matlova *et al.* 2005). A study by Brauer *et al.* (1999) showed that peat samples contained viable MAIC isolates belonging to the genotypes associated with human and animal infections. The high numbers of mycobacteria recovered from potting mix in this project (Fig. 11 and Table 8) support these findings. Further work is needed to assess which MAIC genotypes are predominant in the environment and in MAIC human infections in New Zealand.

4.2 Identification of Mycobacteria

The majority of 16S rDNA identifications were approximately 600-800bp and exhibited greater than 99% identity to known sequences (Appendix 2). All isolates belonged to the genus *Mycobacterium* and most were able to be assigned to a known species.

The native forests samples generated the greatest number of isolates unable to be designated a known species and were simply classified as *Mycobacterium* species (Table 9). This result indicates a high probability of new species in this environment.
To investigate if the *Mycobacterium* species from the different environments were similar, Clustal X Sequence alignment was performed on isolates of similar length. Isolates 74, 77 and 227 were the most noticeably different to the remaining isolates across 408-419 bp region (Appendix 4). Isolates 7, 58, 76, 79, 84, 217, 219, 221, and 222 are likely to be the same isolate as they are very similar throughout the whole sequence (Appendix 4). Isolates 74 and 77 are the same but different to the remaining isolates and isolate 227 is completely different to any other isolates. These results suggest that the16 isolates shown in Table 9 (those unable to be speciated further than *Mycobacterium* species) likely comprise three potentially novel species.

A search for highly similar sequences (MEGABLAST Search) found that isolates 74 and 77 most closely resembled *M. kumamotensense* (Appendix 5). *M. kumamotensense* is an unpublished mycobacterium first isolated in 2005 in Japan (GenBank Accession Number AB239925).

Isolate 227 most closely resembled *M. intracellulare* using MEGABLAST (Appendix 6). However, the isolate did not resemble *M. intracellulare* phenotypically. The remainder of the isolates could not be speciated further than *Mycobacterium* species.

Table 9: 16S rDNA results of isolates most closely resembling *Mycobacterium* species.

Isolate	No. of base pairs	(%) Homology	Source
7	674/675[δ]	99	Urban
13*	266/268	99	Pot mix
58	694/695	99	Urban
64*	400/400	100	Urban
74	717/720	99	Pot mix
76	714/716	99	Pot mix
77	703/705	99	Pot mix
79	670/675	99	Urban
84	706/706	100	Native forest
217	667/668	99	Native forest
219	718/718	100	Native forest
220	716/716	100	Native forest
221	718/718	100	Native forest
222	712/712	100	Native forest
227	729/734	99	Urban
243*	327/333	99	Urban

* Sequences too short to include in Clustal X multiple profile alignment.
δ 674 nucleotides of 675 alignment identical (% indicates degree of homology to *Mycobacterium* species).

4.2.1 Discussion

The mycobacterial cell wall has been the major hindrance in the development of efficient and rapid procedures for the isolation of genomic DNA. The lipophilic cell wall of mycobacteria is responsible for the low permeability of the cell envelope and provides a formidable protective barrier. As a result of this cell envelope the standard methods for isolating DNA from Gram-negative and Gram-positive bacteria are not optimal for mycobacteria (Parish and Stoker 1998). This project used a commercial kit for DNA extraction. The DNA extraction is based on a chemical type of cell disruption (guanidinium-hydrochloride) in combination with the standard treatment of Proteinase-K and organic solvent extraction to remove proteins and cellular debris from the DNA.

Other methods of speciation such as hsp65 sequencing, RFLP analysis and biochemical tests were investigated during the course of this project. RFLP pattern analysis was trialled for *Mycobacterium* identification and the results were compared to the 16S rDNA sequencing results. RFLP analysis showed poor correlation with 16S rDNA results (Appendix 7). These other methods were either technically difficult to perform (RFLP analysis), time consuming (biochemical tests) or the available databases contained limited data or were poorly maintained (hsp65 sequencing and RFLP analysis). For these reasons 16S rDNA sequencing was chosen as the method of identification for NTM.

Although only 500-800 bp of the 16S rDNA were routinely sequenced, this allowed adequate identification for the purposes of this project and many clinical laboratories still choose to sequence only 500 bp for identification. Further work such as full 16S rDNA gene sequencing would be necessary to adequately differentiate closely related species such as those belonging to the MAIC or those belonging to *M. montefiorense/M. triplex* that differ only at nine positions across the whole 16S rDNA gene (Levi *et al.* 2003). For slow-growing mycobacteria, small differences such as these have been considered significant for the identification of a genetically unique and distinct taxon (Levi *et al.* 2003). Based on these findings it is reasonable to assume that some of the isolates in this project are novel. Alternatively, the small differences in base-pairs or the unidentified nucleotide ("N") found in an otherwise neatly generated sequence may be due to intra-species variation. Intra-species variation is a term expressing the concept of microheterogeneity within a species. Usually this denotes differences of less than a few base pairs per 16S rRNA gene sequence. The Family *Mycobacteriaceae* is closely related to the Family *Streptomycetaceae,* known for its microheterogeneity, especially in the *Streptomyces* genus. Many of the newly discovered mycobacteria differ from one another by only a few base-pairs, but even those small changes in the sequence seem to be correlated with unique phenotypic characteristics, clinical significance and niche (Tortoli 2003) as observed with the *Mycobacterium* species in this study.

There are no universally accepted criteria for 16S rRNA gene sequence-based identification of bacterial isolates. The level of sequence homology required between

the isolate under investigation and those in databases to conclude species identification is a matter of debate. This introduces a level of subjectivity in sequence-based identifications and, as a consequence, potential inaccuracies (Boudewijns *et al.* 2006).

Full 16S rRNA gene sequencing, hsp65 sequencing, RFLP analysis, HPLC mycolic acid analysis and biochemical testing are required for the description of a new mycobacterial species. Due to time and cost constraints these tests could not be performed on the isolates from this project. Further work is needed to identify the potentially novel isolates beyond the *Mycobacterium* species level.

Interestingly, some mycobacteria existed in co-culture with other non-acid-fast bacteria and were unable to be separated despite several attempts at purification (data not shown). As a result they were unable to be sequenced accurately and were identified as probable mycobacteria based on their phenotypical and biochemical characteristics. A bacterium dependant on another for growth will be unable to grow independently *in vitro*. Due to time and cost constraints, the identification of these mycobacterial co-cultures remains unknown.

4.3 Antibiotic Susceptibility of Selected Isolates

None of the environmental isolates of MAIC were resistant to clarithromycin as shown in Table 10 (i.e. MIC < 1 μg/ml). Some of the MAIC isolates failed to grow on sub-culture and therefore could not be tested. Approximately one quarter of the environmental MAIC isolates from this project were pigmented.

Table 10 : Susceptibility of MAIC environmental isolates to clarithromycin using the Alamar Blue Assay.

Isolate Number	Source	Pigmentation	MIC (*u*g/mL)	Interpretation
4	Potting mix	No	<1	S
23	Potting mix	No	<1	S
26	Potting mix	Yes	<1	S
39	Potting mix	Yes	<1	S
86	Peat	Yes	<1	S
90	Peat	No	<1	S
91	Peat	No	<1	S
94	Peat	No	<1	S
95	Peat	No	<1	S
96	Peat	No	<1	S
99	Peat	No	<1	S
105	Peat	No	<1	S
149	Peat	Yes	<1	S
153	Peat	Yes	<1	S
182	Peat	Yes	<1	S
205	Urban	No	-	NV
206	Urban	Yes	<1	S
208	Urban	Yes	<1	S
211	Urban	No	<1	S
218	Native	No	<1	S
224	Urban	No	<1	S
231	Urban	No	<1	S
234	Urban	No	<1	S
235	Urban	No	-	NV
241	Urban	No	<1	S
242	Urban	No	<1	S
243	Urban	No	-	NV

S, susceptible; NV, non-viable and unable to be tested.

4.3.1 Discussion

Kiehn *et al.* (1995) found that 86% of MAC isolates recovered from AIDS patients produced a deep yellow pigment. Stormer and Falkinham (1989) showed that 30% of soil MAC isolates produced deep yellow pigments. They also found that pigmented strains tend to grow more rapidly and are less drug resistant than non-pigmented strains of MAC. The pigmented strains of MAIC in this project did tend to grow faster than the non-pigmented strains as noted by Stormer (1989). Although none of the environmental isolates of MAIC demonstrated resistance to clarithromycin, this base-line susceptibility knowledge can be used as a sensitive marker for future antimicrobial resistance studies.

Chapter 5 Final Discussion and Conclusions

The aims of this project were to develop an optimal technique for the isolation of environmental mycobacteria, compare diversity of mycobacterial species from the different environments and to perform susceptibility testing on MAIC isolates. Knowledge of mycobacterial diversity and antimicrobial susceptibility has provided insights into the indigenous mycobacterial populations of New Zealand soil environments. Data gathered on existing mycobacterial populations, their environmental niche and susceptibility to antibiotics can be used to further explore the effect that human impact plays in the epidemiology of NTM.

The project used a combination of several decontamination procedures for the isolation mycobacteria from soil samples, (Isenberg 2004; Kubica 1975; Parashar *et al*. 2004; Ivanien 1995), as no single method was suitable. Many of the isolation methods available were based on the recovery of clinical mycobacteria. These methods were inappropriate because they focused on isolating pathogenic mycobacteria from human samples. Soil differs greatly to human samples in regard to the types of contaminating micro-flora present. Specialised treatments such as pre-incubation and cetrimide washes were necessary to eliminate contaminating micro-flora.

In addition to the differences in decontamination methods, the incubation lengths and temperatures required adjustment. Unlike the tubercle bacilli, the optimal temperature for many environmental mycobacteria is well below 37°C (human body temperature). Many NTM fail to grow at 37°C and/or have extremely slow growth which requires extensive incubation. Extensive incubation is uncommonly practiced in clinical microbiology. Therefore, to isolate environmental mycobacteria the incubation temperatures were lowered to 25-30°C and the incubation was extended for up to 12 weeks. Adaptation of the existing methods enabled successful isolation of diverse environmental mycobacteria, some of which have been described only recently, such as *M. arupense* (2006), *M. florentinum* (2005), *M. parmense* (2004), and *M. montefiorense* (2003).

This project has demonstrated that New Zealand soil environments are an abundant source of mycobacteria, especially MAIC. In addition to soil, mycobacteria are common in dust and organic debris not in contact with soil, as shown by the urban isolations. Humic and fulvic acid rich soils such as peat or potting mix contain large numbers of mycobacteria including those species associated with human disease. A study by Reed *et al.* (2006) found soil to be the major environmental reservoir for MAC associated with human infections. Exposure to water, food, or pets was not associated with infection. Further work is needed to assess if MAIC are also abundant in natural and potable waters in New Zealand. Information regarding which MAIC genotype predominates in the environment and human infections may shed light on the epidemiology and transmission of MAIC. These data could then be used to prevent activities associated with environmental mycobacteria infections in at-risk populations.

Interestingly, *Mycobacterium gordonae* is the second most common species isolated from human infections in New Zealand (Appendix 3). However, it was not isolated from any of the soil environments tested. The natural source of *M. gordonae* infections may be water-borne rather than soil. A reverse situation has been observed with *M. avium* infections in Africa. *M. avium* infection is low amongst African AIDS patients despite its presence in the soil and water (Falkinham 1996). Further investigation is necessary to discover the natural source of *M. gordonae* in the New Zealand environment.

Mycobacteria will continue to impact on human health as more people predispose themselves to environmental mycobacterial infections. This is due to the increasing use of disinfectants to "sterilise" habitats and which can select for mycobacteria by reducing competition, and the increasing numbers of immunocompromised individuals in the population, most notably, AIDS, the elderly, and organ-transplant patients. Conversely, human impact has also influenced the ecology of mycobacteria. An apparent reduction in *M. scrofulaceum* numbers in the human environment and its replacement by *M. avium* is a possible result of chlorination of drinking water (Primm 2004). Also of great interest is the high recovery rate of *M. montefiorense/M. triplex* from the New Zealand environment. These are relatively recently described mycobacteria associated with human and animal disease (Levi *et al.* 2003). This raises

the question of whether these organisms have always existed, but due to their slow growth rate have failed to be commonly recovered or, are they new and emerging pathogens.

Chapter 6 References

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Acknowledgements

I would like to acknowledge Assoc. Prof. Ian Maddox, who has served as an excellent supervisor throughout this entire study. I wish to thank my co-supervisor Dr Peter Wigley of BioDiscovery New Zealand Ltd, whose enthusiasm and intellect I admire. In addition, those staff of BioDiscovery New Zealand Ltd, who assisted me with the everyday requirements for execution of this study. In particular, scientists Damien Wright and Philippa Rhodes deserve mention for their help with the molecular microbiology. I refer as well to Sarah Sorrenson, whose support and editing skills have been greatly appreciated. I am particularly grateful to BioDiscovery for funding this study and to Massey University for allowing me to use equipment and consumables. Finally, I wish to acknowledge Ross Vaughan of the TB Reference Laboratory at Auckland Hospital, for his useful advice on working with mycobacteria.

Appendices

Appendix 1

The complete 16S rRNA gene of *M. tuberculosis* (Genbank Accession Number AM283534).

```
   1  GGAGTGTTTG GGTTTTGTTT GGAGAGTTTG ATCCTGGCTC AGGACGAACG CTGGCGGCGT
  61  GCTTAACACA TGCAAGTCGA ACGGAAAGGT CTCTTCGGAG ATACTCGAGT GGCGAACGGG
 121  TGAGTAACAC GTGGGTGATC TGCCCTGCAC TTCGGGATAA GCCTGGGAAA CTGGGTCTAA
 181  TACCGGATAG GACCACGGGA TGCATGTCTT GTGGTGGAAA GCGCTTTAGC GGTGTGGGAT
 241  GAGCCCGCGG CCTATCAGCT TGTTGGTGGG GTGACGGCCT ACCAAGGCGA CGACGGGTAG
 301  CCGGCCTGAG AGGGTGTCCG GCCACACTGG GACTGAGATA CGGCCCAGAC TCCTACGGGA
 361  GGCAGCAGTG GGGAATATTG CACAATGGGC GCAAGCCTGA TGCAGCGACG CCGCGTGGGG
 421  GATGACGGCC TTCGGGTTGT AAACCTCTTT CACCATCGAC GAAGGTCCGG GTTCTCTCGG
 481  ATTGACGGTA GGTGGAGAAG AAGCACCGGC CAACTACGTG CCAGCAGCCG CGGTAATACG
 541  TAGGGTGCGA GCGTTGTCCG GAATTACTGG GCGTAAAGAG CTCGTAGGTG GTTTGTCGCG
 601  TTGTTCGTGA AATCTCACGG CTTAACTGTG AGCGTGCGGG CGATACGGGC AGACTAGAGT
 661  ACTGCAGGGG AGACTGGAAT TCCTGGTGTA GCGGTGGAAT GCGCAGATAT CAGGAGGAAC
 721  ACCGGTGGCG AAGGCGGGTC TCTGGGCAGT AACTGACGCT GAGGAGCGAA AGCGTGGGGA
 781  GCGAACAGGA TTAGATACCC TGGTAGTCCA CGCCGTAAAC GGTGGGTACT AGGTGTGGGT
 841  TTCCTTCCTT GGGATCCGTG CCGTAGCTAA CGCATTAAGT ACCCCGCCTG GGGAGTACGG
 901  CCGCAAGGCT AAAACTCAAA GGAATTGACG GGGGCCCGCA CAAGCGGCGG AGCATGTGGA
 961  TTAATTCGAT GCAACGCGAA GAACCTTACC TGGGTTTGAC ATGCACAGGA CGCGTCTAGA
1021  GATAGGCGTT CCCTTGTGGC CTGTGTGCAG GTGGTGCATG GCTGTCGTCA GCTCGTGTCG
1081  TGAGATGTTG GGTTAAGTCC CGCAACGAGC GCAACCCTTG TCTCATGTTG CCAGCACGTA
1141  ATGGTGGGGA CTCGTGAGAG ACTGCCGGGG TCAACTCGGA GGAAGGTGGG GATGACGTCA
1201  AGTCATCATG CCCCTTATGT CCAGGGCTTC ACACATGCTA CAATGGCCGG TACAAAGGGC
1261  TGCGATGCCG CGAGGTTAAG CGAATCCTTA AAAGCCGGTC TCAGTTCGGA TCGGGGTCTG
1321  CAACTCGACC CCGTGAAGTC GGAGTCGCTA GTAATCGCAG ATCAGCAACG CTGCGGTGAA
1381  TACGTTCCCG GGCCTTGTAC ACACCGCCCG TCACGTCATG AAAGTCGGTA ACACCCGAAG
1441  CCAGTGGCCT AACCCTCGGG AGGGAGCTGT CGAAGGTGGG ATCGGCGATT GGGACGAAGT
1501  CGTAACAAGG TAGCCGTACC GGAAGGTGCG GCTGGATCAC CTCCTTTCT
```

Primer PB36F (forward) (positions 23-42bp)

Primer 16S2 (reverse) (positions 788-801bp)

Appendix 2

This table contains information on the NTM isolated during the course of the project.

Isolate No.	Medium	Decontam.	Source	Growth in weeks	16S rDNA ID
4	IH	B	Pot mix	4	MAIC 718/718 100%*
7	MB	B	Urban	4	Myco spp. 674/675 99%
12	MB	B	Pot mix	4	*M. montefiorense* 694/696 99%
13	IH	A	Pot mix	4	Myco spp 266/268
15	IH	B	Pot mix	6	*M. arupense* 683/689 99%
19	MB	A	Pot mix	6	MAIC 361/375 96%
23	IH	A	Pot mix	6	*M. nonchromogenicum* 711/720 98%
23	IH	A	Pot mix	6	MAIC 670/671 99%
26	MB	A	Pot mix	6	MAIC 668/677 98%
27	MB	B	Pot mix	4	*M. terrae* 369/375 98%
32	MB	B	Pot mix	3	*M. simiae* 606/634 95%
33	IH	A	Pot mix	4	*M. simiae* 707/710 99%
39	MB	B	Pot mix	2	MAIC 677/677 100%
44	MB	B	Peat	4	*M. septicum* 359/360 99%
49	MB	A	Urban	10	*M. simiae* 702/705 99%
51	MB	B	Pot mix	10	*M. simiae* 713/718 99%
52	MB	A	Pot mix	10	*M. montefiorense* 703/706 99%
53	MB	A	Pot mix	10	*M. monte/ M. triplex* 717/719 99%
57	MB	B	Pot mix	10	*M. monte/ M. triplex* 717/719 99%
58	IH	A	Urban	10	Myco spp 694/695 99%
59	MB	A	Pot mix	10	*M. lentiflavum* 714/718 99%
60	MB	B	Pot mix	10	*M. monte/ M. triplex* 717/719 99%
61	MB	B	Pot mix	10	*M. lentiflavum* 706/710 99%
63	MB	A	Urban	10	*M. simiae* 691/694 99%
64	MB	A	Urban	10	Myco spp. 400/400 99%
66	MB	B	Pot mix	10	*M. montefiorense* 721/724 99%
67	IH	A	Pot mix	10	*M. montefiorense* 709/712 99%
71	MB	B	Pot mix	10	*M. montefiorense* 720/722 99%
73	MB	B	Pot mix	10	*M. montefiorense* 710/712 99%
74	MB	A	Pot mix	10	Myco spp 717/720 99%
75	MB	B	Pot mix	10	*M. simiae* 707/710 99%

Isolate No.	Medium	Decontam.	Source	Growth in weeks	16S rDNA ID
76	MB	A	Pot mix	10	Myco spp 714/716 99%
77	MB	A	Pot mix	10	Myco spp 703/705 99%
78	MB	B	Peat	4	*M. montefiorense* 698/701 99%
79	MB	B	Urban	12	Myco spp 670/675 99%
80	MB	B	Native	8	*M. montefiorense* 705/708 99%
83	MB	B	Peat	4	*M. florentinum 712/714 99%*
84	IH	A	Native	6	*Myco spp 706/706 100%*
85	MB	B	Peat	5	*M. florentinum 696/698 99%*
86	MB	A	Peat	5	MAIC 715/718 99%
87	MB	A	Peat	5	*M. lentiflavum* 709/712 99%
88	MB	A	Peat	5	*M. lentiflavum* 703/706 99%
90	MB	A	Peat	5	MAIC 723/726 99%
91	MB	A	Peat	5	MAIC 730/734 99%
92	IH	A	Peat	5	*M. montefiorense* 710/716 99%
94	MB	B	Peat	5	MAIC 729/735 99%
95	MB	B	Peat	5	MAIC 729/733 99%
96	MB	A	Peat	5	MAIC 716/718 99%
99	MB	B	Peat	5	MAIC 721/735 99%
101	MB	B	Peat	5	MAIC 718/721 99%
102	MB	B	Peat	5	*M. montefiorense* 727/728 99%
105	MB	B	Peat	5	MAIC 726/729 99%
106	MB	B	Peat	5	*M. simiae* 703/708 99%
107	MB	B	Peat	5	*M. cookii* 712/724 98%
109	MB	B	Peat	5	MAIC 726/729 99%
110	MB	A	Peat	5	MAIC 717/719 99%
111	MB	A	Peat	5	MAIC 730/734 99%
112	MB	B	Peat	5	MAIC 724/726 99%
113	MB	B	Peat	5	MAIC 717/719 99%
114	MB	B	Peat	5	MAIC 710/713 99%
116	MB	B	Peat	5	MAIC 660/663 99%
117	MB	A	Peat	5	*M. lentiflavum* 706/712 99%
119	MB	B	Peat	5	*M. lentiflavum* 698/701 99%
120	MB	B	Peat	5	MAIC 731/734 99%
122	MB	B	Peat	5	MAIC 719/722 99%
123	MB	B	Peat	5	*M. montefiorense* 719/719 100%
145	MB	B	Peat	5	*M. montefiorense* 665/666 99%
147	MB	A	Peat	5	*M. cookii* 711/723 98%

Isolate No.	Medium	Decontam.	Source	Growth in weeks	16S rRNA ID
149	IH	A	Peat	5	MAIC 733/738 99%
151	MB	A	Peat	5	MAIC complex 98%
152	MB	A	Peat	5	*M. scrofulaceum* 706/708 99%
153	IH	A	Peat	5	MAIC 736/742 99%
160	MB	A	Peat	5	MAIC 703/703 100%
170	IH	B	Peat	4	*M. montefiorense* 717/717 100%
172	MB	A	Peat	4	MAIC 707/709 99%
175	IH	B	Peat	4	*M. simiae* 691/696 99%
177	MB	A	Peat	4	MAIC 699/700 99%
178	IH	B	Peat	4	*M. parmense* 575/580 99%
180	IH	B	Peat	4	*M. montefiorense* 719/724 99%
182	IH	B	Peat	4	MAIC 721/726 99%
187	IH	B	Peat	4	*M. florentinum* 711/716 99%
194	MB	A	Peat	4	MAIC 387/390 99%
195	IH	B	Peat	4	*M. montefiorense* 712/712 100%
196	MB	A	Peat	4	MAIC 720/722 99%
197	MB	A	Peat	4	*M. scrofulaceum* 703/705 99%
198	IH	A	Peat	4	MAIC 397/402 98%
199	IH	A	Peat	4	*M. montefiorense* 726/727 99%
200	IH	A	Peat	4	*M. montefiorense* 687/697 98%
203	IH	A	Peat	4	MAIC 718/722 99%
204	IH	A	Peat	4	MAIC 722/724 99%
205	MB	B	Urban	2	MAIC 525/539 97%
206	MB	A	Urban	2	MAIC 387/390 99%
207	IH	A	Urban	2	*M. simiae* 694/697 99%
208	MB	A	Urban	4	MAIC 722/728 99%
210	IH	A	Peat	2	*M. lentiflavum* 704/711 99%
211	MB	B	Urban	2	MAIC 721/724 99%
215	MB	A	Urban	2	*M. asiaticum* 715/721 99%
216	MB	B	Native	4	*M. lentiflavum* 689/696 96%
217	MB	B	Native	4	Myco spp 667/668 99%
218	IH	B	Native	4	MAIC 364/372 97%
219	IH	A	Native	4	Myco spp 718/718 100%
220	MB	B	Native	4	Myco spp 716/716 100%
221	MB	B	Native	4	Myco spp 718/718 100%
222	MB	B	Native	4	Myco spp 712/712 100%
223	IH	A	Peat	4	*M. lentiflavum* 716/719 99%

Isolate No.	Medium	Decontam.	Source	Growth in weeks	16S rRNA ID
224	MB	B	Urban	4	MAIC 727/730 99%
225	IH	A	Peat	4	MAIC 724/728 99%
226	IH	A	peat	4	MAIC 716/718 99%
227	MB	B	Urban	4	Myco spp 729/734 99%
228	MB	B	Urban	4	*M. asiaticum* 717/724 99%
229	MB	A	Urban	4	*M. asiaticum* 694/701 99%
230	MB	A	Peat	10	MAIC 720/726 99%
231	MB	A	Urban	4	MAIC 715/718 99%
232	IH	A	Peat	4	*M. montefiorense* 7713/714 99%
234	MB	A	Urban	4	MAIC 729/730 99%
235	MB	B	Urban	4	MAIC 725/728 99%
236	MB	B	Peat	10	*M. montefiorense* 712/712 100%
238	MB	B	Peat	10	*M. montefiorense* 712/712 100%
241	MB	A	Urban	4	MAIC 729/732 99%
242	IH	B	Urban	4	MAIC 653/661 98%
243	MB	A	Urban	4	Myco spp 327/333 99%
247	IH	B	Peat	4	*M. montefiorense* 717/717 100%

MB, Middlebrook 7H10 agar; IH, in-house medium; Myco spp, *Mycobacterium* species; MAIC, *Mycobacterium avium intracellulare* complex.

* i.e. 718 nucleotides of 718 alignment identical (% indicates degree of homology of isolate to known *Mycobacterium* stated).

Appendix 3

123 mycobacteria were submitted for identification and susceptibility testing to the New Zealand TB Reference Laboratory over a three year period (01/01/03-28/04/06). Organisms submitted for identification and susceptibility testing were considered to be clinically significant.

Organism name	Growth rate	No. of Patients
M. abscessus	R	8
Mycobacterium spp.	S	6
M. gordonae	S	19
M. kansasii	S	5
M. marinum	S	2
M. scrofulaceum	S	4
M. terrae complex	S	3
M. xenopi	S	2
MAIC	S	47
M. fortuitum	R	9
M. chelonae	R	12
M. asiaticum	S	1
M. nonchromigenicum	S	1
M. lentiflavum	S	1
M. palustre	S	1
M. branderi	S	1
M. engbaekii	S	1
Total		**123**

S, slow-grower; R, rapid-grower

Appendix 4

CLUSTAL X (1.83) multiple sequence alignment for isolates unable to be speciated further than *Mycobacterium* species by 16S rDNA sequencing. Isolates with complete sequences of similar length only were included in the analysis.

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CLUSTAL X (1.83) multiple sequence alignment


84              AACATGCA-GTCGA-CGGAAAGGCCTCTTCGGAGGTACTCGAGTGGCGAACGGGTGAGTA
76              TAAATGCA-GTCGA-CGGAAAGGCCTCTTCGGAGGTACTCGAGTGGCGAACGGGTGAGTA
219             -----GCAAGTCGAACGGAAAGGCCTCTTCGGAGGTACTCGAGTGGCGAACGGGTGAGTA
221             --CATGCAAGTCGAACGGAAAGGCCTCTTCGGAGGTACTCGAGTGGCGAACGGGTGAGTA
79              -------AAGTCGNACGGAAAGGCCTCTTCNGAGGTACTCGAGTGGCGAACGGGTGAGTA
58              -----TGCAGTCGAACGGAAAGGCCTCTTCGGAGGTACTCGAGTGGCGAACGGGTGAGTA
222             -------CAGTCGAACGGAAAGGCCTCTTCGGAGGTACTCGAGTGGCGAACGGGTGAGTA
7               -------CAGTCGAACGGAAAGGCCTCTTCGGAGGTACTCGAGTGGCGAACGGGTGAGTA
217             -----TGCAGTCGAACGGAAAGGCCTCC-CGGAGGTACTCGAGTGGCGAACGGGTGAGTA
227             ------GCAGTCGAACGGAAAGGCCTCTTCGGAGGTACTCGAGTGGCGAACGGGTGAGTA
74              ------GCAGTCGA-CGGAAAGGCCCTTTCGGGGGTNCTCGAGTGGCGAACGGGTGAGTA
77              ------GCAGTCGAACGGAAAGGCCCTTTCGGGGGTACTCGAGTGGCGAACGGGTGAGTA
                      ****  **********     * * *** ************************

84              ACACG-TGGGTAATCTGCCCTGCACTTCGGGATAAGCCTGGGAAACTGGGTCTAATACCG
76              ACACG-TGGGTAATCTGCCCTGCACTTCGGGATAAGCCTGGGAAACTGGGTCTAATACCG
219             ACACG-TGGGTAATCTGCCCTGCACTTCGGGATAAGCCTGGGAAACTGGGTCTAATACCG
221             ACACG-TGGGTAATCTGCCCTGCACTTCGGGATAAGCCTGGGAAACTGGGTCTAATACCG
79              ACACG-TGGGTAATCTGCCCTGCACTTCGGGATAAGCCTGGGAAACTGGGTCTAATACCG
58              ACACG-TGGGTAATCTGCCCTGCACTTCGGGATAAGCCTGGGAAACTGGGTCTAATACCG
222             ACACG-TGGGTAATCTGCCCTGCACTTCGGGATAAGCCTGGGAAACTGGGTCTAATACCG
7               ACACGATGGGTAATCTGCCCTGCACTTCGGGATAAGCCTGGGAAACTGGGTCTAATACCG
217             ACACG-TGGGTAATCTGCCCTGCACTTCGGGATAAGCCTGGGAAACTGGGTCTAATACCG
227             ACACG-TGGGCAATCTGCCCTGCACTTCGGGATAAGCCTGGGAAACTGGGTCTAATACCG
74              ACACG-TGGGTGATCTGCCCTGCACTCTGGGATAAGCCTGGGAAACTGGGTCTAATACNG
77              ACACG-TGGGTGATCTGCCCTGCACTCTGGGATAAGCCTGGGAAACTGGGTCTAATACTG
                ***** ****  **************  ******************************** *

84              AATATGACCACGAGGCGCATGCCTTGTGGTGGAAAGCTTTTGCGGTGTGGGATGGGCCCG
76              AATATGACCACGAGGCGCATGCCTTGTGGTGGAAAGCTTTTGCGGTGTGGGATGGGCCCG
219             AATATGACCACGAGGCGCATGCCTTGTGGTGGAAAGCTTTTGCGGTGTGGGATGGGCCCG
221             AATATGACCACGAGGCGCATGCCTTGTGGTGGAAAGCTTTTGCGGTGTGGGATGGGCCCG
79              AATATGACCACGAGGCGCATGTCTTGTGGTGGAAAGCTTTTGCGGTGTGGGATGGGCCCN
58              AATATGACCACGAGGCGCATGTCTTGTGGTGGAAAGCTTTTGCGGTGTGGGATGGGCCCG
222             AATATGACCACGAGGCGCATGCCTTGTGGTGGAAAGCTTTTGCGGTGTGGGATGGGCCCG
7               AATATGACCACGAGGCGCATGCCTTGTGGTGGAAAGCTTTTGCGGTGTGGGATGGGCCCG
217             AATACGACCATTTAGCGCATGCTTTATGGTGGAAAGCTTTTGCGGTGTGGGATGGGCCCG
227             AATATGACCTTTAGACGCATGTCTTTTGGTGGAAAGCTTTTGCGGTGTGGGATGGGCCCG
74              GATAGGACCATGGGATGCATGTTCTGTGGTGGAAAGCTTTTGCGGTGTGGGATGGGCCCG
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77          GATAGGACCATGGGATGCATGTTCTGTGGTGGAAAGCTTTTGCGGTGTGGGATGGGCCCG
             *** ****       *****   * **********************************

84          CGGCCTATCAGCTTGTTGGTGGGGTGACGGCCTACCAAGGCGACGACGGGTAGCCGGCCT
76          CGGCCTATCAGCTTGTTGGTGGGGTGACGGCCTACCAAGGCGACGACGGGTAGCCGGCCT
219         CGGCCTATCAGCTTGTTGGTGGGGTGACGGCCTACCAAGGCGACGACGGGTAGCCGGCCT
221         CGGCCTATCAGCTTGTTGGTGGGGTGACGGCCTACCAAGGCGACGACGGGTAGCCGGCCT
79          CGGCCTATCAGCTTGTTGGTGGGGTGACGGCCTACCAAGGCGACGACNGGTAGCCGGCCT
58          CGGCCTATCAGCTTGTTGGTGGGGTGACGGCCTACCAAGGCGACGACGGGTAGCCGGCCT
222         CGGCCTATCAGCTTGTTGGTGGGGTGACGGCCTACCAAGGCGACGACGGGTAGCCGGCCT
7           CGGCCTATCAGCTTGTTGGTGGGGTGACGGCCTACCAAGGCGACGACGGGTAGCCGGCCT
217         CGGCCTATCAGCTTGTTGGTGGGGTGACGGCCTACCAAGGCGACGACGGGTAGCCGGCCT
227         CGGCCTATCAGCTTGTTGGTGGGGTGATGGCCTACCAAGGCGACGACGGGTAGCCGGCCT
74          CGGCCTATCAGCTTGTTGGTGGGGTGATGGCCTACCAAGGCGACGACGGGTAGCCGGCCT
77          CGGCCTATCAGCTTGTTGGTGGGGTGATGGCCTACCAAGGCGACGACGGGTAGCCGGCCT
            *************************** ****************** ************

84          GAGAGGGTGTCCGGCCACACTGGGACTGAGATACGGCCCAGACTCCTACGGGAGGCAGCA
76          GAGAGGGTGTCCGGCCACACTGGGACTGAGATACGGCCCAGACTCCTACGGGAGGCAGCA
219         GAGAGGGTGTCCGGCCACACTGGGACTGAGATACGGCCCAGACTCCTACGGGAGGCAGCA
221         GAGAGGGTGTCCGGCCACACTGGGACTGAGATACGGCCCAGACTCCTACGGGAGGCAGCA
79          GAGAGGGTGTCCGGCCACACTGGGACTGAGATACNGCCCAGACTCCTACGGGAGGCAGCA
58          GAGAGGGTGTCCGGCCACACTGGGACTGAGATACGGCCCAGACTCCTACGGGAGGCAGCA
222         GAGAGGGTGTCCGGCCACACTGGGACTGAGATACGGCCCAGACTCCTACGGGAGGCAGCA
7           GAGAGGGTGTCCGGCCACACTGGGACTGAGATACGGCCCAGACTCCTACGGGAGGCAGCA
217         GAGAGGGTGTCCGGCCACACTGGGACTGAGATACGGCCCAGACTCCTACGGGAGGCAGCA
227         GAGAGGGTGTCCGGCCACACTGGGACTGAGATACGGCCCAGACTCCTACGGGAGGCAGCA
74          GAGAGGGTGTCCGGCCACACTGGGACTGAGATACGGCCCAGACTCCTACGGGAGGCAGCA
77          GAGAGGGTGTCCGGCCACACTGGGACTGAGATACGGCCCAGACTCCTACGGGAGGCAGCA
            ********************************** ************************

84          GTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGGGGGATGACG
76          GTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGGGGGATGACG
219         GTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGGGGGATGACG
221         GTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGGGGGATGACG
79          GTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGGGGGATGACG
58          GTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGGGGGATGACG
222         GTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGGGGGATGACG
7           GTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGGGGGATGACG
217         GTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGGGGGATGACG
227         GTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGGGGGATGACG
74          GTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGGGGGATGACG
77          GTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGGGGGATGACG
            ************************************************************

84          GCCTTCGGGTTGTAAACCTCTTTCAGCAGGGACGAAGC--GCAAG------------TGA
76          GCCTTCGGGTTGTAAACCTCTTTCAGCAGGGACGAAGC--GCAAG------------TGA
219         GCCTTCGGGTTGTAAACCTCTTTCAGCAGGGACGAAGC--GCAAG------------TGA
221         GCCTTCGGGTTGTAAACCTCTTTCAGCAGGGACGAAGC--GCAAG------------TGA
79          GCCTTCGGGTTGTAAACCTCTTTCANCAGGGACGAAGC--GCAAG------------TGA
58          GCCTTCGGGTTGTAAACCTCTTTCAGCAGGGACGAAGC--GCAAG------------TGA
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222          GCCTTCGGGTTGTAAACCTCTTTCAGCAGGGACGAAGC--GCAAG------------TGA
7            GCCTTCGGGTTGTAAACCTCTTTCAGCAGGGACGAAGC--GCAAG------------TGA
217          GCCTTCGGGTTGTAAACCTCTTTCAGCAGGGACGAAGC--GCAAG------------TGA
227          GCCTTCGGGTTGTAAACCTCTTTCACCATCGACGAAGG--TTCGGGTTTTCTCGGATTGA
74           GCCTTCGGGTTGTAAACCTCTTTCAGTATCGGCGAAGCTTGCGGGTTTTCTCGCAGGTGA
77           GCCTTCGGGTTGTAAACCTCTTTCAGTATCGGCGAAGCTTGCGGGTTTTCTCGCAGGTGA
             ************************* * * ***** * ***

84           CGGTACCTGCAGAAGAAGCACCGGCCAACTACGTGCCAGCAGCCGCGGTAATACGTAGGG
76           CGGTACCTGCAGAAGAAGCACCGGCCAACTACGTGCCAGCAGCCGCGGTAATACGTAGGG
219          CGGTACCTGCAGAAGAAGCACCGGCCAACTACGTGCCAGCAGCCGCGGTAATACGTAGGG
221          CGGTACCTGCAGAAGAAGCACCGGCCAACTACGTGCCAGCAGCCGCGGTAATACGTAGGG
79           CNGTACCTGCAGAAGAAGCACCGGCCAACTACGTGCCAGCAGCCGCGGTAATACGTAGGG
58           CGGTACCTGCAGAAGAAGCACCGGCCAACTACGTGCCAGCAGCCGCGGTAATACGTAGGG
222          CGGTACCTGCAGAAGAAGCACCGGCCAACTACGTGCCAGCAGCCGCGGTAATACGTAGGG
7            CGGTACCTGCAGAAGAAGCACCGGCCAACTACGTGCCAGCAGCCGCGGTAATACGTAGGG
217          CGGTACCTGCAGAAGAAGCACCGGCCAACTACGTGCCAGCAGCCGCGGTAATACGTAGGG
227          CGGTAGGTGGAGAAGAAGCACCGGCCAACTACGTGCCAGCAGCCGCGGTAATACGTAGGG
74           CGGTAGGTACAGAAGAAGCACCGGCCAACTACGTGCCAGCAGCCGCGGTAATACGTAGGG
77           CGGTAGGTACAGAAGAAGCACCGGCCAACTACGTGCCAGCAGCCGCGGTAATACGTAGGG
             * *** * ************************************************

84           TGCGAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGTGGTTTGTCGCGTTGTT
76           TGCGAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGTGGTTTGTCGCGTTGTT
219          TGCGAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGTGGTTTGTCGCGTTGTT
221          TGCGAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGTGGTTTGTCGCGTTGTT
79           TGCGAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGTGGTTTGTCGCGTTGTT
58           TGCGAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGTGGTTTGTCGCGTTGTT
222          TGCGAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGTGGTTTGTCGCGTTGTT
7            TGCGAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGTGGTTTGTCGCGTTGTT
217          TGCGAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGTGGTTTGTCGCGTTGTT
227          TGCGAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGTGGTTTGTCGCGTTGTT
74           TGCGAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGTGGTTTGTCGCGTTGTC
77           TGCGAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGTGGTTTGTCGCGTTGTC
             ************************************************************

84           CGTGAAAACCGGGGGCTTAACCCTCGGCGTGCGGGCGATACGGGCAGACTGGAGTACTGC
76           CGTGAAAACCGGGGGCTTAACCCTCGGCGTGCGGGCGATACGGGCAGACTGGAGTACTGC
219          CGTGAAAACCGGGGGCTTAACCCTCGGCGTGCGGGCGATACGGGCAGACTGGAGTACTGC
221          CGTGAAAACCGGGGGCTTAACCCTCGGCGTGCGGGCGATACGGGCAGACTGGAGTACTGC
79           CGTGAAAACCGGGGGCTTAACCCTCGGCGTGCGGGCGATACGGGCAGACTGGAGTACTGC
58           CGTGAAAACCGGGGGCTTAACCCTCGGCGTGCGGGCGATACGGGCAGACTGGAGTACTGC
222          CGTGAAAACCGGGGGCTTAACCCTCGGCGTGCGGGCGATACGGGCAGACTGGAGTACTGC
7            CGTGAAAACCGGGGGCTTAACCCTCGGCGTGCGGGCGATACGGGCAGACTGGAGTACTGC
217          CGTGAAAACCGGGGGCTTAACCCTCGGCGTGCGGGCGATACGGGCAGACTGGAGTACTGC
227          CGTGAAATCTCACGGCTTAACTGTGAGCGTGCGAGCGATACGGGCAGACTAGAGTACTGC
74           CGTGAAAACTCACAGCTCAACTGTGGGCGTGCGGGCGATACGGGCAGACTGGAGTACTGT
77           CGTGAAAACTCACAGCTCAACTGTGGGCGTGCGGGCGATACGGGCAGACTGGAGTACTGT
             ******* *     *** *** * ******* ********** ********

84           AGGGGAGACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGG
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76          AGGGGAGACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGG
219         AGGGGAGACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGG
221         AGGGGAGACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGG
79          AGGGGAGACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGG
58          AGGGGAGACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGG
222         AGGGGAGACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGG
7           AGGCGAGACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGG
217         AGGGGAGACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGG
227         AGGGGAGACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGG
74          AGGGGAGACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGG
77          AGGGGAGACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGG
            ************************************************************

84          TGGCGAAGGCGGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAA
76          TGGCGAAGGCGGGTCTCTGGGCAGTAACTGACGCTGANGAGCGAAAGCGTGGGGAGCGAA
219         TGGCGAAGGCGGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAA
221         TGGCGAAGGCGGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAA
79          TGGCGAAGGCGGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAA
58          TGGCGAAGGCGGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAA
222         TGGCGAAGGCGGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAA
7           TGGCGAAGGCGGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAA
217         TGGCGAAGGCGGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAA
227         TGGCGAAGGCGGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAA
74          TGGCGAAGGCGGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAA
77          TGGCGAAGGCGGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAA
            ************************************** ***********************

84          CAGGATTAGATACCC-------------
76          CAGGATTAGATACCC-GGGTAGTCCAAA
219         CAGGATTAGATACCCCNGGTAGTCCAG-
221         CAGGATTAGATACCC-GGGTAGTCCAC-
79          CAGGATTANATACCCAGGGTAA-TCCA-
58          CAGGATTAGATACCCGGGGTAAATCCAA
222         CAGGATTAGATACCC-------------
7           CAGGATTAGATACCCCGN-TANTCCAA-
217         CAGGATTAGATACCCCGG-TACTCCAA-
227         CAGGATTAGATACCCCGG-TAGTCCAA-
74          CAGGATTAGATACC--------------
77          CAGGATTAGATACCCCGGGTANTCCAA-
            ******** *****
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Highlighted region is at alignment position 409-418bp and indicates a possible DNA insertion. The asterix (*) denote regions of complete homology for the sequences.

Appendix 5

CLUSTAL X (1.83) multiple sequence alignment of isolates 74 and 77 with *M. kumamotonense* (GenBank Accession Number AB239925).

"*Mycobacterium kumamotonense* sp. nov. recovered from clinical specimen and the first isolation report of *Mycobacterium arupense* in Japan: novel slowly growing, nonchromogenic clinical isolates related to *Mycobacterium terrae* complex" Unpublished 2005.

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74              CGAACGGGTGAGTAACACG-TGGGTGATCTGCCCTGCACTCTGGGATAAGCCTGGGAAAC
77              CGAACGGGTGAGTAACACG-TGGGTGATCTGCCCTGCACTCTGGGATAAGCCTGGGAAAC
Mkum            CGAACGGGTGAGTAACACG-TGGGTGATCTGCCCTGCACTCTGGGATAAGCCTGGGAAAC
                ******************* ****************************************

74              TGGGTCTAATACNGGATAGGACCATGGGATGCATGTTCTGTGGTGGAAAGCTTTTGCGGT
77              TGGGTCTAATACTGGATAGGACCATGGGATGCATGTTCTGTGGTGGAAAGCTTTTGCGGT
Mkum            TGGGTCTAATACCGGATAGGACCATGGGATGCATGTTCTGTGGTGGAAAGCTTTTGCGGT
                ************ ************************************************

74              GTGGGATGGGCCCGCGGCCTATCAGCTTGTTGGTGGGGTGATGGCCTACCAAGGCGACGA
77              GTGGGATGGGCCCGCGGCCTATCAGCTTGTTGGTGGGGTGATGGCCTACCAAGGCGACGA
Mkum            GTGGGATGGGCCCGCGGCCTATCAGCTTGTTGGTGGGGTGATGGCCTACCAAGGCGACGA
                ************************************************************

74              CGGGTAGCCGGCCTGAGAGGGTGTCCGGCCACACTGGGACTGAGATACGGCCCAGACTCC
77              CGGGTAGCCGGCCTGAGAGGGTGTCCGGCCACACTGGGACTGAGATACGGCCCAGACTCC
Mkum            CGGGTAGCCGGCCTGAGAGGGTGTCCGGCCACACTGGGACTGAGATACGGCCCAGACTCC
                ************************************************************

74              TACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCG
77              TACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCG
Mkum            TACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCG
                ************************************************************

74              CGTGGGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGTATCGGCGAAGCTTGCGGGT
77              CGTGGGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGTATCGGCGAAGCTTGCGGGT
Mkum            CGTGGGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGTATCGGCGAAGCTTGCGGGT
                ************************************************************

74              TTTCTCGCAGGTGACGGTAGGTACAGAAGAAGCACCGGCCAACTACGTGCCAGCAGCCGC
77              TTTCTCGCAGGTGACGGTAGGTACAGAAGAAGCACCGGCCAACTACGTGCCAGCAGCCGC
Mkum            TTTCTCGCAGGTGACGGTAGGTACAGAAGAAGCACCGGCCAACTACGTGCCAGCAGCCGC
                ************************************************************
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74              GGTAATACGTAGGGTGCGAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGTGG
77              GGTAATACGTAGGGTGCGAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGTGG
Mkum            GGTAATACGTAGGGTGCGAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGTGG
                ************************************************************

74              TTTGTCGCGTTGTCCGTGAAAACTCACAGCTCAACTGTGGGCGTGCGGGCGATACGGGCA
77              TTTGTCGCGTTGTCCGTGAAAACTCACAGCTCAACTGTGGGCGTGCGGGCGATACGGGCA
Mkum            TTTGTCGCGTTGTCCGTGAAAACTCACAGCTCAACTGTGGGCGTGCGGGCGATACGGGCA
                ************************************************************

74              GACTGGAGTACTGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATC
77              GACTGGAGTACTGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATC
Mkum            GACTGGAGTACTGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATC
                ************************************************************

74              AGGAGGAACACCGGTGGCGAAGGCGGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAA
77              AGGAGGAACACCGGTGGCGAAGGCGGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAA
Mkum            AGGAGGAACACCGGTGGCGAAGGCGGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAA
                ************************************************************

74              GCGTGGGGAGCGAACAGGATTAGATACC------------------------------
77              GCGTGGGGAGCGAACAGGATTAGATACCCCGGGTANTCCAA------------------
Mkum            GCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGTACTA
                ****************************
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Mkum, *Mycobacterium kumamotonense*

Appendix 6

CLUSTAL X (1.83) multiple sequence alignment of isolate 227 with *M. intracellulare* (GenBank Accession Number AY859027.1).

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227             --------------GCA-GTCGAACGGAAAGGCCTCTTCGGAGGTACTCGAGTGGCGAAC
AY859027.1      CGTGCTTAACACATGCAAGTCGAACGGAAAGGCCCCTTCGGGGGTACTCGAGTGGCGAAC
                              *** ********** ****** ********** ********** *****

227             GGGTGAGTAACACGTGGGCAATCTGCCCTGCACTTCGGGATAAGCCTGGGAAACTGGGTC
AY859027.1      GGGTGAGTAACACGTGGGCAATCTGCCCTGCACTTCGGGATAAGCCTGGGAAACTGGGTC
                ************************************************************

227             TAATACCGAATATGACCTTTAGACGCATGTCTTTTGGTGGAAAGCTTTTGCGGTGTGGGA
AY859027.1      TAATACCGGATAGGACCTTTAGACGCATGTCTTTTGGTGGAAAGCTTTTGCGGTGTGGGA
                ******** *** ********************************************

227             TGGGCCCGCGGCCTATCAGCTTGTTGGTGGGGTGATGGCCTACCAAGGCGACGACGGGTA
AY859027.1      TGGGCCCGCGGCCTATCAGCTTGTTGGTGGGGTGATGGCCTACCAAGGCGACGACGGGTA
                ************************************************************

227             GCCGGCCTGAGAGGGTGTCCGGCCACACTGGGACTGAGATACGGCCCAGACTCCTACGGG
AY859027.1      GCCGGCCTGAGAGGGTGTCCGGCCACACTGGGACTGAGATACGGCCCAGACTCCTACGGG
                ************************************************************

227             AGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGGG
AY859027.1      AGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGGG
                ************************************************************

227             GGATGACGGCCTTCGGGTTGTAAACCTCTTTCACCATCGACGAAGGTTCGGGTTTTCTCG
AY859027.1      GGATGACGGCCTTCGGGTTGTAAACCTCTTTCACCATCGACGAAGGTTCGGGTTTTCTCG
                ************************************************************

227             GATTGACGGTAGGTGGAGAAGAAGCACCGGCCAACTACGTGCCAGCAGCCGCGGTAATAC
AY859027.1      GATTGACGGTAGGTGGAGAAGAAGCACCGGCCAACTACGTGCCAGCAGCCGCGGTAATAC
                ************************************************************

227             GTAGGGTGCGAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGTGGTTTGTCGC
AY859027.1      GTAGGGTGCGAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGTGGTTTGTCGC
                ************************************************************

227             GTTGTTCGTGAAATCTCACGGCTTAACTGTGAGCGTGCGAGCGATACGGGCAGACTAGAG
AY859027.1      GTTGTTCGTGAAATCTCACGGCTTAACTGTGAGCGTGCGGGCGATACGGGCAGACTAGAG
                ************************************ ********************

227             TACTGCAGGGGAGACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAA
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AY859027.1      TACTGCAGGGGAGACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAA
                ************************************************************

227             CACCGGTGGCGAAGGCGGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAAGCGTGGGG
AY859027.1      CACCGGTGGCGAAGGCGGGTCTCTGGGCAGTAACTGACGCTCAGGAGCGAAAGCGTGGGG
                ************************************************************

227             AGCGAACAGGATTAGATACCCCGGTAGTCCAA-----
AY859027.1      AGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGT
                ********************* *********
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The asterix (*) denote regions of complete homology for the sequences

Appendix 7

Polymorphism Restriction Analysis (PRA) and the online database www.praonline.fiocruz.br were used to generate RFLP patterns (Telenti *et al.* 1993).

Isolate	BstEII digest	HaeII digest	ID by RFLP	ID by 16S rDNA
7	310/235/205/110	140/155 *	NO ID	*Myco spp.* 674/675 99%
12	235/205	130/105	NO ID	*M. montefiorense* 694/696 99%
19	235/205	140/120/100	*M. peregrinum*	MAIC 361/375 96%
23	115/305	60/140/145	NO ID	MAIC 670/671 99%
33	325/115	130/145	NO ID	*M. simiae* 707/710 99%
44	245/220	155/150/100/60	*M. peregrinum*	*M. septicum* 359/360 99%
49	320 125	145 125 80 55	*M. simiae*	*M. simiae* 702/705 99%
63	320 115	125 100	*M. genevense*	*M. simiae* 691/694 99%
77	320 115	125 110 60	*M. gordonae*	*Myco* spp 703/705 99%
85	441	125 145	* *NO ID	*M. florentinum* 696/698 99%
197	441	125 170(?160)	*M. triviale*	*M. scrofulaceum* 703/705 99%
205	441	130/80/60	NO ID	MAIC 525/539 97%
216	441	100 125	*M. szulgai*	*M. lentiflavum* 689/696 96%

BstEII and HaeII, restriction enzymes; ID, identification; NO ID, isolate could not be identified by its band pattern using the online database.
MAIC, *M. avium-intracellulare* complex.

*i.e. 140/155 is a restriction digest banding pattern indicating two bands were observed, one at position 140, the other at position 155. The band pattern is visually determined by comparing the unknown isolates bands to control cultures with known banding patterns or to DNA ladders with known banding patterns.

** The PRA profile for *M. florentinum* was not included in the online database most likely due to the organism being recently described. However, the RFLP pattern was published in International Journal of Systematic and Evolutionary Microbiology (Tortoli *et al.* 2005) and the isolate from this project had the same pattern.