MOLECULAR ANALYSIS OF *MYCOBACTERIUM KANSASII* FROM HUMAN AND POTABLE WATER SPECIMENS

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ABSTRACT

Environmental mycobacteria are a group of human and animal pathogens that have significant impacts on the morbidity and mortality of humans. The prevalence of pulmonary disease due to environmental mycobacteria is increasing. This growth is due to increased identification of mycobacteria as pulmonary pathogens, improvements in methods of detection with culture, as well as an ageing population. Exposure is also likely to be responsible, as the organisms have been isolated from environmental sources such as water, biofilms, soil and aerosols. Humans are exposed to mycobacteria in water through drinking, swimming and bathing. Aerosols generated during these activities can be inhaled, and water can be aspirated when swallowed. *Mycobacterium kansasii* from drinking water samples have been linked to outbreaks of pulmonary disease. However, it is not clear if the organisms found in water samples are the same as those that cause disease in humans.

From this study we were able to successfully type both human and environmental strains of *M. kansasii*. ITS strain types I, IV and V were found among the QLD patient isolates and Brisbane Water isolates. Newer typing methods delivered better strain differentiation than ITS-REA, however they have limitations. Municipal water in Brisbane is unlikely to be the source of infection for patients with *M. kansasii* disease. However, water contamination with *M. kansasii* cannot be excluded from other areas associated with mining and industry.

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LIST OF ABBREVIATIONS

AFB – Acid-Fast Bacilli

BD – Becton, Dickenson and Company, USA

Dubos Broth – an enrichment broth used to store *M. kansasii* isolates

HRM – High Resolution Melt Analysis

ITS-REA – Internally Transcribed Spacer Region with Restriction Enzyme Analysis

MGIT – Mycobacterium Growth Indicator Tube

NTM – Non-tuberculous Mycobacteria

PFGE – Pulsed Field Gel Electrophoresis

PCR – Polymerase Chain Reaction

PRA-*hsp65* – PCR restriction fragment analysis of the 65-kDa heat shock protein

Rep-PCR – Repetitive PCR

RFLP – Restriction Fragment Length Polymorphism

QMRL – Queensland Mycobacterium Reference Laboratory

STATEMENT OF ORIGINAL AUTHORSHIP

The work contained in this thesis partially has not been previously submitted to meet requirements for an award at this higher education institution. To the best of my knowledge and belief, the thesis contains some material that has been previously published or written by another person as this is a part of a much larger research project.

QUT Verified Signature

Signature

Carla Elise Tolson

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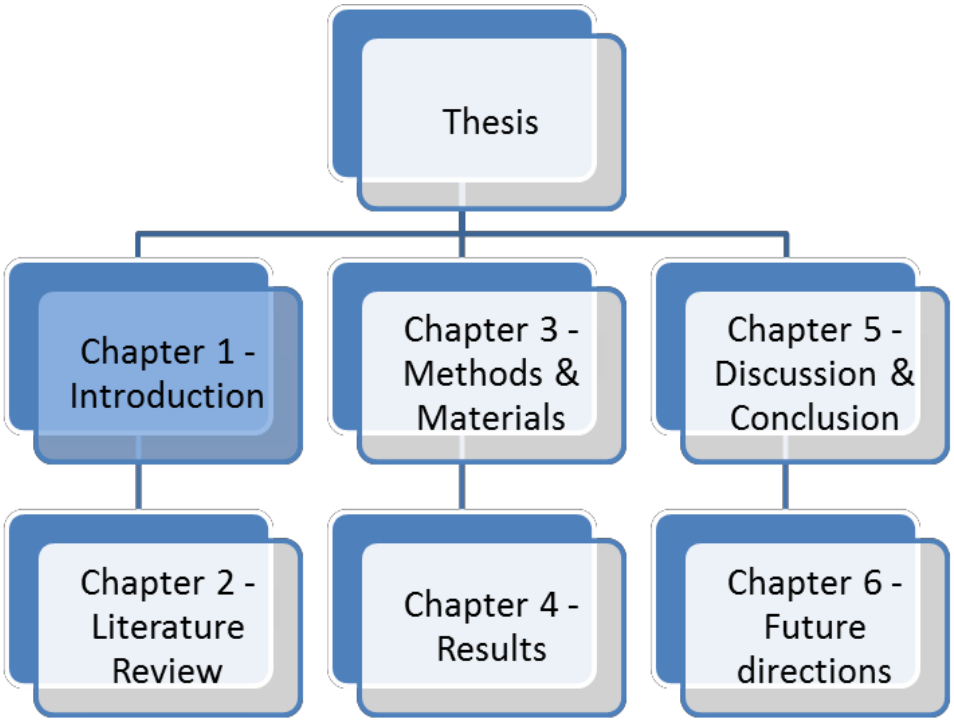
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CHAPTER 1: INTRODUCTION

1.1 Background

Non-tuberculous mycobacteria (NTM) are opportunistic pathogens, and are a major cause of pulmonary and extra-pulmonary disease in humans. They are commonly isolated from environmental sources and are not considered to be transmitted by person-person contact, unlike their relative *Mycobacterium tuberculosis* (Griffith *et al.* 2007). NTM don't always cause significant disease in humans, and their presence is often regarded as colonisation/contamination unless there are clinical and radiological features to help prove disease. Colonisation can be defined as a migration of microorganisms successfully creating a community in a natural environment where they can thrive and survive such as the human pulmonary system. Contamination is seen as this colonised community creating an unwanted presence in any natural environment, hence why these terms have been used interchangeably.

Currently there are more than 150 species of NTM described in the literature (Tortoli 2006). Molecular techniques have improved significantly recently, resulting in an increase of the identification of new species. Presently, the American Thoracic Society and Infectious Disease Association of America (ATS/IDSA) have treatment recommendations for only 20 species of NTM (Griffith *et al.* 2007). However, treatment may not be required for all isolates. From the most recent figures published in Queensland, significant public health notifiable NTM disease has increased from 2.2 (1999) to 3.2 (2005) per 100,000 population (Thomson 2010). Given that the incidence of NTM is increasing in an ageing population (Griffith *et al.* 2007), the importance of further work in establishing the source of this increase is critical. Looking particularly at *M. kansasii*, it has been hypothesised that the source is environmental as organisms have been previously isolated from tap water sources (Engel *et al.* 1980). No definitive link has been established to conclusively prove that water is the source of human disease.

1.2 Context

The major focus of this study is to identify the genotypes of *M. kansasii* found in patients and potable water samples from Brisbane and throughout Queensland, Australia. A problem area of research is the lack of an assay that can produce an accurate strain type in a clinical laboratory that is cost and time effective. Also in Queensland as well globally, there is a lack of information regarding *M. kansasii* infections with respect to the prevalence in the environment and linking this to causing disease in humans. We hypothesise that newly developed DNA-based genotyping methods will enable us to characterise and differentiate clinically and environmentally sourced strains of *M. kansasii.*

1.3 Study Aims

There are two main aims with the following objectives:

Aim 1 – To genotype *M. kansasii* isolates using new and existing genotyping methods

Objective 1.1 – Apply the DiversiLab method to genotyped clinical and environmental *M. kansasii* strains

Objective 1.2 – Genotype clinical and environmental *M. kansasii* strains using an Internal Transcribed Spacer Region and Restriction Enzyme analysis (ITS-REA) method

Objective 1.3 – Develop and apply High Resolution Melt Analysis to genotyped clinical and environmental *M. kansasii* strains

Aim 2 – To compare the strain genotypes of *M. kansasii* isolates from clinical samples to those obtained from Brisbane water and patient home water samples

Objective 2.1 – Compare the discriminatory capability of each genotyping method

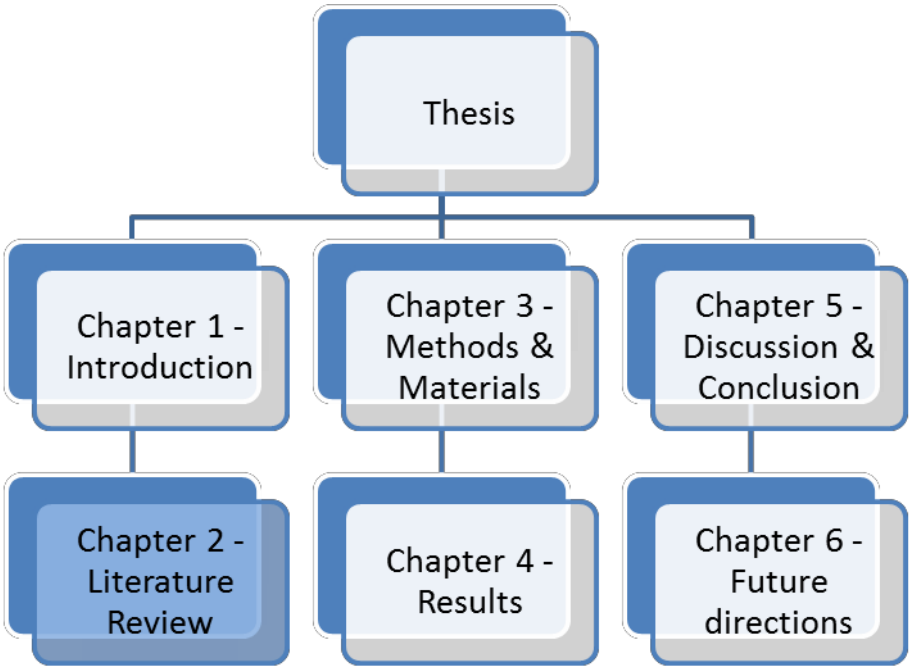
Objective 2.2 – Correlate the clinical *M. kansasii* strain genotypes to strain types from the patient home and Brisbane water samples

1.4 Significance and Scope

Currently worldwide, there are at least seven *M. kansasii* strain types identified using the established typing schemes for this species. Exhaustive typing has been done on type I isolates producing many subtypes, however, questions remain including: are they all pathogenic? Are these pathogenic strains only recovered from humans or are they found in the environment as well? Knowing which strain type the patient has may have an impact on patient therapy (Picardeau *et al.* 1997). Identifying the particular type strain of *M. kansasii* the patient has may help a clinician in managing the patients' infection e.g. the virulence of particular strains may affect the decision to treat. Treatment is a lengthy course of anti-tuberculous drugs described in detail in section 2.1.2.

The reservoir for *M. kansasii* in the environment is still poorly understood and a conclusive relationship between water exposure and infection has not been proven. This study was the first of its kind in Australia where both clinical and water isolates were compared at the same time. The main purpose of this study was to determine the strain similarities of environmental and clinical *M. kansasii* isolates.

There is much to discover with regards to *M. kansasii* strain types as there have been only a handful of papers published specifically on *M. kansasii*. There is literature available on type I strain of *M. kansasii* but very little is known about strain types II, III, IV, V and VI. This begs the question of whether all strain types can cause disease like the most commonly reported type I strain. Considering the recent progress in molecular typing methods, there is a justification to apply these methods to characterise *M. kansasii* strains accurately. Previous methods are very time consuming, difficult to perform and costly. For a routine diagnostic laboratory setting, rapid and cost-effective methods are vital.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CHAPTER 2: LITERATURE REVIEW

2.1 *Mycobacterium kansasii* and its clinical presentation

2.1.1 *M. kansasii* disease

M. kansasii is the fourth most common *Mycobacterium* species isolated in Queensland (Thomson 2010). *M. kansasii* often presents as pulmonary disease however it can cause disseminated disease in immunosuppressed humans (Griffith *et al.* 2007).

Up to 60% of pulmonary isolates identified as non-tuberculous mycobacteria (NTM) are not considered responsible for disease (Thomson 2010). It is believed that some patients are colonized by these bacteria, with little or no impact on the host. Many other isolates are considered contaminants from either patient or laboratory (Falkinham 1996). It is important to differentiate NTM from its relative *M. tuberculosis*, as NTM can clinically present like tuberculosis (TB). Unlike TB, NTM's are considered non-contagious (Falkinham 1996). Figure 1 shows a comparison of a normal X-ray to NTM diseased lung (Figure 2), which has increased opacity in the right upper lobe as indicated by the arrow.

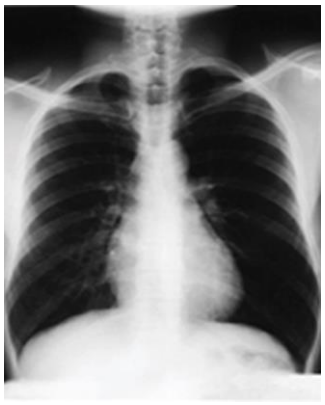


FIGURE 1: NORMAL CHEST X-RAY. IMAGE: IWONA GRODZKA - NPS.ORG.AU

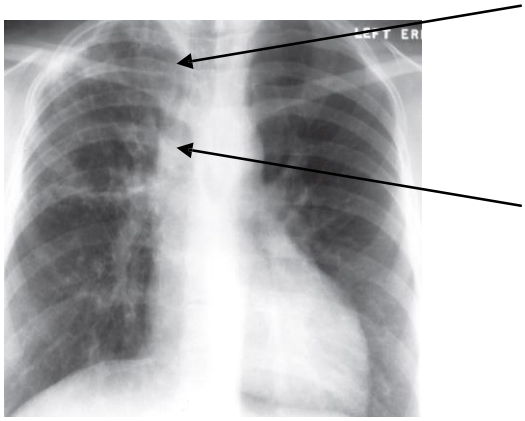


FIGURE 2: PATIENT WITH *M. KANSASII* LUNG DISEASE. IMAGE FROM: (ERASMUS *ET AL.* 1999)

M. kansasii not only causes pulmonary disease, it is also associated with disseminated disease. *M. kansasii* is the second most frequent NTM that causes disseminated disease in patients with Human Immunodeficiency Virus/Acquired

Immune Deficiency Syndrome (HIV/AIDS) (Horsburgh *et al.* 1989). Determining the significance of NTM isolation in any patient is difficult and requires a combination of laboratory, clinical and radiographic criteria (Griffith *et al.* 2007). Because of the high level of difficulty in diagnosing NTM disease, a Special Interest Group in the USA developed the American Thoracic Guidelines: *An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases* (Griffith *et al.* 2007).

Table **1** below shows the criteria required for diagnosing NTM lung disease. Table **2** demonstrates the strength shown for an accurate amount of evidence for a correct diagnosis.

TABLE 1: CRITERIA FOR NTM LUNG DISEASE DIAGNOSIS

Clinical (both required) 1.Pulmonary symptoms, nodular or cavitary opacities on chest radiograph, or a high-resolution computed tomography scan that shows multifocal bronchiectasis with multiple small nodules (A, I)* AND 2.Appropriate exclusion of other diagnoses (A, I)
Microbiologic 1.Positive culture results from at least two separate expectorated sputum samples (A, II). If the results from (1) are nondiagnostic, consider repeat sputum AFB smears and cultures (C, III). OR 2.Positive culture result from at least one bronchial wash or lavage (C, III) OR 3.Transbronchial or other lung biopsy with mycobacterial histopathologic features (granulomatous inflammation or AFB) and one or more sputum or bronchial washings that are culture positive for NTM (A, II) 4.Expert consultation should be obtained when NTM are recovered that are either infrequently encountered or that usually represent environmental contamination (C, III) 5.Patients who are suspected of having NTM lung disease but do not meet the diagnostic criteria should be followed until the diagnosis is firmly established or excluded (C, III) 6.Making the diagnosis of NTM lung disease does not, per se, necessitate the institution of therapy, which is a decision based on potential risks and benefits of therapy for individual patients (C, III)

Adapted from (Griffith *et al.* 2007)

TABLE 2: THE STRENGTH OF RECOMMENDATION BASED ON QUALITY OF EVIDENCE

Categories Reflecting the Strength of Each Recommendation for or against Its Use	
Category	Definition
A B C	Good evidence to support a recommendation for use Moderate evidence to support a recommendation for use Poor evidence to support a recommendation for or against use
Grades Reflecting the Quality of Evidence on Which Recommendations are Based	
Category	Definition

I	Evidence from at least one properly randomised, controlled trial
II	Evidence from at least one well-designed clinical trial without randomisation, from cohort or case-controlled analytic studies (preferably from more than one centre), from multiple time-series studies or from dramatic results in uncontrolled experiments
III	Evidence from opinion of respected authorities, based on clinical experience, descriptive studies, or reports of expert committees.

Adapted from (Griffith *et al.* 2007)

2.1.2 Treatment of *M. kansasii* disease

Current practice for treatment of this infection is a combination therapy of three different antimicrobials for a period of 12 to 18 months or until culture negative after 12 months of treatment (Griffith *et al.* 2007). According to the current guidelines, the regiment consists of: daily isoniazid (300mg/d), rifampicin (600mg/d) and ethambutol (15mg/kg/d). If rifampicin resistance is detected in the patients' isolate, a change in the regime is needed for a remission status. A three drug combination regimen is recommended based on susceptibilities including clarithromycin or azithromycin, moxifloxacin, ethambutol, sulfamethoxazole, or streptomycin (Griffith *et al.* 2007). Like all drugs, there are side-effects and these particular agents can cause serious effects such as ocular toxicity and permanent liver damage (Yee *et al.* 2003). Patients diagnosed with *M. kansasii* require specialised physicians to monitor treatment progress as treatment failures can lead to drug-resistance (Ahn *et al.* 1981).

2.1.3 Environmental "sources" of *M. kansasii*

Many NTM's are free-living saprophytes that have been recovered and isolated from a varied range of environments, including water, soil, dust, and aerosols (Falkinham 2002). McSwiggan and Collins were the first to report the isolation of *M. kansasii* from tap water in England in 1974 (McSwiggan *et al.* 1974). Engel, *et al.* (Engel *et al.* 1980) followed by publishing the first in-depth study determining the presence of *M. kansasii* in water from Rotterdam, The Netherlands. The results found a high number of positive isolations from taps in older buildings rather than newer establishments and only found one positive culture from mains water supply. Falkinham summarised work previously published and stated that *M. kansasii* is commonly found in urban areas rather than in regional districts (Falkinham 1996).

This inference was based on Anh, *et al.* and Wolinsky's research from the 1970's (Ahn *et al.* 1979; Wolinsky 1979). A long-term study testing for *M. kansasii* in tap water found that it was isolated intermittently explaining why some groups had difficulty in isolating this organism (Collins *et al.* 1984).

2.2 Methods used for culturing and identification of *M. kansasii*

2.2.1 Culturing techniques

Culturing and identification techniques have evolved over the last 100 years. Egg based solid medium (Lowenstein-Jensen media - BD) and liquid media (BACTEC™ MGIT™ 960 Mycobacterial Detection System – BD) methods have now replaced mice models and serum methodologies (Idigoras *et al.* 2000). *M. kansasii* grows best at 37^0C but can also grow at 32^0C. These organisms are slow growers and the growth rate in liquid culture takes from one-three weeks whilst solid culture is four-six weeks (Griffith *et al.* 2007).

2.2.2 Identification techniques

There are many ways of identifying Mycobacterium, from the early days of biochemical tests (Tsukamura 1981) to the more recent developments of Polymerase Chain Reaction (PCR). There have been numerous advances in NTM genetic research, in particular, with regards to determining the best genomic region for differentiating NTM strains. There are numerous journal articles published in this field, but the most accepted regions used for identifying NTM, are 16S rRNA (Abed *et al.* 1995; El Amin *et al.* 2000), and Heat Shock Protein *hsp*65 (Devallois *et al.* 1997; Kim *et al.* 2005). 16S rRNA is a suitable target for identification of bacteria, as it is highly conserved yet variable enough to provide differentiation. There are hypervariable regions A and B outside the 16S rRNA region (El Amin *et al.* 2000), which provide sufficient variability to allow these regions to be used for the identification of *Mycobacterium* sp.

Heat shock proteins, or HSP, are a class of proteins with related functions. Their expression increases when cells are exposed to elevated temperatures or other stress. Heat shock proteins help protect other proteins from heat stress (Campbell 2009). This response to heat stress can also be seen in heat-stressed animals and microorganisms (Campbell 2009). HSPs can be found in almost all living organisms, ranging from bacteria to humans (Campbell 2009). *hsp65* is a bacterial protein that has been found to be unique to *Mycobacterium sp.* as previously discussed by (Shinnick *et al.* 1988). Some strains of *M. kansasii* have identical 16S sequences as *M. gastri;* hence *hsp*65 is used to differentiate these strains further. Figure 3 depicts a typical structure of the *hsp*65 gene.

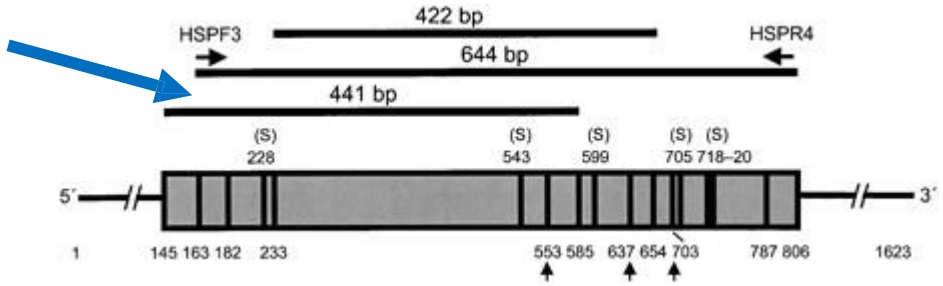


FIGURE 3: DIAGRAMMATICAL REPRESENTATION OF THE *HSP65* GENE. THERE ARE THREE SITES THAT HAVE BEEN USED TO SPECIATE *MYCOBACTERIUM SP.* (KIM *ET AL.* 2005). THE BLUE ARROW INDICATING THE 441 BASE PAIR (BP) REGION OF THE GENE IS THE MOST COMMONLY USED PART OF THE GENE UTILISED FOR SPECIATION.

2.3 Strain types and genotyping methods used for *M. kansasii*

2.3.1 Current genotyping methods

A reproducible typing technique with strain level resolution is essential when comparing patient and environmental isolates (Pfaller *et al.* 2007). Strain typing over a period of time is important as the rate of genomic evolution of *M. kansasii* is not known. There has been a long progression of genotyping methods used to type *M. kansasii* strains.

The first method for strain differentiation of *M. kansasii* was published in 1975 by Engel and Engel described the use of phage typing as an approach to type *M. kansasii* (Engel 1975). From 1975 to 1997, no studies were published showing

improvement in strain differentiation. Picardeau, *et al.* was the first to evaluate many newly described DNA-based techniques for the identification and strain typing of *M. kansasii* (Picardeau *et al.* 1997). The methods investigated included: analysis of restriction fragment length polymorphisms (RFLPs) with the major polymorphic tandem repeat (MPTR) probe and the insertion sequence (IS1652) probe, Southern blot hybridisation, pulsed field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP) analysis, PCR restriction analysis (PRA) and AccuProbe Gen-Probe (Hologic®, USA) identification (Picardeau *et al.* 1997). Overall, results were correlated between the different typing methods. However, two of these methods could only identify two out of the five *M. kansasii* strain types (IS1652 is only present in strain type II & III and AccuProbe could not identify strain type II or IV).

Alcaide, *et al.* (1997) published their study using the same techniques as Picardeau but performed additional assays, these included: *hsp65* PCR followed by restriction fragment length polymorphism (PCR-RFLP) analysis using enzymes *BstE*II *and Hae*III with separation by agarose gel electrophoresis (Telenti *et al.* 1993), *gyrA* intein enzyme digest as well as AccuProbe Gen-Probe (Hologic®) identification. After this, sequencing of the 16S-23S rRNA intergenic spacer (ITS) region of all isolates and PFGE was performed on the type I isolates as per protocol (Burki *et al.* 1995).

Iinuma, *et al*. (1997) was next to publish and used a unique technique to type *M. kansasii* not previously done by either Picardeau or Alcaide. They used Large-Restriction-Fragment analysis (LRF) and investigated the typeability, reproducibility and the discriminatory power of the method when applied to this species. They used five restriction enzymes, eventually choosing the restriction enzyme that gave the greatest discrimination (*Vsp*I). The isolates were run on PFGE to generate 16 to 21 fragments. The *Vsp*I enzyme yielded 21 fragments. These results showed a better discrimination than the previous methods used by Picardeau and Alcaide.

Roth, *et al.* described a novel typing method using the 16S-23S rRNA spacer region as the target (Roth *et al.* 2000). *Hae*III and *Cfo*I restriction enzymes were used to

visualise the fragments using RFLP and agarose gel electrophoresis. This method was successful at discriminating Mycobacteria that previously could not be separated. This method can not only be used to type *M. kansasii* but also can speciate other commonly isolated Mycobacteria such as *M. intracellulare, M. avium,* etc. (Roth *et al.* 2000).

Gaafer *et al*. (2003) took a different approach and published an evaluation of the modifications of the AFLP technique (Gaafar *et al.* 2003). Gaafer focused on the type I *M. kansasii* isolates, discriminating type I strain with a streamlined AFLP technique using a single enzyme, adaptor and primer. This method revealed a high level of heterogeneity between the subspecies that was thought to be clonal. Following on from Gaafer, Zhang *et al*. (2004) performed PRA of the *hsp65* gene and PFGE on isolates from USA (Zhang *et al.* 2004). PRA matched the five strain types described in previous studies (Alcaide *et al.* 1997; Picardeau *et al.* 1997).

The articles mentioned within this literature review, discuss briefly the clinical relevance of the different strain types of *M. kansasii*. Type I has been isolated most commonly world-wide and is also thought to be the strain type that causes disease (Griffith *et al.* 2007). The proportion of the other known strain types vary between countries and this may reflect different environmental conditions that exist (Wu *et al.* 2009). A definitive relationship has not been established yet between strain types to disease (disseminated vs pulmonary). It is assumed that type I causes disease but this could be due to being the most common isolated type (Griffith *et al.* 2007). The relationship between antibiotic susceptibility and strain types is also not fully understood. Susceptibility testing is not routinely performed as a set regime for treatment is used and only further investigated if no clinical improvement is seen after therapy (Griffith *et al.* 2007). The isolates that have had strain typing and susceptibility testing done concurrently, have not been informative in defining a clear answer (Wu *et al.* 2009).

Looking at retrospective studies, Taillard *et al*. (2003) completed a survey in Switzerland using clinical implications rather than evaluating molecular techniques

(Taillard *et al.* 2003). Strain typing was performed using PCR-restriction enzyme analysis of *hsp65* as previously described by (Alcaide *et al.* 1997). No data analysis was demonstrated or provided in the article. Santin, *et al.* (2004) performed a retrospective genotyping study in Catalonia, Spain and found that 97.8% of *M. kansasii* were type I isolates (Santin *et al.* 2004). The isolates were first identified using phenotypic methods and species confirmation by the AccuProbe assay. Following identification, typing was performed using PCR-RFLP analysis of the *hsp65* gene as described in Alcaide *et al*. (1997). Their results showed only two isolated *M. kansasii* strain types, however this study focused on the incidence of *M. kansasii* within HIV and non-HIV populations rather than genotyping isolates.

da Silva Telles, *et al.* (2005) performed antibiotic susceptibilities as well as PRA-*hsp65* on *M. kansasii* clinical isolates in Brazil (da Silva Telles *et al.* 2005). Of the 106 isolates that were typed, 104 were type I, whilst type II and type III had one isolate each, as determined by Telenti's method (1993). This paper places more emphasis on antibiotic testing rather than strain typing but did make a note that the genotypic characterisation of the isolates did not correlate well with clinical disease. It was also mentioned that antimicrobial MIC values identified could not correlate whether the patient would succeed in remission from the infection. From the strains used in the study, only two isolates were not type I strain and no discussion was provided to inform if the two isolates had a unique sensitivity profile. No data analysis or tabulated statistics was provided for the typing of the isolates.

Chimara, *et al.* (2004) performed a similar study to Santin, *et al.* (2004). They identified 189 isolates of *M. kansasii* and performed PRA-*hsp65* using the restriction enzymes *BstEII* and *HaeIII* (Chimara *et al.* 2004). Of the 189 isolates, 182 were type I, one type II, one type III and five were other *Mycobacterium* sp. This retrospective study focused on HIV status, similar to Santin *et al.* (2004). A cause for concern in the accuracy of initial identification was that only phenotypic methods such as cord formation visualised microscopically using Ziehl-Neelsen staining was used. There may have been other species that could have been missed or misidentified using such a crude technique, therefore questioning the validity of their results.

Further retrospective studies were published in foreign languages where the abstract was only available in English. The details of these studies are as follows: Leal Arranz, *et al*. (2005) carried out a clinical and epidemiological retrospective and prospective study of *M. kansasii* in the metropolitan area of Bilbao, Spain (Leal Arranz *et al.* 2005). They discovered that the majority of the isolates were genotype I (98.5%) and amongst type I isolates, eight clones were found. Of these eight clones, 1 and 3 were the most common. The typing methods and all genotypes were not described. Jiménez-Pajares, *et al.* (2005) studied the phenotypic and genotypic characteristics of *M. kansasii* over a four-year period in Spain (Jimenez-Pajares *et al.* 2005). The genotypic characteristics where evaluated using PRA-*hsp65* and restriction enzyme digestion with *BstE*II and *Hae*III using Telenti's method (Telenti *et al.* 1993). They identified 5 out of 6 of the described genotypes with 86.6% strains identified as type I. They concluded that together conventional and molecular methods accurately identify atypical isolates.

Mimura (English abstract only, published in Japanese) investigated the epidemiology of *M. kansasii* infections in Okayama Prefecture, Japan, to consider the mode of infection using PFGE (Mimura 2005). Isolates were typed using the restriction enzyme *Vsp*I as described by Iinuma *et al*. (1997). Tap water from five industrial companies from the same area was collected and cultured to investigate a potential source of infection. The *M. kansasii* isolates were clustered into groups IV and V but the tap water isolates could not be characterised by PFGE. They concluded that the source was living as a common environmental source. The full text of this manuscript would have been useful to assess the strain relatedness between clinical and water isolates, as the translation of the conclusions in the abstract was not informative. These three abstracts made it difficult to derive definitive conclusions. The full text would be needed to review the typing techniques and analysis.

Yoshida, *et al.* published a research paper (Yoshida *et al.* 2007) and a review paper (Yoshida *et al.* 2011) on *M. kansasii* molecular epidemiology, (both articles in

Japanese). The research paper has an abstract summary in English where it was stated that PCR-PRA-*hsp65*, ITS and 16S-23S sequencing, PFGE, RFLP with the MPTR and IS1652 probes were used in their study. Results briefly state that of the 174 isolates tested, 170 were classified as type I, two belonged to type II and one each were type IIb and type VI. The review paper's abstract briefly discussed *M. kansasii* and the epidemiological trends of disease in Japan. Without a full translation, a comparison to the other Japanese studies to correlate results within the country was not possible.

Wu, *et al.* (2009) published a clinical review, antibiotic profiles and molecular analysis of *M. kansasii* isolates in Taiwan (Wu *et al.* 2009). Restriction enzyme digestion PFGE was performed as previously described by (Zhang *et al.* 2004). They observed nine different PFGE patterns with cluster I being the most common. Their results correlated well with Brazilian investigators, but differed to other Western nations. The results are displayed effectively indicative of a robust study.

More recently, Sajduda, *et al.* (2012) used PRA-*hsp65* using capillary electrophoresis with previous identification using LiPA assay (INNO-LiPA MYCOBACTERIA, Innogenetics, Belgium) (Sajduda *et al.* 2012). This is a relatively new approach to strain typing, more rapid and accurate to perform, but it is costly and out of reach of most laboratories. This group also used tap water samples in their study along with human isolates and one animal isolate. Of the 27 strains tested PRA type I dominated with 11 clinical strains, the reference strain and one environmental isolate. Of the remaining 15 isolates, PRA type II included four human, two environmental and one animal strains; type III included five environmental strains, type IV included two environmental strain; and one type V environmental strain was identified. This paper combined both old and new techniques and showed that both could be reproduced and used for other *Mycobacterium sp.* as well.

2.3.2 Current literature on Diversilab and High Resolution Melt Analysis

Diversilab as a tool for strain typing has appeared in the literature only the last eight years. Specifically, there have been fourteen papers published where this method has been applied to both TB and NTM.

Cangelosi, *et al*. (2004) evaluated the rep-PCR method using *M. tuberculosis* and *M. avium* species (Cangelosi *et al.* 2004). Both species results correlated with previous strain typing methods described in the paper. Freeman, *et al*. (2005) used the rep-PCR technique as a confirmation of the Multi-locus repeat units (MIRU) method (Freeman *et al.* 2005). They described Diversilab as a useful tool to show clusters as a quick and relatively cheaper alternative to MIRU. Ashworth, *et al*. (2008) published similar results to Freeman, *et al*. (2005) in which they depicted the rep-PCR method as a reliable detection method for visualising TB outbreaks with a better turnaround time compared to MIRU (Ashworth *et al.* 2008). Al-Hajoj, *et al*. (2010) used rep-PCR as a diagnostic tool to determine the evolution of TB strains in a particular patient in response to treatment (Al-Hajoj *et al.* 2010). Jang, *et al*. (2011) compared RFLP and Diversilab methods (Jang *et al.* 2011). They found that both methods are reproducible; however, Diversilab showed greater diversity amongst previously recognised clonal clusters. All papers showed a good discriminatory power compared to the methods currently accepted as the standard for TB typing.

Currently, there have been nine papers published using Diversilab as a strain typing method for NTM. Wang, *et al*. (2008) used rep-PCR to characterise a pseudo-outbreak of *M. paraffinicum* in a hospital (Wang *et al.* 2008). Gira, *et al.* (2004) used rep-PCR to confirm a patient's infection with the bacteria *M. mageritense* originated from a foot-bath salon (Gira *et al.* 2004). Marshall, *et al*. (2011) used Diversilab to link municipal drinking water as the source of infection for patients with *M. lentiflavum* disease (Marshall *et al.* 2011). There are six papers on *M. abscessus* typing using Diversilab. Some of the articles described problems with reproducibility among runs with Diversilab (Zelazny *et al.* 2009; Thomson *et al.*

2013), where the others showed good reproducibility with this method and were able to demonstrate distinct clustering among the isolates (Harris *et al.* 2012; Cheng *et al.* 2013; Jamal *et al.* 2014; Mougari *et al.* 2014). Considering the contradictions between the publications, this typing method may need some modifications or changes by the company to improve its robustness and reliability.

High-resolution melt analysis (HRM) has been used only in recent years for identification, mutation gene analysis and strain typing of Mycobacteria. To date, there are five papers published using HRM as a rapid diagnostic tool to screen for drug resistance in multi-drug resistant TB. (Ong *et al.* 2010; Ramirez *et al.* 2010; Chen *et al.* 2011; Lee *et al.* 2012; Pholwat *et al.* 2014). There is one paper detecting drug resistance in *M. leprae* using the same technique as used in detecting TB drug resistance (Li *et al.* 2012). They demonstrated that their technique was able to screen rapidly for mutations in gene-specific regions they targeted.

There are two papers published utilising HRM for NTM typing. Castellanos *et al*. (2010) used HRM to rapidly identify *M. avium subspecies paratuberculosis* types and compare to previous data from another method (Castellanos *et al.* 2010). From the results, this method could determine the three prevalent types of this species rapidly and accurately. Won *et al*. (2010) used HRM coupled with a broad-based PCR to rapidly identify NTM in positive blood culture bottles (Won *et al.* 2010). Results showed a 100% concordance with previously validated methods used for initial identification with 46/52 isolates identified.

Overall from the literature, there is little documented evidence of the application of these newer techniques for strain typing *M. kansasii* isolates.

2.3.3 Current known genotypes of *M. kansasii*

From DNA-based studies, the literature suggests that there are between five to seven strain types of *M. kansasii* amongst human and environmental isolations.

Strain type I is the one most commonly isolated world-wide and known to cause disease in humans (Ross *et al.* 1992; Marras *et al.* 2002). Picardeau, *et al.* (1997) was the first to confirm that there are five strain types. Alcaide, *et al.* (1997) was able to reproduce and confirm Picardeau's study using the same methods with additional techniques. Just as Alcaide and co-workers replicated the results, Zhang, *et al.* also attained the same conclusions. Richter, *et al.* identified a new stain type calling it number six (VI) (Richter *et al.* 1999). They used similar techniques to Picardeau, *et al.* (1997) but included additional sequencing to differentiate the strain types. Iinuma, *et al.* (1997) performed large-restriction-fragment analysis to produce results showing greater diversity among strains (sub-types) but were unable to group into the accepted strain types. Taillard, *et al.* (2003) established a seventh strain type by similar methods used by Picardeau, *et al*. (1997). To date, there isn't any further literature to prove or disprove the existence of a seventh strain type.

2.4 Summary and Implications

Table **3** below is a summary of the literature describing the genotyping methods and their advantages as well as disadvantages. Briefly, most of the typing systems provide good discrimination overall, however, disadvantages are the requirement of specialised equipment and length of time required to perform the tests.

TABLE 3: SUMMARY OF PUBLISHED GENOTYPING METHODS USED TO DIFFERENTIATE *M. KANSASII* STRAINS.

Method	Advantages	Disadvantages	Paper
Phage Typing	Need only 10 single colonies to work	Expensive Time consuming Strain discrimination was	Engel, 1975
RFLP with MPTR probe & IS1652 probe; Southern blot analysis PFGE; AFLP analysis PRA; Gen-probe	Many typing techniques used and 5 strains are characterised	Some of these methods could only identify 2/5 strains Time consuming Specialised expensive equipment is needed	Picardeau, 1997
hsp65 PCR-RFLP gyrA intein digest Gen-probe; 16-23S ITS PFGE on type I only	Good discrimination seen with *hsp65* & PFGE	Time consuming Expensive	Alcaide, 1997

LRF analysis	Good discrimination between strains Reproducible	Time consuming Expensive analytical software is required	Iinuma, 1997
16S-23S rRNA spacer region REA	Discovered a 6th strain type. This new technique helped separate out where previous methods could not	Time consuming	Roth, 2000
PRA-*hsp65*	Good discrimination Seventh type described	No analysis shown Time consuming	Taillard, 2003
AFLP of type I only	Simplistic technique using fewer reagents Good discrimination seen amongst type I	Expensive software for analysis is required	Gaafer, 2003
PRA-*hsp65* PFGE	Reproducible results generated. Confirmed previous work (Alcaide & Picardeau)	Time consuming Expensive software needed for accurate analysis	Zhang, 2004
AccuProbe (Hologic) Assay PCR-RFLP of hsp65	Good identification before typing	No visual analysis shown. AccuProbe can miss some strains of *M. kansasii*, maybe hence only finding 2 strain types	Santin, 2004
PRA-*hsp65*	Results visualised well in table and gel format	Initial phenotypic methods used to identify *M. kansasii* found other species when strain typing. May have missed other strains	Chimara, 2004
Unknown (paper in Spanish)	Insufficient material to determine	Insufficient material to determine	Leal Arranz, 2005
PRA-*hsp65*	Insufficient material to determine from the abstract alone	Insufficient material to determine from the abstract alone	Jimenez-Pejares, 2005
PFGE	Insufficient material to determine from the abstract alone	Insufficient material to determine from the abstract alone	Mimura 2005
PRA-*hsp65*	Typed as per Telenti and found 3 strain types	No analysis was shown. More of an emphasis on antibiotic testing than strain typing	da Silva Telles, 2005
PCR-PRA-*hsp65* ITS with 16S-23S rRNA sequencing PFGE	Insufficient material to determine from the abstract alone	Insufficient material to determine from the abstract alone	Yoshida, 2007

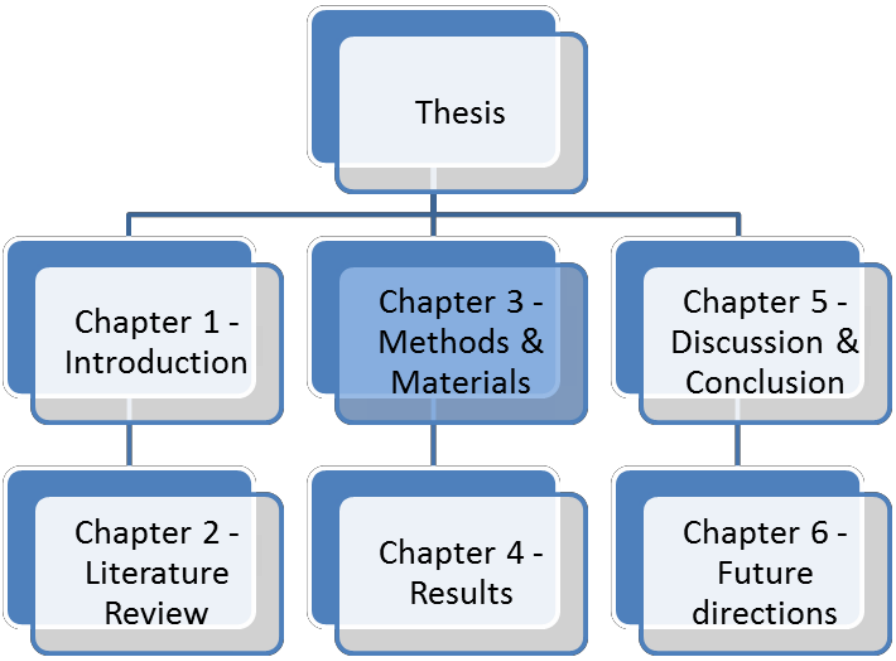
RFLP with MPTR & IS1652 probes			
PFGE	Displayed all data and analysis well Results correlated with the study conducted in Brazil (da Silva Telles 2005)	Observed 9 different patterns but didn't state if they are the common strain "types"	Wu, 2009
A review article which didn't stipulate what molecular testing was performed	Insufficient material to determine from the abstract alone	Insufficient material to determine from the abstract alone	Yoshida, 2011
LiPA assay Identification first PRA-*hsp65* capillary electrophoresis for typing	Accurate identification was done first Used tap water samples along with human and an animal isolate Showed it was reproducible and accurate	No visual analysis shown for *M. kansasii*. Expensive equipment is required as well as highly trained staff	Sadjuda, 2012

2.5 Summary

From these previously published methods, it is clear that a more rapid, accurate and cost effective method is required for *M. kansasii* strain typing. From this the following hypothesis was conceived:

Newly developed DNA-based genotyping methods are able to characterise and differentiate clinically and environmentally sourced strains of M. kansasii.

With this hypothesis in mind, access to the latest equipment enables the development of more robust and accurate genotyping methods. This can then provide a better understanding of the population structure of clinical and environmental *M. kansasii*. This will also provide a basis for establishing a link between environmental *M. kansasii* and human disease.


Thesis
Chapter 1 - Introduction
Chapter 3 - Methods & Materials
Chapter 5 - Discussion & Conclusion
Chapter 2 - Literature Review
Chapter 4 - Results
Chapter 6 - Future directions

CHAPTER 3: METHODS AND MATERIALS

3.1 Participants and Isolates

Isolates were collected over a period of five years from 2005 to 2010. This time interval was selected as a previous study has been conducted on isolates prior to 2005 at the Queensland Mycobacterium Reference Laboratory. These years were also selected to coincide with the Brisbane Water isolates collected from potable water around Brisbane and from patient houses (2006-2010). The water isolates were investigated by Dr Rachel Thomson as part of her PhD project entitled: Characteristics of Nontuberculous Mycobacteria from a Municipal Water Distribution System and Their Relevance to Human Infections (completed 2013). 171 isolates were originally selected for this study (85 patient isolates and 86 water isolates) but after further investigation of the isolates, some had to be excluded as they were either not M. kansasii or not viable. The final number of isolates used in the current study was 139 plus M. kansasii ATCC 12478 control strain.

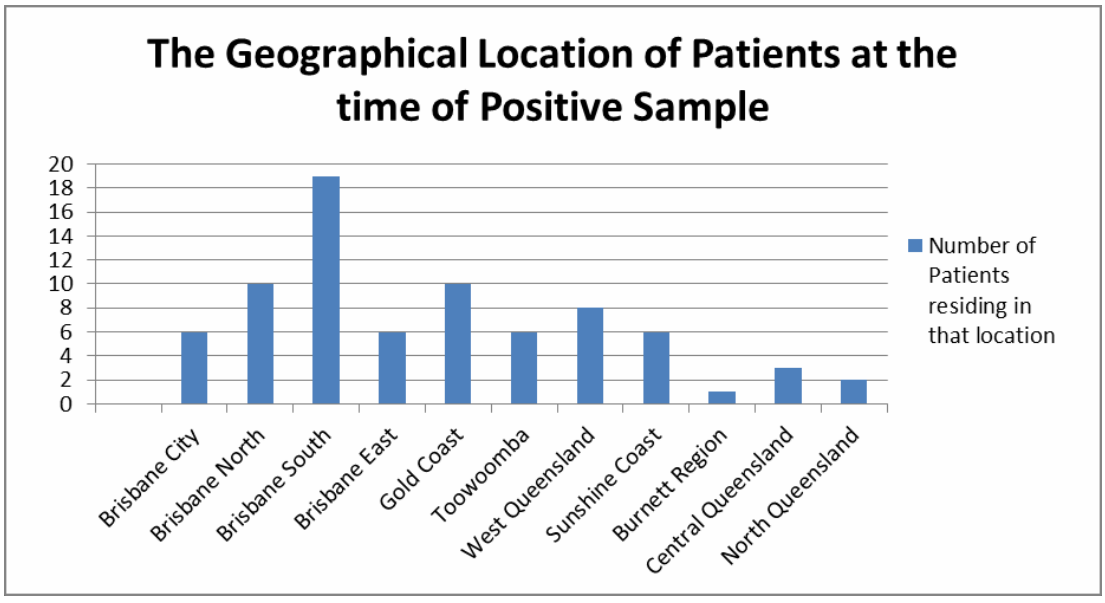


FIGURE 4 ABOVE DEMONSTRATES WHERE THE PATIENT RESIDED AT THE TIME OF SPECIMEN COLLECTION AND SUBSEQUENT ISOLATION OF M. KANSASII. THE X-AXIS IS THE REGIONS IN QUEENSLAND THAT WAS REPRESENTED IN THIS STUDY AND THE Y-AXIS DEMONSTRATES THE NUMBER OF PATIENTS.

3.2 Methodology

3.2.1 Isolation and cultivation methods

Patient isolates were collected and stored at the Queensland Mycobacterium Reference Laboratory. Patient isolates were obtained from a range of specimens including sputum, broncho-alveolar lavage fluid, bronchial-washings, swabs and other fluids.

On receipt of the specimens at QMRL, the specimens were decontaminated with 4% sodium hydroxide for 30 minutes and titrated to a pH7 with 1 molar phosphoric acid to destroy normal respiratory flora and reduce overgrowth by rapid growing bacteria. Specimens were cultured in liquid (BACTEC™ MGIT™ 960 Mycobacterial Detection System, BD) and solid (Lowenstein-Jensen agar - BD) media for up to ten weeks (Somoskovi *et al.* 2000). This ten-week window provides sufficient time to allow very slow growing NTM time to propagate. When a culture was obtained, either in liquid or solid media, smear microscopy was performed by Ziehl-Neelsen (Kinyoun 1915) and observed by oil-immersion light microscopy. Solid and liquid NTM morphology can differ significantly. It is important to distinguish *M. tuberculosis* isolates from NTM. Figure depicts the different morphologies of *M. tuberculosis* and *M. kansasii*.

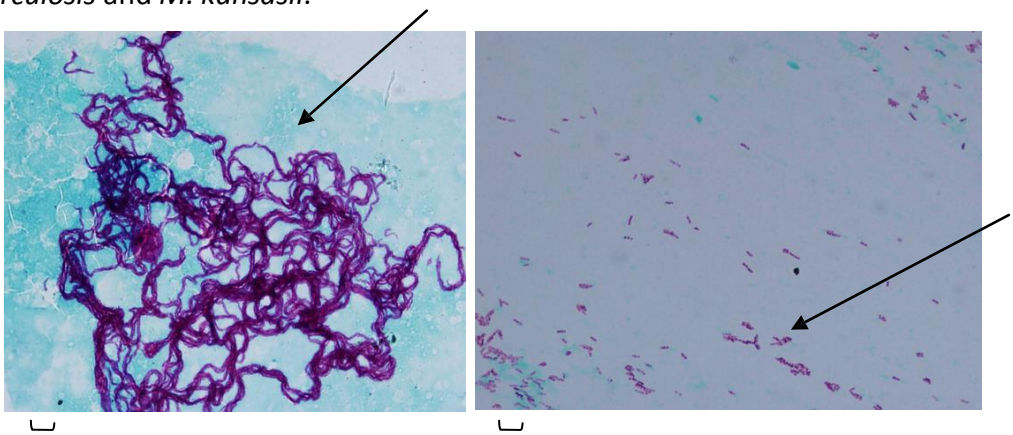


FIGURE 5: SMEAR MICROSCOPY FROM CULTURE, ON THE LEFT IS *M. TUBERCULOSIS* COMPLEX AND ON THE RIGHT IS *M. KANSASII*; VIEW SEEN AT X100 UNDER OIL-IMMERSION. SCALE FOR THE FIGURES ABOVE: 1 = 1 MICRON. AN ARROW HAS BEEN ADDED TO SHOW THE READER WHAT TO LOOK AT SPECIFICALLY. PICTURES WERE TAKEN AND PROVIDED BY THE AUTHOR OF THIS DOCUMENT.

The cord factor (Julian *et al.* 2010) is what differentiates *M. tuberculosis* complex from other species. Cord factor can also be seen in some NTM species (Julian *et al.* 2010) but not as clearly defined as it is for *M. tuberculosis* complex. In NTM species it is seen as loosely corded Acid Fast Bacilli, not the tight serpentine cording associated with *M. tuberculosis* complex. A rapid test to help discriminate *M. tuberculosis* complex from NTM is the MPT64 Antigen assay. Briefly, it is an immunochromatographic identification test that uses a gold labelled antigen which detects the protein MPT64 that is unique to *M. tuberculosis* complex isolates (Ismail *et al.* 2009).

M. kansasii is in the Photochromogen group classified by Runyon (Runyon 1959). From Figure , the growth on solid media to the right is typical for *M. kansasii*; they are defined as buff raised colonies on solid media with the development of pigment after exposure to light. Before pigmentation is present, the colonies are cream in colour, with a very close similarity to *M. tuberculosis* complex on solid media. Pigment colour varies between a bright yellow to a dark orange. Organisms are stored in an enrichment broth (Dubos broth - BD) and frozen at -80^{0}C in aliquots until required.

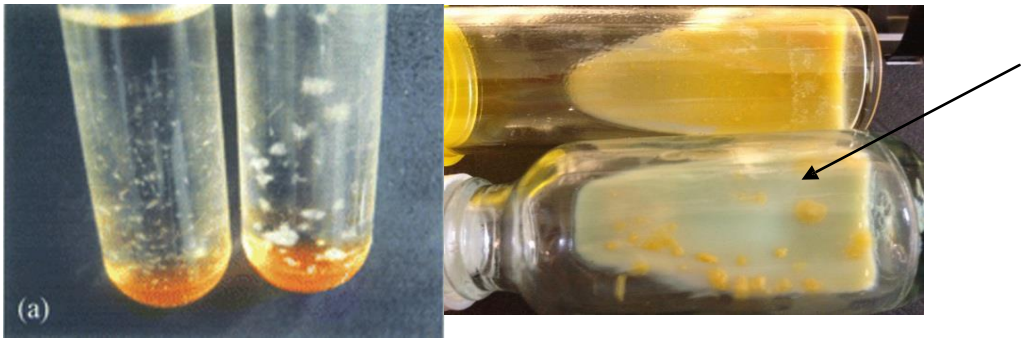


FIGURE 6: MACROSCOPIC VIEW OF *M. KANSASII* IN LIQUID CULTURE ON THE LEFT (MGIT™) AND SOLID MEDIA ON THE RIGHT (LOWENSTEIN-JENSEN). AN ARROW HAS BEEN ADDED TO SHOW THE READER WHAT TO LOOK AT SPECIFICALLY. PICTURES WERE TAKEN AND PROVIDED BY THE AUTHOR OF THIS DOCUMENT.

3.2.2 Identification Techniques

Identification of NTM at QMRL is performed using a variety of molecular methods including the commercially available kit GenoType Mycobacterium CM Line Probe Assay (Hain Lifescience GmbH, Germany), 16S rRNA sequencing and *hsp*65

sequencing. Description of the Line Probe assay is briefly as follows: DNA is extracted from culture, amplified and detected via a reverse hybridisation and alkaline phosphatase reaction on a membrane strip. This assay is robust and time effective but can only differentiate between 14 common species of Mycobacteria including *M. tuberculosis* complex.

When a result cannot be determined by the Line Probe method, 16S rRNA and *hsp*65 sequencing is performed. Both genes effectively differentiate between the species, but there are some species of Mycobacteria that have identical 16S rRNA sequences. For example some strains of *M. kansasii* have been found to be identical to *M. gastri*. When species have identical 16S rRNA sequences, they can be further differentiated using the *hsp*65 gene. The 16S rRNA species-specific primers are:

Forward - BF 5'-AGAGTTGGATCCTGGCTCAG-3'
Reverse - R2 5'-CCTACGAGCTCTTTACG-3' (Abed *et al.* 1995).

For *hsp*65 the primers are:

Forward - A11 5'-ACCAACGATGGTGTGTCCAT-3'
Reverse - A12 5'-CTTGTCGAACCGCATACCCT-3' (Devallois *et al.* 1997).

Details for the sequencing protocol performed are located in Appendix D (QMRL 2014)

Once it has been established that *M. kansasii* has been identified, these isolates are subsequently used for the genotyping assays.

3.2.3 DNA Extraction

Of the three strain typing methods described here, DiversiLab is the only method that requires a very high purity and concentration of DNA. The only kit that has been validated for use with the DiversiLab system is the UltraClean® Microbial DNA

extraction kit (MoBio, USA). The kit contains all reagents and tubes required to complete the protocol. The following modifications were made to the manufacturer's instructions as there were problems with acquiring a high enough concentration of DNA, the modifications made to the protocol are as follows:

- Collect 10µL loop of culture from solid growth.
- Add 50µL of solution MD1 and incubate at 95^0C for 20 minutes.
- Vortex – maximum speed (12,000g) for 30 minutes.

Further explanations of the modifications made to the extraction method are found in Chapter 4.2 on page 36.

3.2.4 Internally Transcribed Spacer Region with Restriction Enzyme Analysis (ITS-REA)

The Internally Transcribed Spacer (ITS) region is a section of non-functional RNA located amongst structural ribosomal RNAs (rRNA) on a common precursor transcript (Barry *et al.* 1991; Gurtler *et al.* 1996). Genes encoding rRNA and spacers transpire in tandem repeats that are thousands of duplicates in length, each separated by regions of non-transcribed DNA. Sequence evaluation of the ITS region is commonly used because it is easy to amplify (this is due to the large copy of rRNA genes) and also has a high level of dissimilarity, even amid closely related species (Ben Amar *et al.* 2012). This is clarified by the comparatively low evolutionary pressure acting on a non-functional sequence.

Restriction Enzyme Analysis (REA) is where an enzyme is used to cut DNA at specific recognised nucleotide sequence known as a restriction site (Roberts *et al.* 1976). Restriction enzymes are commonly classified into three types (type I, II & III) (Kessler *et al.* 1990). The type used determines whether they cut their DNA substrate at the recognition site or if the recognition and cleavage sites are separated from one another (Roberts *et al.* 1976). These enzymes are found in bacteria and probably evolved to provide a defence mechanism against invading viruses (Kruger *et al.*

1983). There are over 3000 restriction enzymes that have been studied in detail, and more than 600 are available commercially (Roberts *et al.* 2007).

The method used in this study was described by Roth *et al.* (Roth *et al.* 2000) and adapted for In-House use by QMRL Supervising Scientist Jim Psaltis (Psaltis 2005). A brief description of the method is as follows:

Mastermix was prepared using the following –

TABLE 4: PREPARATION OF THE PCR REACTION FOR ITS-REA

	For 1x Reaction
PCR Buffer 10x + MgCl2 (Qiagen, Netherlands)	8.0µL
dNTP's diluted 1:10 (GE Healthcare, UK)	4.0µL
SP1 forward Primer (10uM concentration – Sigma Aldrich, USA) 5'ACC TCC TTT CTA AGG AGC ACC	0.5µL
SP2 reverse Primer (10uM concentration – Sigma Aldrich, USA) 5'GAT GCT CGC AAC CAC TAT CCA	0.5µL
0.1% Triton	1.0µL
HotStar TAQ Polymerase (Qiagen, Netherlands)	0.5µL
Rnase & Dnase Free Water	30.5µL
DNA Template	5.0µL
Total Volume	50.0µL

The product was amplified using the following cycling conditions:

TABLE 5: ITS-REA PCR THERMAL CYCLING CONDITIONS

Initial Denaturation	94^0C – 5 minutes	1 cycle
Denaturation	94^0C – 1 minute	45 cycles
Annealing	59^0C – 1 minute	
Extension	72^0C – 1 minute	
Final Extension	72^0C – 1 minutes	1 cycle
Hold	4^0C - Hold	

The PCR amplicons were visualised by gel electrophoresis using 2% agarose gel run at 100 Voltage constant for 60 minutes. The product size should be between 205-320bp.

REA – Three enzymes were used, namely, *Hae*III (*Haemophilus aegyptius*), *Cfo*1 (*Clostridium formicoaceticum*) and *Dde*1 (*Desulfovibrio desulfuricans*). Each enzyme is prepared using the following Mastermix.

TABLE 6: PREPARATION OF THE ENZYME DIGEST FOR EACH OF THE THREE ENZYMES

	For 1x Reaction
For *Hae*III	
Buffer x10 (sure/cut Buffer M)	2.5µL
RE *Hae*III (10u/uL)	0.2µL
Injection water	13.3µL
For *Cfo*1	
Buffer x10 (sure/cut Buffer L)	2.5µL
RE *Cfo*1 (10u/uL)	0.2µL
Injection water	13.3µL
For *Dde*1	
Buffer x10 (sure/cut Buffer H)	2.5µL
RE Dde1 (10u/uL)	0.2µL
Injection water	13.3µL
Template (from ITS PCR)	10µL (for each enzyme)
Total volume	25µL (for each enzyme)

Each enzyme digest reaction was incubated at 37^0C for 90 minutes. Thereafter, the digested products were visualised on a 3% agarose gel, including a low mass ladder.

3.2.5 *M. kansasii* genotyping using DiversiLab

DiversiLab™ (bioMerieux, USA) uses the patent technology of *rep*-PCR. Repetitive elements are interspersed with repetitive sequences and these sequences are characterised as relatively short (usually <500bp), non-coding, dispersed elements in bacterial genomes. The number and spacing of repetitive elements varies from genome to genome. The primers target the repeat region and amplify sequences between the repeats. Multiple fragments of varying lengths are generated and the amplified fragments are separated by size, producing a unique molecular fingerprint. Combining the primers from multiple repetitive elements, results in a higher level of discrimination between species and strains; hence the fingerprint for analysis and comparison. From the fingerprints generated, further analysis can be conducted and produced automatically by the software once the particular fingerprints are selected for investigation. The software constructs dendrograms with the fingerprints aligned in order of relatedness using multiple analysis tools. Other representations of the data produced by the DiversiLab software include similarity matrix, graph overlay and scatter plot.

M. kansasii isolates were genotyped using the DiversiLab method as per the protocol, which is briefly outlined below:

Using the DNA previously prepared, a primary amplification of the repetitive elements using a Mastermix is created. The volumes have been altered and are outlined below:

TABLE 7: MASTER MIX VOLUMES FOR DIVERSILAB

	For 1x Reaction
MasterMix1 (MM1) – from DiversiLab kit	14.4µL
10x PCR Buffer (Applied Biosystems, USA)	2.0µL
Primer Mix – from DiversiLab kit	1.6µL
AmpliTaq DNA Polymerase (Applied Biosystems, USA)	0.4µL
DNA Template	1.6µL
Total Volume	20.0µL

PCR performed using the following thermal cycling conditions:

TABLE 8: DIVERSILAB PCR THERMAL CYCLING CONDITIONS

Initial Denaturation	95^0C – 5 minutes	1 cycle
Denaturation	95^0C – 30 seconds	35 cycles
Annealing	66^0C – 45 seconds	
Extension	72^0C – 60 seconds	
Final Extension	72^0C – 5 minutes	1 cycle
Hold	4^0C – hold	

The lab chip was then loaded and ran as per the manufacturer's instructions.

Fingerprint analysis was performed on these results to create the final dendrogram and strain pattern. Before a report can be generated, Quality Control (QC) was performed on each sample using the DiversiLab software. The software QC feature flags obviously abnormal curves and allows exclusion of these samples. Reports were generated directly after QC has been checked. The software also identifies marker peaks and normalised data automatically. After acknowledging the results for each run, a printable report was generated and the dendrogram with scatter post were created. An example is shown below of what is generated by the software.

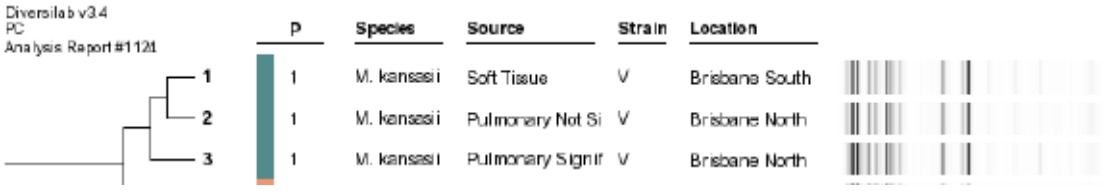


FIGURE 7: DIVERSILAB FINGERPRINT GENERATED BY SOFTWARE.

3.2.6 Genotyping of *M. kansasii* using High Resolution Melt Analysis (HRM)

HRM is an extension of DNA dissociation analyses (Qiagen 2013). It is used to characterise DNA samples according to their dissociation behaviours as they switch from double stranded DNA (dsDNA) to single stranded DNA (ssDNA) with increasing temperatures (CorbettLifeScience 2006). Depending on the G-C content, different

melt curves will be generated for each isolate. Using the Rotorgene 6000 Real Time PCR instrument (Qiagen, Australia), fluorescent signals with much greater optical and thermal precision are generated compared to previous methods (CorbettLifeScience 2006). Prior to performing a HRM analysis, a target sequence must first be purified to a high copy number (CorbettLifeScience 2006). This is done by a PCR procedure in the presence of a dsDNA intercalating fluorescent dye (CorbettLifeScience 2006). The dye (in this case SYBRGreen) does not interact with ssDNA but intercalates with dsDNA and fluoresces brightly in this state (Wittwer *et al.* 2003; CorbettLifeScience 2006). Initially, the fluorescence is high in a melt analysis because the sample starts as dsDNA, but fluorescence diminishes as the temperature is raised and DNA dissociates into single strands (CorbettLifeScience 2006). The observed melt is characteristic for every sample, as seen in Figure .

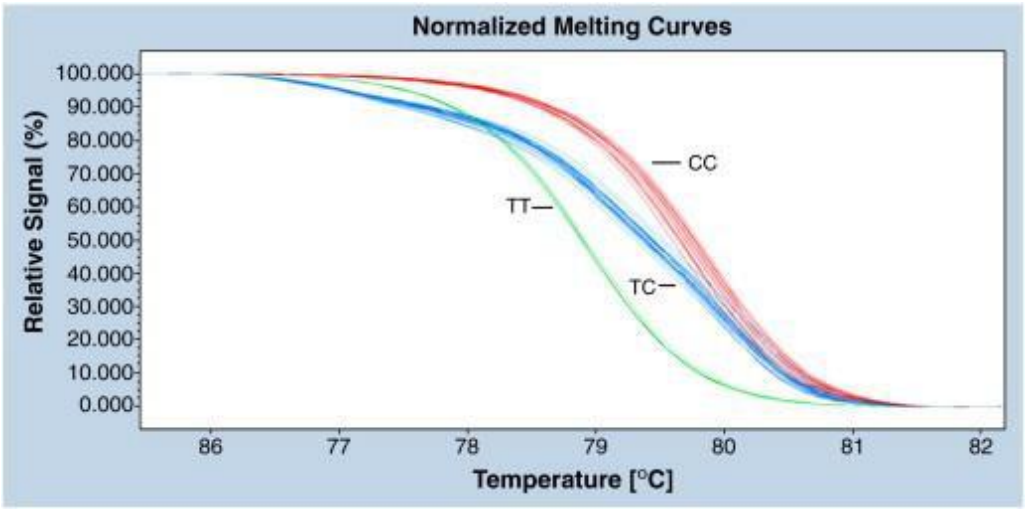


FIGURE 8: HRM MELTING CURVE, IMAGE SOURCE: WWW.SCIENCEDIRECT.COM. THE MELTING CURVE SHOWS THE TEMPERATURES OF WHERE THE DNA DISSOCIATES FROM EACH STRAND.

HRM was performed and outlined briefly:

Prepare Mastermix as outlined in the table below –

TABLE 9: PREPARATION OF HRM REACTIONS

	For 1x Reaction
Type-IT® HRM PCR Kit (Qiagen, Netherlands)	7.5µL
Rnase/Dnase free water	10.0µL
Forward Primer – 16S rRNA* (10µM)	0.25µL
Reverse Primer – 16S rRNA* (10µM)	0.25µL
DNA Template	2.0µL
Total Volume	20.0µL

* 16S rRNA primer sequences are listed in section 3.2.2 above.

Mastermix and DNA template was dispensed into specific Rotorgene compatible tubes. All samples were run in duplicate and included in every run is a positive control, a no-template control and a water control.

Real-time PCR was performed initially followed by HRM using the following thermal cycling conditions:

TABLE 10: HRM PCR THERMAL CYCLING CONDITIONS

Initial Denaturation	95^0C – 10 minutes	1 cycle
Denaturation	95^0C – 10 seconds	40 cycles
Annealing	52^0C – 15 seconds	
Extension	72^0C – 30 seconds	
Hold	72^0C – 5 minutes	1 cycle
HRM	Melt from 72^0C to 95^0C rising by 0.05^0C at each step	

Analysis of results is described further below.

Validation of the HRM curves was done by performing Clustal W analysis of all 16S rRNA sequences for all isolates. A phylogenic tree was drawn to visualise the isolate clusters using the software Geneious version 6.1.4.

The following parameters were used for drawing the tree:

- Perform Clustal W analysis and import the file
- Select Mr Bayes, then Substitution Model JC69

- Rate Variation is Gamma and all other settings are left on Default.
- When interpreting, a Value of 1 means completely identical the Reference Strain ATCC12478.

3.3 Summary of experimental plan

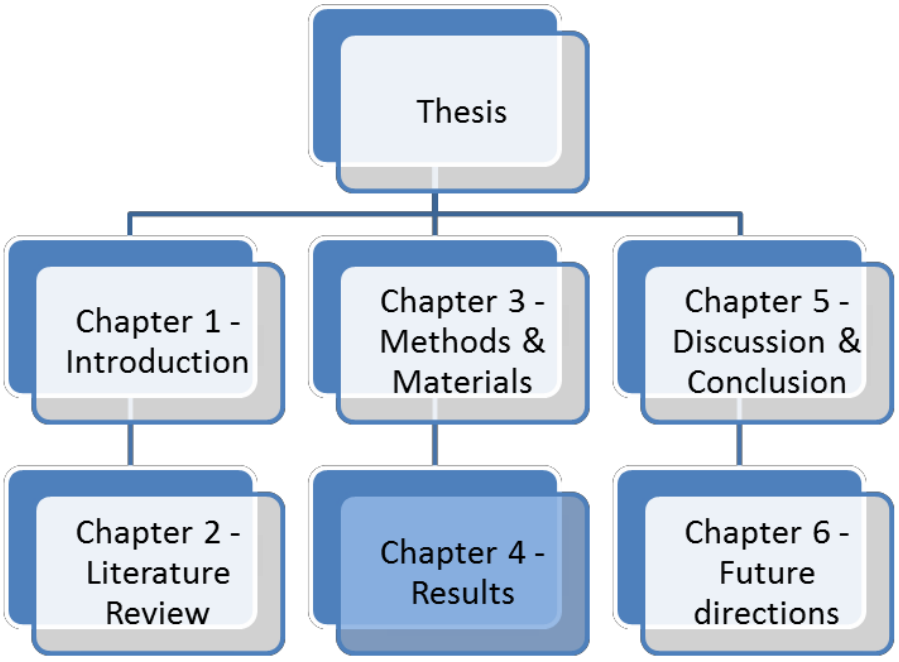
From the results generated from all methods for both clinical and water isolates, a comparison was produced to determine strain types of each isolate. ITS-REA was performed and analysed at first, as a known profile was produced to determine its strain type. To define the strain type, the enzyme digest key defined by Roth, *et al.* which displays the profiles for each *M. kansasii* type, was used (Roth *et al.* 2000).

For DiversiLab, once the QC was performed and completed for each isolate and each isolate has sufficient peaks for an accurate fingerprint, a dendrogram tree was drawn. This was done using the DiversiLab software version 3.4.38 with Pearson correlation co-efficient and unweighted pair group method with arithmetic means (UPGMA). This was used to compare the isolates and establish clonal relationships. DiversiLab similarity cut off values are determined as >97% - indistinguishable, >95% - similar and <95% - different.

HRM analysis was used for the first time to strain type *M. kansasii*. Not only was HRM performed, but a full Clustal W analysis looking specifically at the 16S sequence of all the isolates was also undertaken. From these sequences, a phylogenic tree was drawn using the software program Geneious version 6.1.4.

3.4 Ethics

This project had ethical approval under the following units: The Queensland University of Technology Research Ethics unit (Approval No. 0900000085) and the Human Research Ethics Committee of the Prince Charles Hospital (EC 2617).


Thesis
Chapter 1 - Introduction
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CHAPTER 4: RESULTS

4.1 Resuscitation of isolates

Selected isolates were removed from -80^{0}C permanent storage collection and thawed to room temperature. One Lowenstein-Jensen slope was inoculated with 250µL of Dubos broth (BD, USA) from the storage Cryo tube. Slopes were subsequently incubated at 36^{0}C for up to 6 weeks to allow resuscitation of isolates. Any isolates that were overgrown with non-acid fast organisms were decontaminated and inoculated into fresh Dubos broth and Lowenstein-Jensen slopes. The recovery rate of patient and environmental isolates was greater than 95%. Any isolates that didn't typically resemble *M. kansasii* were subjected to DNA extraction and were further identified using either 16S rRNA or *hsp*65 sequencing. Isolates identified as NTM other than *M. kansasii* were excluded from the study.

4.2 DNA Extraction

As previously stated, the only kit that we could use for DNA extraction that was validated, specifically for the DiversiLab assay, was the UltraClean® Microbial DNA extraction kit (MoBio, USA). In order to obtain high quality as well as a sufficient volume of DNA to perform the genotyping experiments in this study, modifications to the protocol were carried out. Specific modifications included:

1. In step number 4, the incubation temperature and time increased from 65^{0}C to 95^{0}C and from 10 minutes to 20 minutes respectively to allow for cell wall lysis to release the DNA contained.
2. In step number 5, the vortex time was increased from 10 minutes to 40 minutes to aid in cell wall lysis
3. In step number 16, the elution volume of MD5 solution was increased from 35µL to 50µL, to ensure sufficient amount of DNA for all three methods used in this study.

According to the manufactures instruction for DiversiLab, for a successful fingerprint to be generated a concentration of DNA was required to be 25-50ng/uL. Below is a chart to demonstrate the distribution isolate concentration ranges. The X-Axis demonstrates the DNA concentration obtained and the Y-Axis shows the number of isolates for each category of DNA concentration

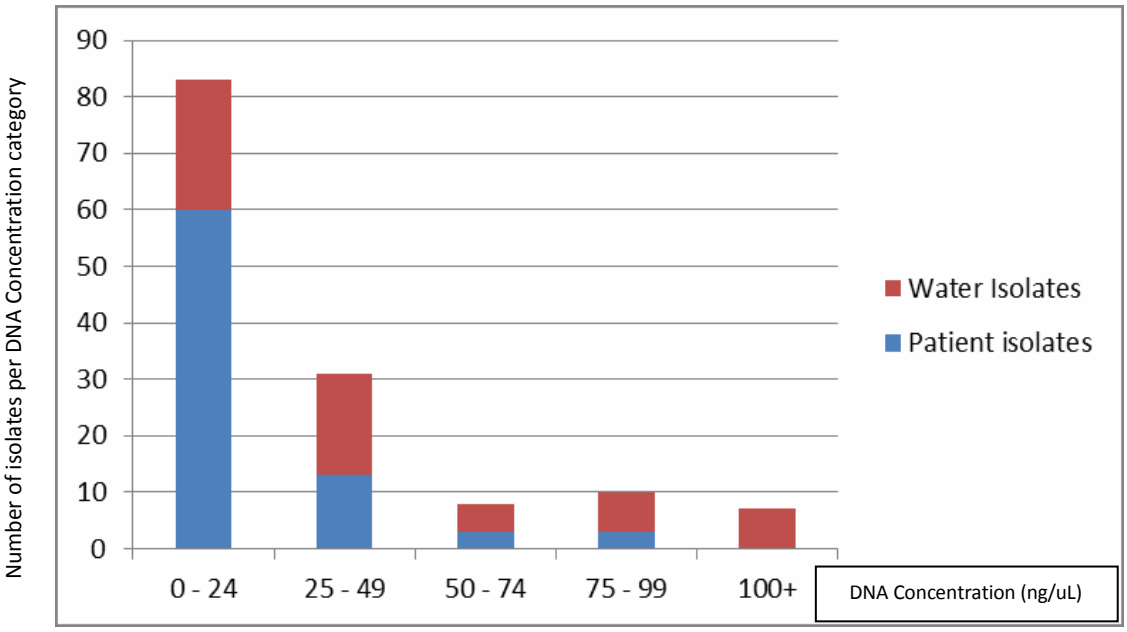


FIGURE 9: DNA CONCENTRATIONS ACQUIRED FROM DNA EXTRACTIONS USING THE MOBIO KIT (NG/UL)
Full results for each patient can be found in the Appendix.

4.3 DiversiLab

The PCR, preparation of DiversiLab chips and fingerprint analysis was performed as per the protocol supplied by bioMerieux, USA. Previous publications have described inconsistencies in the reproducibility of the DiversiLab method, and initially, this was also found in this study. Frequently an error of "low intensity" was received as a result of insufficient DNA (approximately 40% of extractions), in the samples. Modifications to DNA extraction outlined above appeared to help resolve these issues by decreasing the low intensity result to less than approximately 5%.

The DiversiLab v3.4.38 software was used to create a dendrogram of all the isolates (Appendix B). As per the protocol, analysis was performed using the Pearson correlation co-efficient and Unweighted Pair Group Method with Arithmetic Means (UPGMA) to compare isolates and determine clonal relationships. Any isolates that were ≥97% similarity were considered to be indistinguishable. Isolates that showed 95% similarity were considered similar and isolates with ≤95% were considered to be unrelated.

TABLE 11: NUMBER OF ISOLATES FALLING INTO EACH CATEGORY FOR RELATEDNESS WITH DIVERSILAB

	Indistinguishable	Similar	Unrelated	**Strain Clusters
Patient	14	36	24**	19
Environmental	17	12	32**	26

** Strain Clusters – defined as the amount of strain types that are unrelated at a ≤95% correlation. As a summary, there are 19 strain variants amongst the 24 Patient isolates and there are 26 strain clusters amongst the 32 Environmental isolates.

4.4 High Resolution Melt Analysis (HRM)

HRM was performed on all isolates used in this study. The HRM assay was found to be highly sensitive, as only 5-10ng/µL of DNA was required compared to 25-50ng/µL of DNA required for the DiversiLab method. To determine whether the strain type of each isolate, a criteria of either "same" or "different" was used as previously described by (Price *et al.* 2007) and (Stephens *et al.* 2008). A +5 Fluorescent Units (U) or a -5 U was used as the comparator in determining whether the strain was the same or different. When an isolate is greater than 5 U in the difference graph, it is considered to be different. If the isolate is less than 5 U, the isolate is deemed to be the same as the control used to compare. For this study, the ATCC 12478 *M. kansasii* strain was used as the comparator. Below are some examples of these normalised and difference graphs. In Appendix C, the full HRM data is displayed.

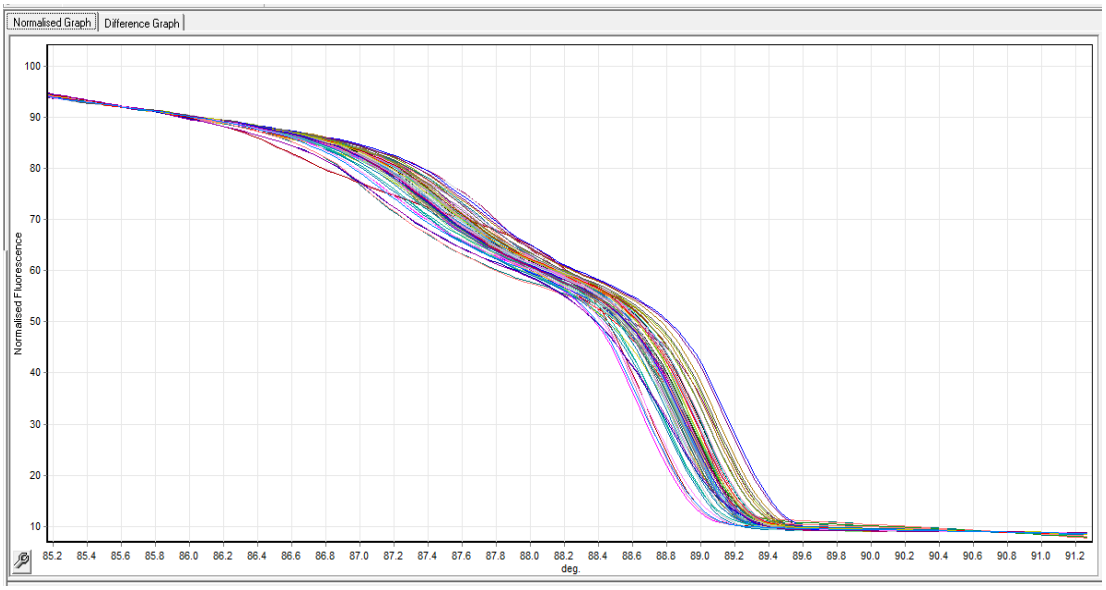


FIGURE 10: NORMALISED HRM CURVES OBTAINED FROM THE ROTORGENE 6000 SOFTWARE OF *M. KANSASII* PATIENT ISOLATES. Y – AXIS IS THE NORMALISED FLUORESCENCE AND X – AXIS IS THE MELTING TEMPERATURE IN DEGREES CELSIUS.

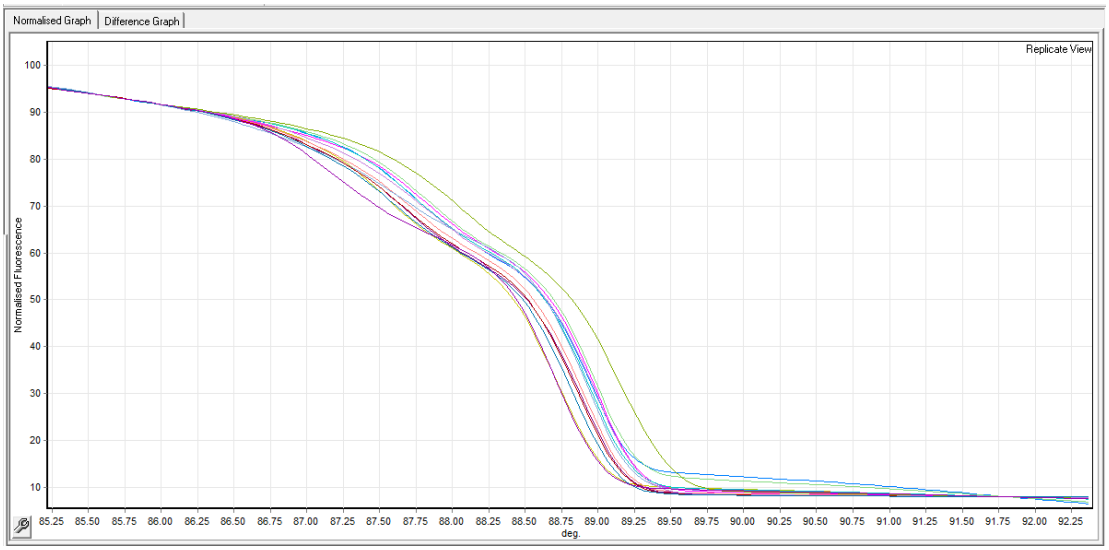


FIGURE 11: NORMALISED HRM CURVES OF WATER *M. KANSASII* ISOLATES. Y – AXIS IS THE NORMALISED FLUORESCENCE AND X – AXIS IS THE MELTING TEMPERATURE IN DEGREES CELSIUS.

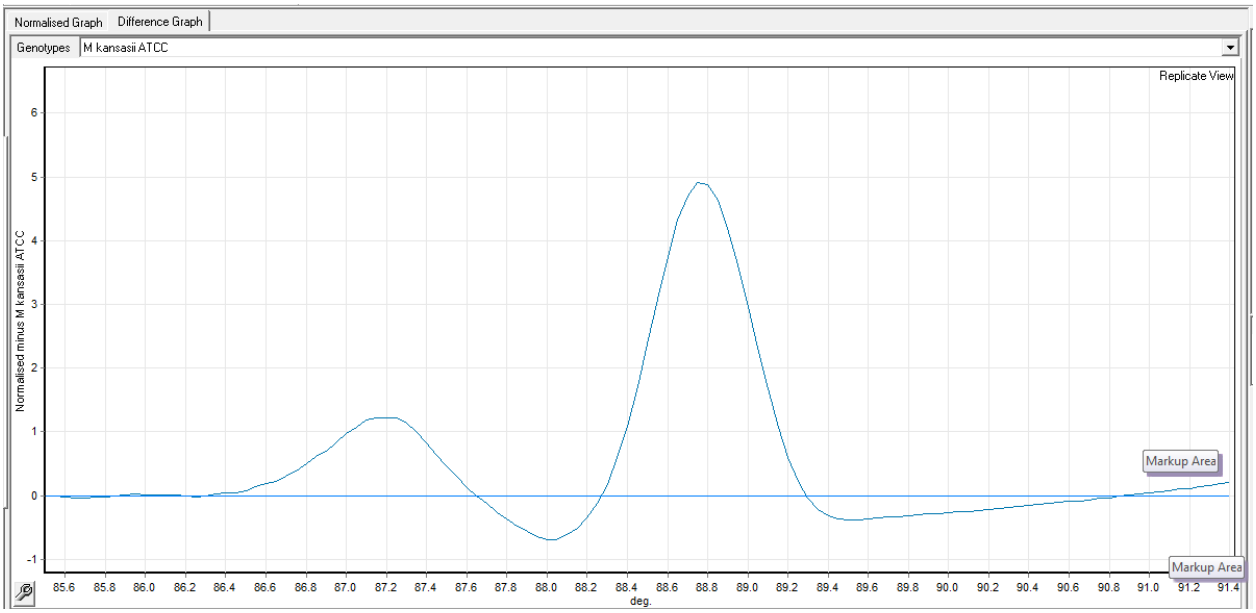


FIGURE 12: A DIFFERENCE CURVE FOR A PATIENT *M. KANSASII* ISOLATE WHICH IS ILLUSTRATING THE "SAME" PROFILE AS THE CONTROL *M. KANSASII* ATCC 12478 STRAIN. Y – AXIS IS THE NORMALISED FLUORESCENCE MINUS THE REFERENCE STRAIN AND X – AXIS IS THE MELT TEMPERATURE IN DEGREES CELSIUS WHERE THE STRAIN DEVIATES FROM THE REFERENCE STRAIN.

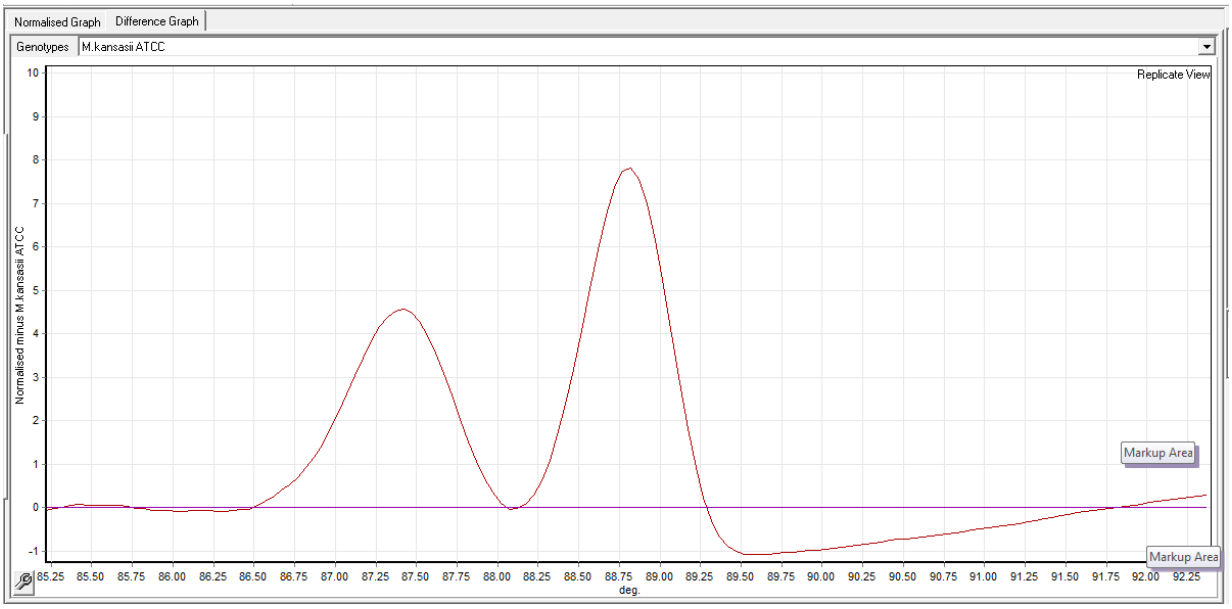


FIGURE 13: DIFFERENCE CURVE FOR A WATER *M. KANSASII* ISOLATE WHICH IS DEMONSTRATING THAT THE ISOLATE IS A "DIFFERENT" PROFILE AND NOT THE SAME STRAIN AS THE CONTROL. Y – AXIS IS THE NORMALISED FLUORESCENCE MINUS THE REFERENCE STRAIN AND X – AXIS IS THE MELT TEMPERATURE IN DEGREES CELSIUS WHERE THE STRAIN DEVIATES FROM THE REFERENCE STRAIN.

4.5 Validation of HRM results

16S rRNA sequencing was performed on each of the isolates in order to validate the separation of the HRM curves. These sequences were aligned using the Clustal

W alignment function of the BioEdit software program (version 7.2.5). A typical output from the alignment is shown in Figure below.

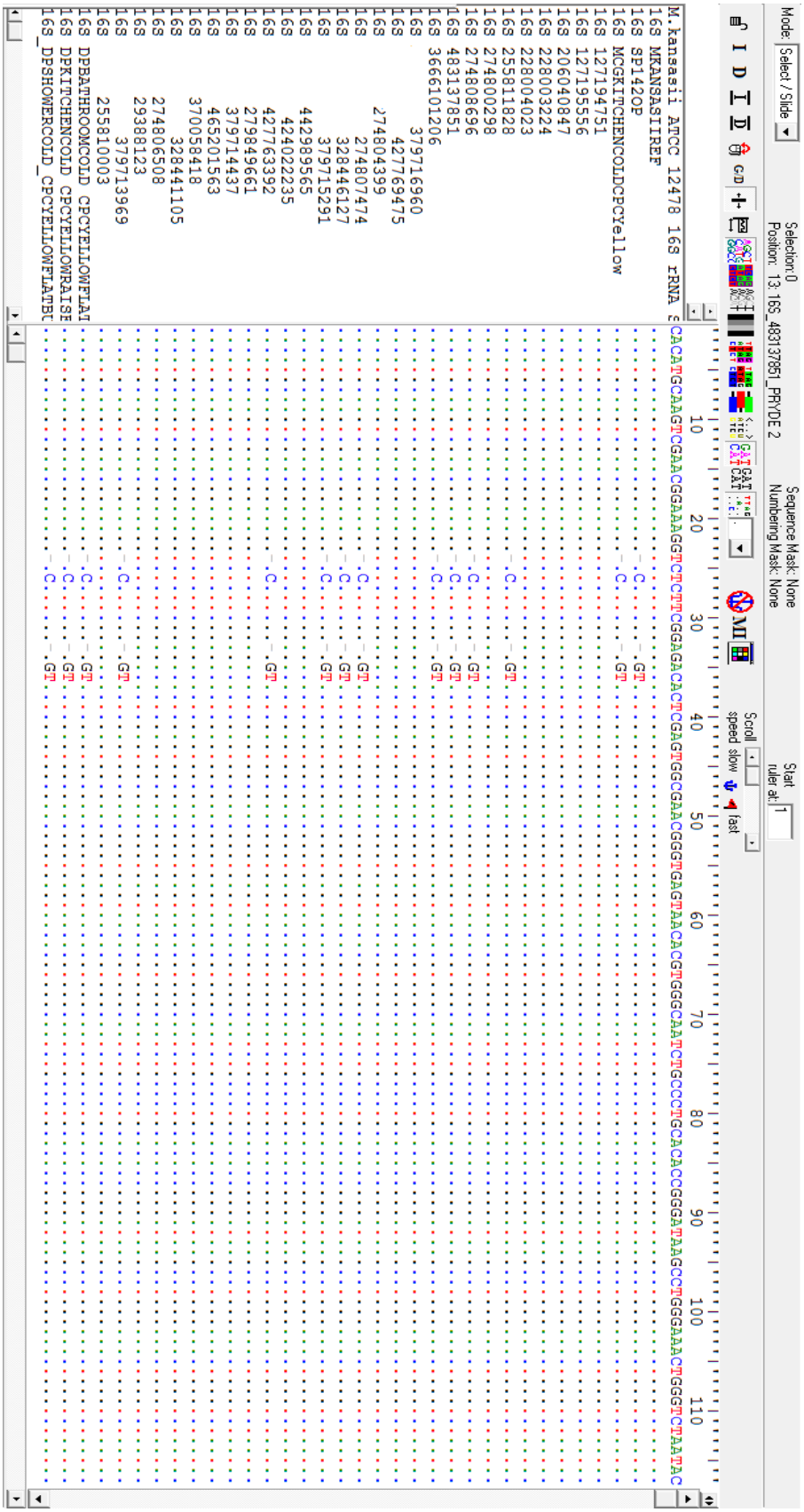


FIGURE 14: CLUSTALW ALIGNMENT OF *M. KANSASII* PATIENT AND WATER ISOLATES. SINGLE NUCLEOTIDE POLYMORPHISMS (SNP) WERE OBSERVED FOR STRAINS THAT DEVIATED FROM THE *M. KANSASII* ATCC 12478 REFERENCE STRAIN. VISUAL INSPECTION OF THESE SEQUENCES DETERMINED THAT STRAIN TYPES I, IV & V'S COULD BE SEPARATED BASED ON EACH CLUSTER'S SNP DIFFERENCES.

A phylogenetic tree was created using the Mr Bayes application as input for the Geneious version 6.1.4 program. The HRM assay was quick to perform (three hour turnaround time per run) on a large set of isolates tested in this study. From the data produced, HRM showed good inter-run reproducibility as seen by the melt curve temperatures being very close together. The complete set of HRM results and associated Phylogenetic tree are listed in Appendix D: HRM Results. Below is a graphical summary of the results for same vs. different as previously described

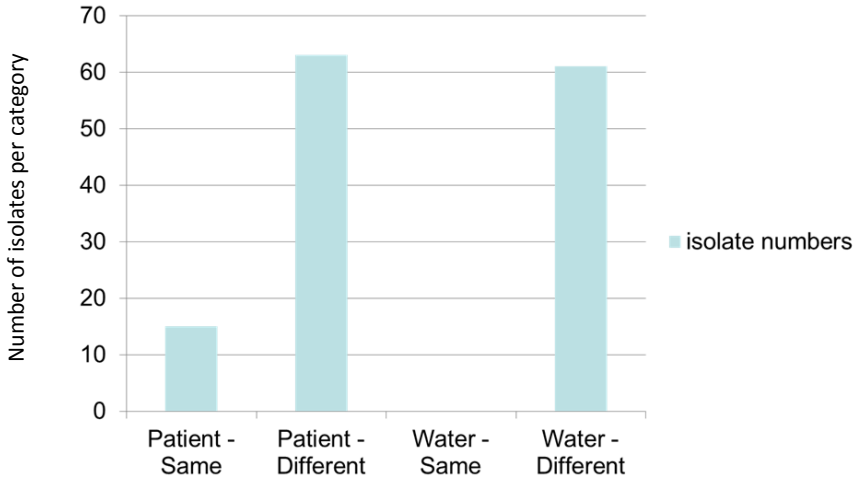


FIGURE 15: GRAPHICAL REPRESENTATION OF SAME VS DIFFERENT NUMBERS OF ISOLATES DETERMINED BY HRM. THIS IS SHOWN FOR BOTH PATIENT ISOLATES AND WATER ISOLATES.

4.6 ITS-REA

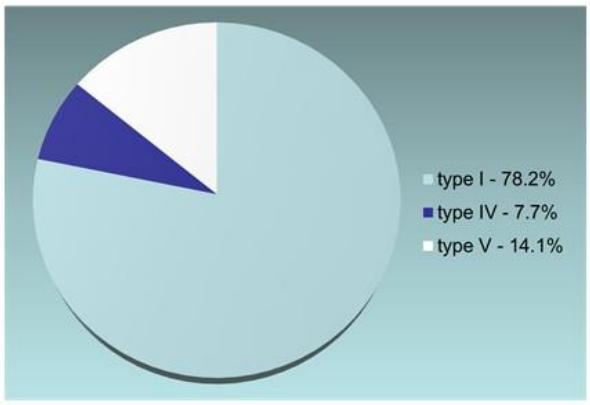
This method was robust and reproducible and it produced clear product differentiation on the electrophoresis gel images. As devised by Roth *et al*. (2000), the strain types each had a clear pattern to distinguish particular strain types from each other (Roth *et al.* 2000). Using gel electrophoresis, each isolate was clustered into a strain type by this method based on its particular 16S-23S sequence. When we use this to compare with the 16S rRNA sequences of *M. kansasii*, most strains only contained one nucleotide change difference. Specifically, this can be observed between nucleotides 461-469 of the 16S rRNA region (Table 12 below). As it is only a single nucleotide difference, it is not a reliable method for strain

differentiation. The 16S-23S showed better differentiation and hence it is superior for subtyping within the *M. kansasii* species. Figure 16 shows the strain type proportion of both the patient isolates and water isolates.

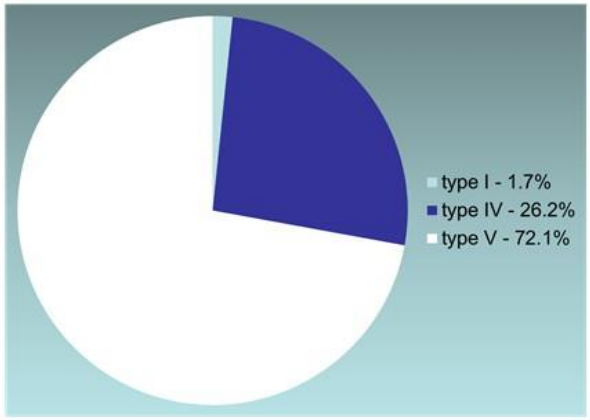
The electrophoresis gel pictures images for isolates tested in this study are located in Appendix B: ITS-REA Enzyme Digest Gel Pictures.

TABLE 12: NUCLEOTIDE CHANGES BETWEEN STRAINS TYPE OF *M. KANSASII* AS ADAPTED FROM (ROTH *ET AL.* 2000)

***M. kansasii* strain type**	**16S rRNA sequence profile nucleotide 461-469 region.**
Type I	CGG CTT **C**TC
Type IV	CGG GTT **T**TC
Type V	CGG GTT **TC**C



Patient strain type proportion



Brisbane water strain type proportion

FIGURE 16: STRAIN TYPE PROPORTION OF PATIENT AND WATER ISOLATES DEMONSTRATED IN A PIE CHART. THE PROPORTION BETWEEN BOTH THE PATENT ISOLATES AND THE BRISBANE WATER ISOLATES ARE SURPRISING. THE PATIENT STRAIN TYPE DOMINANT IS TYPE I BUT THE WATER STRAIN TYPE DOMINANT IS TYPE V.

4.7 *M. kansasii* genotyping results summary

Results from all three genotyping assays (DiversiLab, HRM and ITS-REA) have been presented as a Venn diagram to show each strain type with the three typing methods and how well they correlated with each other. A scatterplot of each patient and water isolates created by DiversiLab software has also been included as another form for visualisation of results. Finally a table representation of the results is also included.

Venn Diagrams are seen below and they illustrate the relationship between each of the assays and demonstrate how many isolates correlate with the other typing methods performed.

Abbreviations:

- ITS-REA: Internally Transcribed Spacer region with Restriction Enzyme Analysis.
  ITS-REA is the assay that grouped the isolates used in this study into the previously identified three main strain types (I, IV & V). This is why a 100% strain type identification using this was standardised.
- HRM: High Resolution Melt analysis.
  HRM showed relatedness to strain type I only and no strain relationship with type IV or V. This was due to the control strain used in this project (only one available) being closely related to strain type I.
- Numbers in the Boxes: demonstrate how many isolates match to the previously assigned strain types using ITS-REA as the basis for the analysis.

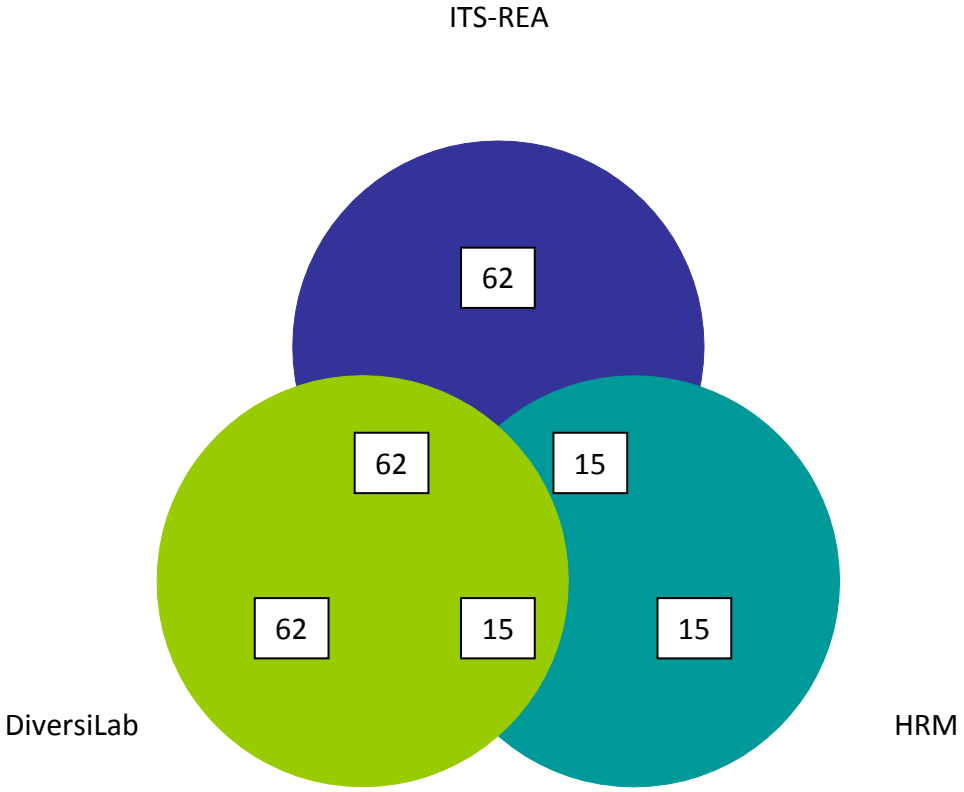


FIGURE 17: VENN DIAGRAM OF THE NUMBER OF *M. KANSASII* PATIENT AND WATER ISOLATES COMBINED FOR ITS-REA STRAIN TYPE I. SEE PAGE 46 FOR THE ABBREVIATIONS AND DEFINITIONS.

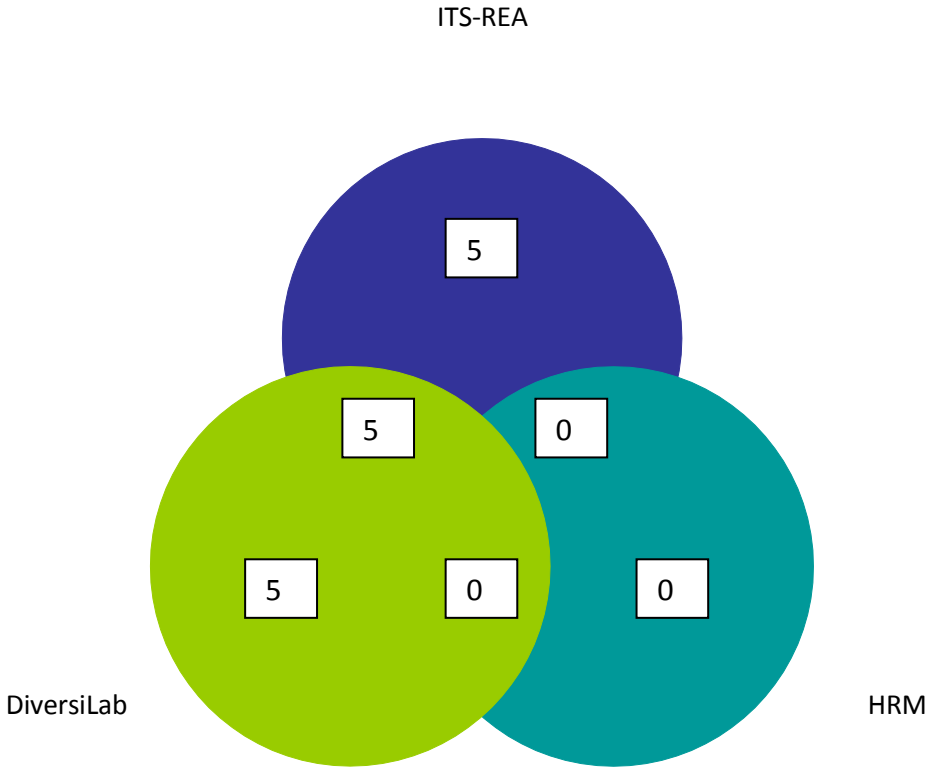


FIGURE 18: VENN DIAGRAM OF THE NUMBER OF *M. KANSASII* PATIENT AND WATER ISOLATES COMBINED FOR ITS-REA STRAIN TYPE IV. SEE PAGE 46 FOR THE ABBREVIATIONS AND DEFINITIONS.

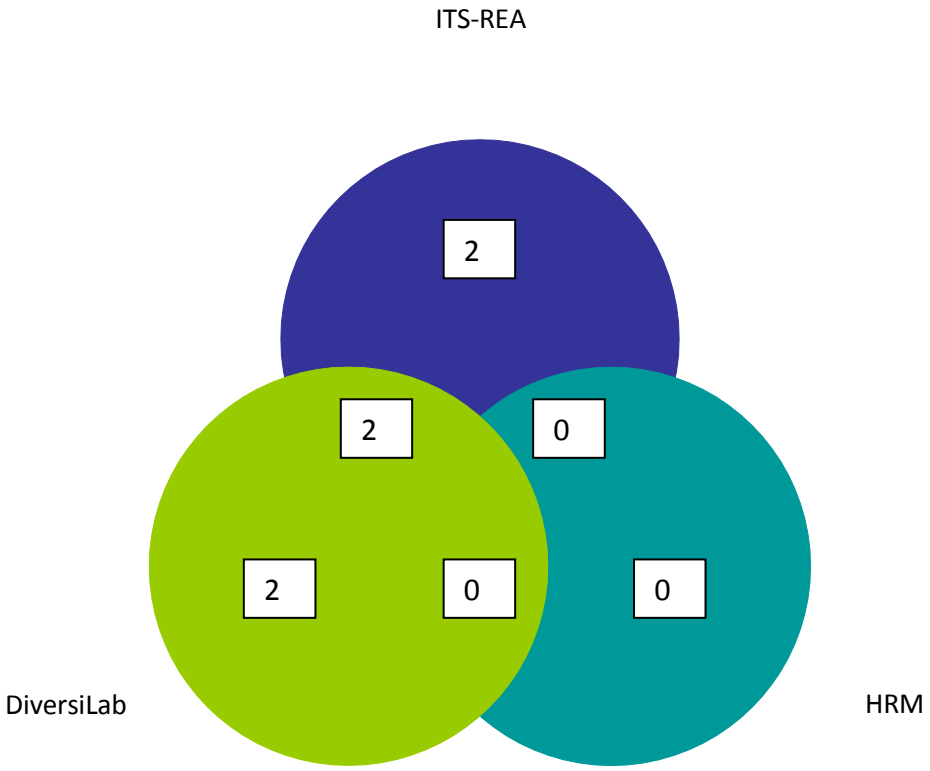


FIGURE 19: VENN DIAGRAM OF THE NUMBER OF *M. KANSASII* PATIENT AND WATER ISOLATES COMBINED FOR ITS-REA STRAIN TYPE V. SEE PAGE 46 FOR THE ABBREVIATIONS AND DEFINITIONS.

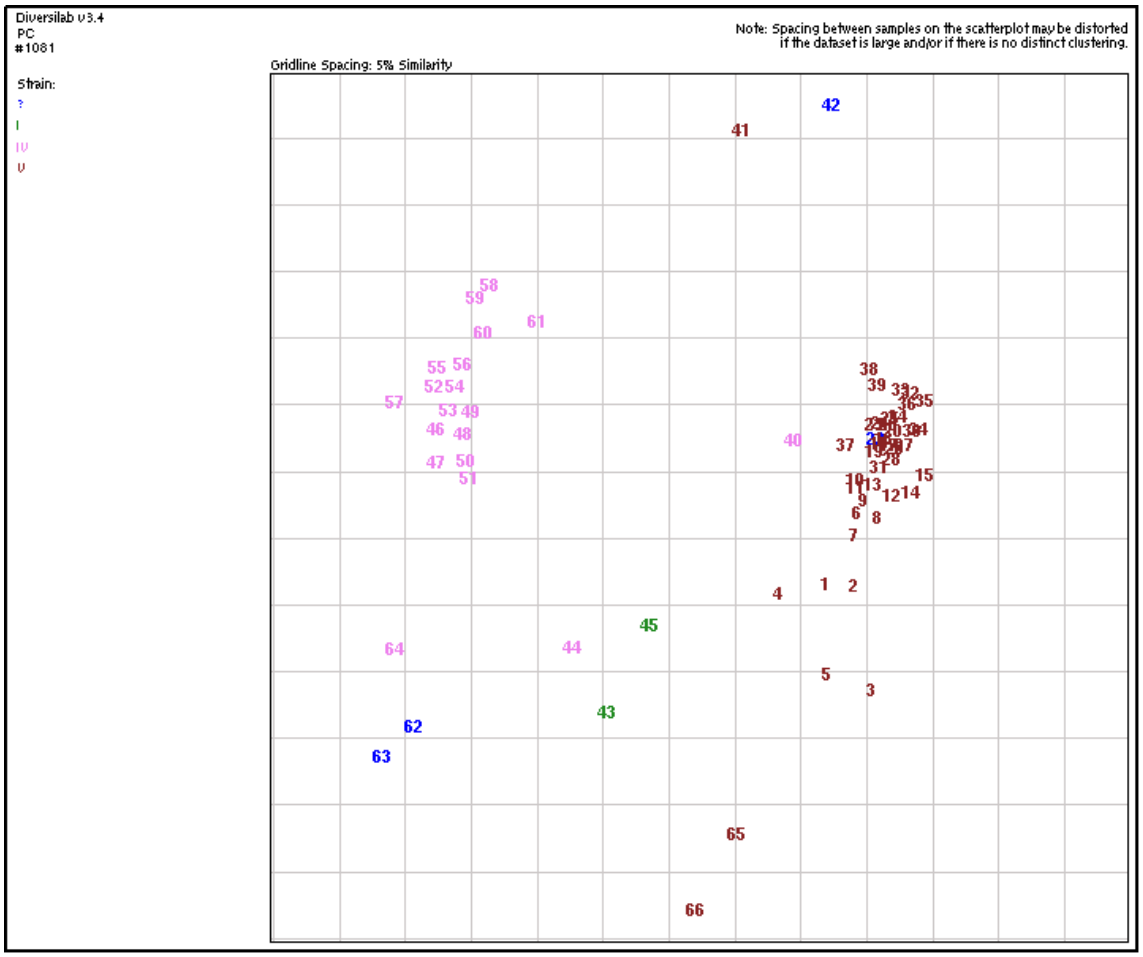


FIGURE 20: SCATTERPLOT OF *M. KANSASII* WATER ISOLATES. THE STRAIN TYPES DETERMINED BY THE ITS-REA ARE COLOUR CODED (I, IV & V) AND ISOLATES WERE GROUPED BY THEIR INDIVIDUAL FINGERPRINT GENERATED FROM DIVERSILAB SOFTWARE.

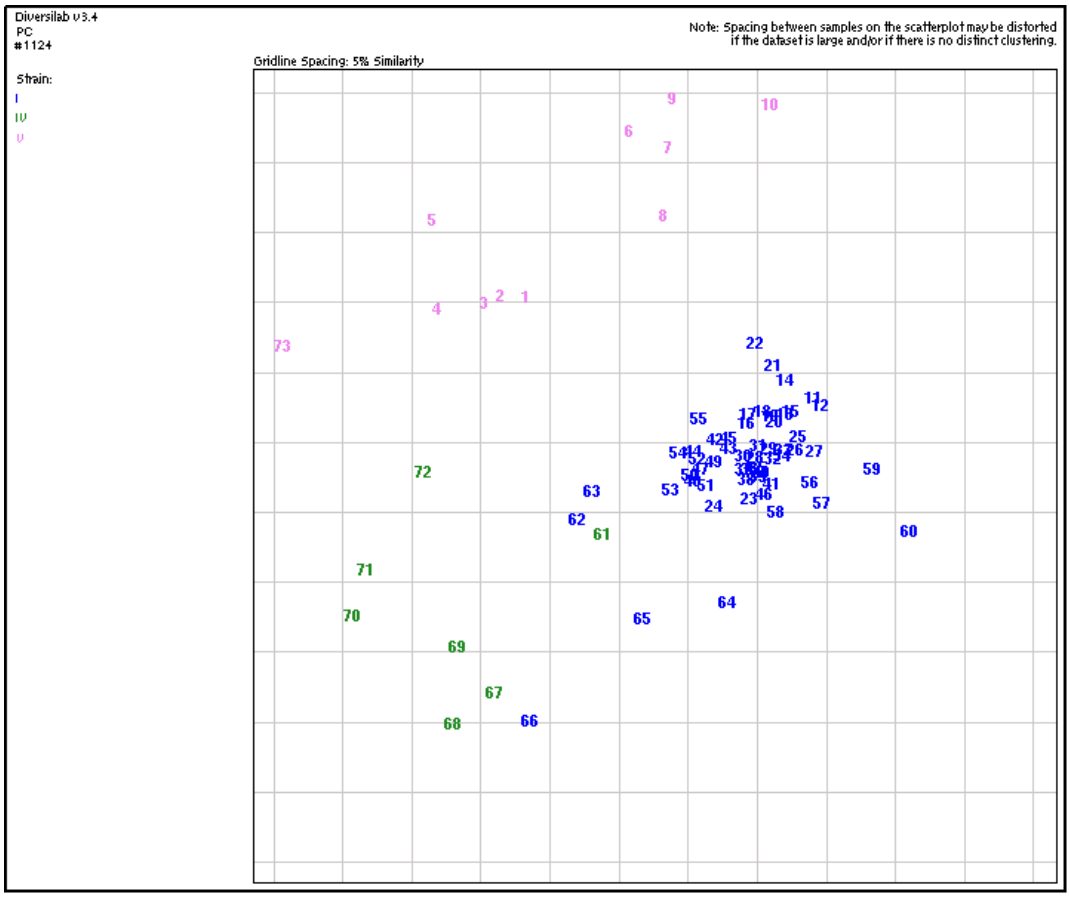
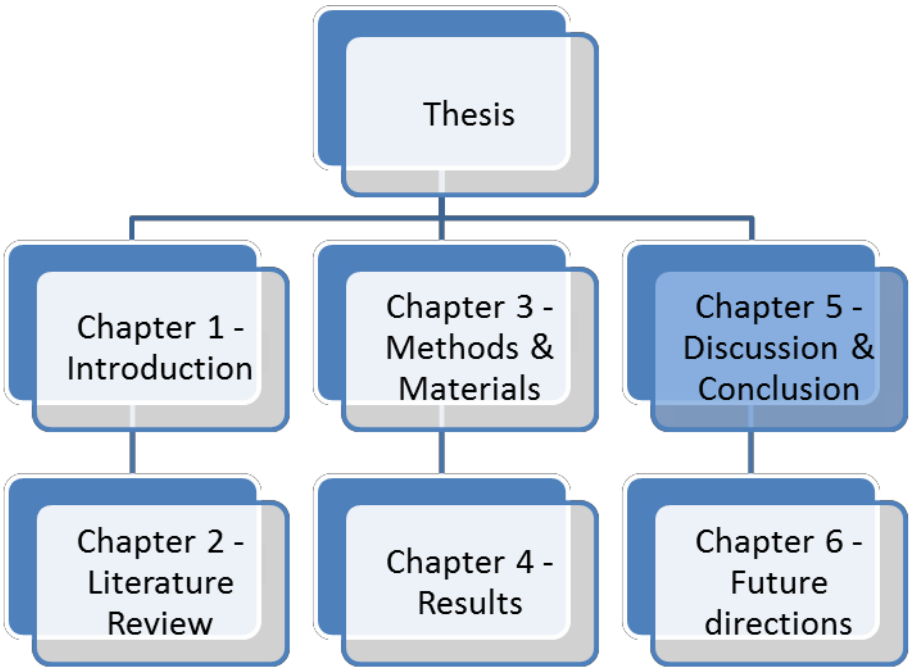


FIGURE 21: SCATTERPLOT OF *M. KANSASII* PATIENT ISOLATES. THE STRAIN TYPES DETERMINED BY THE ITS-REA ARE COLOUR CODED (I, IV & V) AND ISOLATES WERE GROUPED BY THEIR INDIVIDUAL FINGERPRINT GENERATED FROM DIVERSILAB SOFTWARE.

Method	ITS-REA	DiversiLab	HRM
Number of types produced	3	31	Same or Different
Typeability	100%	80-100% (after re-extraction)	95-100%
Speed (TAT)	2 days	5 hours	4 hours
Cost (per isolate)	$30~	$48~	50 cents~
Skill level required	Basic-Intermediate	Intermediate	Basic-intermediate

TABLE 13: COMPARISON OF THE THREE M. KANSASII GENOTYPING METHODS EXAMINED

As a summary of the figures and tables provided to show the visual analysis, Figure 16-18 are Venn diagrams to show how each strain type of *M. kansasii* matches up per strain typing method. Figure 19 and 20 are Scatterplots generated by DiversiLab Software. It is an excellent visual tool to show how much diversity there is between every isolate both Patient and Environmental. Table 13 is a summary handy for a Diagnostic Laboratory as it demonstrates Turn-Around-Time for the assay as well as the Cost, Typeability and Sensitivity.


Thesis
Chapter 1 - Introduction
Chapter 3 - Methods & Materials
Chapter 5 - Discussion & Conclusion
Chapter 2 - Literature Review
Chapter 4 - Results
Chapter 6 - Future directions

CHAPTER 5: DISCUSSION & CONCLUSIONS

5.1 Previously used typing methods

Strain typing has progressed over time as highly-advanced technologies have become available to better differentiate and subtype species of Mycobacteria. The first typing method described used bacterial phages to strain-type Mycobacteria (Engel 1975). Currently, DNA-based assays are considered the gold standard as the demand for more rapid and cost effective methods increases.

From the previously discussed methods found in the literature for *M. kansasii*, each assay had positive and negative attributes with regards to reproducibility, specificity and sensitivity. Engel's technique required specialised equipment and many weeks to perform the assay (Engel 1975). In addition, phage typing doesn't provide the level of specificity required to accurately and robustly type NTM isolates. Picardeau *et al*. (1997) were the first to investigate a variety of different DNA-based assays for typing bacterial isolates. RFLP with the MPTR probe, PFGE, AFLP & PRA demonstrated excellent reproducibility and typeability across all strain types. The IS1652 probe could only detect types II & III but could discriminate sub-groups of type II better than any other assay and AccuProbe couldn't detect types II, III or IV. Each of the assessed assays had positive and negative aspects with regards to specificity and sensitivity and the requirement of specific equipment for some of these methods. Overall, Picardeau *et al*. demonstrated that the previously established five strain types of *M. kansasii* were identified by the assays previously discussed. Some of the methods revealed polymorphisms, which then allowed for further strain differentiation.

Alcaide *et al*. (1997) used a specific housekeeping gene *hsp*65 with restriction fragment length polymorphism PCR, followed by enzyme digest and visualisation of products on an agarose gel. They also evaluated other techniques similar to Picardeau (1997) and obtained similar results overall. Again, most of these techniques could not be used for this study due to the lack of specific expensive

equipment required to perform the assays such as PFGE tanks with current/voltage controlling apparatus and gel quantification analysis software and equipment. Similarly, methods described by Iinuma *et al*. (1997) who used large restriction fragment length polymorphisms, digested with *Vsp*I and visualised on PFGE could not be applied in this study due to the lack of specialised equipment. They demonstrated varieties of different subtypes within known types *M. kansasii* strains as well successfully replicating their results. Some subtypes were also compared using 16S rRNA sequencing and it was noted that all isolates tested from one subtype classification had identical sequences. A disadvantage to this assay is the length of time required to obtain results. It takes many days to perform this test from start to finish as a high quality DNA product yield is required initially.

Other groups that used the same techniques as described by the Picardeau & Iinuma researchers were: Gaafar *et al*. (2003), Taillard *et al*. (2003) and Zhang *et al*. (2004). There were also some retrospective studies performed by Santin *et al*. (2004) and Chimara *et al*. (2004), where the focus of these studies were to report on the demographics of infection with regards to HIV status of the patients whose isolates were used in the study. From the results published, they concurred to a degree with our results. They each described strain type I as the most dominant type in each study, which matched our findings from this research. Most described type II and III isolates, however these were not found amongst the Queensland isolates studied. We could suggest that maybe these particular strains are geographic specific as they've not been isolated in Queensland before (Psaltis 2005).

The assays discussed above have demonstrated advantages and disadvantages with regards to reproducibility, specificity and sensitivity. Apart from not having access to the equipment for most of the methods described previously, it was necessary to develop and validate more accurate and robust typing methods. These new methods are required to provide a greater level of strain discrimination, improve

cost effectiveness and ability to attain rapid strain typing results with equipment available in a routine diagnostic laboratory.

5.2 The main aims produced by this study

There were two main aims in this study which were:

1. *To genotype* M. kansasii *isolates using new and existing genotyping methods;* and
2. *To compare the strain genotypes of* M. kansasii *isolates from clinical samples to those obtained from Brisbane water and patient home water samples*.

In addressing these aims, the following conclusions can be made:

Aim 1 –

The DiversiLab method was used to produce genotypic fingerprints and dendrograms for all isolates tested in this study. There were some issues with the reproducibility of the assay, consistent with those previously described (Zelazny *et al*. (2009), Cheng *et al.* (2013) and Thomson *et al.* (2013). Using this technique as the only diagnostic tool for *Mycobacterium* sp. is a possibility but could be limited due to the reproducibility related to the difficulty in obtaining sufficient DNA required. The DiversiLab software program is user-friendly and can provide a wide range of analytical tools, such as the fingerprint dendrograms as demonstrated, as well as scatterplots and pyramid plots, all at the touch of a button.

In order to obtain sufficient, high quality DNA for the DiversiLab method, the extraction protocol had to be altered significantly. If the manufacturer could guarantee a reproducible assay with consistent results and competitive pricing, then DiversiLab would be a better alternative to PFGE or other genotyping methods.

Initially, the development and application of HRM as a strain typing method was difficult to do, as this method is novel for genotyping *M. kansasii* isolate. Factors in determining how to make this assay viable included – looking specifically at the test conditions, the primer selection as well as how to perform analysis validation. However, the only downfall of the HRM genotyping method is that it requires comparison to known strain types in order to assign unknown strains to a specific type. Sequencing analysis of the 16S rRNA regions showed SNPs unique to each strain of *M. kansasii*. The other benefits of this assay are: it is inexpensive (approximately 50 cents per test), running time from start to finish is only 3 hours and the analysis is quick and easy to perform, as it doesn't require highly skilled staff to operate or analyse the results. This assay certainly has the potential to be the new gold standard for *Mycobacterium sp.* genotyping in the future.

The ITS-REA method proved to be most robust and consistent genotyping method of the three methods tested in this study. DNA concentration was not a concern as results were obtained irrespective of the quantity and quality of the DNA. However, a drawback of this method is that it was difficult to analyse the gel electrophoresis images for some isolates, and it was difficult to visualise very faint bands generated by enzyme restriction. There was no readily available software program to analyse the ITS-REA data and hence the results could only be interpreted by visualising the restriction enzyme bands on the gel. This can lead to misidentification of the numbering of DNA fragment base pairs. However, together with the DiversiLab and HRM results, accurate strain types could be assigned by ITS-REA. Another disadvantage of the ITS-REA method is that it is time-consuming and not feasible to use in a routine diagnostic laboratory, however, the advantage is that it is a robust method providing consistent inter-run results.

Aim 2 –

The discriminatory power of each assay varied greatly. DiversiLab was the most discriminatory, producing intricate dendrograms which showed differing strains in

more detail compared to the other genotyping methods. The dendrogram tree gave a percentage similarity which made it easy to detect the clonal strains. After final analysis, 31 strains were identified by the DiversiLab method, which was more strain types than the other methods. HRM has a high discriminatory potential as well, as it can determine one SNP difference when compared to a reference strain. If more reference strain types were available, it would have been possible to optimise this assay for routine genotyping in a diagnostic laboratory. ITS-REA only has the capability for determining the six known strain types of *M. kansasii*, but it also can determine other species of mycobacteria (whereas the DiversiLab and HRM can demonstrate differences within the six strain types). DiversiLab also has the capacity to determine different species by showing outliers in the dendrogram with significant degrees of difference.

The strain types obtained from this study are consistent with those reported in other regions of the world. Strain type I has been shown to be the cause of the majority of clinically significant disease in humans. The most commonly observed patient isolate strains observed in this study was strain type I and some type IV and V strains were also found. However, the water isolates showed a different picture with only one type I isolate found and the rest of the isolates were type IV and V. This suggests that water is not likely to be a common source of infection for *M. kansasii* in human disease. Other areas of the world have found type II, III and VI and very few strain types IV and V in water (Zhang *et al.* 2004). This may mean that the environmental strains could be location specific.

As a summary for comparing the attributes of each genotyping method for both clinical and environmental samples, all methods could identify strain type I. DiversiLab and ITS-REA could identify strain types IV and V, but HRM identification could not be validated for those strains due to the lack of appropriate reference strains for these genotypes. As we have a large enough sample size to use as a validation, it would be the next logical step forward for implementation. Extrapolating from the data, the strain types that were identified by ITS-REA to be type IV and V did appear to cluster tightly using HRM analysis. Therefore, this study found that each typing method was able to correctly characterise

each of the major strains types found globally.

5.3 Optimisation of DNA yield from *M. kansasii* isolates

Of the three assays used for strain typing, only one required a specific extraction kit. The DiversiLab assay recommends that only the UltraClean® Microbial DNA extraction kit (MoBio, USA) be used. This is due to the high degree of purity required to give reliable fingerprint results. However this proved to be inadequate and modifications had to be made to the extraction protocol to achieve a high DNA concentration.

Initially, when the manufacturer's instructions were followed precisely, problems were encountered with low DNA yield (i.e. <10ng/µL). It was clear that modifications to the method were necessary to increase DNA concentration. After discussions with the company's technical support, the incubation temperature, the incubation time and the vortex time were increased. These changes made a significant improvement to the DNA yield with increases in concentration from <10ng/µL to 50ng/µL.

5.4 ITS-REA typing of *M. kansasii* isolates

The method gave a broad level of species discrimination using the 16S-23S spacer region, not seen previously with the 16S rRNA region. As Roth discussed, some species of Mycobacteria have the same 16S region (Roth *et al.* 2000). Using the 16S-23S spacer region provided better discrimination between many *Mycobacterium* species. When restriction enzyme analysis was applied, sub-species of some of these Mycobacteria were discovered. Roth *et al*. (2000) demonstrated that this method had a high level of discrimination between subtypes of *M. kansasii*. Using the algorithm published by Roth *et al.* (2000), isolates could be allocated a strain type with easy interpretation. Using the ITS-REA typing method enabled the exclusion of some isolates in this study that were not *M. kansasii* but *M. gastri* (species that share identical 16S rRNA sequences).

The ITS-REA assay was selected for this study as it has been used successfully in the QMRL

laboratory (Psaltis 2005), allowing an analysis of trends in *M. kansasii* strain types overtime; The patient isolate selection in this study was from 2005 - 2010, specifically selected in order to correlate this study's findings to those found by Psaltis's previous study (2005). From Psaltis's research, strain types I, IV, V & VI were described for patient isolates from 1975-2004. Compared to the results obtained in this study, only strain types I, IV & V were found but no type VI.

Worldwide, the distribution of previously defined strain types has shown that *M. kansasii* type I is the most dominant (Alcaide *et al.* 1997; Iinuma *et al.* 1997), however, in this study, only three strain types were found in Queensland. The absence of strain types II and or III could be due to the climatic differences and the geographical location of Australia. This assay could also be used as an identification method to speciate other mycobacteria. In this study, there were a few isolates (seven isolates) that didn't quite fit the typical ITS-REA pattern for *M. kansasii*; however, further identification using the Roth *et al*. (2000) algorithm found that these isolates were in fact not *M. kansasii* but other *Mycobacterium* species.

5.5 DiversiLab rep-PCR typing of *M. kansasii*

DiversiLab is a relatively new strain typing method. One of the first publications that compared this method to an established typing method, Pulse Field Gel Electrophoresis (PFGE) was published in 2005 (Ross *et al.* 2005). They found that DiversiLab had good intra- and inter-run reproducibility, a rapid turnaround time and standardised results.

This method was selected for use in this study due to the rapid turnaround time for an accurate strain typing result. A fingerprint can be achieved in one day instead of several days compared to PFGE.

DiversiLab has not been applied to *M. kansasii* previously, however, it has been used to genotype other mycobacterial species such as *M. tuberculosis* and *M. avium* (Cangelosi *et al.* 2004).

While there has been some work published on the utility of DiversiLab as a typing tool, the current study has shown that there was difficulty with the reproducibility of the method. This may be due to the fact that *M. kansasii* in particular have a very dry and buff colony morphology type, which may be difficult to lyse and hence the DNA yield is low and not of sufficient quality. In the QMRL laboratory, other mycobacterial species such as the rapidly growing *M. abscessus* are easier to extract DNA from. These organisms form their mycolic acid wall structure rapidly, and hence, it is easier to lyse the cell wall to attain a high yield and good quality DNA. The issue of sufficient high quality DNA is a limitation of the DiversiLab system, and it is recommended that at least 25-50ng/µL of high quality DNA is used. This requirement is required to produce reproducible typing results. Even with this in mind, it was still difficult to attain the minimum amount of DNA concentration. This is more than likely the main reason for variation between runs in this study.

Overall, it was difficult to determine whether the results obtained in this study correlate with other findings as currently there are no other reports on the application of the DiversiLab method to genotype *M. kansasii* isolates. What can be deduced is that the same difficulties experienced by other researchers were also found in this study. Also, it was observed that this method is a more accurate way of typing *M. kansasii* isolates than ITS-REA. This study found that the DiversiLab method is a rapid and an improved genotyping method compared to previous methods, such as PFGE. This is due to fact that the DiversiLab method provides a detailed fingerprint. In concordance with the ITS-REA results, the fingerprints generated agreed with the ITS-REA strain types, and hence, strain type numbers I, IV and V were assessed using the DiversiLab software. In addition, the software enables the detection of sub strain-type populations. This allows for further differentiation of strains within each of the strain type groups, therefore DiversiLab is a better typing tool than ITS-REA. The DiversiLab software can be used to visualise the data to show particular clusters and further details about the isolates can be added such as source (environmental/clinical; pulmonary vs extrapulmonary, etc.) allowing visual strain comparisons. An advantage of the DiversiLab is the capability of pooling isolates into one dendrogram, unlike the ITS-REA method where the limitation is the gel size, and in most cases multiple gels have to be used when genotyping large numbers of isolates.

5.6 High Resolution Melt Analysis of *M. kansasii* isolates

From published literature, HRM hasn't been used as a typing method for Mycobacteria but has been used more as a diagnostic tool to determine whether a resistance trait is present. This study is a first where HRM was used as a *M. kansasii* strain typing tool, and hence, further validation was required to determine the robustness of the method in a diagnostic setting. The 16S rRNA region was used as the target region for typing *M. kansasii* isolates, and this region is consistently used in the routine diagnostic laboratory at QMRL.

The specificity of the HRM method used in this study was limited by access to *M.* kansasii type strains. The HRM assay determined whether unknown test isolates were the same or different type to the control *M. kansasii* type strains. None of the water isolates matched the control strain. This is expected because the isolates were predominantly types IV & V. Of the 61 type I patient isolates tested, only 15 matched (24.59%) the *M. kansasii* type strain. This could be due to the enhanced sensitivity of the HRM method compared to ITS-REA, and that type I sub-strains were found in this study. This has been shown for other typing methods by (Alcaide *et al.* 1997; Iinuma *et al.* 1997). When comparing the HRM results with the DiversiLab patterns, the same low percentage of type I isolates matching the *M. kansasii* type strain was found. The DiversiLab also demonstrated a degree of difference amongst the type I strains. With respect to DiversiLab, the degree of separation seen amongst the type I isolates was only a small percentage. HRM was similar with its results as well. When comparing the known type I strains with the
M. kansasii type strain, most isolates were only slightly greater or slightly lesser than the 5 U of deviation (just out of set limits).

This study found that HRM was a robust and sensitive genotyping tool, which was quick and could easily be applied in a routine diagnostic laboratory. This method requires a single piece of equipment to perform both the PCR and HRM reactions (Rotorgene, Qiagen), the reagents required are minimal, the cost is low and results can be obtained in one day.

The validation results obtained in this study showed that HRM can determine *M. kansasii* strain type I isolates. *M. kansasii* type I strains are the most dominant strains worldwide (Alcaide *et al.* 1997; Iinuma *et al.* 1997), and therefore, HRM is ideally suited to rapidly screen *M. kansasii* isolates in the routine diagnostic laboratories worldwide, which will assist clinicians to determine appropriate treatment for the patient. There are two notable limitations which limit the potential of this assay. Firstly, each run performed can only be analysed on a single run basis. Unlike the DiversiLab software where multiple runs can be combined to give a full profile, the HRM software doesn't have the capabilities to merge multiple runs together. If isolates from different runs need to be compared, the only way possible is to re-run the isolates together. Secondly, only one control strain is available. For every run performed, multiple controls are a necessity to complete a full analysis.

5.7 Conclusions

TABLE 14: SUMMARY OF THE METHODS USED - ADVANTAGES AND DISADVANTAGES OF EACH METHOD.

	Advantages	Disadvantages
ITS-REA	•Robust •Gold Standard	•Costly •Time consuming
DiversiLab	•Quick turnaround time •Good analysis capabilities	•Costly •High quantity of DNA required
HRM	•Quick turnaround time •Cheap assay	•Can't analysis large sample numbers •Can only analyse by using "same vs different

ITS-REA method used in this study was useful in broadly determining the *M. kansasii* strain diversity in Queensland compared to previous international benchmarks. When comparing patient *M. kansasii* isolates to environmental isolates, all three methods proved to be effective genotyping methods. However, each of the methods had advantages and disadvantages, but essentially, each of these methods could be used routinely in a diagnostic setting. ITS-REA is laborious and takes approximately two days to obtain a result, where both the DiversiLab and HRM methods can be done in one day from DNA extraction to obtaining results. Both DiversiLab and HRM require specialised platforms, which are not available in all routine diagnostic laboratories. The greatest advantage offered by the DiversiLab genotyping method is that it can provide clinicians with strain type information indicative of either colonisation or disease, which will impact on patient treatment. A limitation of the ITS-REA method is that it only provides a broad strain type number, whereas both DiversiLab and HRM methods are able to sub-type *M. kansasii* isolates within each ITS-REA group, providing enhanced strain discrimination.

When considering the cost of each of the three methods, ITS-REA costs approximately $30 per isolate, DiversiLab is the most expensive at $48 per isolate and HRM is the most inexpensive at only 50 cents per isolate. For a diagnostic laboratory where budgets are crucial, HRM would be the obvious genotyping method to use. Besides the cost factor, the quick turn-around time of DiversiLab and HRM to identify strain types is important. As previously stated, differentiating between colonisation vs. disease could potentially impact therapy. It is also important to differentiate between relapse vs. reinfection for patients who culture multiple isolates over time. Using either assay, a strain comparison can be determined quickly. Rapid strain differentiation may also be of particular use in the setting of an outbreak, where a particular source is suspected. To determine what type of infection the patient has, multiple isolates need to be run in parallel. With DiversiLab and HRM, this is possible.

There were few limitations in this study. With regards to strain typing, the limitations included obtaining high DNA yield required for the DiversiLab method. Not knowing how much culture was required at the beginning of the DNA extraction was challenging. Due to the high fatty acid bi-lipid layer of mycobacteria, as well as *M. kansasii* being a very dry buff colony morphology type, made it difficult to lyse the cells to release the DNA. At the same time, it was also difficult to ensure the quality of the DNA was not affected once the cells were lysed. For example, shearing of the DNA during the extraction procedure had to be monitored closely during the bead-beating step. It is therefore challenging to ensure good quality and sufficient DNA was available to use for the genotyping methods.

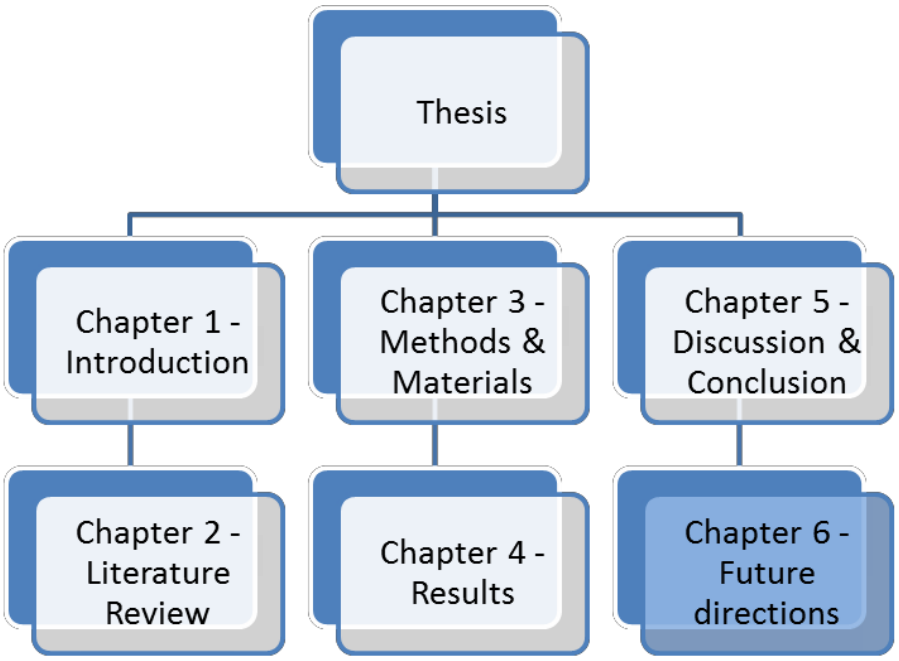
Further limitations included not having additional *M. kansasii* reference strain types available for use. These strains would have ensured the robustness of the HRM assay in enabling accurate strain differentiation and strain identification. Without access to a comprehensive collection of *M. kansasii* strain types, it is difficult to establish these methods in a routine diagnostic laboratory.

This is the first study to compare three different genotyping techniques applied to *M. kansasii* isolates from both clinical and environmental sources. Results from this study have enabled the determination of the most timely, robust and cost-effective genotyping method for *M. kansasii* isolates from diverse sources. HRM showed potential for a rapid result of same or different, but DiversiLab proved to be a better typing method giving better diversity amongst the defined isolates.

Overall, ITS-REA was an important assay to perform for this research. Without the results, we could not determine or validate the results that we obtained from the two other assays. HRM could only determine an isolate to be either “same” or “different”. As disappointing the test results were, this test could be fine-tuned and developed further to be a rapid assay in a diagnostic setting. The only limitation that can be foreseen is having enough data to create an extensive library to

compare the test to. DiversiLab was by far the most superior assay as the discrimination power was superior to the other assays. The software makes it user friendly to analyse and compare isolates against other previous isolates. This has the potential for clinicians to retrospectively look at isolates from other patients and look for the occurrence of outbreaks and possible clusters.

As hypothesised in this study, it was possible to differentiate and characterise all *M. kansasii* strains used in this study. The DiversiLab method was by far the best at differentiating strains, with ITS-REA providing a broad strain "baseline" for strain typing. HRM could be used if sufficient reference/known strain types are used as controls for the assay.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CHAPTER 6: FUTURE DIRECTIONS

From the results generated by this study, we have established recommendations for future work:

- To investigate other environmental sources of infection, for example soil and dust. These environmental sources of *M. kansasii* infections have not been determined in Queensland.
- Following further optimisation of these assays, they could be implemented in the routine diagnostic laboratory. Knowledge of *M. kansasii* strain types could possibly assist clinicians in appropriate patient treatment options.
- Collecting and filtering water from other parts of Queensland, or even other parts of Australia should be done to determine the broader distribution of environmental *M. kansasii* strain types.
- When mapping of the patients' addresses, it was found that they resided in small area clusters (Chou *et al.* 2014). Further investigation into their water sources or even other potential sources of infection is warranted.

Future directions of this research could entail the following:

- Performing Whole Genome Sequencing of the *M. kansasii* isolates would enable detailed strain specific and sub-type identification of type I isolates in particular, given that these strains are the dominant types found in most parts of the world. In addition, other genetic targets could be identified on the genome that could enable improved strain typing of *M. kansasii* compared to ITS or 16S rRNA.
- Acquiring supplementary reference strains to further validate the HRM would be useful and could therefore be implemented into a diagnostic laboratory.

- Further collection and filtering of water samples from the clustal patient areas such as Roma and Toowoomba in Queensland could be performed. These areas where high levels of *M. kansasii* isolation from patients have occurred warrant further investigation.
- Virulence factors have not been published previously. With regards to strain types of *M. kansasii* further investigation into whether one strain type is more likely to cause disease than other types would be clinically relevant.

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Appendices

APPENDIX A1: THE GEOGRAPHICAL LOCATION BY SUBURB AND POSTCODE OF PATIENTS' FROM WHOM *M. KANSASII* WAS ISOLATED FROM

AUSLAB#	Patient Name	Patient Location	Postcode
395755535	PaOk	Brisbane City	4005
395709239	PaOk	Brisbane City	4005
379716960	HaAm	Brisbane City	4005
255811828	RaMo	Brisbane City	4007
370058418	BrCl	Brisbane North	4030
394353072	DeWa	Brisbane North	4030
275130015	ChMa	Brisbane North	4031
352222085	GeTe	Brisbane North	4032
228004760	EiVe	Brisbane North	4034
465201563	JaCl	Brisbane North	4034
451496292	DeRo	Brisbane North	4034
337068637	ViSm	Brisbane North	4035
274807474	LeBe	Brisbane South West	4077
127195556	PeHu	Brisbane City	4101
255810003	RoDo	Brisbane City	4101
207238565	GrCl	Brisbane South	4103
328445213	GrCl	Brisbane South	4103
127198945	KaHa	Brisbane South	4103
431019367	AlSe	Brisbane North	4104
356665897	LuPe	Brisbane South	4109
468263113	AnVa	Brisbane South	4110
417675063	AnVa	Brisbane South	4110
328441086	BeSo	Brisbane South East	4121
483137851	DoPr	Brisbane South East	4121
328444903	BrHa	Brisbane South East	4122
420525523	PaGr	Brisbane South East	4151
461586782	PaGr	Brisbane South East	4151
228003390	JoSt	Brisbane South East	4152
274806508	PeDa	Brisbane South East	4157
328446127	SuBe	Brisbane East	4159
379715291	SuBe	Brisbane East	4159
329116087	PeHe	Brisbane East	4160
329119359	PeHe	Brisbane East	4160
272993344	PeFr	Brisbane East	4172
442989565	GoBl	Brisbane East	4179
206040847	GlKe	Gold Coast	4207
427761068	KeEc	Gold Coast	4210
385497420	PaRe	Gold Coast	4211
274804399	GiAv	Gold Coast	4214
29386800	GiOs	Gold Coast	4214
328449089	GiOs	Gold Coast	4214
465207950	GiOs	Gold Coast	4214

218688812	NaSa	Gold Coast	4215
274808696	EiBr	Gold Coast	4223
427763392	EiBr	Gold Coast	4223
277121288	HaSo	Brisbane South West	4301
424022235	BeBo	Brisbane South West	4301
29388123	MiDa	Brisbane South West	4305
484665030	DaGo	Brisbane South West	4305
439710005	JaSh	Brisbane South West	4340
224345408	WiEl	Toowoomba	4350
228004023	LiMa	Toowoomba	4350
261241461	TaJe	Toowoomba	4350
312561032	GaWi	Toowoomba	4352
275135168	GaWi	Toowoomba	4352
320215986	GaWi	Toowoomba	4352
29387870	GeGo	Western Queensland	4427
228003224	MeLu	Western Queensland	4455
274241613	NoOw	Western Queensland	4455
379849661	ViCh	Western Queensland	4455
379714437	ViCh	Western Queensland	4455
328441105	JoCo	Western Queensland	4455
427766149	LeWh	Western Queensland	4455
427769475	MuAn	Western Queensland	4455
313939095	LeYo	Brisbane North	4503
465247398	RaGo	Brisbane North	4508
127194751	RoFl	Brisbane North West	4520
379713969	AlDo	Sunshine Coast	4551
383998336	ZoFu	Sunshine Coast	4557
398259809	AmWe	Sunshine Coast	4562
402734769	GrJo	Sunshine Coast	4566
486287283	GrJo	Sunshine Coast	4566
453520236	FrMc	Sunshine Coast	4566
336402419	DaMa	Burnett Region	4610
366610206	LaPe	Central Queensland	4670
425454927	NaKu	Central Queensland	4680
274800298	GaHa	Central Queensland	4700
255812452	LeWe	North Queensland	4805
275135278	IaRa	North Queensland	4810

APPENDIX A2: DNA CONCENTRATIONS FOR ALL THE ISOLATES TESTED IN THIS STUDY.

AUSLAB#	Patient Name	DNA Concentration (ng/µL)	Sample Name	DNA Concentration (ng/µL)
395755535	PaOk	12.1	JK KITCHEN COLD +CPC RAISED BUFF YELLOW	115.4
395709239	PaOk	20.1	JK KITCHEN COLD +CPC RAISED BUFF YELLOW	27.5
379716960	HaAm	15.2	JK BATHROOM COLD +CPC ORANGE RAISED NM	87.9
255811828	RaMo	36.2	JK BATHROOM TAP B +CPC ORANGE RAISED NM	86.1
370058418	BrCl	53.9	JK SHOWER HOT +CPC YELLOW RAISED M	125.1
394353072	DeWa	18.7	JK SHOWER COLD +CPC YELLOW RAISED NM	8.9
275130015	ChMa	8.6	NW SHOWER COLD +CPC YELLOW BUFF FLAT	34.7
352222085	GeTe	34.6	MCG KITCHEN COLD WHITE FLAT BUFF	71.8
228004760	EiVe	27.5	MCG KITCHEN COLD +CPC YELLOW RAISED	24.1
465201563	JaCl	14.9	MD AEROSOL PLATE CREAM FLAT BUFF - LIKE	25.6
451496292	DeRo	78.8	DP KITCHEN +CPC YELLOW RAISED M	39.8
337068637	ViSm	8.5	DP BATHROOM COLD +CPC YELLOW FLAT BUFF	36.7
274807474	LeBe	24.8	DP SHOWER COLD +CPC YELLOW FLAT BUFF	34.7
127195556	PeHu	26.3	LYH1 SHOWER COLD OADC + PANTA XMGIT 9/9	14.4
255810003	RoDo	17.9	SP2 +CPC #1	91.3
207238565	GrCl	47.4	SP2 +CPC #2	125.6
328445213	GrCl	10.5	SP3 7H11 +CPC BUFF YELLOW	34.0
127198945	KaHa	19.0	SP7 XMGIT? 8/8	16.6
431019367	AlSe	11.3	SP18 +CPC	159.1
356665897	LuPe	22.8	SP19 +CPC 13/6/07	10.6
468263113	AnVa	18.5	SP24 +CPC 13/6/07 (11/7)	23.0
417675063	AnVa	60.1	SP24	52.2
328441086	BeSo	54.7	SP32 +CPC WHITE BUFF FLAT 30/8/07	17.6
483137851	DoPr	22.3	SP32 O+C 29/8	17.5
328444903	BrHa	8.7	SP33 O+P+C 4/9	144.2
420525523	PaGr	10.2	SP33 +CPC WHITE LARGE FLAKY 20/6 (SU)	17.5
461586782	PaGr	79.1	SP41 +CPC 14/6/07 (12/7)	45.6
228003390	JoSt	37.3	SP49	96.3
274806508	PeDa	19.4	SP57 +CPC 1/8/07 YELLOW FLAT BUFF	15.9
328446127	SuBe	17.3	SP67 O+C 29/8	55.3
379715291	SuBe	22.3	SP89 +PLATE	85.6
329116087	PeHe	11.9	SP89 21/12	12.8
329119359	PeHe	12.8	SP102 O+P 29/8	159.8
272993344	PeFr	18.0	SP104 +CPC LARGE WHITE FLAT 20/6	23.1
442989565	GoBl	39.8	SP104 O+P+C 5/8	90.9

AUSLAB#	Patient Name	DNA Concentration (ng/µL)	Sample Name	DNA Concentration (ng/µL)
206040847	GlKe	7.7	SP105 +CPC 12/6/07	78.2
427761068	KeEc	11.8	SP112	27.0
385497420	PaRe	20.0	SP112 +CPC 12/4	32.5
274804399	GiAv	10.6	SP119 +CPC FLAT LARGE	24.4
29386800	GiOs	16.7	SP119 +CPC 14/06/07 (11/7)	32.4
328449089	GiOs	21.4	SP127 XMGIT 5/8/07 (14/12)	11.8
465207950	GiOs	15.7	SP147 O+P+C 4/9	10.8
218688812	NaSa	32.5	SP152 +CPC LARGE BUFF #1	35.5
274808696	EiBr	9.1	SP152 +CPC LARGE BUFF #2	64.4
427763392	EiBr	26.5	SP154 +CPC CREAM/WHITE FLAT BUFF 9/8/0	8.9
277121288	HaSo	10.9	SP155 +CPC WHITE/CREAM RAISED V BUFF 9/8/07 (30/11)	60.3
424022235	BeBo	20.3	SP155 +O 4/9	12.2
29388123	MiDa	11.1	SP157 +CPC WHITE 31/7/07 (4/12)	22.4
484665030	DaGo	22.3	SP157 WHITE 31/7/07 (4/12)	47.0
439710005	JaSh	16.1	SP175 O+P+C 10/9	36.7
224345408	WiEl	14.2	SP178 +CPC BUFF	22.9
228004023	LiMa	16.2	SP142 O+P 5/9	22.1
261241461	TaJe	21.4	SP183 +CPC WHITE 28/11 (14/12)	17.4
312561032	GaWi	9.7	SP183 +CPC SMALLER YELLOW 28/11 (14/12)	46.5
275135168	GaWi	11	SP217 +CPC CREAM FLAT M 7/8/07	12.4
320215986	GaWi	23.8	SP218 WHITE 7/8/07 913/12)	9.4
29387870	GeGo	27	SP251 +CPC X7H11 BUFF LARGE	16.3
228003224	MeLu	12.8	SP260 +CPC WHITE FLAT BUFF 8/8/07	11.9
274241613	NoOw	25.7	SP281 O+C 29/8	148.6
379849661	ViCh	8.8	SP311 O+P+C 4/9	17.0
379714437	ViCh	21.1		
328441105	JoCo	7.6		
427766149	LeWh	33.5		
427769475	MuAn	20.4		
313939095	LeYo	16.3		
465247398	RaGo	11.1		
127194751	RoFl	38.2		
379713969	AlDo	22.0		
383998336	ZoFu	8.2		
398259809	AmWe	12.8		
402734769	GrJo	16.4		
486287283	GrJo	11.2		
453520236	FrMc	24.9		
336402419	DaMa	13.6		
366610206	LaPe	8.2		
425454927	NaKu	18.3		
274800298	GaHa	85.4		
255812452	LeWe	15.4		
275135278	IaRa	10.2		

APPENDIX A3: ENVIRONMENTAL WATER ISOLATES – *PLEASE SEE KEY BELOW TABLE FOR DEFINITIONS OF EACH COLUMN

Plate Location*	Sample Name*	HaeIII*	CfoI*	DdeI*	Strain Type*	DiversiLab Pattern#*	HRM Strain Match*	Sequencing results for 16S rRNA*
A1	JK KITCHEN COLD +CPC RAISED BUFF YELLOW	No restriction	pattern A	pattern A	V	3	No	*M. kansasii*
B1	JK KITCHEN COLD +CPC RAISED BUFF YELLOW	No restriction	No restriction	pattern A	I	27	No	*M. kansasii*
C1	JK BATHROOM COLD +CPC ORANGE RAISED NM	No restriction	pattern A	pattern A	V	3	No	*M. kansasii*
D1	JK BATHROOM TAP B +CPC ORANGE RAISED NM	No restriction	pattern A	pattern A	V	3	No	*M. kansasii*
E1	JK SHOWER HOT +CPC YELLOW RAISED M	No restriction	pattern A	pattern A	V	1	No	*M. kansasii*
F1	JK SHOWER COLD +CPC YELLOW RAISED NM	No restriction	pattern A	pattern A	V	3	No	*M. kansasii*
G1	NW SHOWER COLD +CPC YELLOW BUFF FLAT	95,85,42	pattern A	pattern A	IV	16	No	*M. kansasii*
H1	MCG KITCHEN COLD WHITE FLAT BUFF	No restriction	pattern A	pattern A	V	11	No	*M. kansasii*
A2	MCG KITCHEN COLD +CPC YELLOW RAISED	95,85,42	pattern A	pattern A	IV	14	No	*M. kansasii*
C2	MD AEROSOL PLATE CREAM FLAT BUFF - LIKE	95,85,42	pattern A	pattern A	IV	16	No	*M. kansasii*
D2	483137851 DP patient isolate	No restriction	pattern A	pattern A	V	28	No	*M. kansasii*
E2	DP KITCHEN +CPC YELLOW RAISED M	No restriction	pattern A	pattern A	V	3	No	*M. kansasii*
F2	DP BATHROOM COLD +CPC YELLOW FLAT BUFF	No restriction	pattern A	pattern A	V	7	No	*M. kansasii*
H2	DP SHOWER COLD +CPC YELLOW FLAT BUFF	No restriction	pattern A	pattern A	V	3	No	*M. kansasii*
A3	LYH1 SHOWER COLD OADC + PANTA XMGIT 9/9	No restriction	pattern A	pattern A	V	2	No	*M. kansasii*
B3	SP2 +CPC #1	No restriction	pattern A	pattern A	V	4	No	*M. kansasii*
C3	SP2 +CPC #2	No restriction	pattern A	pattern A	V	4	No	*M. kansasii*
D3	SP3 7H11 +CPC BUFF YELLOW	No restriction	pattern A	pattern A	V	1	No	*M. kansasii*
E3	SP7 XMGIT? 8/8	No restriction	pattern A	pattern A	V	9	No	*M. kansasii*
F3	SP18 +CPC	No restriction	pattern A	pattern A	V	5	No	*M. kansasii*
G3	SP19 +CPC 13/6/07	No restriction	pattern A	pattern A	V	2	No	*M. kansasii*
H3	SP24 +CPC 13/6/07 (11/7)	No restriction	pattern A	pattern A	V	3	No	*M. kansasii*
A4	SP24	No restriction	pattern A	pattern A	V	3	No	*M. kansasii*
B4	SP32 +CPC WHITE BUFF FLAT 30/8/07	95,85,42	pattern A	pattern A	IV	14	No	*M. kansasii*
C4	SP32 O+C 29/8	No restriction	pattern A	pattern A	V	2	No	*M. kansasii*

Plate Location*	Sample Name*	HaeIII*	CfoI*	DdeI*	Strain Type*	DiversiLab Pattern#*	HRM Strain Match*	Sequencing results for 16S rRNA*
D4	SP33 O+P+C 4/9	95,85,42	pattern A	pattern A	IV	23	No	*M. kansasii*
E4	SP33 +CPC WHITE LARGE FLAKY 20/6 (SU)	No restriction	pattern A	pattern A	V	2	No	*M. kansasii*
F4	SP41 +CPC 14/6/07 (12/7)	No restriction	pattern A	pattern A	V	3	No	*M. kansasii*
G4	SP49	95,85,42	pattern A	pattern A	IV	21	No	*M. kansasii*
H4	SP57 +CPC 1/8/07 YELLOW FLAT BUFF	95,85,42	pattern A	pattern A	IV	20	No	*M. kansasii*
A5	SP67 O+C 29/8	95,85,42	pattern A	pattern A	IV	15	No	*M. kansasii*
B5	SP89 +PLATE	No restriction	pattern A	pattern A	V	3	No	*M. kansasii*
C5	SP89 21/12	No restriction	pattern A	pattern A	V	3	No	*M. kansasii*
D5	SP102 O+P 29/8	95,85,42	pattern A	pattern A	IV	16	No	*M. kansasii*
E5	SP104 +CPC LARGE WHITE FLAT 20/6	95,85,42	pattern A	pattern A	IV	19	No	*M. kansasii*
F5	SP104 O+P+C 5/8	95,85,42	pattern A	pattern A	IV	16	No	*M. kansasii*
G5	SP105 +CPC 12/6/07	No restriction	pattern A	pattern A	V**	12	No	*M. kansasii*
H5	SP112	No restriction	pattern A	pattern A	V	2	No	*M. kansasii*
A6	SP112 +CPC 12/4	No restriction	pattern A	pattern A	V	3	No	*M. kansasii*
B6	SP119 +CPC FLAT LARGE	No restriction	pattern A	pattern A	V	3	No	*M. kansasii*
C6	SP119 +CPC 14/06/07 (11/7)	No restriction	pattern A	pattern A	V	4	No	*M. kansasii*
D6	SP127 XMGIT 5/8/07 (14/12)	95,85,42	pattern A	pattern A	IV	17	No	*M. kansasii*
E6	SP147 O+P+C 4/9	No restriction	pattern A	pattern A	V	3	No	*M. kansasii*
F6	SP152 +CPC LARGE BUFF #1	No restriction	pattern A	pattern A	V	3	No	*M. kansasii*
G6	SP152 +CPC LARGE BUFF #2	No restriction	pattern A	pattern A	V	3	No	*M. kansasii*
H6	SP154 +CPC CREAM/WHITE FLAT BUFF 9/8/0	No restriction	pattern A	pattern A	V	3	No	*M. kansasii*
A7	SP155 +CPC WHITE/CREAM RAISED V BUFF 9/8/07 (30/11)	No restriction	pattern A	pattern A	V	2	No	*M. kansasii*
B7	SP155 +O 4/9	No restriction	pattern A	pattern A	V	10	No	*M. kansasii*
C7	SP157 +CPC WHITE 31/7/07 (4/12)	95,85,42	pattern A	pattern A	IV	16	No	*M. kansasii*
D7	SP157 WHITE 31/7/07 (4/12)	No restriction	pattern A	pattern A	V	4	No	*M. kansasii*
F7	SP175 O+P+C 10/9	95,85,42	pattern A	pattern A	IV	17	No	*M. kansasii*
G7	SP178 +CPC BUFF	No restriction	pattern A	pattern A	V	4	No	*M. kansasii*
H7	SP142 O+P 5/9	No restriction	pattern A	pattern A	V	18	No	*M. kansasii*
A8	SP183 +CPC WHITE 28/11 (14/12)	95,85,42	pattern A	pattern A	IV	15	No	*M. kansasii*

Plate Location*	Sample Name*	HaeIII*	CfoI*	DdeI*	Strain Type*	DiversiLab Pattern#*	HRM Strain Match*	Sequencing results for 16S rRNA*
B8	SP183 +CPC SMALLER YELLOW 28/11 (14/12)	95,85,42	pattern A	pattern A	IV	22	No	*M. kansasii*
D8	SP217 +CPC CREAM FLAT M 7/8/07	No restriction	pattern A	pattern A	V	2	No	*M. kansasii*
E8	SP218 WHITE 7/8/07 913/12)	No restriction	pattern A	pattern A	V	2	No	*M. kansasii*
F8	SP251 +CPC X7H11 BUFF LARGE	No restriction	pattern A	pattern A	V	8	No	*M. kansasii*
H8	SP260 +CPC WHITE FLAT BUFF 8/8/07	No restriction	pattern A	pattern A	V**	13	No	*M. kansasii*
A9	SP281 O+C 29/8	No restriction	pattern A	pattern A	V	5	No	*M. kansasii*
B9	SP311 O+P+C 4/9	No restriction	pattern A	pattern A	V	6	No	*M. kansasii*
C9	M. KANSASII ATCC 12478 POSITIVE CONTROL	No restriction	No restriction	pattern A	I	25	NA	*M. kansasii*
D9	M. KANSASII ATCC 12478 POSITIVE CONTROL	No restriction	No restriction	pattern A	I	25	NA	*M. kansasii*
E9	M. KANSASII ATCC 12478 POSITIVE CONTROL	No restriction	No restriction	pattern A	I	25	NA	*M. kansasii*
F9	NEGATIVE CONTROL							

*Key definitions of each identifier are as follows:

- *Plate Location* – When the ITS-REA was first performed, each isolate was run once and had its own unique position in the 96well plate. This identifier corresponds to the position on the electrophoresis gel images as seen in the **Appendix B: ITS-REA Enzyme Digest Gel Pictures**.
- *Sample Name* – identifier for that particular sample
- *Hae*III/*Cfo*1/*Dde*1 – ITS-REA enzyme result determined from the electrophoresis gel images; looking specifically at the characteristic banding pattern produced by each enzyme.
- *Strain type* – the ITS-REA profile determined by the 3 enzyme digests. These were compared to (Roth *et al.* 1998) and a typical strain type was delegated.
- *DiversiLab Pattern #* – the cluster number assigned by the DiversiLab software. The specific fingerprints created were aligned into a dendrogram and the isolates which were >97% in similarity were given the same pattern number.

- *HRM Strain Match* – comparison of each isolate to the *M. kansasii* ATCC strain and determined whether they were the same strain or a different strain.
- *Sequencing results for 16S rRNA* – 16S rRNA was performed on each isolate for two reasons: firstly to ensure the isolate was *M. kansasii* and it was pure and secondly the results were needed for the validation of HRM.
- ** - the pattern from DiversiLab was very different to the other type V isolates suggesting a new novel species.

APPENDIX A4: PATIENT ISOLATES – *PLEASE SEE KEY BELOW TABLE FOR DEFINITIONS OF EACH COLUMN

Plate Location*	AUSLAB#*	Patient Name*	HaeIII*	CfoI*	DdeI*	Strain Type*	DiversiLab Pattern#*	HRM Strain Match*	Sequencing results for 16S rRNA*
B1	127194751	RoFl	No restriction	No restriction	pattern A	I	3	No	*M. kansasii*
C1	127195556	PeHu	No restriction	No restriction	pattern A	I	4	No	*M. kansasii*
E1	207238565	GrCl	No restriction	No restriction	pattern A	I	8	No	*M. kansasii*
F1	328445213	GrCl	No restriction	No restriction	pattern A	I	3	No	*M. kansasii*
G1	206040847	GlKe	No restriction	No restriction	pattern A	I	7	No	*M. kansasii*
H1	228003224	MeLu	No restriction	No restriction	pattern A	I	7	No	*M. kansasii*
A2	224345408	WiEl	No restriction	No restriction	pattern A	I	2	No	*M. kansasii*
B2	228004023	LiMa	No restriction	No restriction	pattern A	I	4	No	*M. kansasii*
C2	228004760	EiVe	No restriction	No restriction	pattern A	I	3	Yes	*M. kansasii*
D2	255810003	RoDo	No restriction	No restriction	pattern A	I	3	No	*M. kansasii*
F2	255811828	RaMo	95,85,42	pattern A	pattern A	IV	37	No	*M. kansasii*
G2	127198945	KaHa	95,85,42	pattern A	pattern A	IV	34	No	*M. kansasii*
H2	255812452	LeWe	No restriction	No restriction	pattern A	I	3	No	*M. kansasii*
A3	272993344	PeFr	No restriction	No restriction	pattern A	I	2	No	*M. kansasii*
B3	277121288	HaSo	No restriction	No restriction	pattern A	I	3	No	*M. kansasii*
C3	261241461	TaJe	No restriction	No restriction	pattern A	I	1	No	*M. kansasii*
D3	274806508	PeDa	No restriction	No restriction	pattern A	I	1	No	*M. kansasii*
E3	228003390	JoSt	95,85,42	pattern A	pattern A	IV	33	No	*M. kansasii*
F3	274804399	GiAv	No restriction	No restriction	pattern A	I	1	No	*M. kansasii*
G3	29386800	GiOs	No restriction	No restriction	pattern A	I	2	No	*M. kansasii*
H3	328449089	GiOs	No restriction	No restriction	pattern A	I	2	No	*M. kansasii*
A4	465207950	GiOs	No restriction	No restriction	pattern A	I	6	No	*M. kansasii*
B4	274241613	NoOw	No restriction	No restriction	pattern A	I	6	No	*M. kansasii*
C4	275130015	ChMa	No restriction	No restriction	pattern A	I	19	No	*M. kansasii*
D4	274807474	LeBe	No restriction	pattern A	pattern A	V	24	No	*M. kansasii*
E4	275135278	IaRa	No restriction	No restriction	pattern A	I	6	Yes	*M. kansasii*
F4	274808696	EiBr	No restriction	pattern A	pattern A	V	26	No	*M. kansasii*
G4	427763392	EiBr	No restriction	pattern A	pattern A	V	29	No	*M. kansasii*
H4	312561032	GaWi	No restriction	No restriction	pattern A	I	1	No	*M. kansasii*
A5	275135168	GaWi	No restriction	No restriction	pattern A	I	3	No	*M. kansasii*
B5	320215986	GaWi	No restriction	No restriction	pattern A	I	3	No	*M. kansasii*

C5	329116087	PeHe	No restriction	No restriction	pattern A	I	2	No	*M. kansasii*
D5	329119359	PeHe	No restriction	No restriction	pattern A	I	2	No	*M. kansasii*
E5	313939095	LeYo	No restriction	No restriction	pattern A	I	8	No	*M. kansasii*
F5	29387870	GeGo	No restriction	No restriction	pattern A	I	6	No	*M. kansasii*
G5	29388123	MiDa	No restriction	No restriction	pattern A	I	3	No	*M. kansasii*
A6	379849661	ViCh	No restriction	No restriction	pattern A	I	3	No	*M. kansasii*
B6	379714437	ViCh	No restriction	No restriction	pattern A	I	6	No	*M. kansasii*
C6	337068637	ViSm	No restriction	No restriction	pattern A	I	9	No	*M. kansasii*
D6	356665897	LuPe	95,85,42	pattern A	pattern A	IV	36	No	*M. kansasii*
E6	352222085	GeTe	No restriction	pattern A	pattern A	V	23	No	*M. kansasii*
F6	328441086	BeSo	No restriction	pattern A	pattern A	V	23	No	*M. kansasii*
G6	328441105	JoCo	No restriction	No restriction	pattern A	I	3	No	*M. kansasii*
H6	328444903	BrHa	No restriction	No restriction	pattern A	I	6	No	*M. kansasii*
A7	328446127	SuBe	No restriction	pattern A	pattern A	V	25	No	*M. kansasii*
B7	379715291	SuBe	No restriction	pattern A	pattern A	V	25	No	*M. kansasii*
C7	366610206	LaPe	No restriction	pattern A	pattern A	V	24	No	*M. kansasii*
D7	370058418	BrCl	No restriction	No restriction	pattern A	I	3	No	*M. kansasii*
F7	431019367	AlSe	No restriction	pattern A	pattern A	V	27	No	*M. kansasii*
G7	395755535	PaOk	No restriction	No restriction	pattern A	I	5	Yes	*M. kansasii*
H7	395709239	PaOk	No restriction	No restriction	pattern A	I	3	Yes	*M. kansasii*
A8	439710005	JaSh	No restriction	No restriction	pattern A	I	5	No	*M. kansasii*
B8	398259809	AmWe	95,85,42	pattern A	pattern A	IV	32	No	*M. kansasii*
C8	385497420	PaRe	No restriction	No restriction	pattern A	I	5	No	*M. kansasii*
D8	402734769	GrJo	No restriction	No restriction	pattern A	I	5	No	*M. kansasii*
E8	486287283	GrJo	No restriction	No restriction	pattern A	I	21	No	*M. kansasii*
F8	425454927	NaKu	No restriction	No restriction	pattern A	I	5	No	*M. kansasii*
G8	427761068	KeEc	No restriction	pattern A	pattern A	V	30	No	*M. kansasii*
H8	379713969	AlDo	95,85,42	pattern A	pattern A	IV	35	No	*M. kansasii*
A9	453520236	FrMc	No restriction	No restriction	pattern A	I	20	No	*M. kansasii*
B9	427766149	LeWh	No restriction	No restriction	pattern A	I	3	No	*M. kansasii*
C9	465201563	JaCl	No restriction	No restriction	pattern A	I	11	No	*M. kansasii*
D9	465247398	RaGo	No restriction	No restriction	pattern A	I	3	No	*M. kansasii*
E9	420525523	PaGr	No restriction	No restriction	pattern A	I	1	Yes	*M. kansasii*
F9	461586782	PaGr	No restriction	No restriction	pattern A	I	13	Yes	*M. kansasii*
G9	484665030	DaGo	No restriction	No restriction	pattern A	I	12	Yes	*M. kansasii*
H9	468263113	AnVa	No restriction	No restriction	pattern A	I	17	Yes	*M. kansasii*
A10	417675063	AnVa	No restriction	No restriction	pattern A	I	3	Yes	*M. kansasii*
C10	427769475	MuAn	No restriction	No restriction	pattern A	I	6	No	*M. kansasii*
D10	451496292	DeRo	No restriction	pattern A	pattern A	V	23	No	*M. kansasii*
E10	442989565	GoBl	No restriction	No restriction	pattern A	I	3	No	*M. kansasii*
F10	274800298	GaHa	No restriction	No restriction	pattern A	I	6	Yes	*M. kansasii*
G10	383998336	ZoFu	No restriction	No restriction	pattern A	I	10	Yes	*M. kansasii*
H10	218688812	NaSa	No restriction	No restriction	pattern A	I	7	No	*M. kansasii*
A11	336402419	DaMa	No restriction	No restriction	pattern A	I	14	Yes	*M. kansasii*
B11	424022235	BeBo	No restriction	No restriction	pattern A	I	15	Yes	*M. kansasii*
C11	379716960	HaAm	No restriction	No restriction	pattern A	I	16	Yes	*M. kansasii*
D11	394353072	DeWa	No restriction	No restriction	pattern A	I	2	Yes	*M. kansasii*

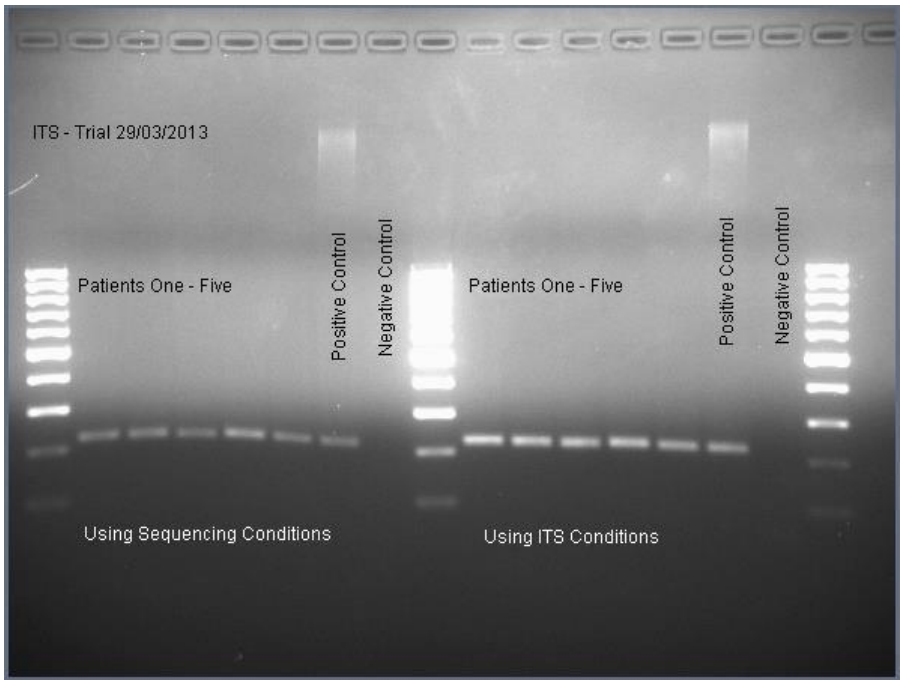
E11		M. KANSASII ATCC 12478 POSITIVE CONTROL	No restriction	No restriction	pattern A	I	18	NA	*M. kansasii*
F11		M. KANSASII ATCC 12478 POSITIVE CONTROL	No restriction	No restriction	pattern A	I	18	NA	*M. kansasii*
H11		NEGATIVE CONTROL							

*Key definitions of each identifier are as follows:

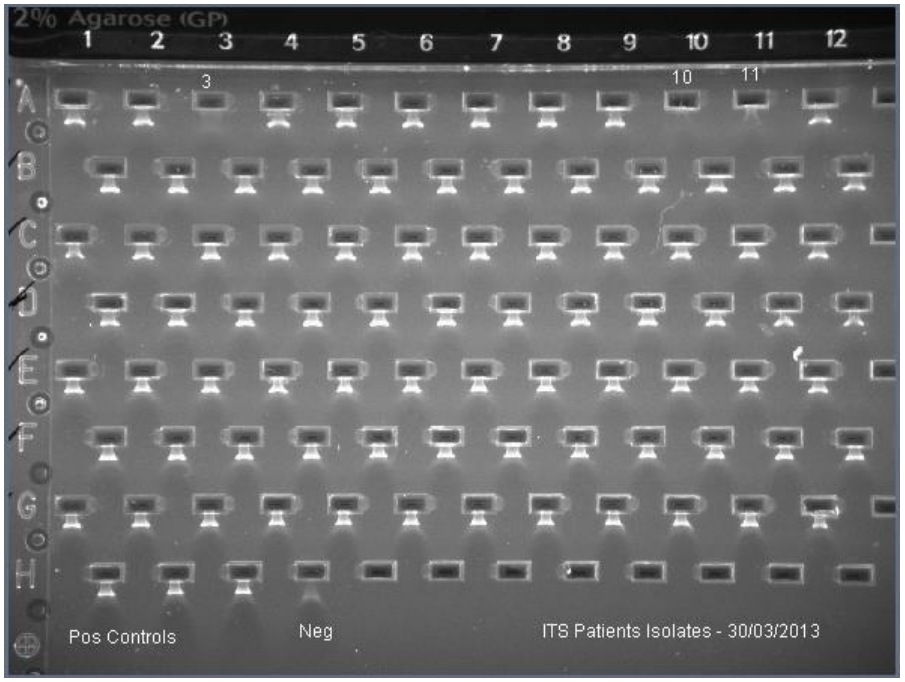
- *Plate Location* – When the ITS-REA was first performed, each isolate was run once and had its own unique position in the 96well plate. This identifier corresponds to the position on the electrophoresis gel images as seen in the **Appendix B: ITS-REA Enzyme Digest Gel Pictures**.
- *Auslab #* - a unique code that is used to identify and monitor patient samples in the Pathology Queensland.
- *Patient Name* – patient isolates that have been de-identified.
- *Hae*III/*Cfo*1/*Dde*1 – ITS-REA enzyme result determined from the electrophoresis gel images; looking specifically at the characteristic banding pattern produced by each enzyme.
- *Strain type* – the ITS-REA profile determined by the 3 enzyme digests. These were compared to (Roth *et al.* 1998) and a typical strain type was delegated.
- *DiversiLab Pattern #* – the cluster number assigned by the DiversiLab software. The specific fingerprints created were aligned into a dendrogram and the isolates which were >97% in similarity were given the same pattern number.
- *HRM Strain Match* – comparison of each isolate to the *M. kansasii* ATCC strain and determined whether they were the same strain or a different strain.
- *Sequencing results for 16S rRNA* – 16S rRNA was performed on each isolate for two reasons: firstly to ensure the isolate was *M. kansasii* and it was pure and secondly the results were needed for the validation of HRM.

Appendix B: ITS-REA Enzyme Digest Gel Pictures

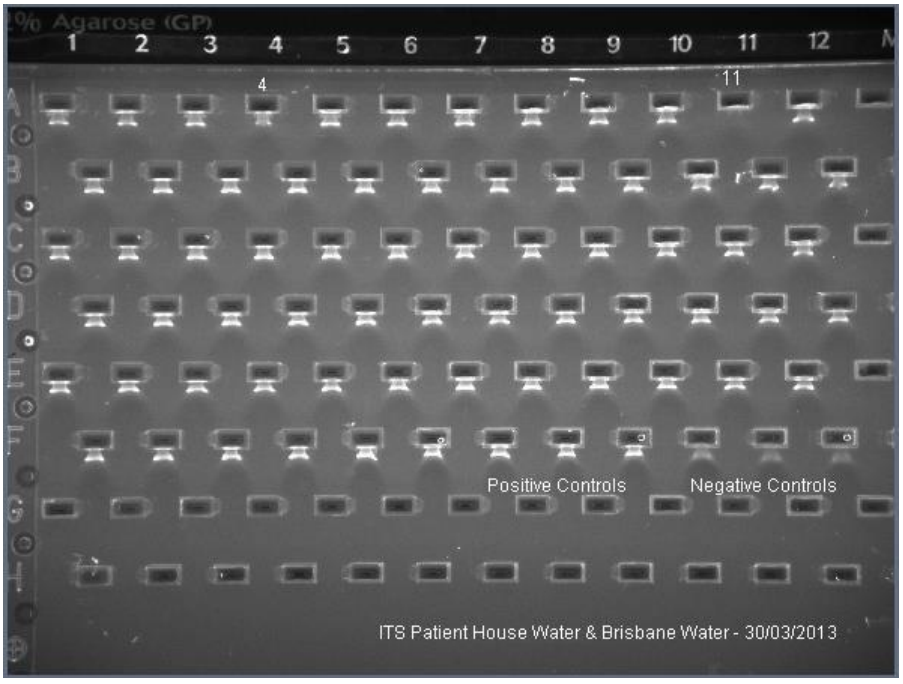
ITS Primer Trial with different thermal cycling conditions



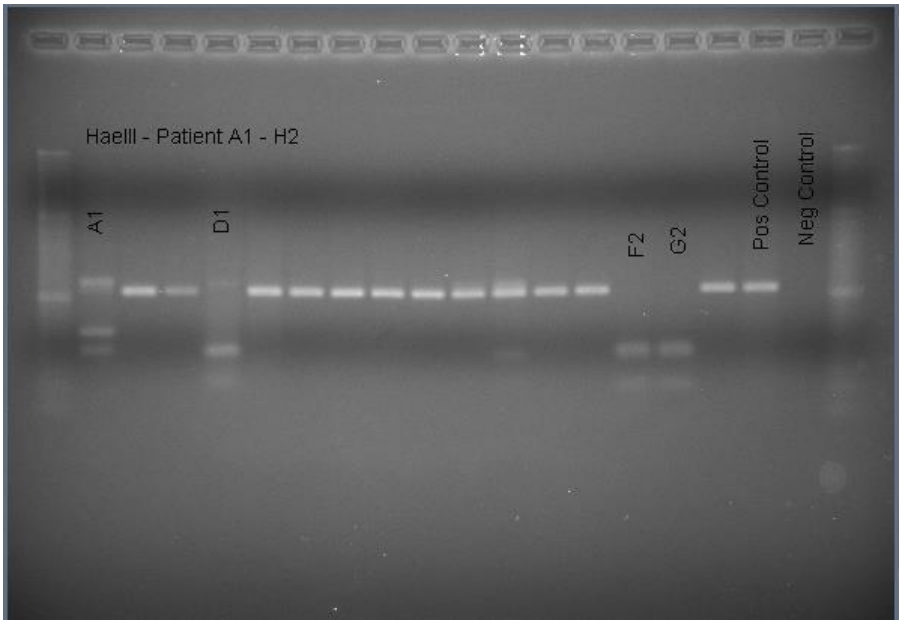
ITS Primary Amplification product check – Patient



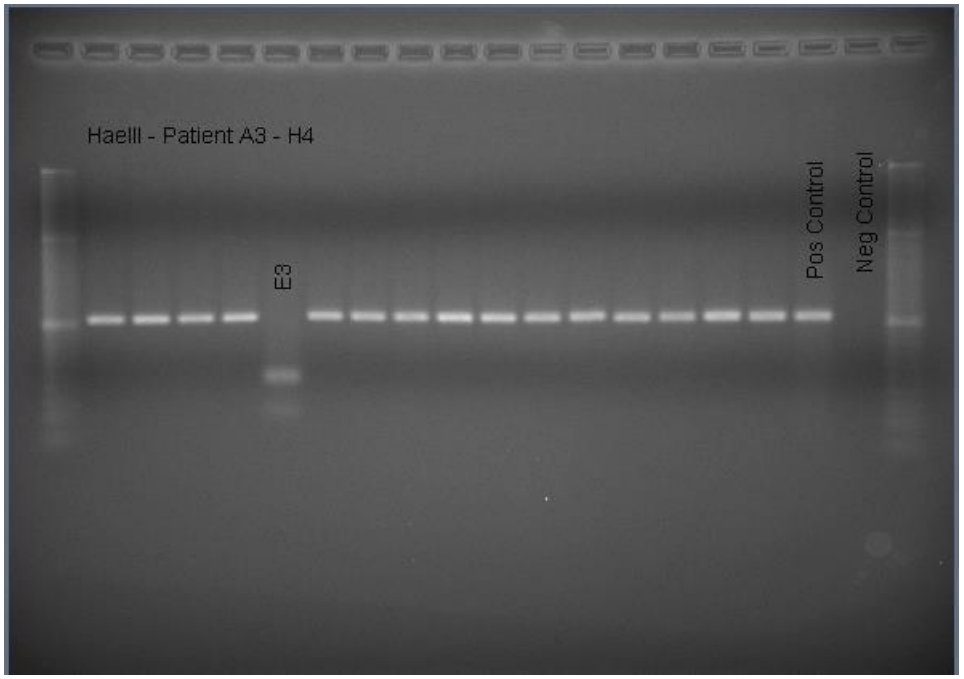
ITS Primary Amplification product check – Water



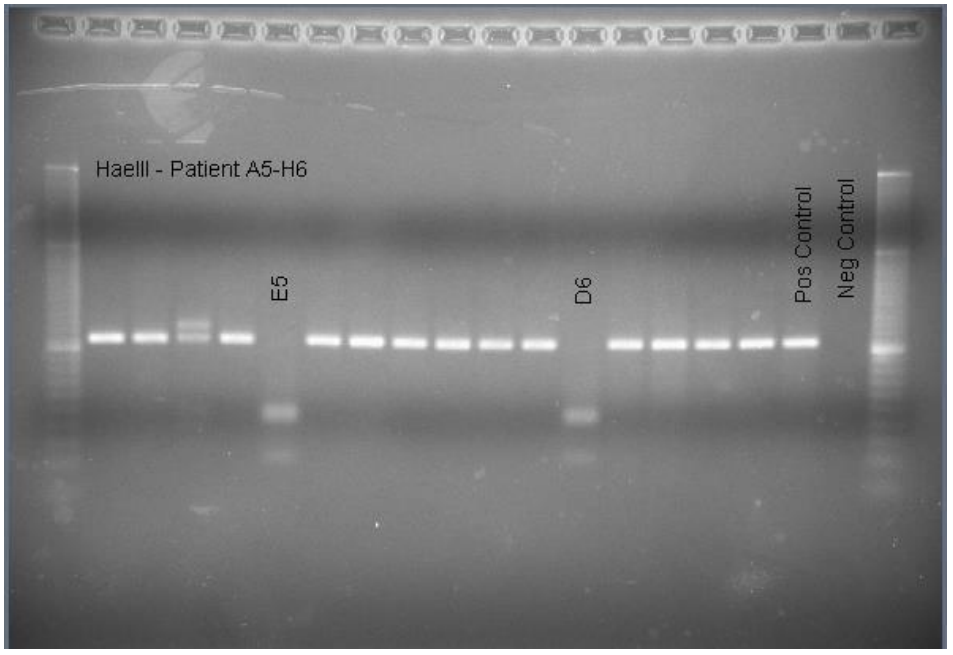
HaeIII – Patient Gel 1



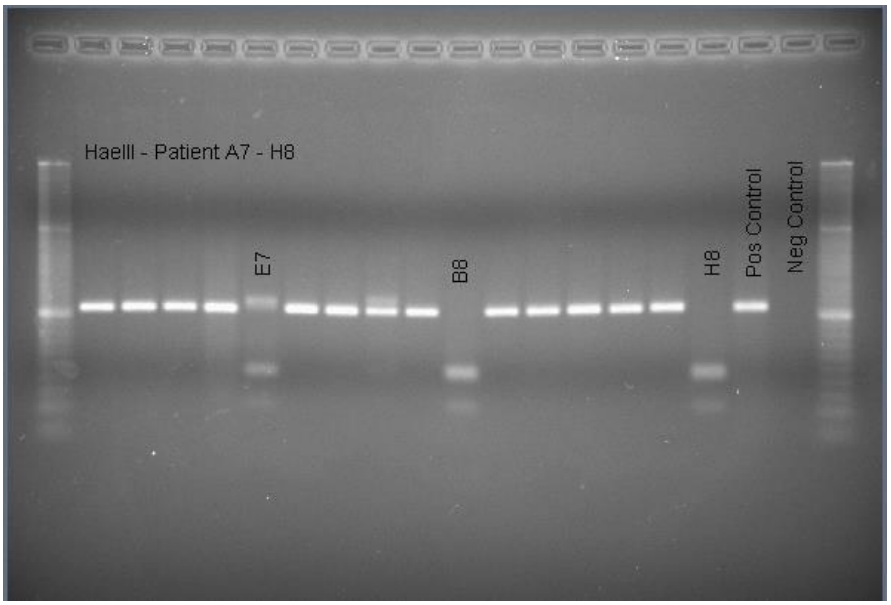
HaeIII – Patient Gel 2



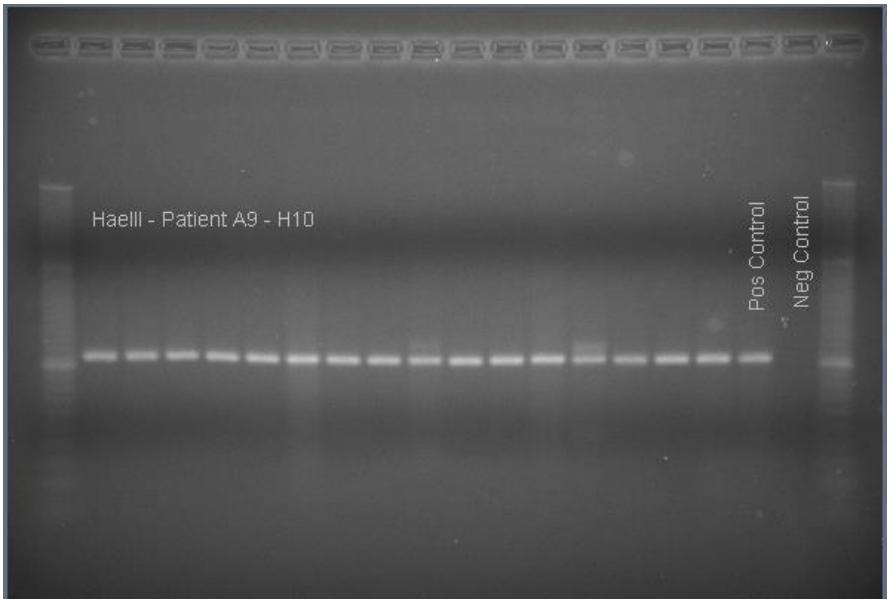
HaeIII – Patient Gel 3



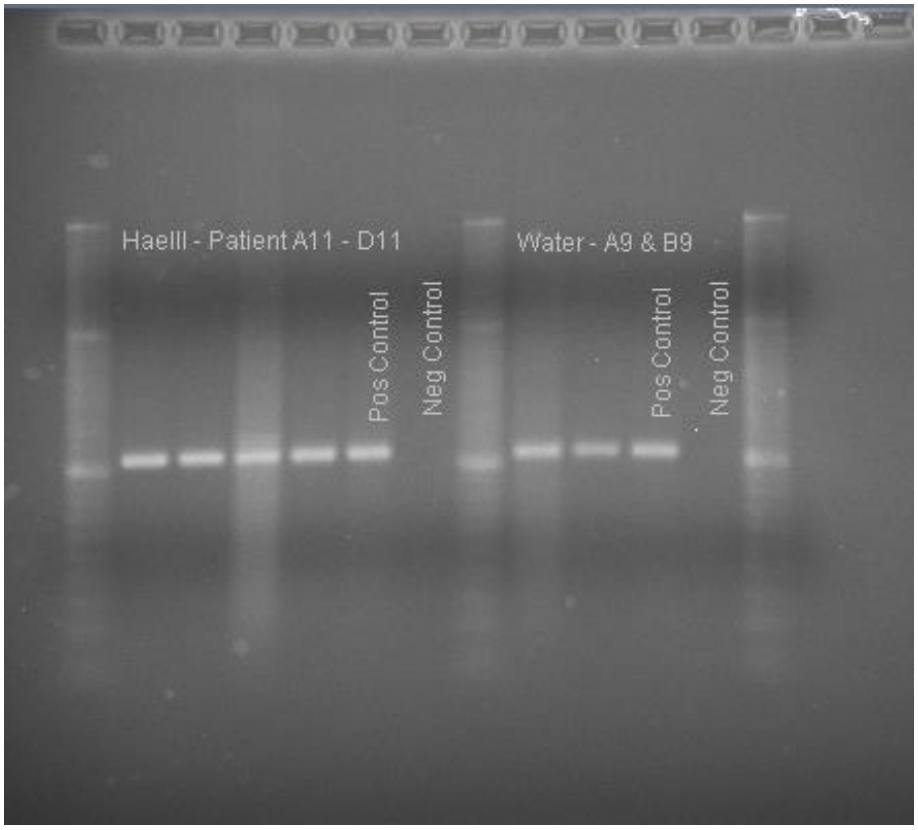
HaeIII – Patient Gel 4



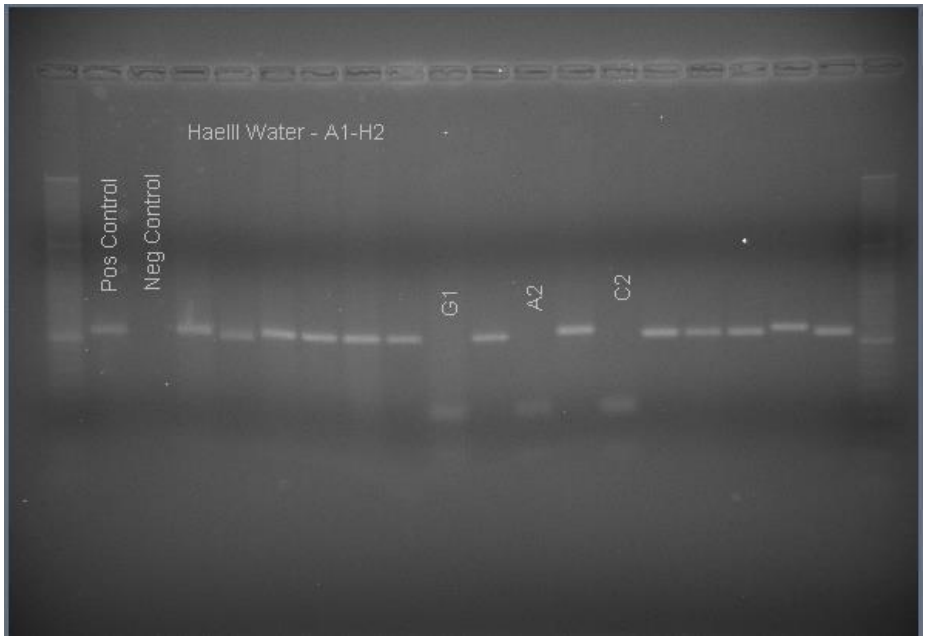
HaeIII – Patient Gel 5



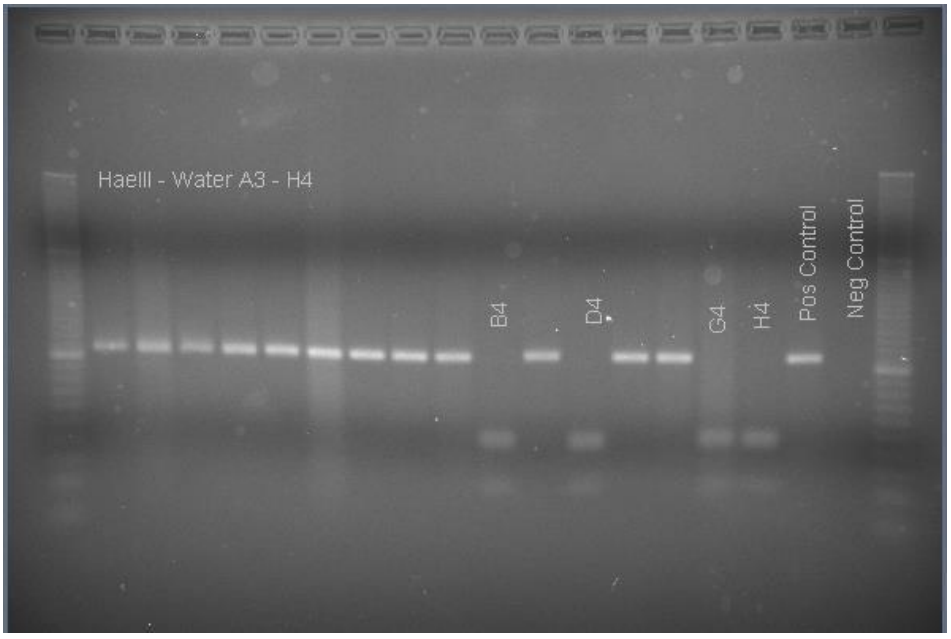
HaeIII – Patient Gel 6 & Water Gel 5



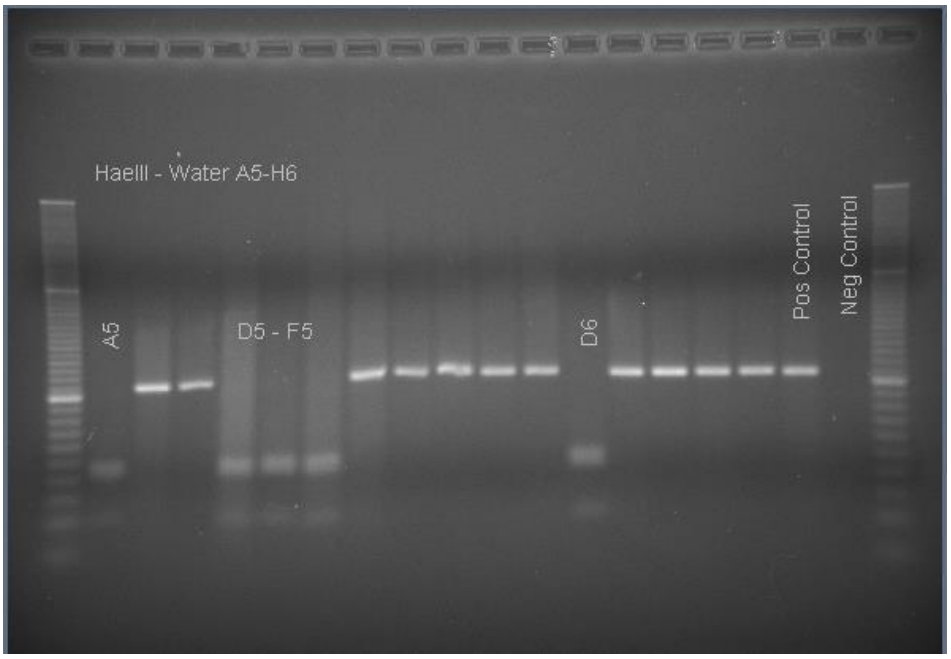
HaeIII – Water Gel 1



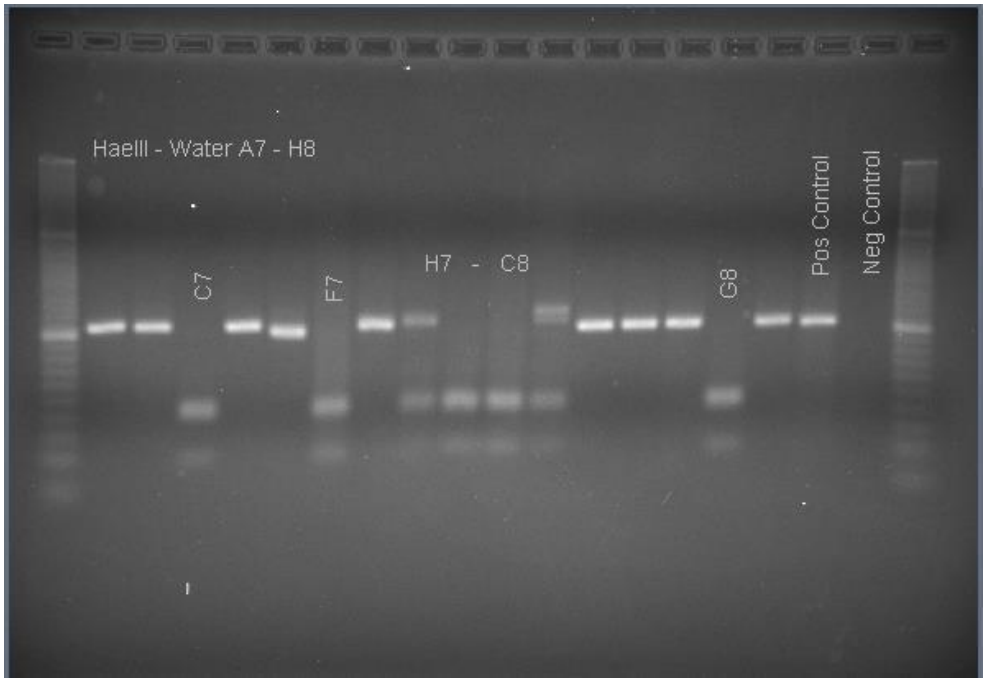
HaeIII – Water Gel 2



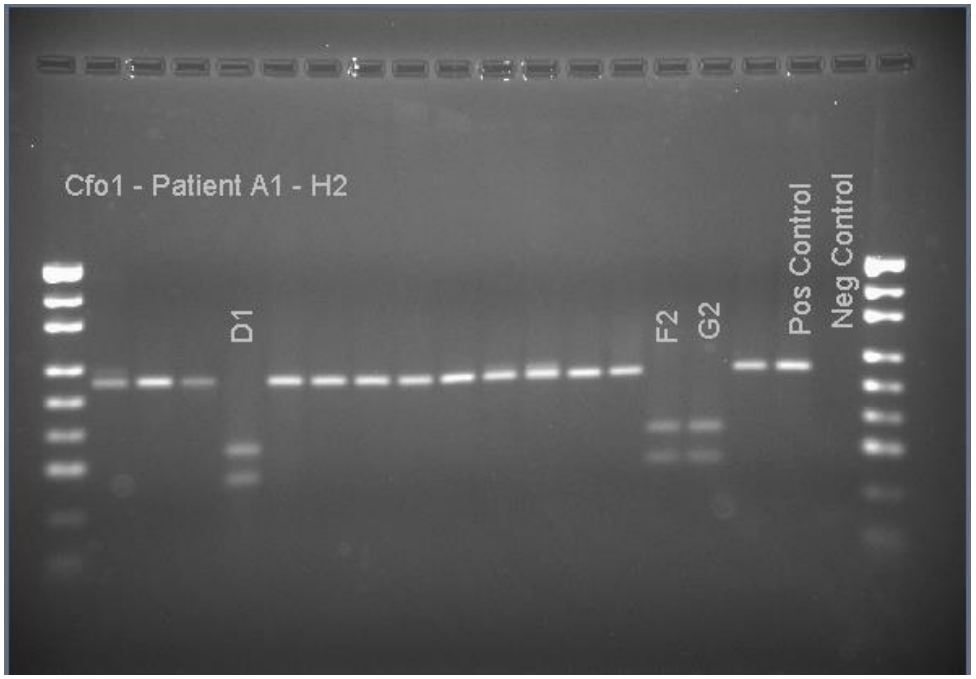
HaeIII – Water Gel 3



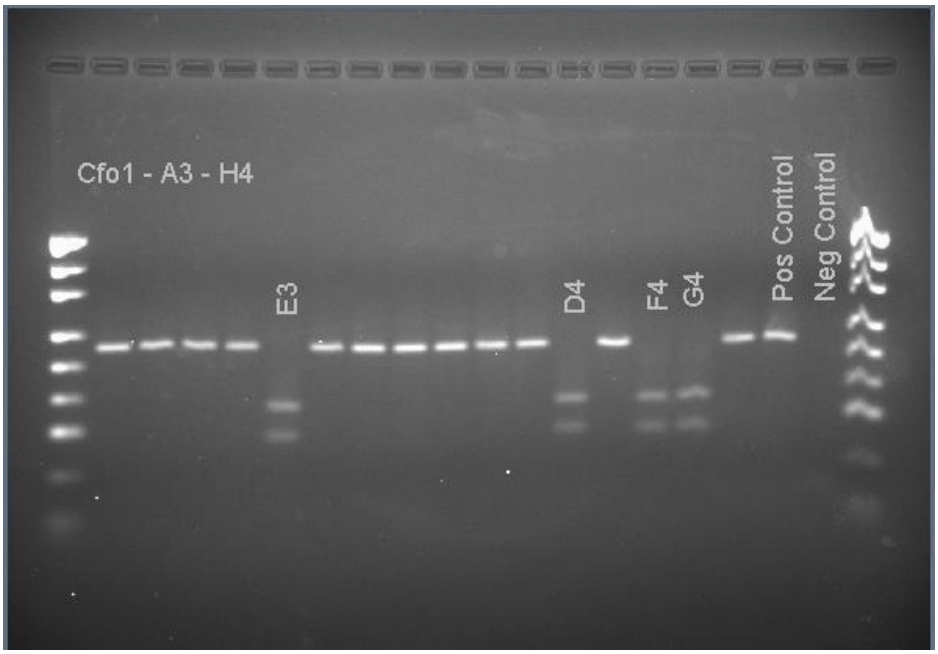
HaeIII – Water Gel 4



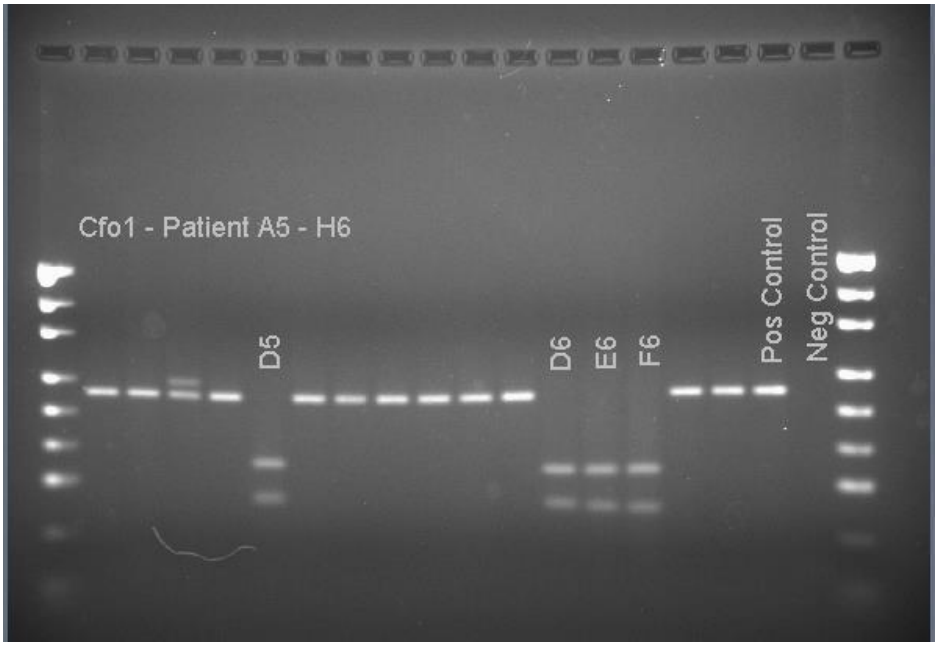
CfoI – Patient Gel 1



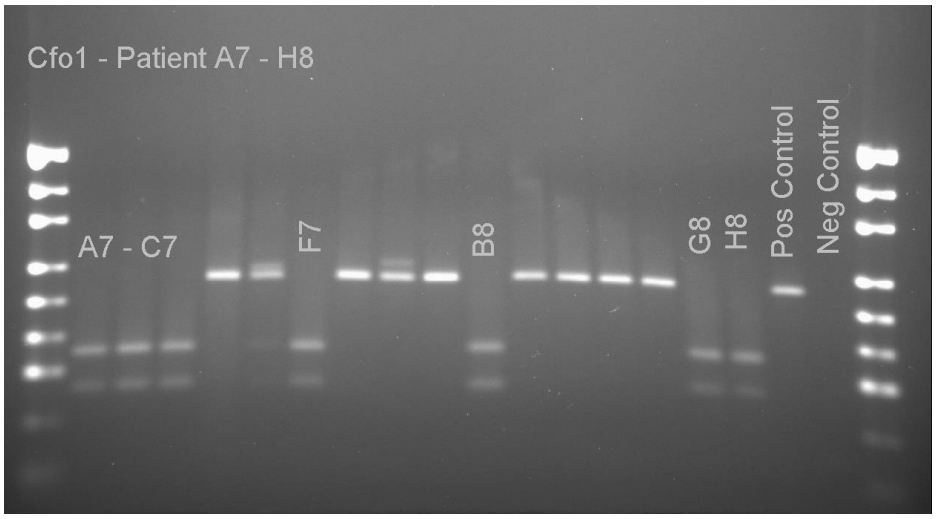
Cfo1 – Patient Gel 2



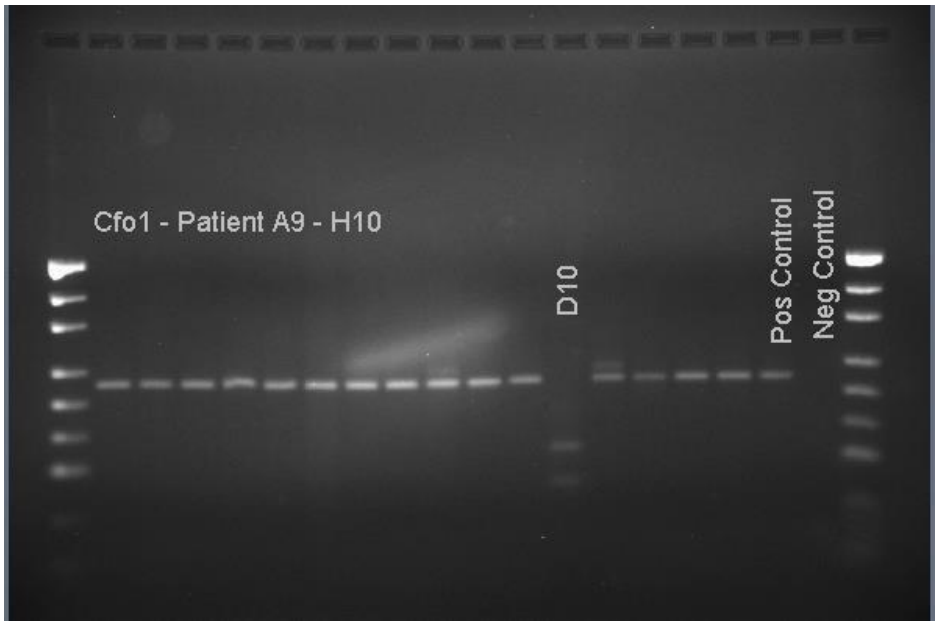
Cfo1 – Patient Gel 3



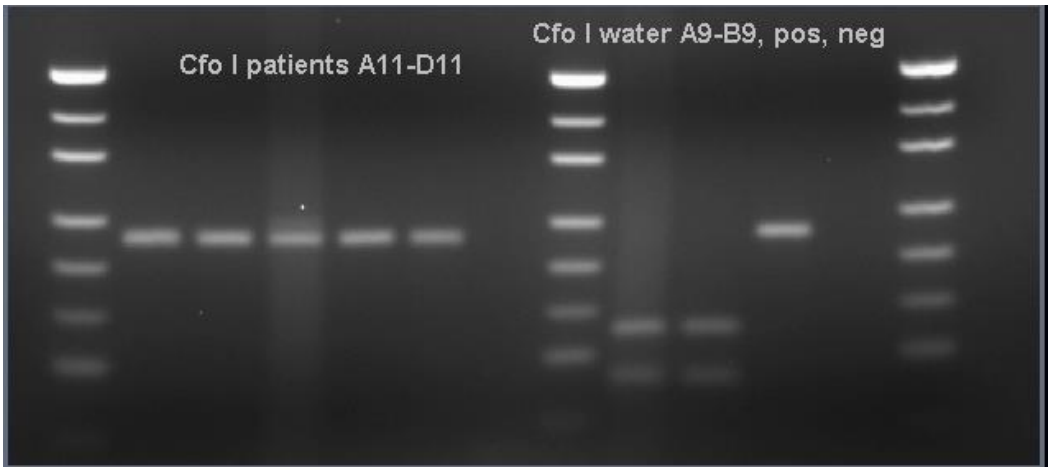
Cfo1 – Patient Gel 4



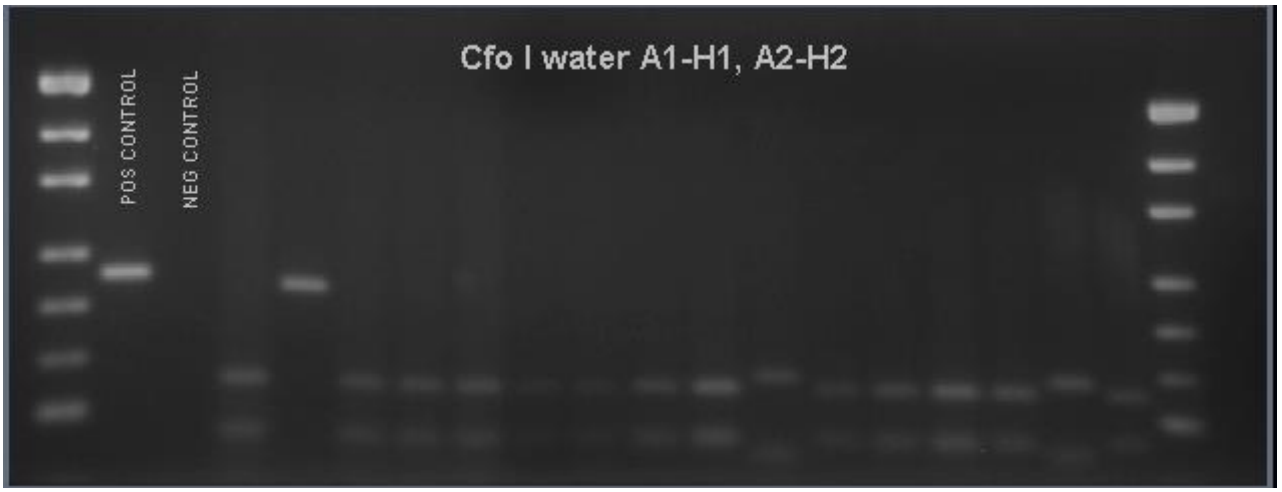
Cfo1 – Patient Gel 5



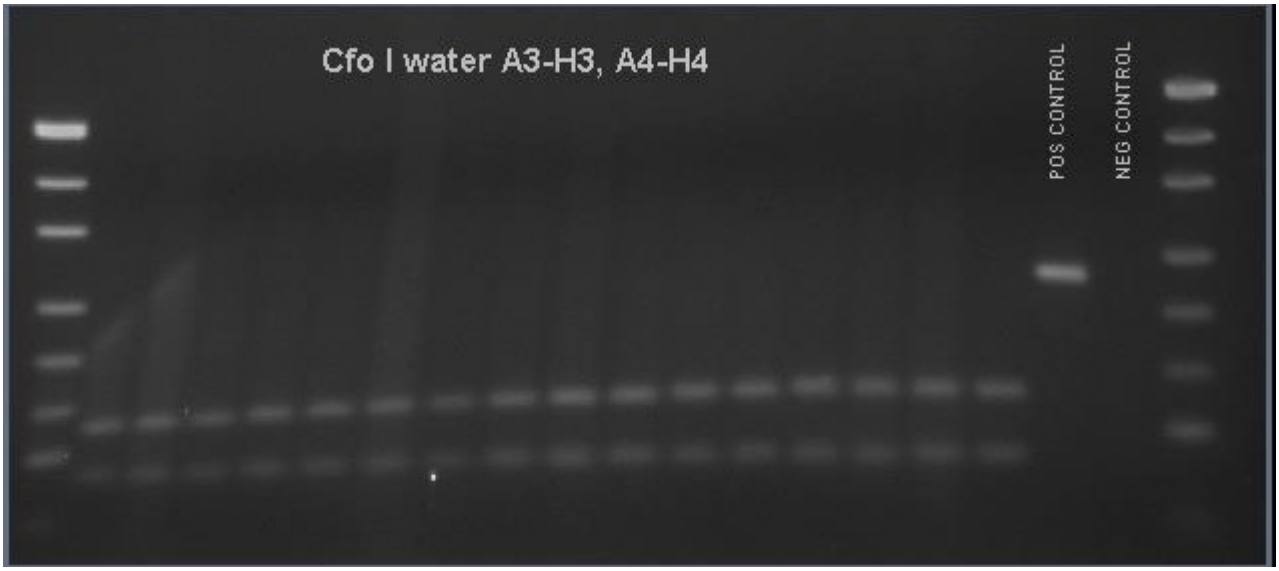
Cfo1 – Patient Gel 6 & Water Gel 5



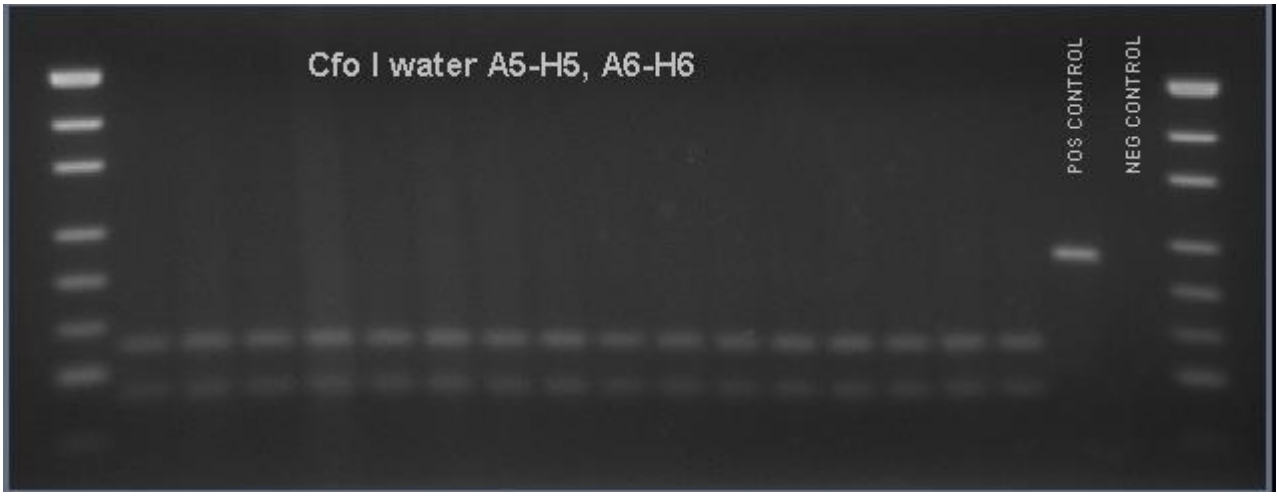
Cfo1 – Water Gel 1



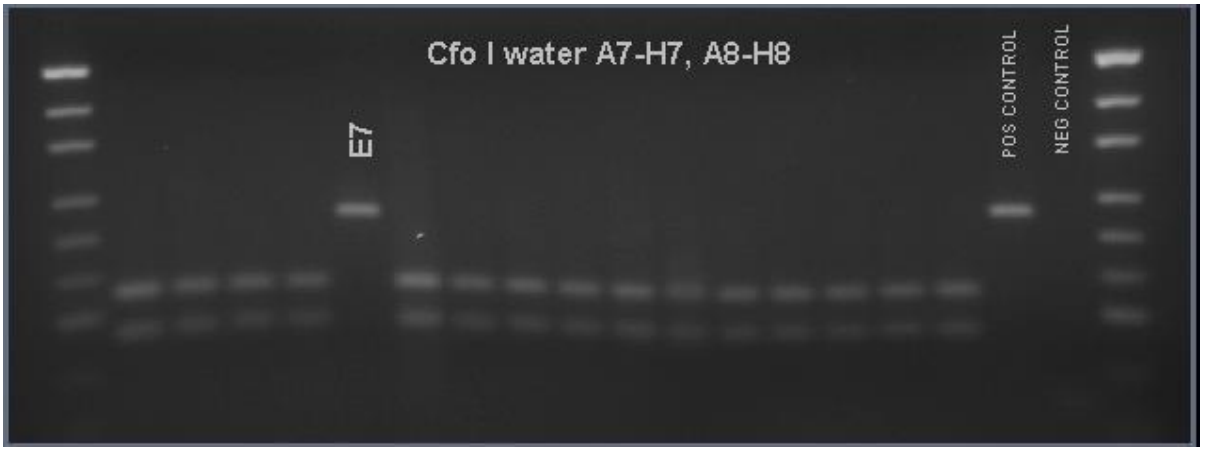
Cfo1 – Water Gel 2



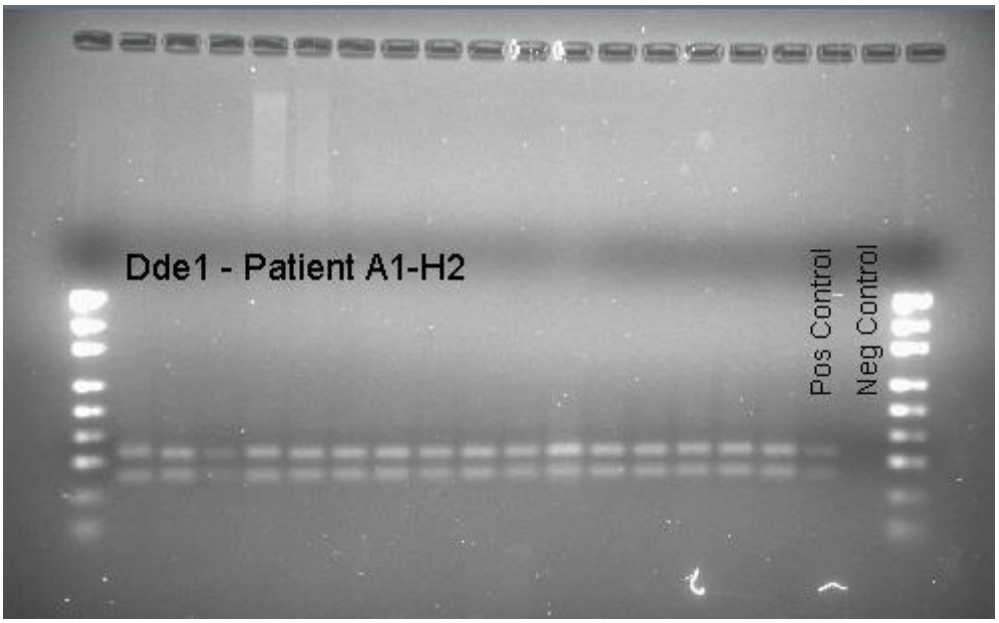
Cfo1 – Water Gel 3



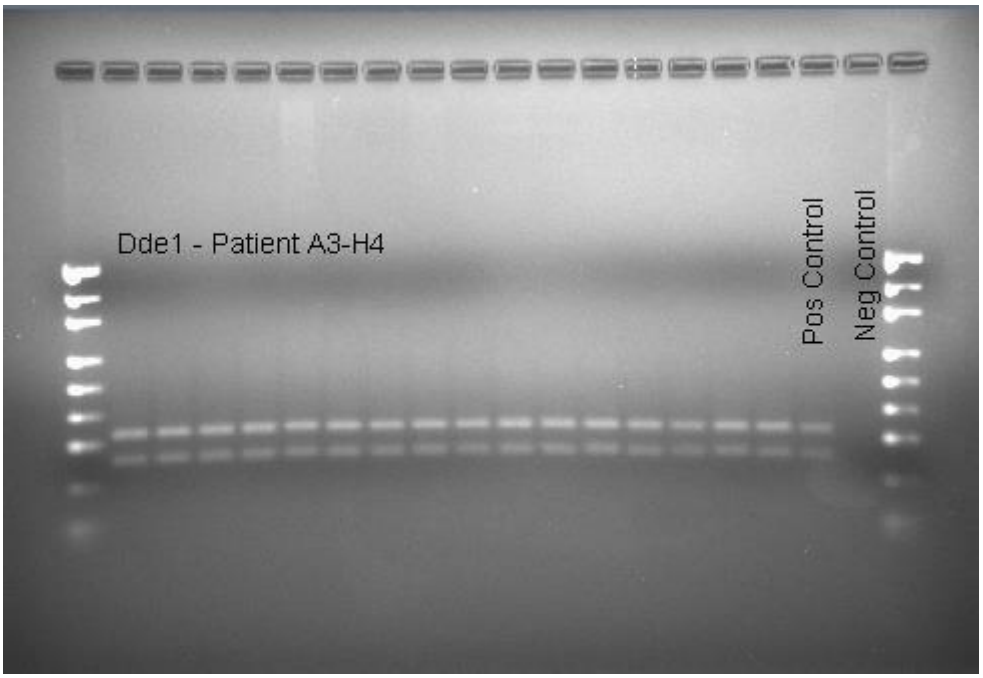
Cfo1 – Water Gel 4



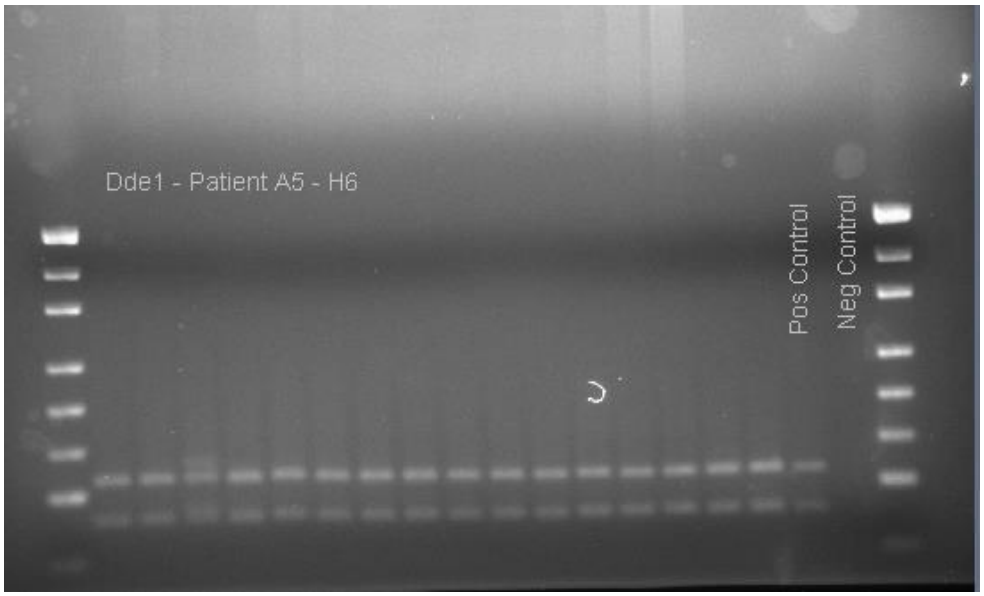
Dde1 – Patient Gel 1



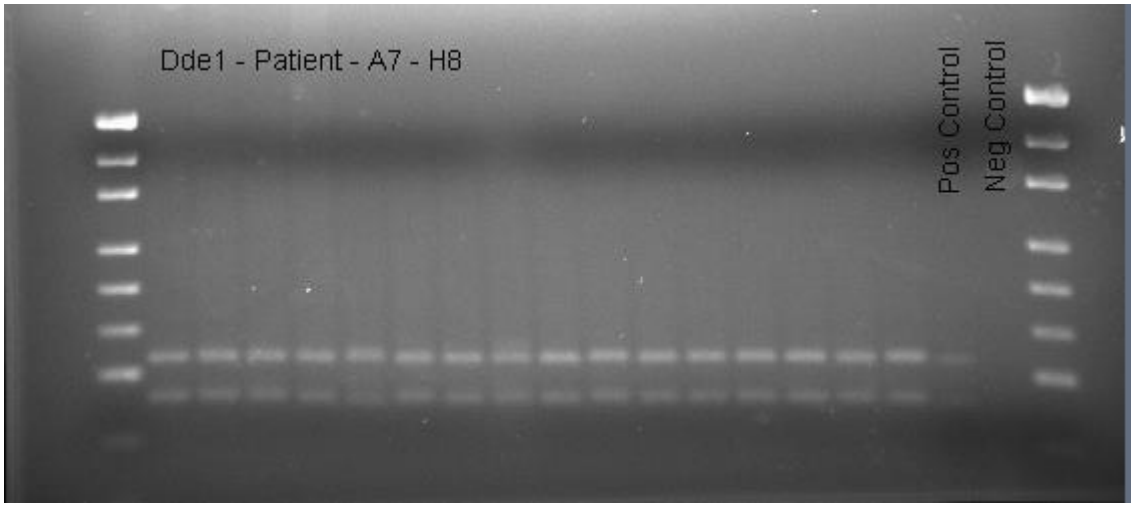
Dde1 – Patient Gel 2



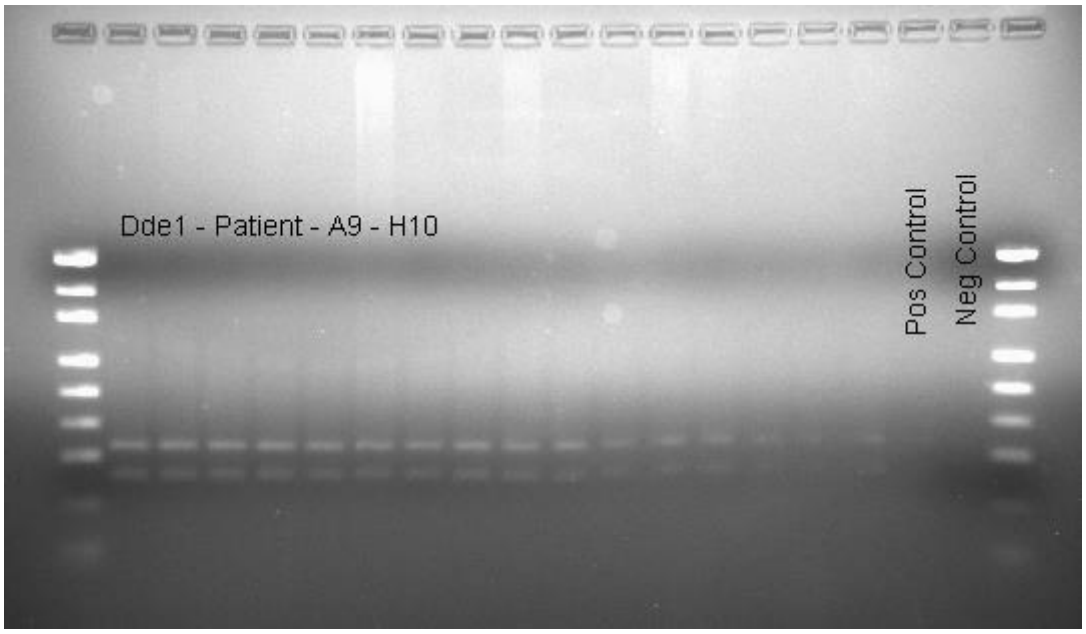
Dde1 – Patient Gel 3



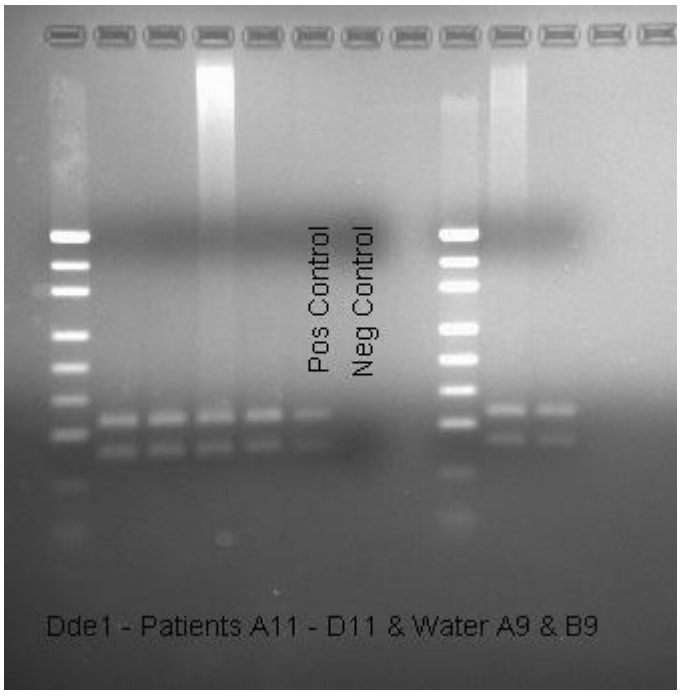
Dde1 – Patient Gel 4



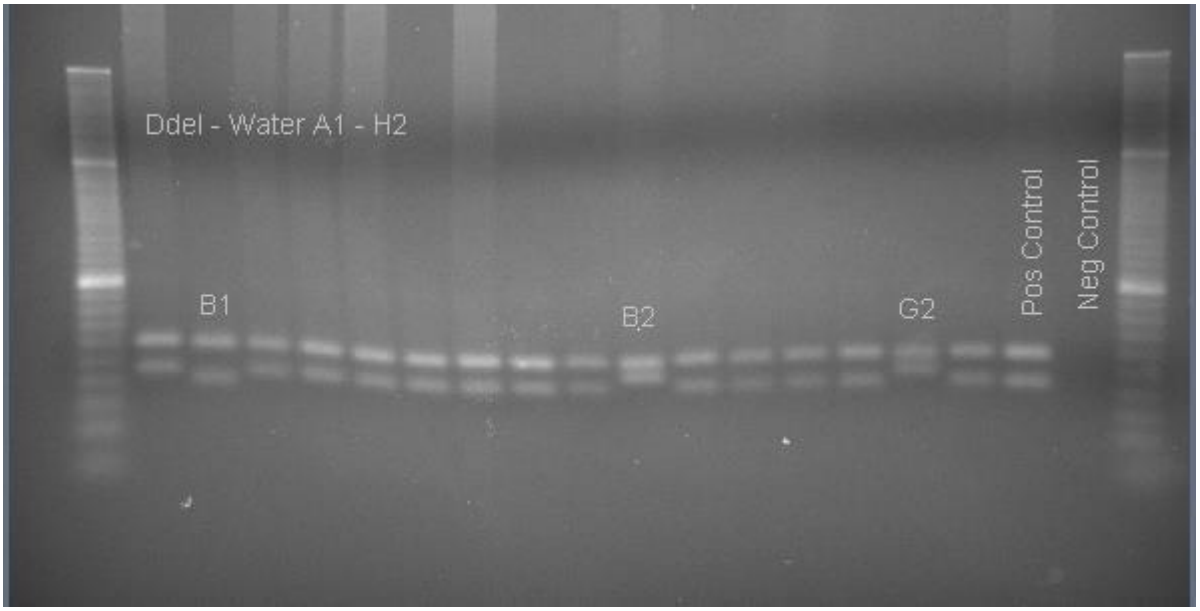
Dde1 – Patient Gel 5



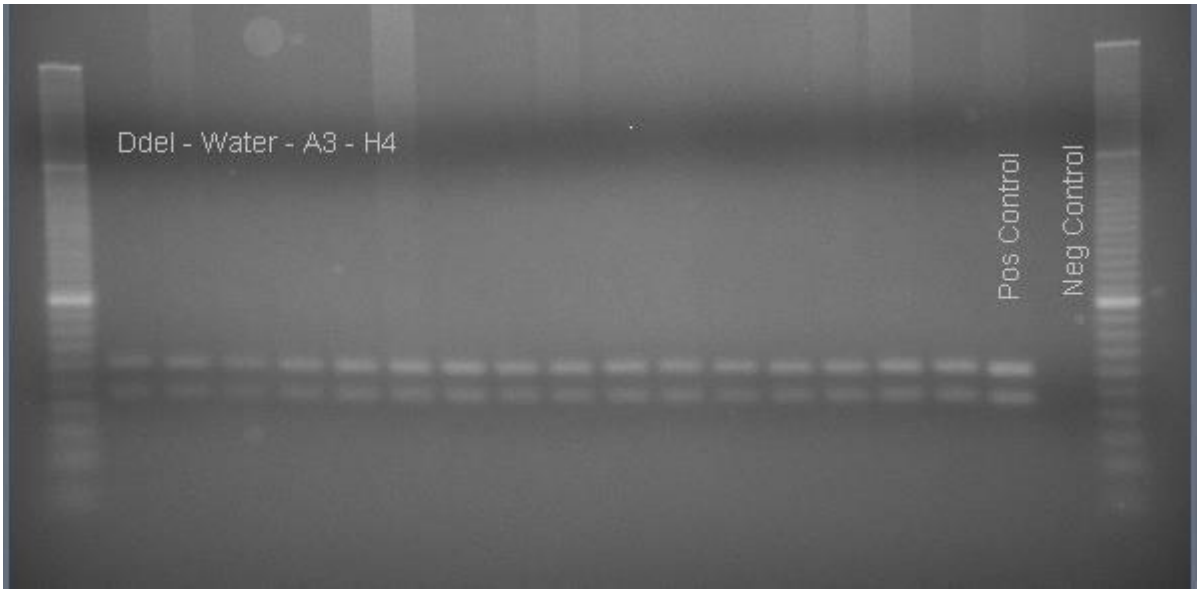
Dde1 – Patient Gel 6 & Water Gel 5



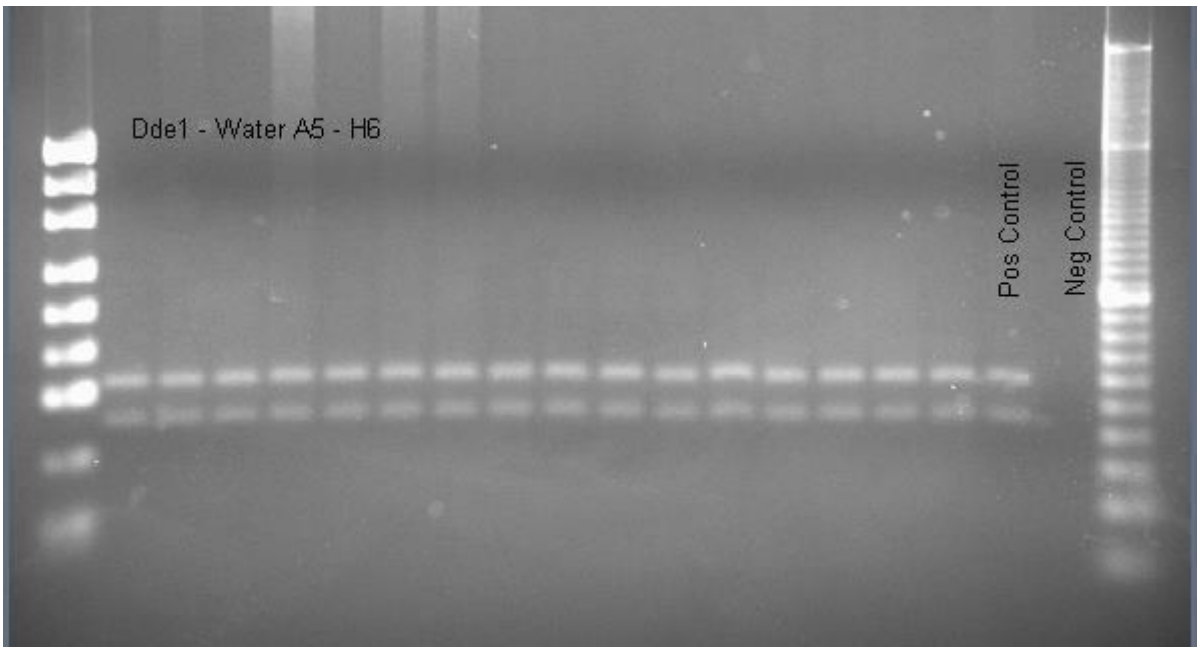
Dde1 – Water Gel 1



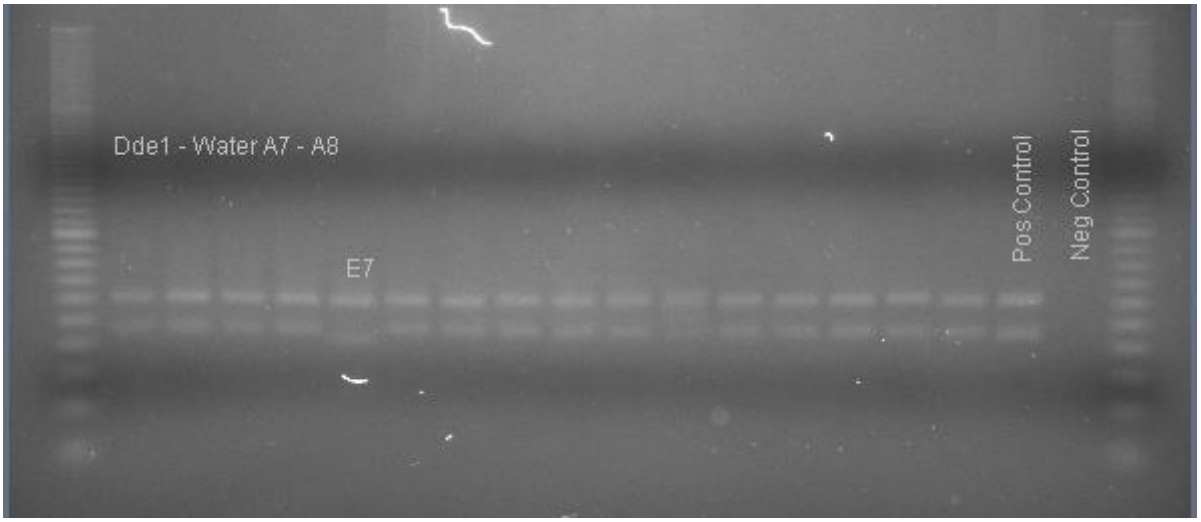
Dde1 – Water Gel 2



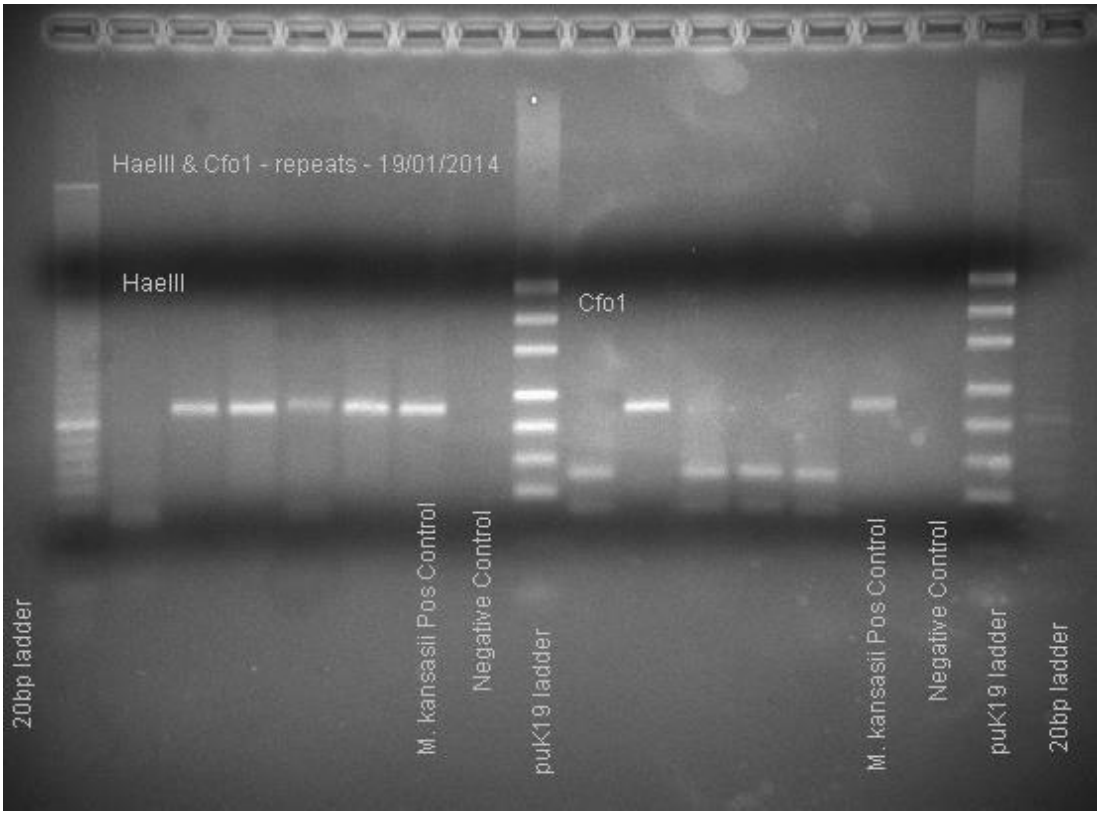
Dde1 – Water Gel 3



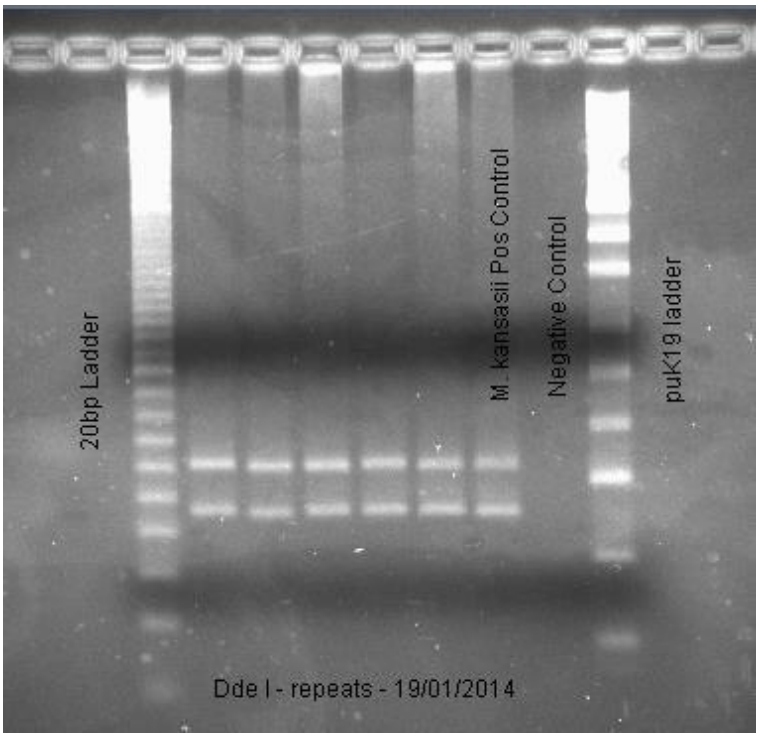
Dde1 – Water Gel 4



HaeIII & Cfo1 – Repeat Gel



Dde1 – Repeat Gel



Appendix C: DiversiLab Results from bioMerieux software printout

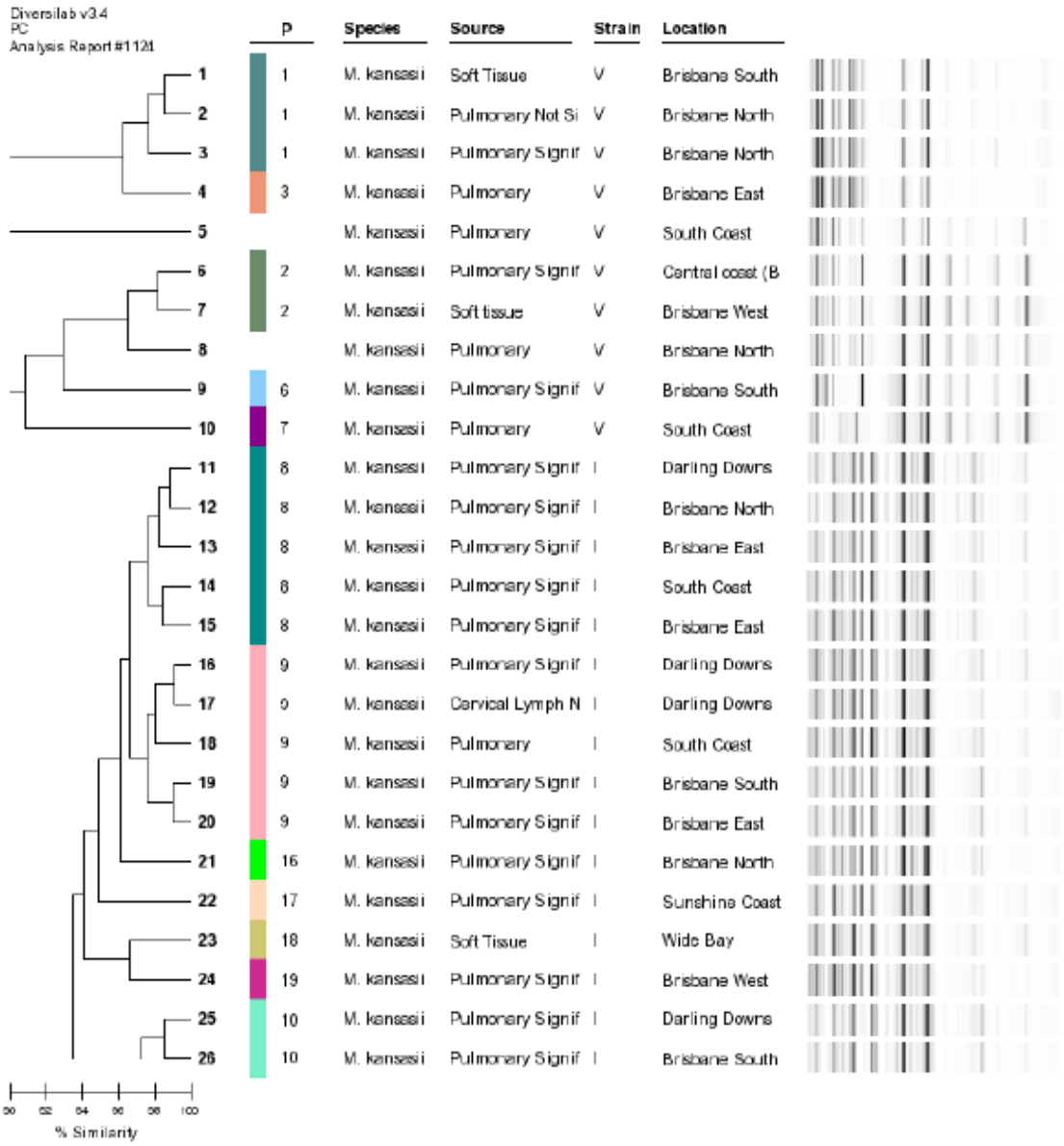
DiversiLab Patient Results

Pattern	Key	Species	Source	Strain	Location	Note
1	1	M. kansasii	Soft Tissue	V	Brisbane Sou	
	2	M. kansasii	Pulmonary No	V	Brisbane Nor	
	3	M. kansasii	Pulmonary Si	V	Brisbane Nor	
3	4	M. kansasii	Pulmonary	V	Brisbane Eas	
	5	M. kansasii	Pulmonary	V	South Coast	
2	6	M. kansasii	Pulmonary Si	V	Central coas	
	7	M. kansasii	Soft tissue	V	Brisbane Wes	
	8	M. kansasii	Pulmonary	V	Brisbane Nor	
6	9	M. kansasii	Pulmonary Si	V	Brisbane Sou	
7	10	M. kansasii	Pulmonary	V	South Coast	
8	11	M. kansasii	Pulmonary Si	I	Darling Down	
	12	M. kansasii	Pulmonary Si	I	Brisbane Nor	
	13	M. kansasii	Pulmonary Si	I	Brisbane Eas	
	14	M. kansasii	Pulmonary Si	I	South Coast	
	15	M. kansasii	Pulmonary Si	I	Brisbane Eas	
9	16	M. kansasii	Pulmonary Si	I	Darling Down	
	17	M. kansasii	Cervical Lym	I	Darling Down	
	18	M. kansasii	Pulmonary	I	South Coast	
	19	M. kansasii	Pulmonary Si	I	Brisbane Sou	
	20	M. kansasii	Pulmonary Si	I	Brisbane Eas	
16	21	M. kansasii	Pulmonary Si	I	Brisbane Nor	
17	22	M. kansasii	Pulmonary Si	I	Sunshine Coa	
18	23	M. kansasii	Soft Tissue	I	Wide Bay	
19	24	M. kansasii	Pulmonary Si	I	Brisbane Wes	
10	25	M. kansasii	Pulmonary Si	I	Darling Down	
	26	M. kansasii	Pulmonary Si	I	Brisbane Sou	
	27	M. kansasii	Pulmonary Si	I	Brisbane Nor	
11	28	M. kansasii	Pulmonary	I	Brisbane Nor	
	29	M. kansasii	Pulmonary Si	I	South Coast	
	30	M. kansasii	Pulmonary No	I	Darling Down	
	31	M. kansasii	Pulmonary Si	I	Ipswich & We	
	32	M. kansasii	Pulmonary Si	I	Darling Down	
	33	M. kansasii	Pulmonary Si	I	Brisbane Wes	
	34	M. kansasii	Pulmonary	I	Darling Down	
	35	M. kansasii	Pulmonary Si	I	Brisbane Nor	
	36	M. kansasii	Pulmonary Si	I	Brisbane Sou	
	37	M. kansasii	Pulmonary Si	I	Brisbane Nor	
	38	M. kansasii	Pulmonary Si	I	Brisbane Wes	
	39	M. kansasii	Pulmonary Si	I	Brisbane Nor	
	40	M. kansasii	Pulmonary No	I	NQ	
	41	M. kansasii	Soft Tissue	I	Darling Down	
12	42	M. kansasii	Pulmonary Si	I	Brisbane Nor	
	43	M. kansasii	Pulmonary Si	I	South Coast	

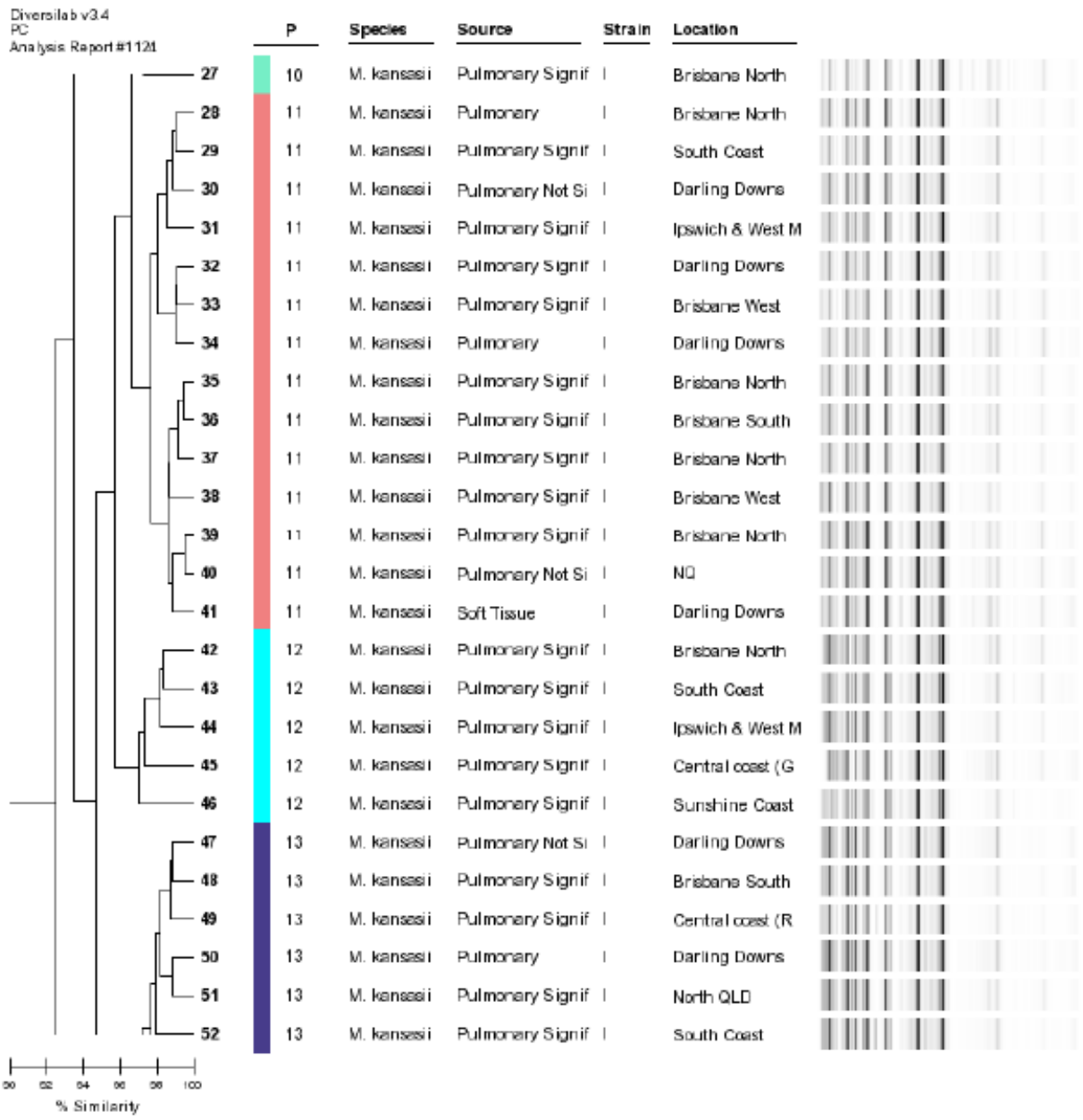
Fingerprint Dendrogram

	44	M. kansasii	Pulmonary Si	I	Ipswich & We	
	45	M. kansasii	Pulmonary Si	I	Central coas	
	46	M. kansasii	Pulmonary Si	I	Sunshine Coa	
13	47	M. kansasii	Pulmonary No	I	Darling Down	
	48	M. kansasii	Pulmonary Si	I	Brisbane Sou	
	49	M. kansasii	Pulmonary Si	I	Central coas	
	50	M. kansasii	Pulmonary	I	Darling Down	
	51	M. kansasii	Pulmonary Si	I	North QLD	
	52	M. kansasii	Pulmonary Si	I	South Coast	
	53	M. kansasii	Pulmonary	I	Darling Down	
	54	M. kansasii	Pulmonary Si	I	Darling Down	
20	55	M. kansasii	Pulmonary Si	I	Wide Bay	
14	56	M. kansasii	Pulmonary Si	I	Darling Down	
	57	M. kansasii	Pulmonary Si	I	North QLD	
	58	M. kansasii	Pulmonary Si	I	Brisbane Sou	
21	59	M. kansasii	Pulmonary Si	I	Ipswich & We	
22	60	M. kansasii	Pulmonary Si	I	Brisbane Nor	
15	61	M. kansasii	Pulmonary Si	IV	Brisbane Nor	
	62	M. kansasii	Pulmonary Si	I	Brisbane Sou	
23	63	M. kansasii	Pulmonary Si	I	Sunshine Coa	
24	64	M. kansasii	ATCC	I		
25	65	M. kansasii	Pulmonary Si	I	Brisbane Nor	
26	66	M. kansasii	Pulmonary No	I	Brisbane Sou	
27	67	M. kansasii	Pulmonary No	IV	Sunshine Coa	
28	68	M. kansasii	Pulmonary Si	IV	Brisbane Eas	
29	69	M. kansasii	Pulmonary Si	IV	Brisbane Sou	
30	70	M. kansasii	Pulmonary	IV	Sunshine Coa	
31	71	M. kansasii	Pulmonary Si	IV	Brisbane Sou	
32	72	M. kansasii	Pulmonary No	IV	Wide Bay	
33	73	M. kansasii	Pulmonary No	V	South Coast	

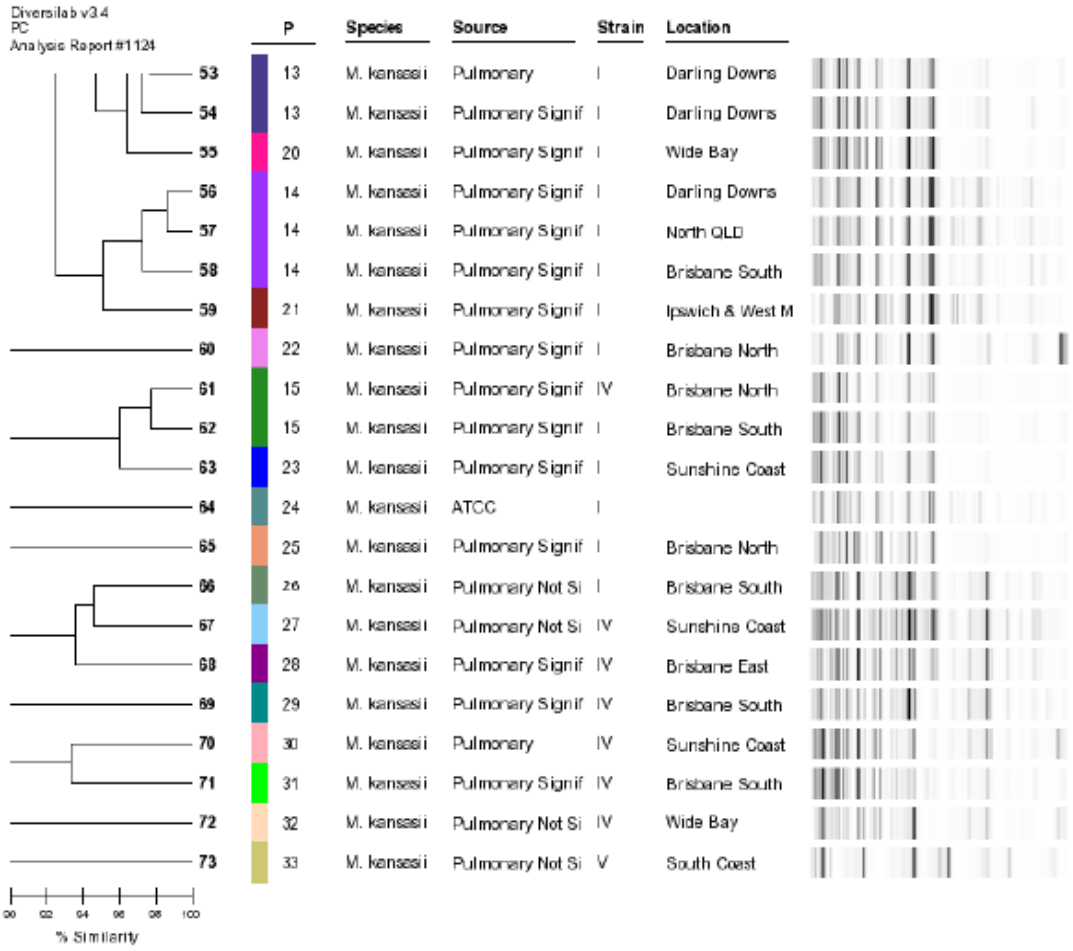
Fingerprint Dendrogram continued



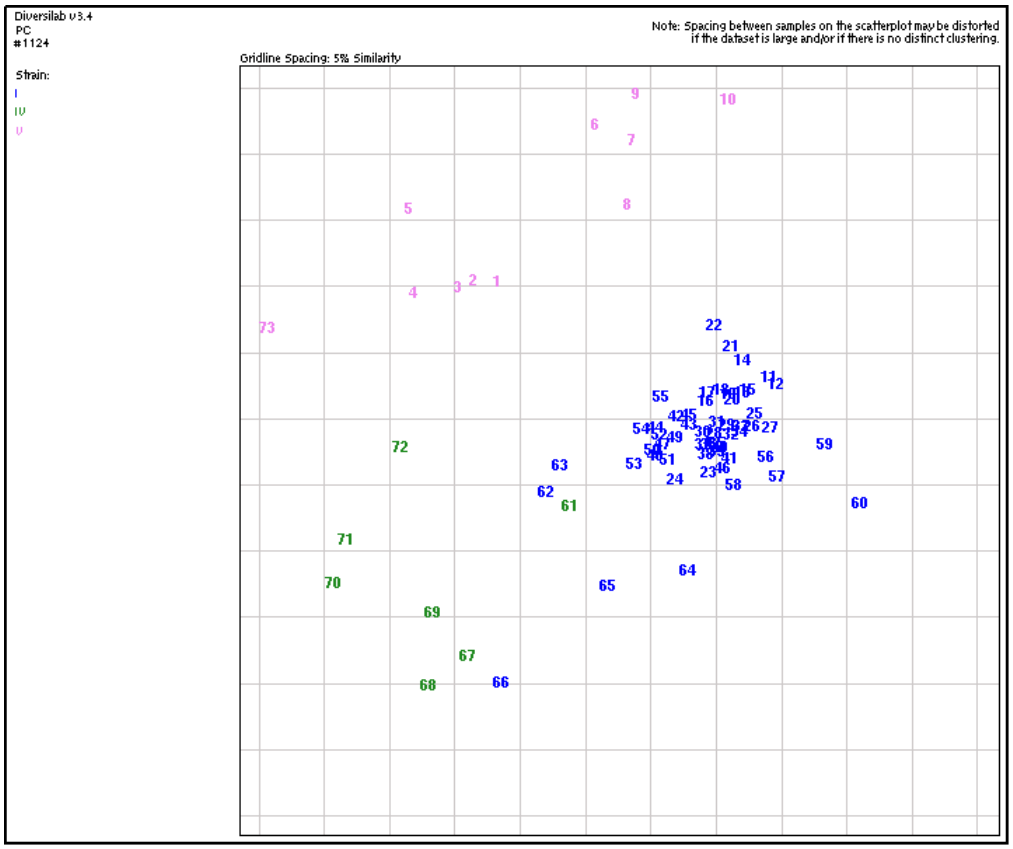
Fingerprint Dendrogram



Fingerprint Dendrogram continued



Fingerprint Dendrogram continued

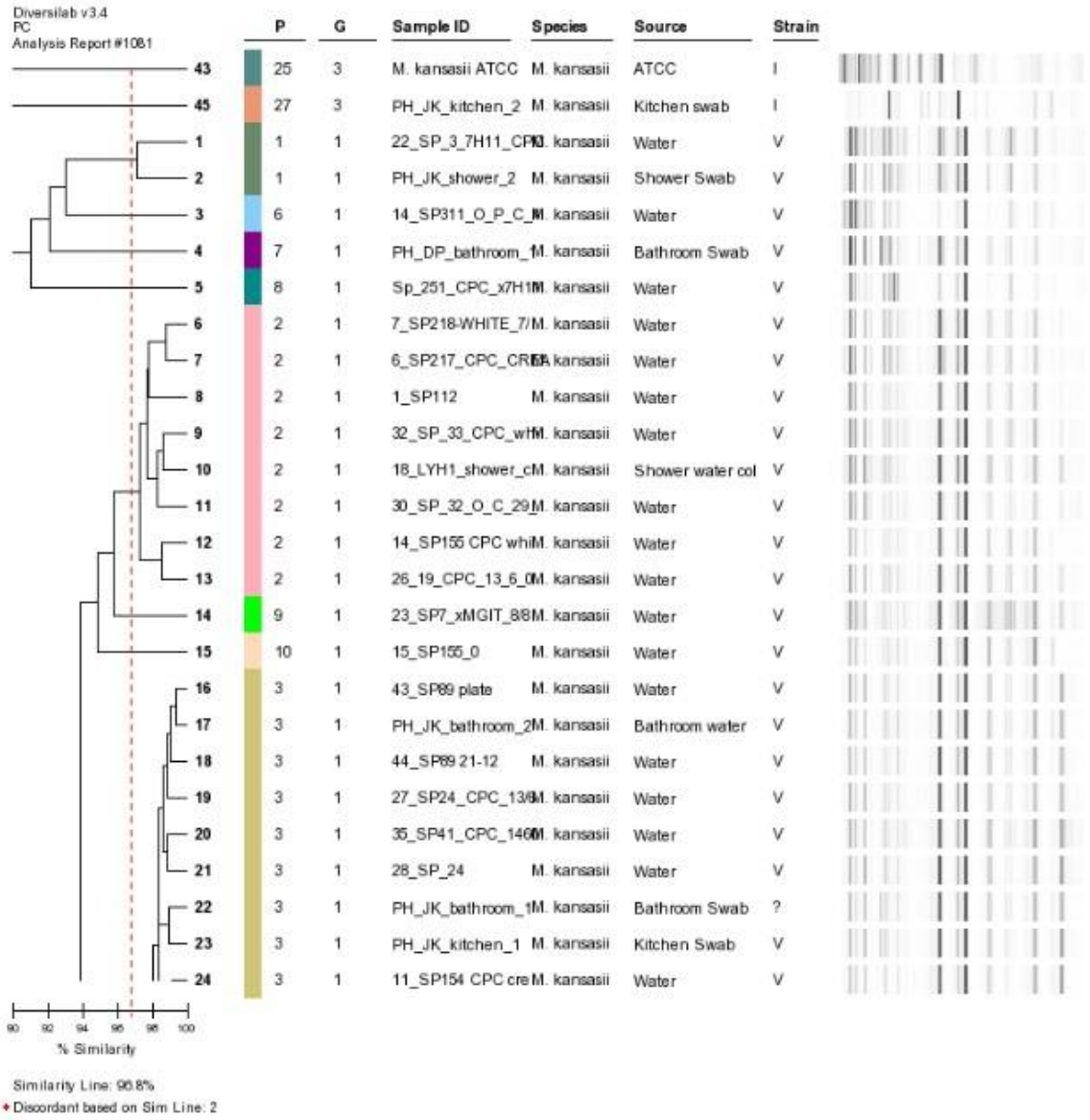


Scatterplot of Patient results

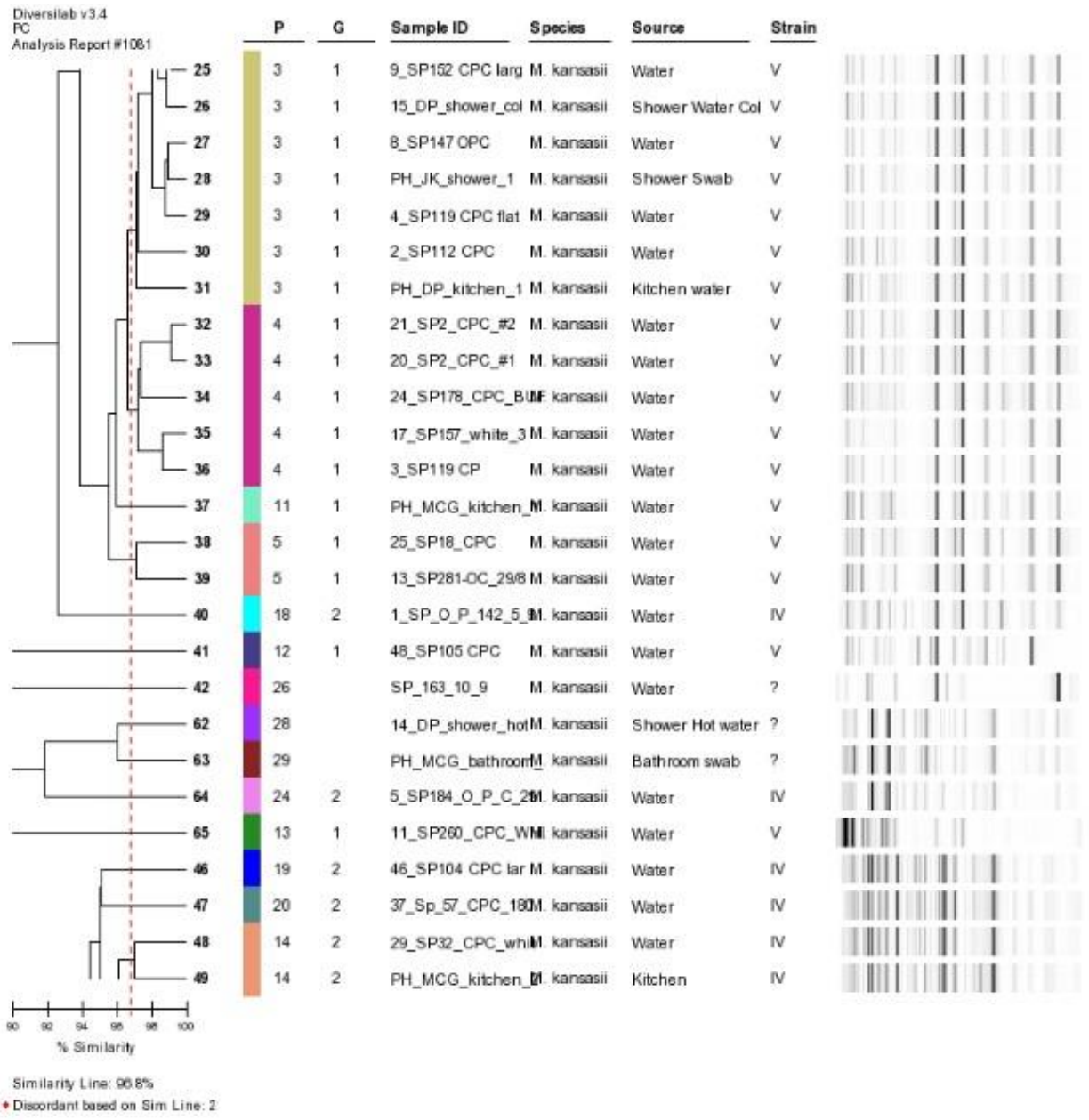
DiversiLab Water Results

Group	Pattern	Key	Sample ID	Species	Source	Strain		Note
1	1	1	22_SP_3_7H11	M. kansasii	Water	V		
		2	PH_JK_shower	M. kansasii	Shower Swab	V		
	6	3	14_SP311_O_P	M. kansasii	Water	V		
	7	4	PH_DP_bathro	M. kansasii	Bathroom Swa	V		
	8	5	Sp_251_CPC_x	M. kansasii	Water	V		
	2	6	7_SP218-WHIT	M. kansasii	Water	V		
		7	6_SP217_CPC_	M. kansasii	Water	V		
		8	1_SP112	M. kansasii	Water	V		
		9	32_SP_33_CPC	M. kansasii	Water	V		
		10	18_LYH1_show	M. kansasii	Shower water	V		
		11	30_SP_32_O_C	M. kansasii	Water	V		
		12	14_SP155 CPC	M. kansasii	Water	V		
		13	26_19_CPC_13	M. kansasii	Water	V		
	9	14	23_SP7_xMGIT	M. kansasii	Water	V		
	10	15	15_SP155_0	M. kansasii	Water	V		
	3	16	43_SP89 plat	M. kansasii	Water	V		
		17	PH_JK_bathro	M. kansasii	Bathroom wat	V		
		18	44_SP89 21-1	M. kansasii	Water	V		
		19	27_SP24_CPC_	M. kansasii	Water	V		
		20	35_SP41_CPC_	M. kansasii	Water	V		
		21	28_SP_24	M. kansasii	Water	V		
		22	PH_JK_bathro	M. kansasii	Bathroom Swa	?		
		23	PH_JK_kitche	M. kansasii	Kitchen Swab	V		
		24	11_SP154 CPC	M. kansasii	Water	V		
		25	8_SP152 CPC	M. kansasii	Water	V		
		26	15_DP_shower	M. kansasii	Shower Water	V		
		27	8_SP147 OPC	M. kansasii	Water	V		
		28	PH_JK_shower	M. kansasii	Shower Swab	V		
		29	4_SP119 CPC	M. kansasii	Water	V		
		30	2_SP112 CPC	M. kansasii	Water	V		
		31	PH_DP_kitche	M. kansasii	Kitchen wate	V		
	4	32	21_SP2_CPC_#	M. kansasii	Water	V		
		33	20_SP2_CPC_#	M. kansasii	Water	V		
		34	24_SP178_CPC	M. kansasii	Water	V		
		35	17_SP157_whi	M. kansasii	Water	V		
		36	3_SP119 CP	M. kansasii	Water	V		
	11	37	PH_MCG_kitch	M. kansasii	Water	V		
	5	38	25_SP18_CPC	M. kansasii	Water	V		
		39	13_SP281-OC_	M. kansasii	Water	V		
	12	41	48_SP105 CPC	M. kansasii	Water	V		
	13	65	11_SP280_CPC	M. kansasii	Water	V		
2	18	40	1_SP_O_P_142	M. kansasii	Water	IV		
	19	48	48_SP104 CPC	M. kansasii	Water	IV		
	20	47	37_Sp_57_CPC	M. kansasii	Water	IV		
	14	48	29_SP32_CPC_	M. kansasii	Water	IV		
		49	PH_MCG_kitch	M. kansasii	Kitchen	IV		
	15	50	2_SP183_CPC_	M. kansasii	Water	IV		
		51	41_SP87 OC	M. kansasii	Potable wate	IV		
	16	52	16_SP157_CPC	M. kansasii	Water	IV		
		53	PH_MD_aeroso	M. kansasii	Shower Aeros	IV		
		54	47_SP104 OPC	M. kansasii	Water	IV		
		55	45_SP102 OP	M. kansasii	Water	IV		
		58	PH_RW_shower	M. kansasii	Shower Swab	IV		
	21	57	38_SP_48	M. kansasii	Water	IV		
	17	58	23_SP175_O_P	M. kansasii	Water	IV		
		59	5_SP127 xMGI	M. kansasii	Water	IV		
	22	60	3_SP183_smal	M. kansasii	Water	IV		
	23	61	31_SP_33_O_P	M. kansasii	Water	IV		
	24	64	5_SP184_O_P_	M. kansasii	Water	IV		
	26	42	SP_183_10_9	M. kansasii	Water	?		
3	25	43	M. kansasii	M. kansasii	ATCC	I		
	27	45	PH_JK_kitche	M. kansasii	Kitchen swab	I		
	28	62	14_DP_shower	M. kansasii	Shower Hot w	?		
	29	63	PH_MCG_bathr	M. kansasii	Bathroom swa	?		

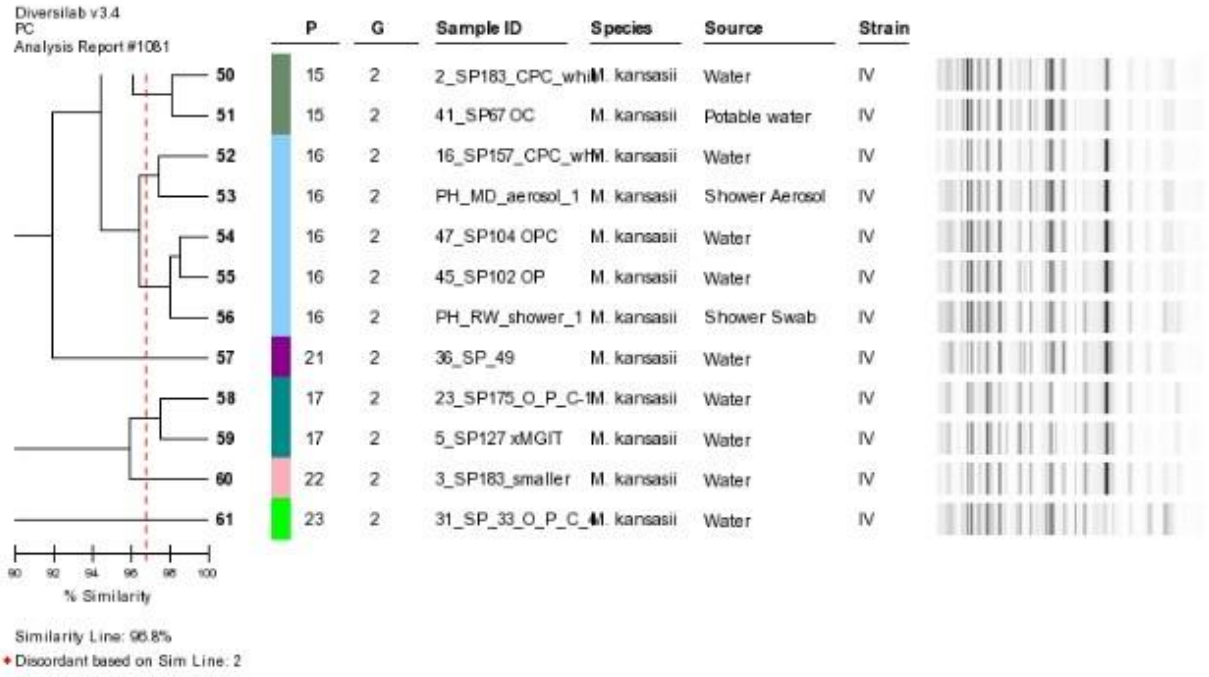
Fingerprint Dendrogram



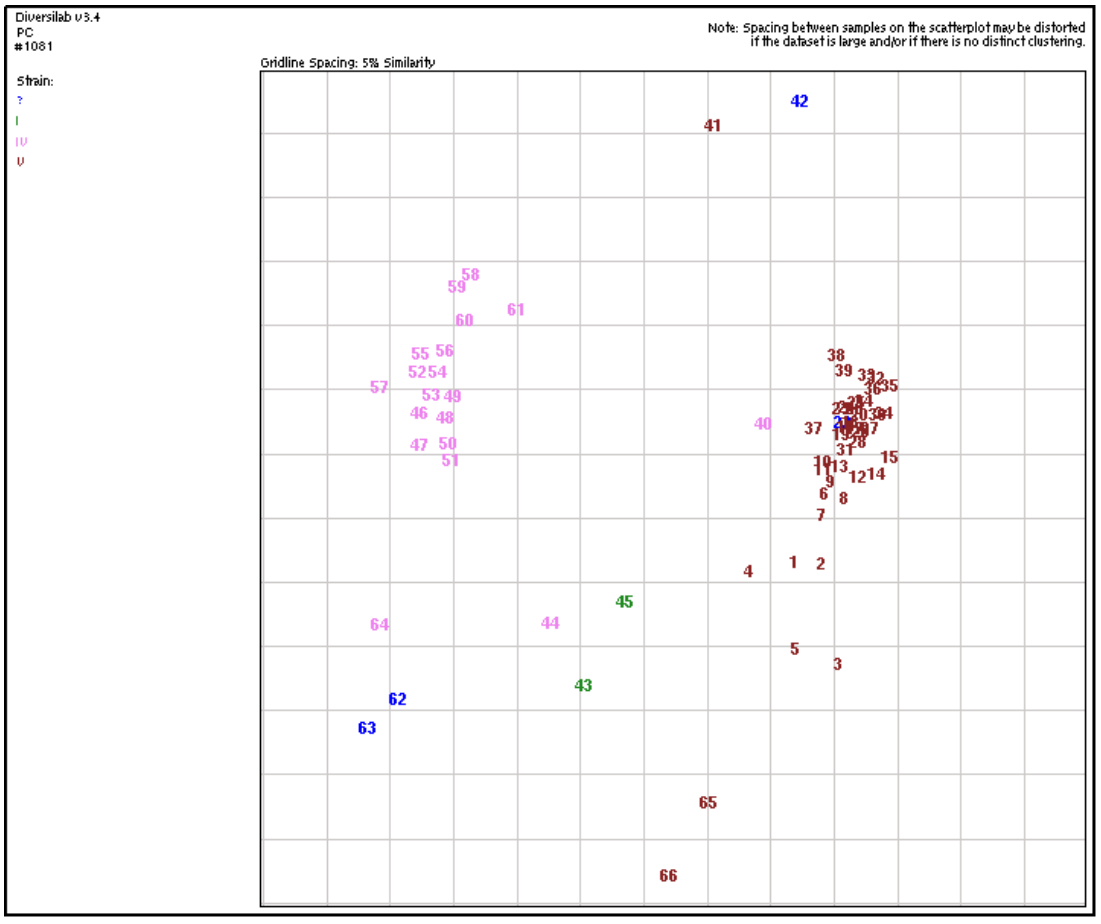
Fingerprint Dendrogram



Fingerprint Dendrogram continued



Fingerprint Dendrogram continued



Scatterplot of Water isolates

Appendix D: HRM Results

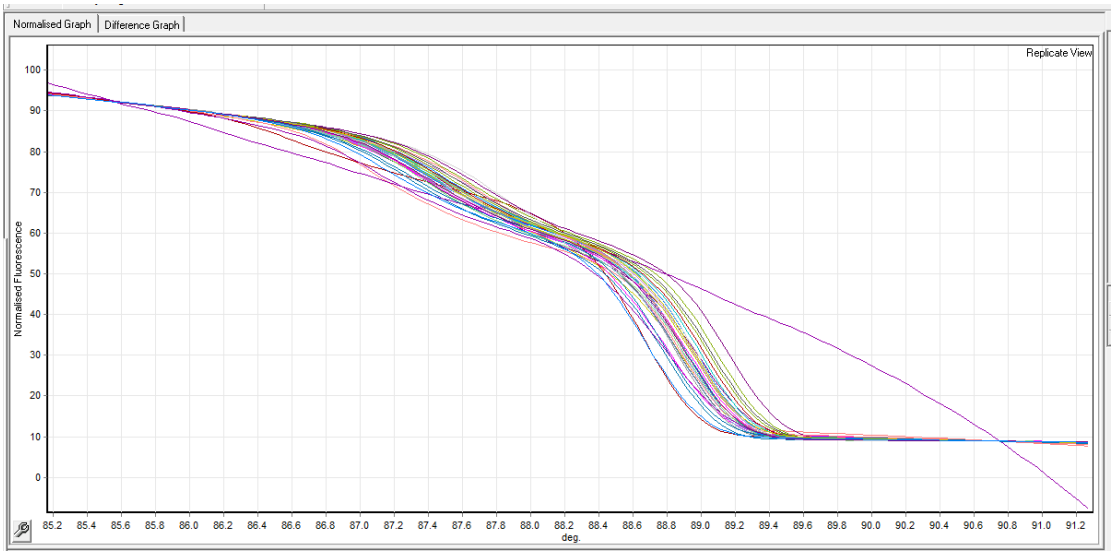
In this Appendix, all HRM results are listed below.

Screenshots from Rotor-Gene Q Series Software

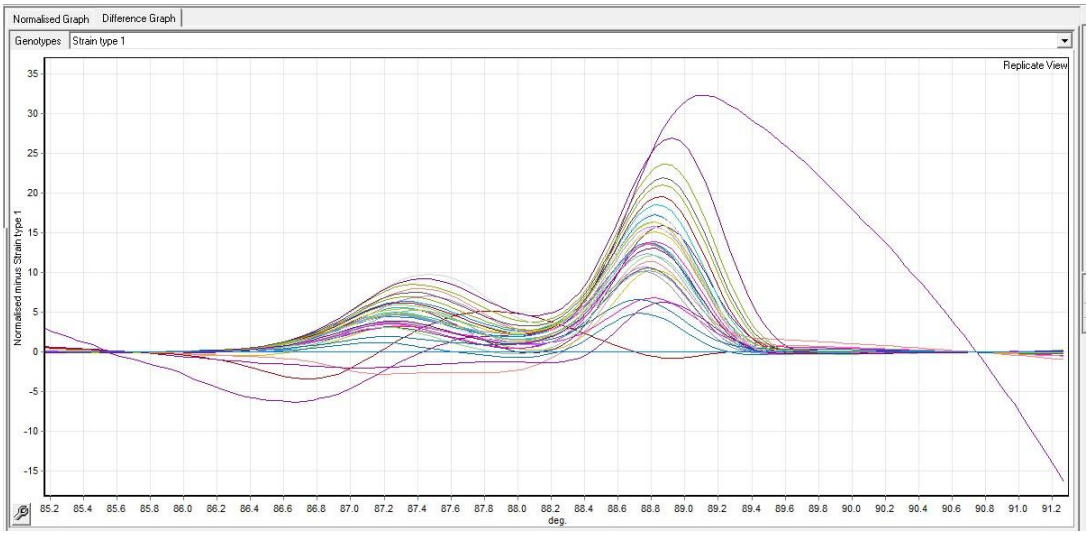
For the Normalised graphs below: Y – Axis is the Normalised Fluorescence and X – axis is the Melting Temperature in degrees Celsius.

For the Difference graphs below: Y – Axis is the Normalised Fluorescence minus the Reference Strain and X – axis is the Melt Temperature in degrees Celsius where the strain deviates from the Reference strain.

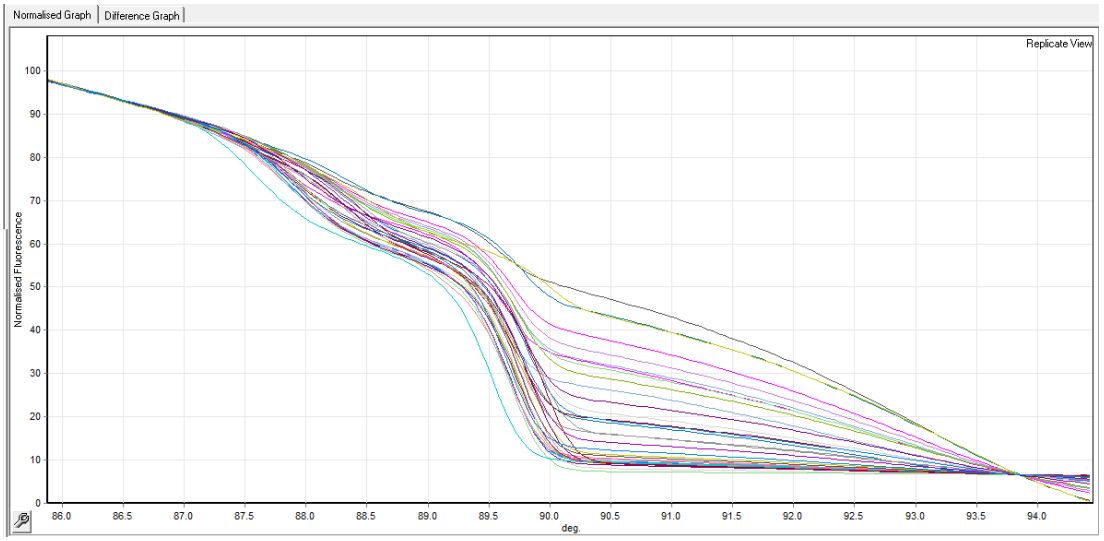
Run 1 – Normalised graph – Patient isolates



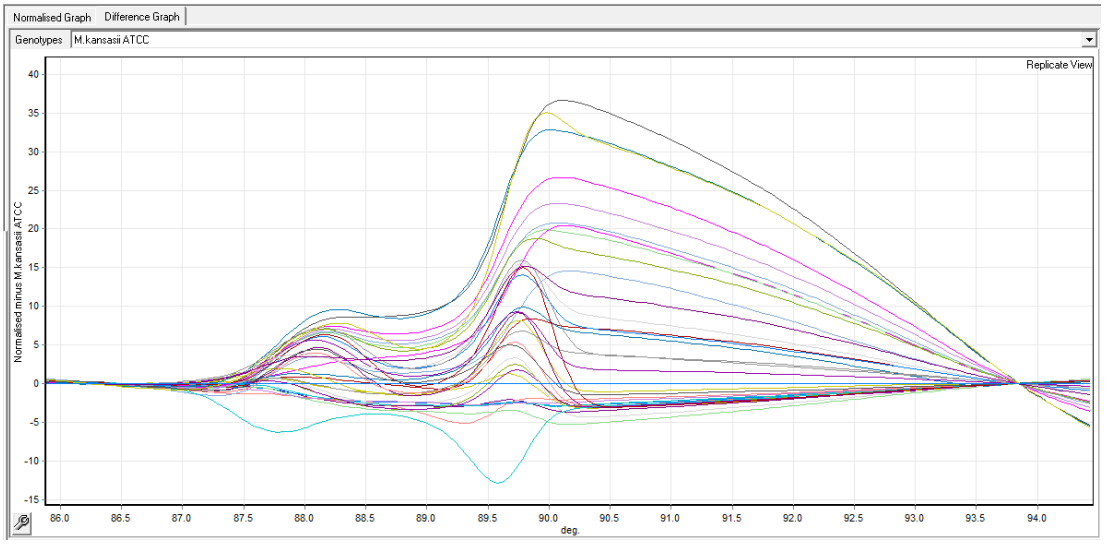
Run 1 – Difference Graph – Patient isolates



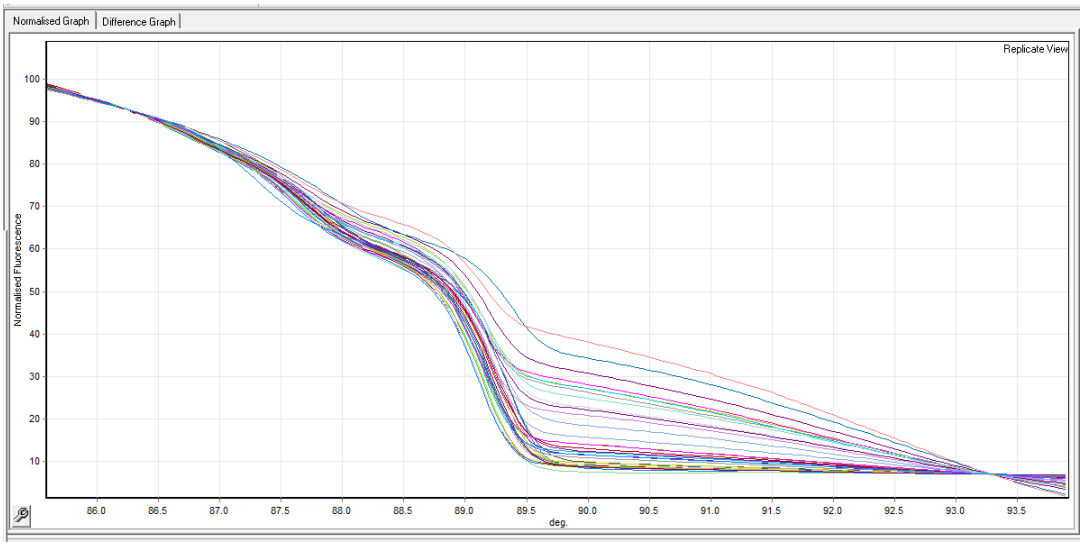
Run 2 – Normalised Graph – Patient isolates



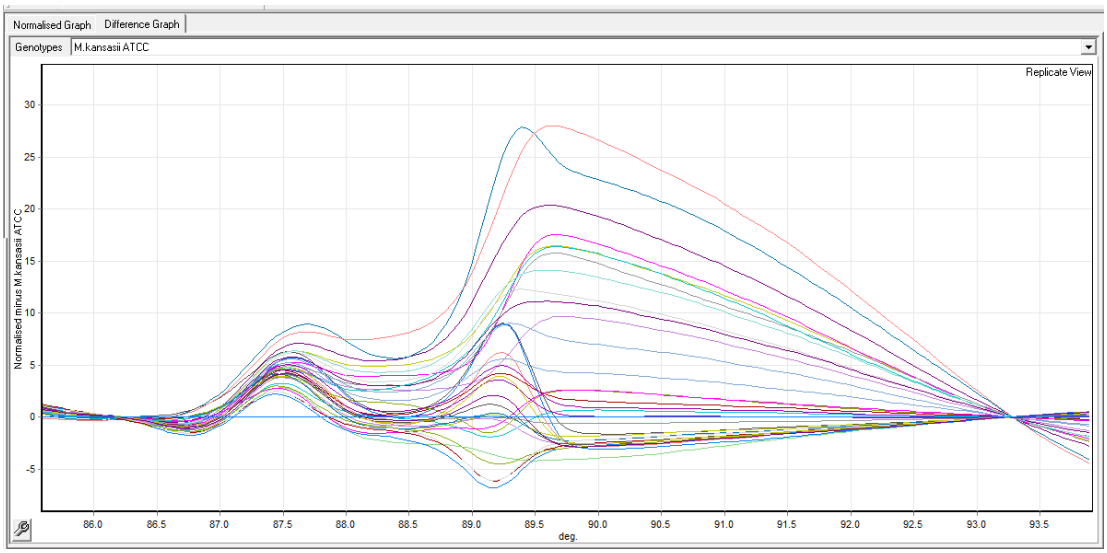
Run 2 – Difference Graph – Patient isolates



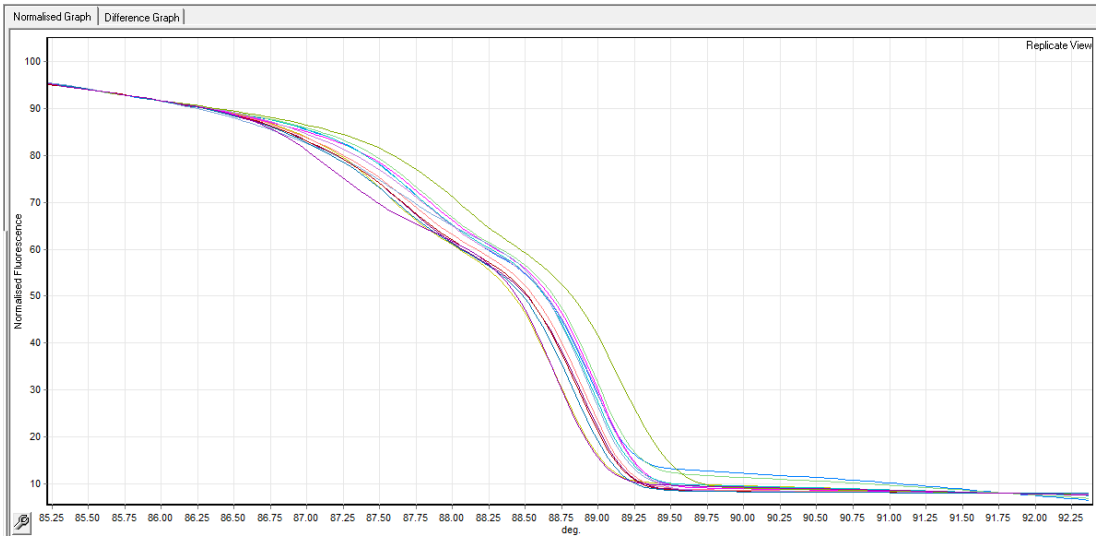
Run 3 – Normalised Graph – Water isolates



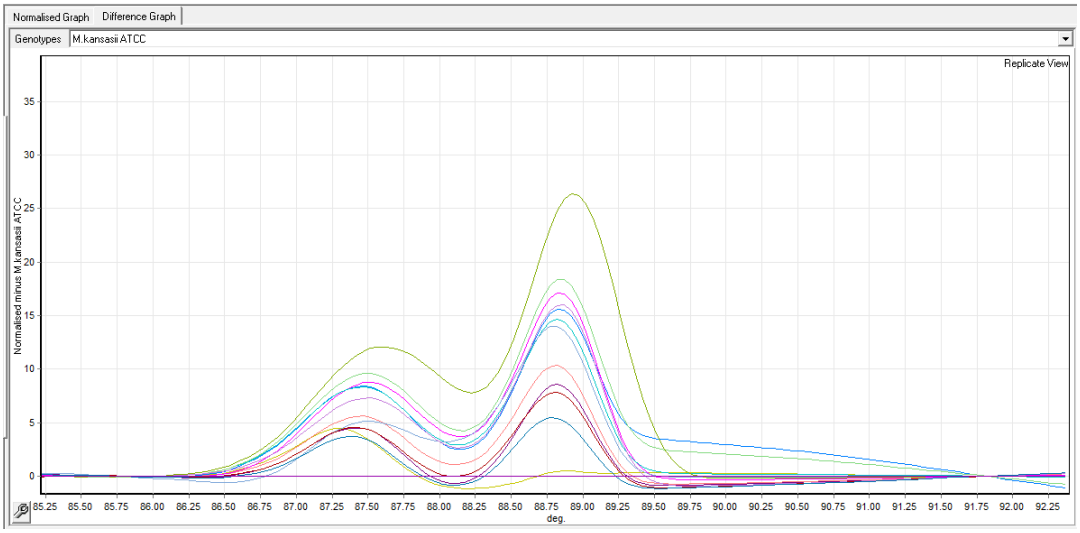
Run 3 – Difference Graph – Water isolates



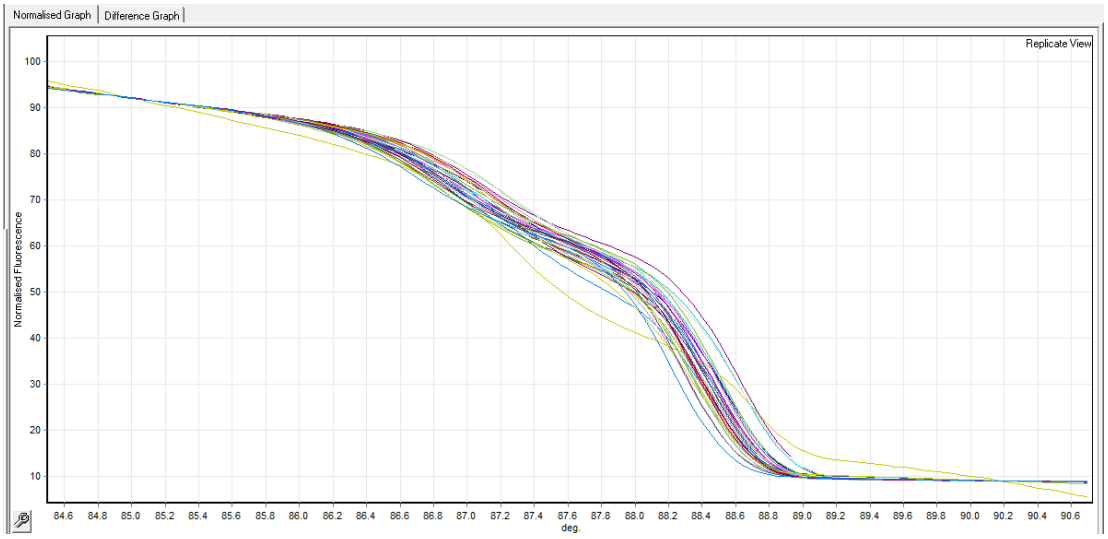
Run 4 – Normalised Graph – Water isolates



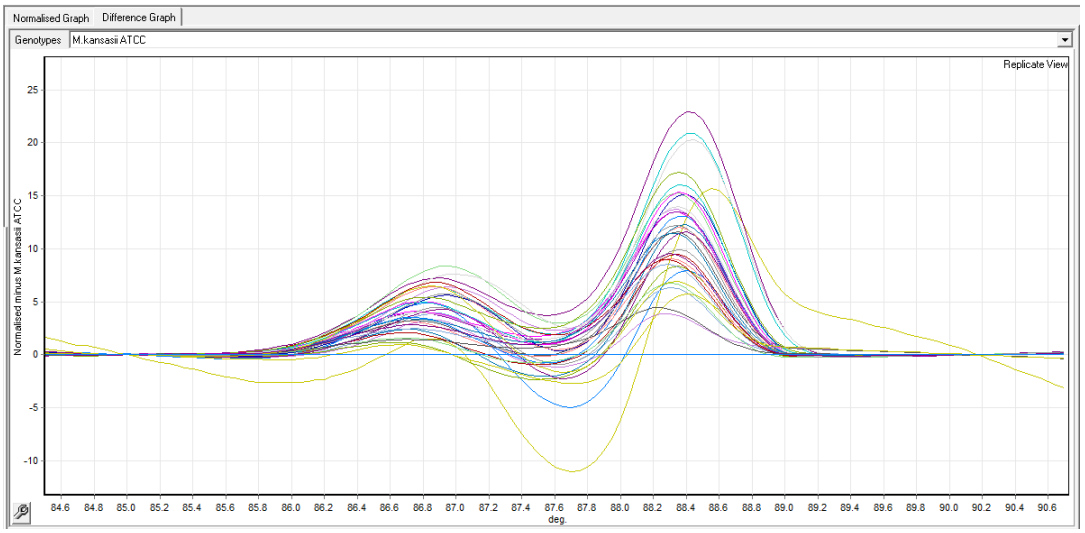
Run 4 – Difference Graph – Water isolates



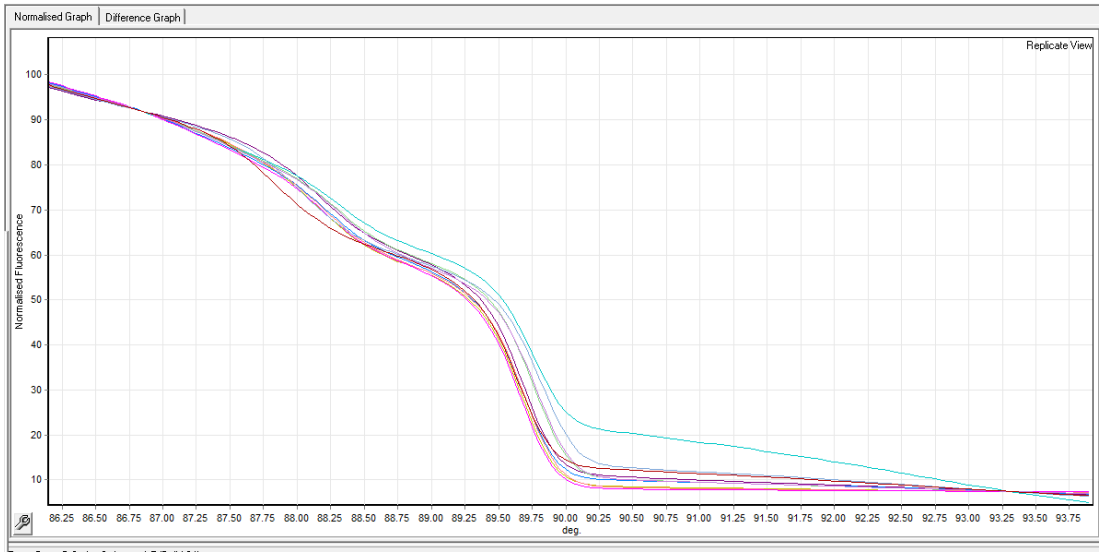
Run 5 – Normalised Graph – Patient isolates



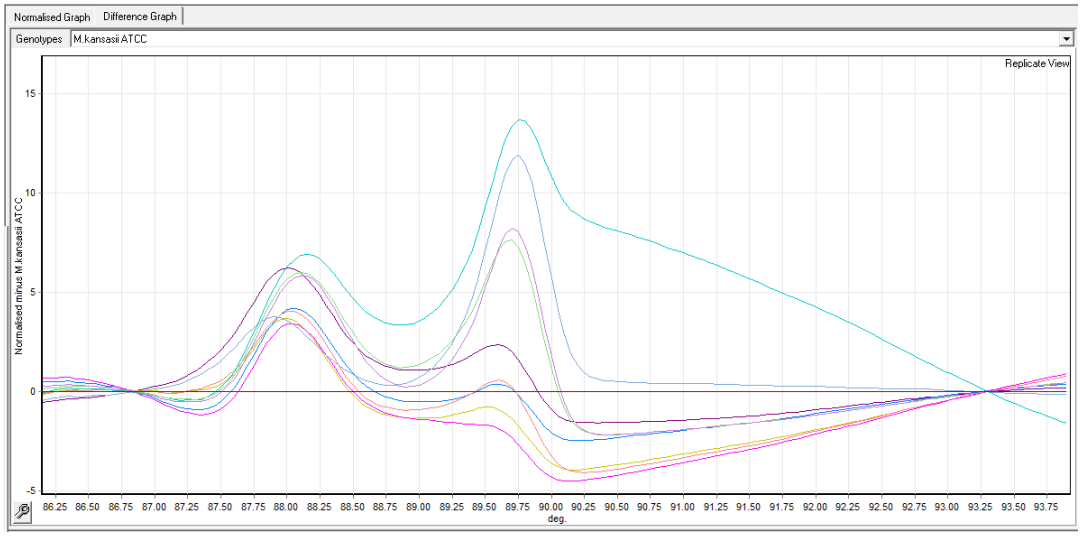
Run 5 – Difference Graph – Patient isolates



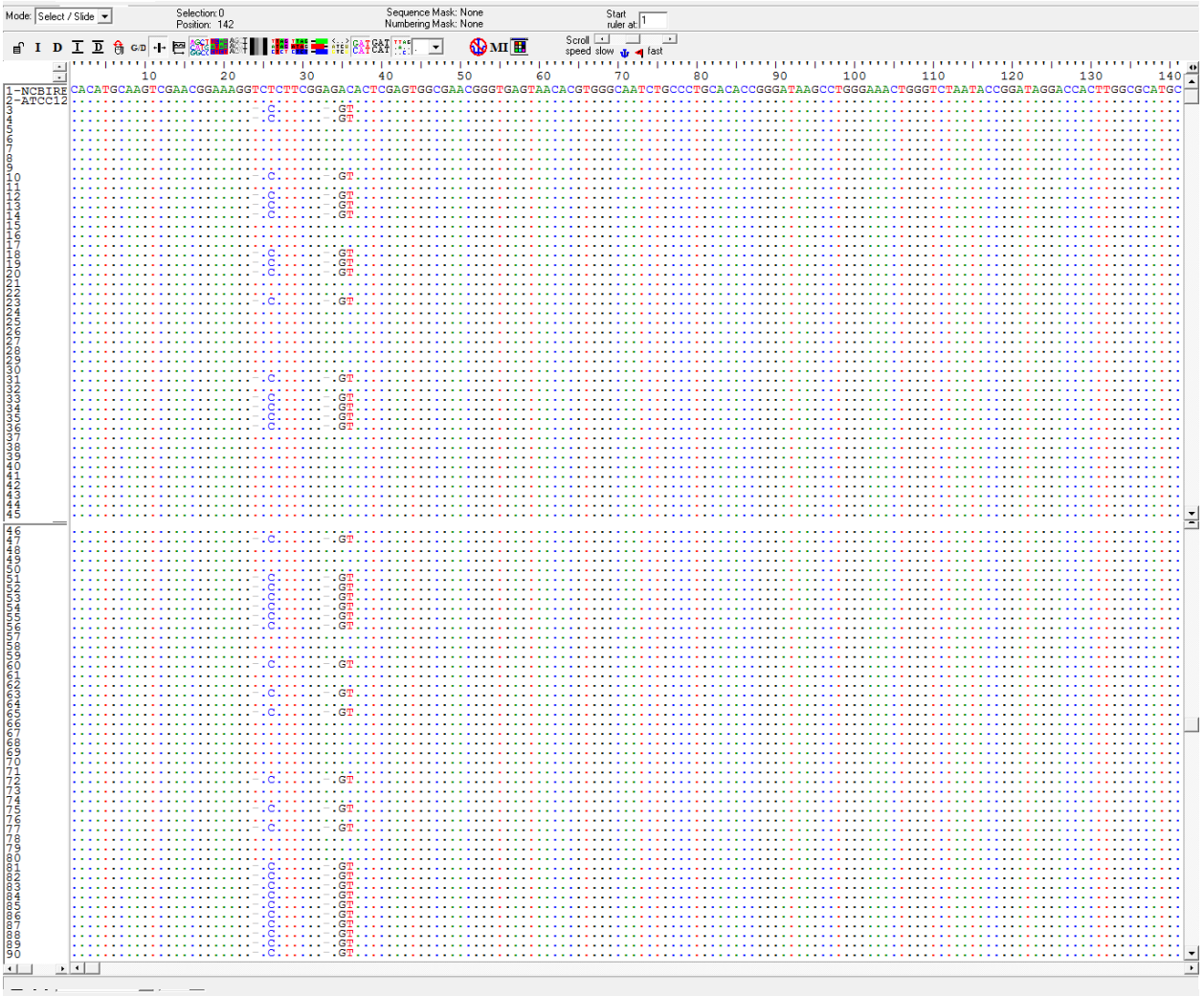
Run 6 – Normalised Graph – repeats from previous failed samples, both Patient and Water isolates

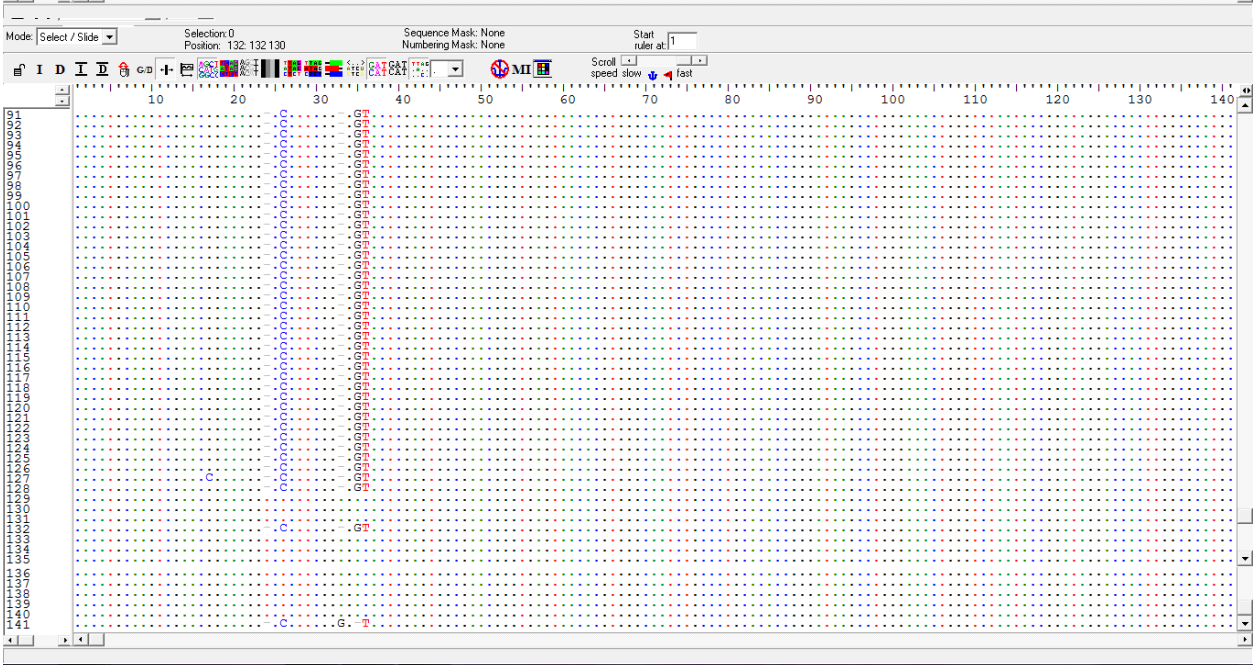


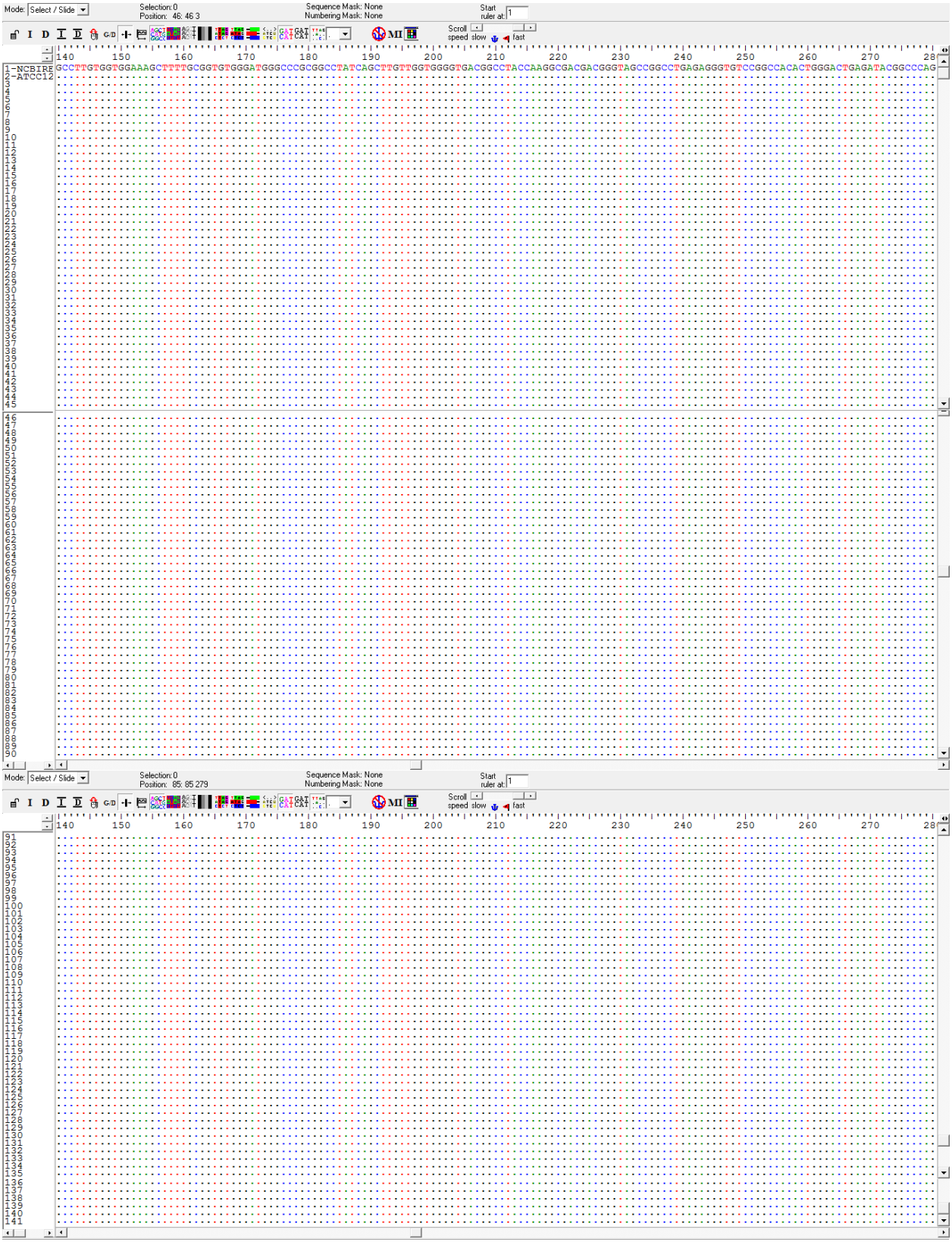
Run 6 – Difference Graph – repeats from previous failed samples, both Patient and Water isolates



Clustal W Analysis Screenshots from BioEdit






Mode: Select / Slide
Selection: 0
Position: 46: 46 3
Sequence Mask: None
Numbering Mask: None
Start ruler at: 1
Scroll speed slow fast
1-NCBIRE GCCTTGTGGTGGAAAGCTTTTGCGGTGTGGGATGGGCCCGCGGCCTATCAGCTTGTTGGTGGGGTGACGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGTCCGGCCACACTGGGACTGAGATACGGCCCAG
2-ATCC12
Mode: Select / Slide
Selection: 0
Position: 85: 85 279
Sequence Mask: None
Numbering Mask: None
Start ruler at: 1
Scroll speed slow fast


Mode: Select / Slide
Selection: 0
Position: 423
Sequence Mask: None
Numbering Mask: None
Start ruler at: 1
Scroll speed slow fast
1-NCBIRE GACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGGGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCACCATCGACGAAGGTCCGGGTTCTCTCGGATTGACGG
2-ATCC12
Selection: 0
Position: 90: 90 382
Sequence Mask: None
Numbering Mask: None
Start ruler at: 1
Scroll speed slow fast
Mode: Select / Slide
Selection: 0
Position: 484
Sequence Mask: None
Numbering Mask: None
Start ruler at: 1
Mode: Select / Slide
Selection: 0
Position: 66: 66
Sequence Mask: None
Numbering Mask: None
Start ruler at: 1
Scroll speed slow fast

Figtree Dendrograms from 16S rRNA Sequences

Top circle = Strain type V; Middle circle = Strain type IV; Bottom circle = Strain type I

Appendix E: DNA Sequencing Protocol

DNA Sequencing for Identification

1 Purpose

This method describes the procedure for amplification and sequencing of 16S rRNA and *hsp*65 gene fragments from presumptive Mycobacterium and aerobic Actinomycetes isolates. These sequences can then be searched against databases to provide an identification for the isolate.

2 Scope

This document is for use by staff of the Queensland Mycobacterium Reference Laboratory who have a background in molecular biology and who meet the requirements of the PCR suite.

This method relies on partial sequencing of genes rather than sequencing complete genes. The partial regions used are sufficient for discrimination of mycobacterium due to the conserved nature of these regions amongst species[5]. As such, this method is only intended to be used for identification or exclusion of mycobacterium and Nocardia species.

3 Principle

16S rRNA Sequencing

Transcription of the 16S rRNA produces the small rRNA sub unit of the bacterial ribosome. The ribosomal structure is essential for bacterial function and is highly conserved amongst bacteria. However there are hypervariable regions within the gene that can vary without affecting the ribosomal structure. These regions are often detectably different between species and can serve as a tool to classify and identify organisms.
Using UNEX primers to amplify a 500bp region from a cultured isolate, the product is then cleaned and sequenced on an ABI Prism 3730DNA sequencer (Applied Biosystems)[1]. Sequences are then compared against a database (most often NCBI public database) to identify similar or identical sequences. Phenotypic information about an isolate is also considered together with the database search results when forming an identification.

*hsp*65 Sequencing

The protein encoded by *hsp*65 is another conserved region suitable as a target for the identification of bacteria, in particular Mycobacteria. Amplification of a 440bp fragment using PCR with target specific primers are

followed by product purification and sequencing as mentioned above. Sequences are then compared against a database (most often NCBI public database) to identify similar or identical sequences. Phenotypic information about an isolate is also considered together with the database search results when forming an identification.

4 Definitions

*hsp*65-Heat shock protein that is 65 kilodaltons in molecular weight
PCR -Polymerase Chain Reaction
16S rRNA-Gene for 16S Ribosomal RNA
bp-Base Pair
NCBI-National Center for Biotechology Information

5 Requirements

5.1 Reagents

Qiagen 10 X PCR buffer + Magnesium chloride reagent
Qiagen dNTP mix
Taq polymerase
Agarose gel powder
1X TAE electrophoresis buffer
Ethidium bromide
EXOSAP-IT
6X loading dye and 100bp DNA Ladder in Loading Dye
Big Dye Terminator Mix
5x Sequencing buffer
Molecular Grade Water
Trigene disinfectant
Invitrogen Bufferless-gel (96-well) platform

16S Sequencing Primers

Sequencing Primer BF 5'-GTAAAACGACGGCCAGTAGAGTTGGATCCTGGCTCAG-3'
Sequencing Primer R2 5'-CCTACGAGCTCTTTACG-3

Hsp-65 Sequencing Primers

Sequencing Primer *hsp*65F (A11)
5'- GTAAAACGACGGCCAGTACC AAC GAT GGT GTG TCC AT - 3`
Sequencing Primer *hsp*65R (A12) 5'- CTTGTCGAACCGCATACCCT -3

5.2 Equipment

96-well PCR plate & PCR lids
PCR racks
Vortex mixer
Microcentrifuge
Thermal Cycler

Microcentrifuge tubes, 1.5ml conical Variable
pipettes and plugged disposable tips
Invitrogen 2% Agarose gel cassettes
Invitrogen Mother Base for 96-well cassettes

6 Safety

The majority of the tasks performed in these PCR methods are carried out in the PCR Suite on Level 4. The integrity of the workspaces within PCR Suite is dependent upon unidirectional airflow and strict observation of guidelines (QIS 24964).

Staff must have participated in a PCR suite induction session before working in the PCR Suite and procedures must be carried out in dedicated workspaces in PCR Suite using the allocated equipment only. Correct Personal Protective Equipment (PPE) must be used.

Warning: Ethidium bromide is a teratogen. Nitrile gloves need to be worn while handling this chemical and caution is advised.

7 Sampling and Sample Preparation

Pure isolate of the test organism is required for sequencing work.

For DNA template preparation see QIS# 17626

8 Procedure

1 In Auslab, review the patients on the **Mycobacteria for rRNA Seq Workflow List** (MYCSEQ) and print.
2 Obtain the DNA from storage (e.g. from PC2 fridge # RBR.B07.L05.RM062.FRT2)
3 Fill in patient details and number of tests being performed including a positive and negative control (see Appendix 1 DNA Sequencing For Identification Worksheet)
4 Save the worksheet under the current M: and year file pathway and print.
5 Book sufficient thermal cyclers for the test.

8.1 Mastermix Preparation (Primary PCR)

1 Remove reagents from freezer and allow them to thaw.
2 Record lot numbers and expiry dates on worksheet.
3 Prepare a working dNTP dilution (1:10) as well as the primers (BF & R2 or hsp65F (A11) & hsp65R (A12) at a (1:10) dilution.
4 Vortex reagent tubes well.
5 Label a 96-well plate for the Primary Amplification with the date.
6 Prepare the master mix in a sterile microcentrifuge tube. Vortex well.
7 Dispense 48µl aliquots of the master mix into each well of the primary amplification plate and close the plate with 8-strip PCR caps.

8 Return reagents to freezer storage and disinfectant work area and record on decontamination log (QIS 25291)

8.2 DNA Addition: (PCR Loading Room)

1 Add 2µl of the DNA template to the wells in the PCR plate containing primary amplification master mix corresponding to the order on the worksheet.
2 Disinfectant work area and record on decontamination log.

8.3 Primary PCR: DNA Amplification

1 Turn on thermal cycler and locate programme in user file MRL (Seq Primary).
2 Thermal cycler conditions are:

94°C 5mins1 cycle

94°C 1min
55°C 1min 35 cycles
72°C 1min

72°C 10min 1 cycle

15°C Soak

8.4 Primary PCR: PCR Product Check

Refer to QIS# 18899

1 Prepare the required number of electrophoresis baths with 1X TAE buffer.
2 Prepare the number of 2% electrophoresis gels with 1X TAE and 3µl Ethidium bromide required for both the Primary Amplification and Product Purification.
3 Dispense 2µl aliquots of 6X gel loading dye onto Parafilm for all samples including the Negative control.
4 Add 8µl primary PCR product to the 6X gel loading dye and mix gently.
5 Transfer 10µl mixture to a well in the gel.
6 Load the first and last well with 5µl 100bp DNA Ladder.
7 Electrophorese the samples for 40mins at 100V / constant Amp.
8 Transfer gel to the Bio-Rad GelDoc system. If Primary Amplification PCR has been successful, intense bands for each test sample and the Positive control well will be evident at about 500bp (16S) and about 440bp (*hsp*65), and there will be no product band in the negative control lane.
9 Photograph gel using GelDoc and also save a copy onto the following path on M:\MRL\Sequencing.

8.5 Primary PCR: Product Purification

1 Using a new 96 well plate, dispense 4µl of EXOSAP-IT into sufficient wells for each PCR reaction with product. Samples that have no product, e.g.: negative control, have no further work performed on them.
2 Add 10µl of Primary Amplification PCR product to the corresponding wells of the plate.
3 Put the plate in thermal cycler. Find folder "*MRL*" and choose program "*exosap*".
Thermal cycler conditions are:

37°C 15 min
80°C 15 min
4°C soak

8.6 Product Purification: PCR Product Check

1 Use the Invitrogen Bufferless gel system: using the 8-channell Multi-channel pipette, place 5µL of the purified product into each well. Do not add Loading dye. Once this is done turn on the Mother Base and set the time for 5 mins, press START. When the 5 mins has elapsed, press the START button to stop the gel running.
2 Photograph gel using GelDoc and also save a copy onto the following path on M:\MRL\Sequencing.
3 Store the purified product at 0-4°C if required that day, or at -20°C until required.

8.7 Sequencing PCR Reaction Set -up

1 Complete preparation of the sequencing Mastermix by adding Big Dye Terminator and Sequencing Buffer (see Appendix 1).
2 Label a 96-well plate with the code "_<initials of person performing test>_< date of submission in yyyy/mm/dd format>, for example: if operator with initials AA is submitting a plate on the first of June 2013 – AA_20130601.
3 Dispense 9µl of the Mastermix and 0.5µl of purified product to the corresponding wells of the plate and seal plate.
4 Return reagents to freezer storage and wipe bench with Trigene disinfectant and record on decontamination log (QIS#: 25291).

8.8 Sequencing PCR: DNA Amplification

1 Select the program "seq" under the MRL folder on the thermal cycler. The thermal cycling conditions are:

30 cycles of
95°C 20sec
50°C 15sec
60°C 60sec

2 Using Sequencing submission sheet (QIS#: 25291), complete the spreadsheet with required data and following the prompts email the sheet to the Sequencing Service.

9 Calculations and Results

9.1 Calculations and Results – Mycobacteria species

1. Sequence results can be found at M:\Sequencing_Service\Sequencing_Data\MRL_2014
2. The Sequencing service provides a report on the quality of each sequence. Sequences must meet the following requirements MM18-A :
    a. Average QV>20
    b. >300bp useable sequence
    c. Signal:noise > 100:1
    d. < 0.5% ambiguous base calls
3. Open the NCBI homepage: https://www.ncbi.nlm.nih.gov and sign in under QMRL with NCBI Username : pathmrl
4. Open the nucleotide BLAST platform
5. Add sequential sample sequences in FASTA format to the Query Sequence window.
    a. Cut and paste location/AUSLAB numbers to identify each sequence.
    b. Visualise each sequence using appropriate software, e.g. Finch TV
    c. Clean sequence before pasting into BLAST e.g.: remove primer sequences, adjust ambiguous base calls
6. Run the BLAST search using “Others (nr etc.)” as the Database Search Set with Program Selection optimized for “Highly similar sequences (megablast)”.
7. Once the BLAST results are available, open the “Recent Results” tab and save the run as a “Saved Strategies”.
8. Return to the BLAST results and open the Sequencing for Identification worksheet (Appendix 1) in another window.
9. Record each samples sequencing identification in the worksheet. For guides on interpreting BLAST results, see the following:
    a. BLAST
    b. MM18-A5
10. Review results for each patient with regards to patient history, culture morphology, time of growth etc. Any conflicting information may need investigation and a supervisor should be consulted.
11. Ensure that the rest of the worksheet is completed and product check gel photos are attached.
12. At this stage the results should be checked with another scientist for correct transcription and interpretation. The other scientist should confirm:
    a. The run meets quality criteria
    b. Correct identifications have been recorded on the worksheet(check BLAST results)
    c. Identifications fit with other information
    d. Reagent QC and product check information is complete

e.The worksheet is signed off

13.After the worksheet is checked, enter the results into AUSLAB specimen notes, recording the region sequenced, the identification and the percentage match for each result i.e.: either 100%, 99% or <99%
14.Enter the organism identification on the "Tests for Mycobacteria" page.
15.Further tests may be required, e.g.: sequencing of another region, morphology check. If this is the case, add the appropriate test code/ worklist code.
16.If no further tests are required, delete the "Further tests are being carried out" comment and replace with the appropriate comment. Ie: either "susnp\" or "tind\" shortcuts.
17. Ensure each specimen page has the MYCOID code added.
18. Ensure each specimen page has the "mtf1\" short cut entered in the last field of the page.
19. If the report for a specimen is complete, validate all "AFB" pages except the main page, then add to the MYCSIGN list. The report is then reviewed by the Chief Scientist before being released.
20. Remove the entry from the MYSEQ list

9.2 Calculations and Results – Nocardia species

1.Sequence analysis is performed as for Mycobacteria species. Follow steps 9.1.1 – 9.1.13. Entering the results for Nocardia species into AUSLAB is different to Mycobacterium results mainly because of the lack of an AFB code (and hence AFB pages) for these results.
2.Each presumptive Nocardia should have a "SITE" code and associated AUSLAB page. Enter the identification into an available organism field on this page. If the correct organism is not listed in AUSLAB, it may be necessary to type the result into the comment field and enter Nocardia sp. in the organism field.
3.All Nocardia sp have an identification and sensitivities performed. Ensure that each Nocardia is on either the MYCNOC (sensitivities to be performed) or the MYCACT (sensitivities already performed) lists.
4.Nocardia results are reviewed by a PC3 staff member before being sent to the MYCSIGN list.
5.Remove each entry from the MYCSEQ list.

10 Method Performance

When reporting the results, the following criteria should be followed:

- The identification is only accepted when the percentage match from the sequence is ≥99%.
- If it is less than this (i.e. ≤98%), the identification can only be given as *M. species*.
- If an unusual Mycobacterium is identified by sequencing, the details are entered into AUSLAB specimen notes. Taking into account all the details e.g. clinical history, site of isolate and morphological features.

Refer to Chief Scientist or supervisor. If the isolate is considered an environmental organism and not known to cause disease, it is also called *M. species*.

11 Quality Controls and Acceptance Criteria

Results are valid if the:

- Positive Control is Positive – Positive control used are *M. intracellulare* (ATCC 13950) for 16S and *M. abscessus* (ATCC 19977) for *hsp*65
- Negative Control is Negative – Negative control used a reaction blank to rule out any source of contamination within the reagents used.

12 Reporting

After results are entered in to AUSLAB, the report is reviewed by the QMRL Chief Scientist before being released. The clinical relevance of the organism identification is not commented on in the laboratory report; however each report provides information on how to contact the Clinical Microbiologist for further information.

13 Records

The Sequencing Service maintains a permanent electronic record of all sequence files.
Electronic copies of worksheets are kept at "M:\MRL\Sequencing" for the current year while worksheet hardcopies are kept for a minimum of three years in the QMRL bookshelf.
Patient records are maintained on AUSLAB by the Health Services Information Agency.

14 References

1 ABI PrismTM Big DyeTM Terminator Cycle Sequencing Kit. Version 3.1 PE Applied Biosystems

2 Parish T and Stoker NG (ed) Mycobacteria Protocols Vol 101 (1998) Humana Press Totowa, new Jersey

3 Rogall T, Flohr T, Bottger EC 1990. Differentiation of Mycobacterium species by direct sequencing of amplified DNA. J. Gen. Microbiol. 136: 1915-1920

4 Rapid Identification of Mycobacteria to Species Level by PCR-Restriction Fragment Length Polymorphism Analysis of the *hsp65* Gene and Proposition of an Algorithm To Differentiate 34 Mycobacterial Species. Devallois et al. Journal of Clinical Microbiology. 1997:2969-2973.

5 CLSI. Interpretive *Criteria for Identification of Bacteria and Fungi by DNA Target Sequencing*; Approved Guideline. CLSI document MM18-A. Wayne, PA: Clinical and Laboratory Standards Institute; 2008

6 NCCLS. *Nucleic Acid Sequencing Methods in Diagnostic Laboratory Medicine; Approved Guideline*. NCCLS document MM9-A. NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2004.

7 Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. (1990) "Basic local alignment search tool." J. Mol. Biol. 215:403-410

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15 Associated Documents

QIS#17626 Preparation of Crude DNA Template for PCR
QIS#18899 Electrophoresis and Documentation of PCR Products
QIS#29382 PCR Suite Sequencing Sample Submission Worksheet
QIS#30690 PCR Suite Laboratory Manual
QIS#15078 Microbiology Safety Manual
QIS# 24964 PCR Suite Contamination Control Procedures
QIS# 25291 Work Area/Equipment Decontamination Log

Appendix F: Dubos Broth Media Recipe

Dubos Broth

PURPOSE
Universal broth culture medium for Mycobacteria.

INGREDIENTS	Storage	Amount
Dubos Broth base (Difco 238510)	RT	3.25g
Rabbit Serum	-20^{0}C	50mL
Glycerol (Ajax 242-2.5GL)	RT	25mL
RO water		425mL

pH: 6.6 $\pm$ 0.2

PREPARATION
1. Add the Glycerol and the Dubos Broth base to the RO water and mix well.
2. Autoclave 121°C for 15 min.
3. Cool to approximately 50°C in a water bath.
4. Add the Rabbit Serum and mix thoroughly.
5. Dispense in 2mL amounts into 7mL sterile plastic bottles.
6. Label DUBOS include the expiry date.

HAZARDS
Dubos Broth base: Irritant

SHELF LIFE
The prepared medium may be stored for up to 3 months at 2-8°C.

QUALITY CONTROL
M fortuitum ATCC 6841 (58446-0605) producing turbid Growth.

M.intracellulare ATCC 15985 (58446-0584) producing turbid Growth.

REFERENCES
Difco & BBL Manual: Manual of Microbiological Culture Media (Second Edition - 2009).