Evaluation of conventional and molecular strategies for the rapid diagnosis and molecular characterisation of strains of *Mycobacterium tuberculosis*

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Abbreviations

AFB	Acid fast bacilli
AIDS	Acquired immune deficiency syndrome
BCG	Bacillus Calmette Guerin
CDC	Centre for Disease Control
Ct	Threshold cycle
dNTPs	Deoxynucleotide triphosphates
DOTS	Directly observed treatment shortcourse
DR	Direct repeat
DVR	Direct variable repeat
ETR	Exact tandem repeat
GC	Guanine cytosine ratio
HIV	Human immunodeficiency virus
IUATLD	International Union against Tuberculosis and Lung Disease
IS*6110*	Insertion sequence 6110
LCR	Ligase chain reaction
LCx-MTB	Abbott Diagnostics LCx *Mycobacterium tuberculosis* ligase chain reaction assay.
LJ	Lowenstein-Jensen medium
MAC	*Mycobacterium avium* complex
MIRU	Mycobacterial interspersed repetitive units
MPTR	Major polymorphic tandem repeats
MTB	*Mycobacterium tuberculosis*
NTM	Nontuberculous mycobacteria
PAB	Protein antigen B
PCR	Polymerase chain reaction

RFLP	Restriction fragment length polymorphism
RT-PCR	real-time PCR
SSCP	Single stranded conformational polymorphism
TAE	Tris acetic acid
TB	Tuberculosis
Tm	Melting temperature
UNG	uracil N-glycosylase
VNTR	Variable numbers of tandem DNA repeats
WHO	World Health Organization
ZN	Ziehl Neelson

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Abstract

Laboratory diagnosis of tuberculosis is often difficult and time consuming. This study has evaluated some new strategies for improved isolation and detection of *Mycobacterium tuberculosis* in clinical specimens. This work was conducted over several years examining samples from the a high tuberculosis prevalence population in Jeddah, Saudi Arabia and in the low tuberculosis prevalence setting in Queensland, Australia. Commercial nucleic acid amplification technologies were evaluated and compared with in-house real-time quantitative PCR strategies for both pulmonary and extrapulmonary specimens and for paraffin embedded tissue samples. The study examined strategies for the detection of multidrug resistance strains through the use of Lipa assay to detect mutations in the *rpo*B gene. Variable numbers of tandem DNA repeat (VNTR) typing was applied to samples from Saudi Arabia and Queensland, Australia to assess their discriminatory power and to demonstrate the diversity and uniqueness of strains of *M. tuberculosis* in distinct geographical regions. A combination of VNTR typing targeting six ETR loci and an additional three polymorphic MIRU loci was applied to a strains of MTB to enhance discrimination of strains. The results demonstrated that culture remains the "gold standard" for diagnosis and that a liquid culture system is essential for timely isolation of mycobacteria. Direct nucleic acid techniques are valuable diagnostic tools in samples where AFBs can be demonstrated but have markedly reduced sensitivity in AFB smear negative MTB culture positive samples. A combination of VNTR and MIRU typing provides excellent discrimination of strains of *Mycobacterium tuberculosis.* This stable typing strategy relies on PCR which allows for real-time epidemiology of transmission to be monitored.

Chapter 1: General Introduction

In recent years there has been a resurgence of tuberculosis which has been a companion of the human race since prehistoric times. Tuberculosis is reappearing in many countries as a public health crisis. Thus, if not an emerging disease, it is an important reemerging disease, and though ancient, is not a disease of the past (Navin *et al.* 2002). In fact, tuberculosis is slowly and inexorably spreading. Victims of tuberculosis are everywhere in the world, but more than 90% of them are in the Third World. (Chretien 1998). It can strike anyone, anywhere, at any time, but is nevertheless most active amongst the poorest nations and societies. Tuberculosis is still a common disease and poses a major public health problem in both developed and developing countries due to their communicability. The World Health Organisation estimates that there were 8.4 million new tuberculosis cases in 1999, up from 8.0 million in 1997. (Anon. 2001). This is largely due to a 20% increase in incidence in African countries most affected by the epidemic of HIV/AIDS. Each year more than 3 million deaths are directly attributed to the disease (Nolte *et al.* 1995).

The causative agent of tuberculosis in humans is the *Mycobacterium tuberculosis* complex which consists of *M. tuberculosis*, *M. africanum*, *M.bovis*, and *M. microti* (Nolte *et al.* 1995). These organisms are acid-fast, aerobic, non-motile, non-spore forming, slow-growing bacteria. Tuberculosis has increasingly gained in importance since its resurgence in the nineteen eighties in many parts of the world, the cause of which has been multifactorial. The most important factor is due to suboptimal tuberculosis control programmes, whether due to lack of resources as seen in developing countries or due to a breakdown in existing tuberculosis control programmes as seen in the United States (Brundney *et al.* 1992). Factors such as the the

impact of HIV infection in sub-Saharan Africa, the emergence of multi-drug resistant tuberculosis, and increased migration and population displacement has exacerbated the problem (Bloom 1994).

M. tuberculosis is spread by aerosolized droplet nuclei with the respiratory tract being the initial site of infection. *M. tuberculosis* can cause disease in almost every organ system but most predominantly in the lungs and are classified as either extrapulmonary or pulmonary tuberculosis (Leowski 1988). The most effective way to combat the spread of organisms and disease is through rapid diagnosis, thorough contact tracing and prompt initiation of appropriate therapy. In 1993, the Centres for Disease Control and Prevention made recommendations that the results from concentrated acid fast smears should be reported within 24 hours of specimen collection, culture in liquid media should be performed in order to detect the growth of *M. tuberculosis* within 10 to 14 days, and antimicrobial susceptibility tests should be complete within 15 to 30 days after collection of the initial diagnostic specimen (Tenover *et al.* 1993). These recommendations were apparently relaxed to results in less than 21 days in a 1997 commentary (Styrt *et al.* 1997). The question arises as to whether this standard can be reasonably achieved using current "state of the art" diagnostic methods. In this study, conventional and new molecular technologies were investigated in a routine mycobacteriology laboratory at the King Khalid National Guard Hospital in Jeddah, Saudi Arabia over a two-year period from January 1996 to January 1998. Clearly from the literature, there is no simple diagnostic solution for the identification *M. tuberculosis* in clinical specimens and combinations of various technologies are required in order to achieve the CDC prevention recommendations.

A steady decrease in the incidence of infections with *M. tuberculosis* in the developed world had been reported (Leowski 1988). However, more recently, there has been resurgence of infections many attributed to AIDS patients and the emergence of

multiple-drug resistant *M. tuberculosis* (Bloom *et al.* 1992). This problem is exacerbated in developing countries where overcrowding and poor nutrition contribute to reduced immunity and increased susceptibility to infection. In Saudi Arabia, accurate data on the incidence of tuberculosis is not available although it is estimated to be 30 cases per 100,000 persons with the highest figures in Jeddah. These figures represent an incidence 2-4 times higher than that of developed countries (Al-Kassimi 1994). Resistant strains of *M. tuberculosis* are common in Saudi Arabia with resistance to isoniazid being most common (Al-Orainey *et al.* 1989). Rifampicin has been widely used in the treatment of tuberculosis and for other endemic conditions including brucellosis and leishmaniasis. Thus, rifampicin resistance is prevalent (Al-Orainey *et al.* 1989). In the Saudi Arabian patient population, patients with positive AFB smears and pulmonary symptoms with or without radiological findings are frequently started on anti-tuberculosis therapy pending the results of culture which may take up to 12 weeks. It has been observed in the Southern region of Saudi Arabia that up to 50% of healthy individuals harbor NTM in their mouths and throats (Nsanze *et al.* 1993). The high incidence of NTM complicates the diagnosis of tuberculosis and frequently results in the unnecessary administration of anti-tuberculosis drugs. Tests which can provide a high specificity for MTB in smear positive specimens will therefore prove cost effective in reducing use of anti-tuberculosis drugs, contact investigations and isolation facilities.

1.1 Laboratory Diagnosis

Definitive diagnosis of respiratory tract tuberculosis is made by first concentrating, digesting and decontaminating respiratory tract specimens (expectorated or induced sputa, bronchoalveolar lavage or gastric lavage) and then performing both acid fast microscopy and culture on the processed specimens. Acid-fast microscopy results can be reported in 24 hours or less but the method lacks sensitivity and is unable to

distinguish between different species of mycobacteria (Kent *et al.* 1985). Growth on selective agar is slow, with visible colony growth taking at least three weeks and usually 4-6 weeks. Traditional methods for diagnosis of tuberculosis rely on microscopy using Zeihl-Neelson or Auramine stains to demonstrate acid fastness and culture on to selective media for isolation of *M. tuberculosis*. Both the acid fast stains and cultural methods are insensitive. It is estimated that 5,000-10,000 organisms per ml of sputum are required for smear positivity (Kent *et al.* 1985) and that only 50% of clinically diagnosed cases are culture positive (Daniel 1990).

The Bactec TB-460 system (Becton Dickinson, Sparks, Md.) uses a radiometric method of detection of mycobacterial growth and has been widely used to detect positive cultures more rapidly than conventional culture. However, on average the time required to detect growth is 13 days (Abe *et al.* 1992) but can be up to 15 to 22 days (Woods 1993). The BACTEC 460 system has been marketed since 1977 (Nolte *et al.* 1995). The Middlebrook 12B medium for this system contains ^{14}C-labelled palmitic acid as the substrate. During mycobacterial growth, ^{14}C-labelled CO_2 is produced and released into the headspace air of the vials. A $^{14}CO_2$ detection device allows early determination of mycobacterial growth. However, this system requires radioactive reagents, causing waste problems, and vials have to be handled and punctured for readings at least eight times during the six to eight weeks of incubation, requiring a considerable amount of work and increasing the risk of cross-contamination. Other limitations include the inability to observe colony morphology, mixed cultures and hazards of using needles. In Saudi Arabia, the use of radioactive materials in the laboratory setting is strongly discouraged and alternative non-radioactive liquid culture systems have been pursued. Certain improvements have been reported with manual systems, such as the Septi-Check system (Becton Dickinson) (Sewell *et al.* 1993), the

MB redox system (Biotest, Heidelberg, Germany) (Naumann *et al.* 1997) and the Mycobacteria growth indicator tube (MGIT, Becton Dickinson: Pfyffer *et al.* 1997). However, all of these systems still require much handling. In 1995, the BacT/Alert (Organon Teknika) fully automated system for isolation of mycobacteria from both respiratory specimens and normally sterile body fluids (other than blood) was developed and approved by the Food and Drug Administration in July 1996 (Rohner *et al.* 1997). Both the BACTEC and MB/BacT systems detect microbial growth by monitoring changes in CO_2 as organisms grow in the culture bottles. The primary differences are that the BacT/Alert uses a colorimetric detection method, tests each bottle every 10 minutes and uses a growth detection algorithm that monitors each bottle for an increased rate of change and/or sustained increase in CO_2 concentration (Wilson *et al.* 1992). The Bactalert system has a marked advantage over the BACTEC 460 system in that it is a completely automated, non-radioactive system which employs continual, non-invasive monitoring of cultures.

Liquid culture coupled with radiometric or colorimetric detection methods offer excellent sensitivity but, the slow rate of growth of the organism, requiring on average at least 13 days for detection of *M.tuberculosis,* and definitive species identification requiring biochemical tests, nucleic acid hybridization or chemical analysis of mycolic acids (Thibert *et al.* 1993) makes for a delayed diagnosis. Direct nucleic acid hybridization, however, lacks the sensitivity of culture methods.

1.2 Immunopathology

Tuberculosis is a unique infection which requires cellular immune responses for its control (Collins 1982). The main defense of the lung is the alveolar macrophage and following inhalation into the alveolar spaces, tubercle bacilli are phagocytosed. Infected macrophages may spread throughout the body, probably randomly according to

blood flow, and can lodge in virtually any organ. The most important areas are those that favour bacillary growth: the lymph nodes, kidneys, epiphyseal areas of the long bones, vertebral bodies, meninges and, most importantly, the apical-posterior areas of the lungs (Bloom 1994). *M.tuberculosis* has evolved the ability to grow in unactivated alveolar macrophages and can grow unimpeded both in the initial focus and in the lymphohaematogenous metastatic foci for 4-8 weeks until cell mediated immunity develops (Wardle 1995). All people have a population of lymphocytes capable of recognising tubercle antigen processed by macrophages. When such lymphocytes encounter the macrophage-antigen complex, they are activated to divide, producing a progeny of similarly reactive cells. These in turn replicate and produce cytokines with the capacity to attract, retain, and stimulate macrophages at the antigen site. Activated macrophages develop high concentrations of lytic enzymes that greatly increase their mycobactericidal activity and also cause tissue necrosis when released from degenerating cells (Bloom 1994). When the population of activated lymphocytes reaches a certain size, cutaneous delayed reactivity to tuberculin or tissue hypersensitivity occurs, generally between 6-14 weeks after infection. At the same time as enhanced macrophage microbiocidal activity, cell mediated immunity appears. Not all persons infected with *M.tuberculosis* will develop disease. In healthy well-nourished subjects, approximately 95% of primary infections heal and remain healed throughout life (Onwubalili 1995). However, after even an inapparent tuberculous infection heals, the lungs may contain one or more small encapsulated caseous foci. In such foci, tubercule bacilli may persist in a dormant or nonmetabolizing state. The bacilli may remain viable in the host for life and cause active disease when the hosts immune function is compromised. Several conditions associated with reduced cellular immunity predispose to disease. These include malnutrition, uraemia, diabetes, leukaemia, lymphomas, alcoholism, immunosuppressive therapy and intercurrent

infection with HIV (Bloom 1994). It is the presence of these bacilli that necessitates prolonged (6-month) courses of chemotherapy, with the resulting problems of cost, compliance and drug resistance. It is not certain that drugs able to kill dormant or stationary-phase mycobacteria can be devised, since most microbicidal agents depend on actively metabolizing or dividing cells. The tubercule bacilli are believed to persist within macrophages as forms with unusual cell walls. Persistence of viable tubercule bacilli may also be the reason why the positive tuberculin reaction is usually maintained for life. Each time the bacillus multiplies, the immune system may be stimulated. (Bloom 1994). The epidemiological and clinical consequences of infection with *M.tuberculosis* are dependant on interplay of host, environmental and bacterial factors. Two bacterial properties that affect the transmissibility and virulence can be measured epidemiologically and clinically: (1) infectivity, the capacity of an organism to establish an infection in the human host and (2) pathogenicity, the capacity of the bacterium to produce disease. The success of *M.tuberculosis* as a pathogen is attributed directly to its ability to manipulate the phagosome that it resides in and to prevent the normal maturation of this organelle into an acidic, hydrolytic compartment. As the macrophage is key to clearing the infection, the interplay between the pathogen and its host cell reflects a constant battle for control (Russell 2001).

1.3 Disseminated Tuberculosis

There are three distinct patterns of disseminated tuberculosis, *viz.* acute miliary tuberculosis, cryptic miliary tuberculosis and chronic disseminated tuberculosis. Lymphohematogenous spread of *M. tuberculosis*, usually at the time of primary infection and less commonly from established foci, can affect almost any organ. Tuberculosis of the lymph nodes is the commonest of the several manifestations of disseminated tuberculosis (Thompson *et al.* 1992). *M. bovis* was once considered a common cause of

cervical adenitis but, by 1910, *M. tuberculosis* was identified as the cause of 70% of mycobacterial cervical lymphadenitis in children and, in 1951, *M. tuberculosis* was shown to be responsible for all except a few cases of lymph node disease (Park *et al.* 1910, Lester 1951). Diseases caused by atypical mycobacteria became increasingly apparent as tuberculosis rates in developed countries declined and reports of cervical lymphadenitis caused by atypical mycobacteria began to appear in the 1950s (Prissick *et al.* 1956). Changes in the pattern and frequency of tuberculosis have strikingly altered the aetiology of mycobacterial lymphadenitis. The possibility that the natural population of the *M.avium* complex and *M.scrofulaceum* is changing is supported by evidence that there has been a shift in the frequency of isolation of non-tuberculous mycobacteria recovered from children suffering from cervical lymphadenitis. Up to 1979, *M. scrofulaceum* was the most frequent cause of cervical lymphadenitis in children (Wolinsky 1979). In contrast, recent reports have established that *M.avium* is the most common causative agent of lymphadenitis in children in both the United States (Wolinsky 1995) and England (Colville 1993). From the review of the literature, it appears that in adults not infected with HIV, the disease is caused predominantly by *M. tuberculosis*. In areas where the incidence of both tuberculosis and HIV infection is high, notably Africa, mycobacterial lymphadenopathy is caused almost exclusively by *M.tuberculosis* (Morrissey *et al.* 1992). In developed countries where the incidence of tuberculosis is comparatively low, HIV patients, are more susceptible to disseminated mycobacterial infections caused by *M.avium* and up to 50% of AIDS patients in the United States and Europe were infected with non-tuberculous mycobacteria (Falkingham 1996). However, it cannot be assumed that all cervical lymphadenitis in children is caused by atypical mycobacteria because up to 10 % may be caused by tubercle bacilli. In Saudi Arabia, the incidence of tuberculosis is 2 to 4 times that of developed countries (Milaat *et al.* 1994, Al-Kassimi 1994) and the incidence of HIV infection low. In Saudi Arabia, there are few reports on the epidemiology of

cervical lymphadenopathy in either adults or children. In 1984, Thabit *et al.* reported that approximately 50% of neck masses were associated with tuberculous lymphadenopathy. Similar figures have been reported from neighbouring Sudan where lymphadenopathy is a common clinical problem (Kheiry *et al.* 1992). As both tuberculous and non-tuberculous lymphadenopathy call for different treatment modalities, careful laboratory diagnosis is warranted.

The most common clinical presentation of tuberculous lymphadenitis is that the patient observes a lump in the neck which is often painless. Occasionally the node many be painful and there may be fever in the early stages of the illness. The node is firm and mobile at first, but becomes fixed when periadenitis supervenes. Abscess formation, involvement of the skin and sinus formation may follow. During treatment, some of the nodes may enlarge and give rise to abscess or sinus formation. Furthermore, new nodes may appear. This is rarely due to the failure of treatment but is believed to be associated with variation of tuberculin sensitivity or with a secondary bacterial infection from the upper respiratory tract. Sinuses may persist for many months before healing finally occurs.

The location of tuberculous adenitis was cervical or supraclavicular in over 90 % of cases, in 80 % only a single node was involved, and evidence of other tuberculosis was present in 18% (pulmonary, 7%; mediastinal nodes 9%; other 2 %) (Summers *et al.* 1980). Diagnosis was made by biopsy and AFB were demonstrated on stains in slightly over half of the cases. The only major diagnostic difficulty is granulomatous lymphadenitis due to mycobacteria other than *M.tuberculosis*, the clinical and histological features of which can be identical, and differentiation is possible only by culture and not by histology. For optimal histological diagnosis of mycobacterial lymphadenopathy, three main diagnostic criteria should be demonstrated: (a) presence of AFB in sections, (b) presence of caseating

necrosis (c) epithelioid granulomata. However, the majority of histological diagnoses are made without all of these criteria being achieved. In a review of 89 cases of extrapulmonary tuberculosis, Cutler *et al.* (1994) determined that only 6/89 (7%) met all three of the diagnostic criteria. In the majority of cases (60/89; 67%), both caseating necrosis and granulomata were demonstrated. In the remaining 20/89 cases (22%), however, histological diagnosis was made on the presence of granulomata alone. Granulomas characterised by epithelioid multi-nucleate giant cells as seen in tuberculosis are also seen in a range of infectious and noninfectious diseases including cat scratch disease, fungal and helminth infections, sarcoidosis and neoplasms (Aranaz *et al.* 1996) and more specific diagnostic criteria are necessary for accurate diagnosis.

The technological advances in the rapid diagnosis of infectious diseases have been applied to mycobacteria. Nucleic acid probes and polymerase chain reactions have been developed to detect mycobacterial DNA in clinical specimens, which allow for the detection of low numbers of both viable and non-viable bacilli (D'Amato *et al.* 1995, Kolk *et al.* 1992, Carpentier *et al.* 1995). Since AFBs are demonstrated in less than half of lymph node biopsies histologically diagnosed as tuberculous lymphadenopathy (Freidig *et al.* 1986), their utility is limited when assessing the sensitivity and specificity of a molecular assay. However, of greater interest will be to determine the sensitivity and specificity of a molecular method in comparison with histological diagnosis which at present must represent the single most diagnostic test for tuberculous lymphadenopathy although fraught with limitations.

1.4 Treatment

Before the availability of effective drug therapy, approximately half of patients with active pulmonary tuberculosis died within two years, a quarter recovered, and a quarter survived with chronically active disease. Treatment regimens are divided into the initial

phase (two months)) and the continuation phase (four to six months). During the initial phase the bactericidal effect of treatment leads to rapid bacteriological sputum conversion and improvement of clinical symptoms. During the continuation phase, consisting usually of fewer drugs given either daily or intermittently, the sterilising effect of therapy eliminates remaining bacilli and prevents relapse. The objectives of standard treatment regimens are to cure all infectious and non-infectious tuberculosis patients and to prevent relapses and the selection of resistant bacilli in infectious patients. In smear negative pulmonary and extrapulmonary cases (non-infectious cases), the risk of selection for resistant bacilli is small: the appropriate short-course chemotherapy includes three drugs in the initial phase and two drugs in the continuation phase. In smear-positive pulmonary tuberculosis (infectious cases), however, there is a risk of selecting resistant bacilli. Therefore, new cases should be given four drugs during the initial phase and two drugs in the continuation phase. This regimen is as effective for patients with sensitive organisms as for those with primary drug resistance (Anon. 2001). The drugs used in the initial phase are Isoniazid (H), Rifampicin (R), Pyrazinamide (Z) with Ethambutol (E) or Streptomycin (S). The drugs used in the continuation phase are combinations of H and E, or H and R. Previously treated cases (relapse, failure, return after interruption of therapy) should be given first 5 and then 4 drugs in the initial phase and three drugs in the continuation phase. Using these drugs in conjunction with each other reduces antitubercular therapy from 18 months to 6 months. Therefore, the emergence of strains resistant to to any of these drugs causes major concern, as it leaves only drugs that are far less effective, have more toxic side effects, and result in higher death rates.

1.5 Directly Observed Treatment

Patients must take all their medication daily or intermittently so that they become non-infectious and are cured with a minimum risk of future relapse. Yet it is well documented that at least 30% of patients receiving self-administered treatment in the initial phase will not adhere to treatment. The best way to ensure patients compliance with therapy is to observe patients taking their drugs. This is directly observed treatment. The World Health Organization (WHO) has established Observed Treatment Short-Course (DOTS) as a key strategy to tuberculosis control (Kochi 1991). The WHO case-management strategy has five elements: (1) a political commitment to a national and permanent TB programme; (2) Case detection by microscopy in symptomatic patients; (3) implementation of directly observed, standardized, free-of-charge, short-course chemotherapy (4) regular supply of all essential anti-TB drugs and (5) monitoring of case detection and treatment outcomes. Administration of DOTS appears to be the most effective way to ensure a decrease in primary resistance, acquired resistance and relapses (WHO 2001). During 1999, 127 countries (an increase of 8 countries from 1998) were implementing DOTS strategy at least as a part of their national tuberculosis control programmes achieving good cure rates.(Anon. 2001).

1.6 Molecular Diagnosis

An alternative to amplification of mycobacteria by culture is amplification of mycobacterial nucleic acid followed by specific identification by hybridization.

A number of nucleic acid amplification methods are being developed for mycobacterial testing including polymerase chain reaction (PCR) and ligase chain reaction (LCR). Any of these methods could significantly reduce the time required for diagnosis of infection with *M.tuberculosis*, perhaps to one day. It is not clear how these methods will be accepted in the routine clinical microbiology laboratory; hence, more information on the clinical utility of these methodologies is required.

1.7 Ligase Chain Reaction

Ligase chain reaction (LCR) is a probe amplification technique first described in 1989 (Wu and Wallace 1989). LCR amplification is based on sequential rounds of template dependant ligation of two adjacent oligonucleotide probes. Linear amplification of the specific probes is achieved when a single pair of oligonucleotides probes are ligated. Exponential amplification is achieved when two pairs of oligonucleotides probes, one complementary to the upper strand and one complementary to the lower strand are used. In the first step of LCR, two sets of oligonucleotide probes are allowed to anneal to their target DNA at 65°C. When complementary base pairing occurs, thermostable ligase (Barany 1991) will link the adjacent probes to yield a longer product. The reaction is then heated to 94°C to denature the ligated product and cooled to 65°C to allow annealing and ligation. The process is then repeated to achieve exponential amplification of ligated products (Podzorski *et al.* 1995). Two factors can critically affect the outcome of this reaction, particularly in the first cycles where the DNA in the specimen is the primary target for ligation. Firstly, the specificity of DNA ligase in the first cycles of the reaction where single base-pair mismatches prevents the adjacent probes from being ligated. In subsequent cycles where the ligation products form the predominant templates, the reaction is not susceptible to single base pair discrimination. Secondly, if there is ligation of probes independent of the target DNA early in the reaction these may serve as templates for amplification and result in blunt-end ligation products (Wu and Wallace 1989).

This procedure was further modified in 1991 to overcome the problems with blunt-end ligation products and the intrinsic specificity of the reaction in detecting single base mismatches. The four oligonucleotide probes are designed in pairs that hybridize to

complementary single-stranded target sequences exposed after the DNA is denatured by heating to 95°C. When a pair of probes has hybridised to the target sequence on a single strand of DNA, there is a gap of a few nucleotides between the probes. The addition of a DNA polymerase into the reaction acts to fill in this gap between the probes by incorporating nucleotides. Once the gap is filled, ligase can covalently join the pair of probes to form an amplification product that is complementary to the original target sequence and can itself serve as a target in subsequent cycles of amplification (Birkenmeyer *et al.* 1991).

Abbott Diagnostics (Chicago Ill.) have obtained the rights to this technology (Weiss 1991) and have adapted their IMx instruments referred to as LCx for the detection of amplified ligated target DNA. Methodologies using LCR were initially developed for the detection of *Chlamydia trachomatis* (Dille *et al.* 1993) and *Neisseria gonorrhoea* (Ching *et al.* 1995) in clinical specimens. Using culture as the "gold standard" for detection of *C. trachomatis*, LCR and Amplicor PCR have a specificity of 99.6% compared to culture (de Barbeyrac *et al.* 1995). This methodology has now been applied for the detection of *Mycobacterium tuberculosis* in respiratory specimens. The LCR target nucleic acid sequence for the *M.tuberculosis* LCR assay is found within the single copy chromosomal gene of *M. tuberculosis* which encodes for protein antigen b (Bengard *et al.* 1989). This gene sequence appears to be specific to the *M. tuberculosis* complex and has been detected in all *M. tuberculosis* complex strains examined to date (Sjobring *et al.* 1990). A preliminary report has shown that the LCR assay for the direct detection of *M.tuberculosis* in respiratory specimens has a high degree of specificity and sensitivity in smear positive respiratory samples, however, the sensitivity of the test was diminished in smear negative specimens (Ausina *et al.* 1997).

1.8 Amplicor Polymerase Chain Reaction

Polymerase Chain Reaction is a technique for the *in vitro* amplification of specific DNA target sequences by the simultaneous primer extension of complementary strands of DNA. The PCR method was devised and named by Mullis and colleagues at the Cetus Corporation (Mullis *et al.* 1987). Polymerase chain reaction (PCR) has recently been used in the diagnosis of many infectious diseases by enabling specific detection of small numbers of nucleic acid molecules (Hayden *et al.* 1991). The diagnosis of tuberculosis is one of the more appropriate applications for PCR as undiagnosed cases have a potentially fatal outcome. Several investigators have therefore applied PCR for the direct detection of *M. tuberculosis* in clinical specimens (Altamirano *et al.* 1992, Eisenach *et al.* 1991). Contamination of samples during amplification giving false positive results is a major problem and must be taken into consideration (Noordhoek *et al.* 1994).

The Amplicor *M. tuberculosis* PCR assay (Roche Molecular Systems, Branchburg, N.J.) has overcome many of the contamination problems through the incorporation of AmpErase in the assay prior to amplification. Amperase contains the enzyme uracil N-glycosylase (UNG), which recognises and catalyses the destruction of deoxyuridine-containing DNA. Deoxyuridine is always present in amplicons because of the use deoxyuridine triphosphate in place of thymidine triphosphate in the amplification reagent (Longo *et al.* 1990). AmpErase prevents any residual uracil-containing amplicons created during previous cycles from being amplified again (Beavis *et al.* 1995). The Amplicor PCR assay has good correlation with conventional culture although inconsistent results have been detected (Beavis *et al.* 1995, D'Amato *et al.* 1995, Moore *et al.* 1995). These have been attributed to the presence of inhibitors, low organism numbers resulting in sample variation and the detection of non-viable organisms (Beavis *et al.* 1995)). The Amplicor PCR assay has the ability to detect

DNA from both viable and non-viable organisms and can detect *M. tuberculosis* after therapy has been initiated. (Beavis *et al.* 1995) PCR is an extremely specific and sensitive method of DNA amplification that is capable of identifying as little as a single copy of a given DNA sequence (Schluger *et al.* 1994). Since no "gold standard" methodology for diagnosing *M. tuberculosis* infection exists, laboratory diagnosis is complex (Narita *et al.* 1992). The utility of molecular methods for tuberculosis depends on the clinical situation in which the assays are performed. In patients with a history of infection, these molecular techniques may not be able to distinguish active from prior infection, particularly in patients with bronchiectasis (Schluger *et al.* 1994). Rapid results with these molecular methods may have large impact on hospital costs with respect to the management and isolation of patients suspected of having tuberculosis. Clarridge *et al.*, in a study investigating 623 samples culture positive for AFB, determined that if all patients who had AFB positive sputum smears were placed under AFB isolation precautions, 28% of these would be isolated unnecessary as they did not have *M.tuberculosis* and 29% of patients infected with tuberculosis would not be isolated because of negative AFB smears (Clarridge *et al.* 1993).

1.9 Real-time PCR detection

Technological advances at Applied Biosystems (Foster City, Ca) have made fully automated real-time detection of specific products possible. The 7700 sequence detection system integrate four major elements; (1) fluorogenic chemistry for target specific oligonucleotide probes, (2) exploitation of the polymerisation dependant 5' nuclease activity of DNA, (3) instrumentation to measure fluorescence signal with a closed PCR reaction vessel, and (4) software that processes and analyses the data. (Bassam *et al.* 1996). The ABI Prism 7700 Sequence Detection System (TaqMan) has been shown to be a rapid and sensitive method for quantification of PCR products (Heid

et al. 1996). The system uses a fluorogenic probe, and the amount of fluorescence detected is proportional to the amount of accumulated PCR product. Quantification of PCR products occurs in real time during the amplification process, with no post-amplification handling being necessary. This eliminates potential sources of carryover contamination and reduces handling time. (Desjardin *et al.*1998).

More investigations are required to interpret nucleic acid amplification methods with culture positive and smear negative sputum samples. Although rapid diagnosis is essential for individual patient management, further work is required to understand sources of infection and means of transmission in order to control the disease.

1.10 Detection of multi-drug resistance

A particularly pernicious feature of the current epidemic is the concurrent increase in the prevalence of disease caused by strains of MTB that are resistant to multiple first-line anti-tuberculous drugs. Multiple-drug resistant tuberculosis is difficult to treat effectively and to cure. As a result, such patients may remain contagious for prolonged periods, leading to increased risk of transmission to contacts (such as family and health care workers), and subsequently into the community, hence, further compromising our ability to control the present epidemic and the occurrence of future cases (Hyman *et al.* 1993).

1.11 Types of resistance

WHO/IUALTD studies use the term *primary resistance* to define the presence of drug resistance to one or more anti-tuberculous drugs in a new tuberculosis patient who presents to a treatment centre. This category includes those patients with primary resistance as well as those patients with undisclosed acquired resistance who either do not remember prior treatment, refuse to divulge the information on past treatment or had an incomplete clinical history recorded. Initial resistance does not include chronic

patients. Primary resistance is defined as the presence of drug resistance to one or more anti-tuberculous drugs in a tuberculous patient who has never received prior anti-tuberculous therapy. It is caused by infection with drug-resistant organisms from another patient who had acquired resistance either due to inadequate chemotherapy or because of infection with primary drug-resistant organisms (Vareldzis *et al.* 1994). The WHO/IUATLD (Anon. 2001) now recommend the use of the term *resistance among new cases* when patients deny any prior anti-TB treatment and, in countries where adequate documentation is available, no documented evidence of prior treatment exists. In the United States from 1982 to 1991, the incidence of primary drug resistance has increased from 0.5 to 3.1% (Anon. 1992). The frequency of primary drug resistance to one or more drugs is estimated to be about 5% in technically advanced countries while it is substantially higher in some developing countries. Although Saudi Arabia has no reliable TB notification system (Al-Kassimi *et al.*1993), primary drug resistance is estimated to be approximately 15% with rifampicin resistance being most prevalent (Ellis *et al.* 1996, Jarallah *et al.* 1992).

Ninety percent of rifampicin resistant strains are also isoniazid resistant. (Drobniewski *et al.* 1998). This high prevalence of resistance to both rifampicin and isoniazid makes rifampicin a suitable marker to monitor multi-drug resistant MTB (Drobniewski *et al.* 1998). Acquired resistance is defined as resistance to one or more anti-tuberculous drugs which arises during the course of treatment usually as a result of delayed diagnosis, non-adherence to the recommended regime following diagnosis or faulty prescribing (Vareldzis *et al.* 1994). Acquired resistance can be determined only in countries with the resources to perform serial susceptibility testing. (Anon. 2001). A proxy for acquired drug resistance is to measure *Resistance among previously treated patients*. The WHO/IUATLD recommend that this group be subdivided into four groups

as follows; (1) treatment failures; (2) treatment relapse; (3) return for treatment following treatment default; and (4) chronic cases. (Anon. 2001)

1.12 Mechanism of resistance

Of concern is the emergence of drug resistant strains in recent years, and efforts have been made to unravel the mechanisms of action and to understand the molecular basis of resistance to these antibiotics. Deviations in structural genes encoding target proteins may lead to differences in secondary structure of such proteins, and therefore loss of functionality. These differences cause insufficient binding of the antibiotic to its target, and ultimately lead to antibiotic resistance (Pretorius *et al.* 1996).

The term multidrug resistance refers to simultaneous resistance to at least rifampicin an isoniazid (Vareldzis *et al.* 1994). Genetic and molecular analysis of drug resistance in MTB suggests that resistance is usually acquired by the bacilli either by alteration of the drug target through mutation (Spratt 1994), or by titration of the drug through overproduction of the target (Davis 1994). Resistant mutations to any single antibiotic occur readily at random in bacilli undergoing replication and may be selected for by not using the appropriate combination of drugs. In tubercle bacilli, the sites of resistance to TB drugs are chromosomally located and are not plasmid borne. Thus, the likelihood of occurrence of a mutant resistant simultaneously to two drugs is the product of individual probabilities (Vareldzis *et al.* 1994). It appears clear there is no novel resistance determinant causing multi-drug resistance but rather an accumulation of individual mutational events in genes encoding drug targets (Morris *et al.* 1995). Development of resistance to rifampicin in MTB follows a single step high-level resistance pattern. Mutants arise spontaneously in strains not exposed previously to the antibiotic at a rate of one mutation per 10^7 to 10^8 organisms (Telenti *et al.* 1993 a). Because patients with cavitary pulmonary lesions may harbour 10^6 to 10^8 tubercle

bacilli, any patient treated with only a single effective drug has a very high probability that the resistant ones will be present and will continue to grow. Conversely, a patient treated with two or more drugs, in concentrations to which the strain is susceptible, has a very high probability of escaping the fate of incurable drug-resistant tuberculosis. The role of chemotherapy is paramount in production of all clinically important drug resistance (Rieder 1994).

Drug resistance and the highly contagious nature of tuberculosis highlight the need for better surveillance programs and means of rapidly detecting resistant strains. It is now known that high-level resistance to isoniazid is associated with mutations to the catalase peroxidase gene *katG.* The mechanisms of action of isoniazid, as well as the mechanisms conferring isoniazid resistance, are complex and not completely understood. Activation of isoniazid to an unstable electrophilic intermediate requires the enzyme catalase peroxidase (KatG, coded by *kat*G). However, mutations in the *kat*G gene only correlate with less than 70 % of isoniazid resistant strains.(Kapur *et al.* 1995, Morris *et al* 1995) . Efforts to determine the factors involved in resistance to isoniazid led to the discovery of the *inh*A locus, which was proposed as the primary target for coresistance to isoniazid and ethionamide (Banjeree *et al.* 1994). InhA is an enoyl-ACP reductase which catalyses an early step in fatty acid synthesis among enterobacteria. A T→G transversion, observed in a few of the resistant strains, at position 280 in the *inh*A gene, results in ser94 to ala94 replacement (Banerjee *et al.* 1994). This replacement, thought to alter the binding affinity of InhA to NAD(H), ultimately results in isoniazid resistance (Dessen *et al.* 1995). Mutations in the *inh*A locus and isoniazid resistance have only shown approximately 10% correlation (Kapur *et al.* 1995, Morris *et al.* 1995). Isoniazid resistance has also been reported with mutations in *aphC* which encodes the alkyl hydroperoxide reductase C (Telenti *et al.* 1997), and *oxyR* which is the oxidative stress regulator (Drobniewski *et al.* 1998). To

detect isoniazid resistance multiple systems are required that are capable of detecting multiple sites simultaneously to predict isoniazid resistance only in approximately 85% of resistant cultures (Telent *et al.* 1997). It has been shown that resistance to rifampicin and streptomycin arise from missense mutations in the genes encoding for bacterial RNA polymerase and certain ribosomal subunits respectively (Heym *et al.* 1993).

It is known from studies on *E. coli* that resistance to rifampicin occurs due to missense and other mutations occurring in a discrete region of the *rpoB* gene. Telenti *et al.* (1993a) cloned and sequenced the cognate region of the *M.tuberculosis* gene by using sequence information available from the *M.leprae rpoB* gene (Honore *et al.* 1993). The data was used to formulate oligonucleotide primers for amplification and sequencing of a 411-bp fragment of *rpoB* from 66 rifampicin resistant and 56 rifampicin susceptible strains recovered from patients from several continents (Musser 1995). Approximately 95% of rifampicin resistant isolates had mutations in the 69-bp region corresponding to codons 511 to 533 of the *rpoB* gene previously recognised as a prominent region for mutations conferring rifampicin resistance in *E.coli.* (Waterson *et al.* 1998). Fifteen distinct mutations involving eight conserved amino acids clustered in this 69-bp region have been identified (Musser 1995). The most commonly reported mutations have been missense mutations and amino acid substitutions at one of two positions (residues 526 and 531) and these account for 80% of the resistant strains (Musser 1995). Additional mutations have subsequently been reported in the 81bp Rifampin resistance-determining region (RRDR) of the *rpo*B gene corresponding to codons 507 to 533 (*E.coli* numbering system) (Mani *et al.* 2001). Different groups from diverse regions of the world have thus far reported around 65 substitutions, 12 deletions, and 4 insertions in the RRDR region of the *rpoB* gene. (Mani *et al* 2001). Further mutations which also confer rifampicin resistance have been reported at codons 381 (Taniguchi *et al.* 1996),

481 (Nash *et al.* 1997), 505 (Matsiota-Bernard *et al.*), 508 (Matsiota-Bernard *et al.*), and 509 (Nash *et al.* 1997). The mechanism of resistance in the remaining 5% of isolates remains undecided but suggests that at least one additional molecular mechanism mediates rifampicin resistance in *M.tuberculosis.* Potential mechanisms include alterations in rifampicin permeability and mutations in other RNA polymerase subunits (Musser 1995).

1.13 Rifampicin Resistance

The gold standard method for the detection of mutations is DNA sequencing as it provides definitive identification of any mutation present. In the case of rifampicin, the presence of mutations appears to be fully predictive of resistance. This is not necessarily the case with other genes associated with drug resistance as silent mutations occur that do not lead to any significant change in phenotype e.g. katG gene, a common site for mutations within the C-terminal region has little effect on the activity of the catalase enzyme (Drobniewski *et al.* 1998). DNA sequencing requires a high level of technical competence and although this technique can provide accurate sequence data within 24 - 48 hours the tests are costly and relatively expensive to maintain making this technique not cost effective for most laboratories.

As an alternative to DNA sequencing Heteroduplex analysis, PCR single stranded conformational polymorphism (SSCP), and mismatch analysis have been used to detect mutations in the *rpoB* gene of MTB. Heteroduplex analysis uses amplified DNA from the test strain and DNA from a reference rifampicin susceptible strain mixed, denatured and cooled to produce a double stranded hybrid through complementary base pairing. In a resistant strain where mutations have occurred, there will be a mismatch of the two strands and the resulting heteroduplex will run with a different mobility to the

homoduplex (where the test strain has no mutations) on a denaturing electrophoresis gel system (Williams *et al.* 1994).

SSCP utilises the complex tertiary structure of single stranded DNA to detect mutations in the *rpoB* gene. PCR is utilised to amplify the 81bp region of the *rpoB* gene of a test strain and the product is denatured to a single stranded DNA. If any two stands of DNA differ by one or more base pairs, they will fold into different tertiary structures with different mobility on a polyacrylamide gel (Telenti *et al.* 1993b).

The mismatch assay is based on the ability of double stranded RNA to withstand digestion with the RNA polymerase, RNase A. Target DNA is amplified by using primers which incorporate T7 RNA polymerase and SP6 RNA polymerase promoters in opposite directions allowing RNA to be transcribed by using the PCR product as a template. A rifampicin-sensitive wild-type strain (H37Ra) is also amplified by using the same primers but with SP6 and T7 promoters incorporated in the strands complementary to the test strain. The test PCR product and reference PCR product are combined in a transcription reaction using either T7 or SP6 RNA polymerase. The complementary transcripts from the test and reference products are allowed to hybridise, and the resulting hybrids are treated with RNase. Any mutation in the test transcript will not pair with the reference transcript, and so the hybrid will be cleaved at that point. Undigested transcripts and cleavage products can be detected by analysis using agarose gel electrophoresis (Nash *et al.* 1997).

The Innolipa Line probe assay using solid phase hybridisation analysis has been developed commercially by Innogenetics, (Ghent, Belgium). This system uses PCR to amplify the 260bp region the *rpoB* gene with the resulting biotinylated PCR products being reverse hybridised to oligonucleotide probes immobilised as parallel lines on a membrane strip. The oligonucleotides include five overlapping probes corresponding to

sequences present in the wild-type, rifampicin sensitive gene (SI-S5), together with four overlapping probes (R2, R4a, R4b and R5) corresponding to the most frequently observed resistance mutations including the two commonest His 536→Tyr and Ser 531→Leu which account for up to 73% of rifampicin resistance (Cooksey *et al.* 1997) . The LiPA test is an easy-to-use format for the rapid detection of rifampicin resistance. The test, available in a kit format, is especially useful in laboratories that are not capable of carry out DNA sequencing. (De Beenhouwer *et al.* 1995, Rossau et al. *1997*). In this study, In the United States, the assay was used for the rapid identification of rifampicin resistance in a collection of 51 rifampicin resistant cultures. Nine distinct rpoB mutations were detected and concordance with standard phenotypic rifampicin susceptibility results was 90.2%. (Cooksey *et al.* 1997).

Of all these assays described for the detection of resistance mutations the Line Probe assay is probably the simplest post PCR procedure to perform and interpretation of the banding pattern allows for quick identification of the most common resistance mutations. This method has been applied successfully for the detection of mutations both in cultures and clinical specimens (De Beenhouwer *et al.* 1995). Although providing clear results SSCP, heteroduplex analysis and RNA mismatch analysis are unable to identify the actual mutation. However, silent mutations or missense mutations not directly related to rifampicin resistance may occur. This means that phenotypically rifampicin susceptible strains with the silent mutations would yield an altered SSCP pattern and heteroduplex analysis and be subsequently interpreted as resistant (Kim *et al.* 1997). In addition, these methods have not been applied successfully for the detection of resistance mutations in clinical samples and are more technically demanding than the Line Probe assay (Waterson *et al.* 1998). To date no assessment of the types of rifampicin mutations observed in Saudi Arabia has been undertaken.

Identification of the various types of mutations observed in Saudi Arabia may elucidate clustering of specific mutations and contribute to epidemiological investigations and mechanisms of transmission.

1.14 Molecular epidemiology

The control of mycobacterial disease requires powerful methods for detecting cases and tracing sources of infection, so that effective treatment can be correctly targeted and suitable public health measures implemented. Conventional epidemiological investigations have relied on phenotypic markers such as biochemical reactions, specific antigens, phage sensitivity and antibiotic resistance patterns which largely show low discrimination among strains (Butcher *et al.* 1996). Phage typing was the only method available for typing isolates of *M.tuberculosis* until the advent of the genotypic methods. A panel of phages were initially tested by a World Health Organisation working group in 1971 and initially showed poor interlaboratory reproducibility. An improved scheme was developed by Jones *et al.* which used four major phages to divide strains into three main groups. These three groups could be further subdivided by six auxiliary phages. Phage typing, however, requires technical skill for reproducibility and interpretation and its use limited in epidemiological studies (Jones *et al.* 1978).

The recent application of discriminatory molecular methods for typing of *M.tuberculosis* strains plays a potentially important role in the investigation of community or nosocomial outbreaks investigations, confirming laboratory contamination and tracking sources of transmission. It is important to consider whether the disease results from reactivation of a previous infection or from active transmission and to understand the risk factors associated with transmission of tuberculosis so that health care resources can be used optimally and control measures implemented. (Gutierrez *et al.* 1998). Over the past few decades, numerous outbreaks of tuberculosis

have been reported in hospitals, prisons, schools and homeless shelters. In some outbreaks, the transmission of *M. tuberculosis* was limited whereas in others, there were high rates of transmission (Valway *et al.* 1998). Similarly, transmission has been reported after minimal exposure to an infectious patient. The variability of transmission rates has been attributed to the environment in which the outbreak occurred and to the clinical characteristics of the source patient. Sreevatsan *et al.* studied selected *M.tuberculosis* from Texas and New York associated with increased rates of *IS6110*-based clustering, a potential measure of increased virulence (Sreevatsan *et al.* 1997). In that study, *M.tuberculosis* complex were classified into three distinct groups based on single nucleotide polymorphisms in the catalase-peroxidase (*katG*463) and gyrase (*gyrA*95) gene sequences. (Sreevatsan *et al.* 1997). Genotypic Group 1 organisms included *M.africanum, M.bovis, M.tuberculosis* and *M.microti.* Groups 2 and 3 only included strains of *M.tuberculosis.* Strains devoid of *IS6110* and the Beijing Family strains including the W strain also made up Group 1 strains. Rhee *et al.* used molecular beacon technology to determine that genotypes classified using the katG-463 and gyrA-95 based system, were not associated with increased infectivity or pathogenicity (Rhee *et al.* 1999)

1.15 Restriction fragment length polymorphism

Repeated DNA sequences have been found to occur in the chromosomes of a wide variety of bacteria. The identification and characterisation of a series of repeated DNA fragments in the *M.tuberculosis* genome that show polymorphism between unrelated strains, has opened a new avenue for epidemiological study of tuberculosis. These include the insertion sequence, IS*6110*, the direct repeat (DR) sequence (Hermans *et al.* 1992), the polymorphic GC-rich repetitive sequences (Ross *et al.* 1992), the major polymorphic tandem repeats (Hermans *et al.* 1992) and an element containing repeats of

GTG nucleotide triplets (GTG)5 oligonucleotides (Wild *et al.* 1994). Although these elements have shown some value in epidemiological studies, they have not proved as discriminatory as IS*6110* restriction fragment length polymorphism (IS*6110*-RFLP) in strain differentiation. Three copies of the element (originally designated IS*6110*, IS*986* and IS*987*) have been isolated from different *M.tuberculosis* or *M.bovis* strains. These have been sequenced and found to differ in only a few nucleotides. IS*6110* is present in the other species of the MTB complex although not in more distantly related mycobacteria (Stanley *et al.* 1998). The DNA fingerprinting method based on (IS*6110*-RFLP) is the method most extensively used and is the current standard method for *M.tuberculosis* strain differentiation (van Embden *et al.* 1993). This method has been shown to be a reliable, stable and reproducible method for differentiating *M.tuberculosis* strains (Goyal *et al.* 1994). In principle, this method involves the extraction of chromosomal DNA from mycobacteria harvested from culture, the digestion of this DNA using the restriction enzyme *Pvu*II, which cleaves mycobacterial DNA at a point within the insertion sequence IS*6110*, and the separation of the resulting bands on an electrophoretic gel. Following electrophoresis the separated DNA fragments are denatured and blotted onto a nylon membrane, the position of the fragments is determined by hybridisation with a single-stranded 245bp DNA probe complimentary to a region of the insertion sequence downstream of the *Pvu*II cleavage site. A characteristic profile of bands is produced reflecting the number of IS*6110* copies and their position within the chromosome, each copy producing a separate band. These RFLP patterns can then be analysed by computer, so that related strains can be identified easily and potential outbreaks quickly recognised. However, the IS*6110* RFLP technique has its own limitations as it requires culturing, DNA extraction, southern hybridisation and results can be obtained only weeks or even months after the initial presentation of the patient at clinic. In addition, the *M.tuberculosis* strains from

various parts of the world differ in the proportion of strains with few copies of IS*6110*. For example, in Spain and in parts of Africa there are few strains with less than five copies of IS*6110* while such low copy strains make up a large proportion of the isolates from Southeast Asia. Discrimination with the RFLP technique is poor in these IS*6110* low copy strains of MTB; hence, the interpretation of clustering among these strains requires the use of a secondary or alternate typing method (Yuen *et al.* 1993).

DNA fingerprinting of *Mycobacterium tuberculosis* isolates has been used increasingly in epidemiological studies. Restriction fragment length polymorphism (RFLP) analysis based on the genetic marker insertion sequence IS*6110* is most frequently used to distinguish *M.tuberculosis* isolates. (van Embden *et al.* 1993). Insertion sequences (IS elements) are mobile DNA elements capable of transposition into diverse sites within the genome. It has been shown that the transposition rates of insertion elements are much higher than the rates of other heritable changes in the genome, such as nucleotide mutations, whereby, IS elements may transpose (jump) to a new locus by a replicative mechanism. (Stanley *et al.* 1996). They are parasitic DNA elements which are maintained in bacterial populations even under adverse natural selection because they can replicate and transpose independently of chromosome replication. Thus IS insertion patterns are subject to continuous variation during the history of a bacterial strain. The insertion sequence IS*6110* has been of particular interest because of its potential diagnostic and epidemiological applications. IS*6110* is a 1360-bp element with imperfect 28-bp inverted repeats at either end. Flanking the element are 3-bp repeats, probably resulting from target site duplication. Analysis of strains of *M.tuberculosis* and *M.bovis* possessing only one copy of IS*6110* have shown that it is inserted conservatively into one of an array of 36-bp directly repeated (DR) sequences with unique intervening sequences between the DRs. (Hermans *et al.* 1991). The most common insertion site is the direct repeat between spacer 24 and 25 (Benjamin *et al.*

2001). Investigations of a single strain with multiple copies of the IS*6110* show distinct insertion sites suggesting that random transposition events have occurred in this strain (Mendiola *et al.* 1992). The extent of random versus site-specific transposition remains unclear. Different sites within the genome of *M.tuberculosis* have been reported as hot spots for the integration of IS*6110* (Fomukong *et al.* 1998). These include the *ipl* locus (Fang and Forbes 1997), the DK1 locus and the *dnaA-dnaN* region (Kupepina *et al.* 1998). This suggests that the integration of IS*6110* is not a truly random event and the frequency of transposition is influenced by the site of insertion within the mycobacterial genome (McHugh *et al.* 1998). The identification of IS*6110* insertion hot spots may complicate the interpretation of IS*6110* RFLP data. For strains containing low copy numbers of IS*6110* integration hot spots may produce false clusters which must be subdivided by a second typing method independent of IS*6110* (Barlow *et al.* 2001). Different *M. tuberculosis* strains show great variability in the chromosomal copy number and location of IS*6110* against a stable genetic background. Consequently, restriction fragments carrying the element are highly pleomorphic. Appropriate interpretation of RFLP patterns may elucidate much about the epidemiological and evolutionary genetics. This application is based on the assumption that persons infected with strains of *M.tuberculosis* that have identical genotypes ("fingerprints") are epidemiologically linked, whereas those with different genotypes are unrelated. Studies from many countries report "clustering" of isolates, a cluster being defined as two or more isolates with fingerprints that are identical or at least similar. It is generally assumed that that the proportion of clustered isolates in a population reflects the amount of recent transmission of *M.tuberculosis.* (Glynn *et al.* 1999). This assumption relies on the assertions that the DNA genotype of a given strain remain constant and that changes in genotypes over time generate considerable genotypic diversity within a population. (Yeh *et al.* 1998). For correct interpretation of molecular typing in the

epidemiology of tuberculosis, it is essential to know the rate at which IS*6110* RFLP patterns change, as an estimated rate of change may or may not support the utility of IS*6110* typing for identifying tuberculosis cases associated with recent transmission. (de Boer *et al.* 1999). If IS*6110*-based genotypes change rapidly, it would obscure epidemiologic links and underestimate transmission, whereas, if genotypes change too slowly, then IS*6110*-based RFLP analysis would link cases that are only distantly related, overestimating transmission. The extent of the difference between two strains is therefore a function not only of the evolutionary or epidemiological distance between them but also the rate of transposition of IS*6110* (Dale *et al.* 1999). In 1994, Cave *et al.* examined sequential IS*6110* genotypes of *M.tuberculosis* in 18 patients and found that the genotypes were identical for 17 of them. In contrast, in a study of 49 patients in San Francisco, Yeh *et al.* (1998) found that 29% of serial isolates of patients whose cultures spanned greater than 90 days had changed RFLP patterns. The primary conclusion that can be drawn from the latter study is that DNA genotypes of *M.tuberculosis* change at a relatively rapid rate. This rate of change suggests that strains with identical genotypes are likely to be epidemiologically linked and supports the use of RFLP for tracking transmission. In contrast, Warren *et al.* (1996) have postulated that that IS*6110*-based RFLP analysis overestimates recent transmission by grouping distantly related strains with genotypes that have remained identical for large amounts of time. If few strains predominate over a long time-period, clustering cannot be assumed to represent recent transmission. The strain variation seen in a population will reflect the number of introductions of *M.tuberculosis* into the population (very many in Europe over many centuries, relatively few in parts of Africa) and the stability and relative fitness of the strains (Glynn *et al.* 1999). Fewer introductions of *M.tuberculosis* may explain the relative homogeneity of isolates seen in Ethiopia and Tunisia compared to the Netherlands. (Hermans *et al.* 1995) In contrast, the widespread occurrence of the

"Beijing family" of strains in Asia is likely to be due to stability of pattern and/or relative fitness of the strain. (van Soolingen *et al.* 1995). An alternative hypothesis is that certain RFLP types are endemic within a region during long periods and that remote transmission of such strains with a period of latent disease prior to reactivation could result in matching types among long-standing residents of the area (Bradden *et al.* 1997). In 1998, Bishai *et al.* tested this hypothesis in the city of Baltimore, USA by examining the demographic and behavioural traits of clustered patients who lacked epidemiological links and found that they most resembled patients with DNA fingerprint matches and firm epidemiologic links who almost certainly had had recently transmitted tuberculosis. This study suggests that remote transmission does not account for a significant proportion of clustering in Baltimore. There are still unresolved questions about the stability of strains. The rates of change of molecular markers have been loosely described in terms of 'molecular clocks" by analogy with those used to describe the molecular evolution of proteins. In addition, the discriminatory power of IS*6110* as an epidemiological marker greatly depends on the IS copy number per strain, and isolates harbouring low copy numbers of IS*6110* can be incorrectly grouped in the same cluster. It is widely recognised that strains with low number copies of IS*6110* (fewer than five) show little polymorphism and identical patterns are commonly found for strains from apparently unconnected patients (Dale *et al.* 1999).

Evaluation of persons exposed to newly reported cases of tuberculosis is undertaken with the dual goals of finding other new cases and also identifying persons with tuberculous infection who are candidates for preventative therapy. (Behr *et al.* 1998). In some studies, it has been possible to establish epidemiological links between clustered cases, and in these circumstances it is reasonable to accept that the cases are part of the same chain of transmission either directly or indirectly from common sources. In 1994, Small *et al.* investigated patients in the San Francisco area and found only 10% of

epidemiological links by RFLP typing were also found by conventional contact tracing investigations. Only 5 % of strains linked by RFLP were confirmed by contact tracing investigating patients in the Netherlands. (van Duetekom *et al.* 1997). An important theme from these studies is that despite diligent efforts, most case clusters are not recognised by the traditional tuberculosis-control strategies such as contact investigation. This relatively low reliability of conventional contact tracing may reflect the importance of casual contact in the transmission of *M.tuberculosis*. Population based studies using RFLP typing of *M.tuberculosis* have indicated an unexpectedly high degree of ongoing transmission in regions with low incidence suggesting that apparently casual contact can be sufficient to transmit infection (Yaganehdoost *et al.* 1999).

1.16 PCR based typing methods

Since the RFLP typing method requires cultured isolates to provide sufficient DNA, various investigators have concentrated on developing typing techniques based on PCR which potentially may be applied directly to clinical specimens. Three approaches have been used to detect polymorphisms: first the use of non-specific primers, second the use of primers based on the polymorphism of the IS*6110* flanking regions and thirdly the use of primers based on the direct repeat region. Most of the PCR-based techniques have been developed in order to amplify polymorphic DNA regions flanking IS*6110* by PCR with oligonucleotide primers to the end of IS*6110* (Ross and Dwyer, 1993). A simplified version of this IS*6110*-based PCR was developed using a single oligonucleotide primer complementary to the inverted repeat of IS*6110*. The 3' end of the primer is directed outwards from both sides of the element (Niemark *et al.* 1996). This DNA fingerprinting assay allows for a characteristic fingerprint from a single reaction tube and has been applied in the typing of cultures, in direct respiratory

specimens and in positive liquid BACTEC cultures (Otal *et al.* 1997). This method is of value in providing rapid information in outbreak situations and in ruling out contamination in a clinical laboratory, however, its limited discriminatory power limits its use in epidemiological investigations (Otal *et al.* 1997). Like the standard RFLP assay, the IS*6110*-based PCR tests are dependant on the presence of multiple copies of the insertion element for their increased discriminatory power and are unlikely to be of great benefit in resolving low copy IS*6110* strains of *M.tuberculosis*.

In 1991, Hermans *et al.* described a unique chromosomal locus in *M.bovis* BCG containing a large number of 36bp direct repeats (DR) which are interspersed by unique spacer sequences, varying in size from 35-41 bp. One DR plus the adjacent unique spacer sequence is termed a "direct variable repeat" (DVR) and is numbered according to the archetype DVRs in *M.bovis* BCG P3. (Groenen *et al.* 1993). When the DR regions of several isolates were compared, it was observed that the order of spacers was about the same in all isolates but deletions and insertions of DVRs occurred. The polymorphism in various isolates comprises the absence or presence of one or more DVRs. DRs are invariably and exclusively present in strains of the *M.tuberculosis* complex. In a study investigating over 1000 *M.tuberculosis* strains, Kamberbeek *et al.* (1997) found all strains to contain this multicopy target. Analysis of part of the DR cluster in various *M.tuberculosis* strains showed that this chromosomal region undergoes frequent genetic rearrangements with the number of DRs being variable in different isolates of *M.tuberculosis*. The polymorphism observed in this region is attributed to two types of genetic rearrangements which occur in this region. (Groenen *et al.*1993). One type consists of the variation of one or a few discrete, contiguous DRs plus spacer sequences. This variation is probably driven by homologous recombination between adjacent or distant DRs. The other type of polymorphism is probably driven

by transpositional events of the insertion sequence, IS*6110*, which is almost invariably present in the DR cluster of *M.tuberculosis* strains. Kamerbeek *et al.* (1997) developed a novel approach to exploit the polymorphism in the DR region by developing a PCR based method to amplify and label all DNA sequences in the DR region. The method referred to as spacer oligotyping or spoligotyping detects the presence or absence of spacer DNA of known sequence in an isolate in two steps. PCR is used to amplify the spacers between the DRs. The reverse primer used in the PCR is biotin labelled, so that all reverse strands synthesised are labelled. Individual spacers are then detected by hybridisation of the biotin-labelled PCR product containing spacer DNA to a membrane to which 37 oligonucleotides derived from spacers in *M.tuberculosis* H37Rv and six spacers from *M.bovis* BCG have been covalently linked. The resulting patterns look reminiscent of a bar code. (Kamerbeek *et al.* 1997). Compared to traditional IS*6110* fingerprinting, the degree of strain differentiation of *M.tuberculosis* by spoligotyping was lower for strains carrying five or more IS*6110* copies and higher for *M.tuberculosis* strains harbouring fewer than five IS*6110* copies. More recently, (Wilson *et al.* 1998) compared these three major strategies for molecular fingerprinting and found that using the standard spacer regions as probes, spoligotyping was not discriminatory enough to be used as a sole typing method but was of value when used in conjunction with other techniques. Similarly, IS*6110*-based PCR generates only one to three bands after agarose gel electrophoresis, and isolates cannot be clustered by identity of a single band only (Wilson *et al.* 1998). Although spoligotyping and IS*6110*-based PCR are simpler to perform than RFLP their reduced discriminatory power makes their utility limited to a primary screening method or alternatively used together in a dual typing strategy (Wilson *et al* 1998).

1.17 Variable numbers of Tandem DNA repeats

Genetic loci containing variable numbers of tandem repeats (VNTR loci) form the basis for human gene mapping and identification, forensic analysis and paternity testing. A multiplex PCR assay co-amplifying nine STR loci and Amelogenin now forms the standard method for DNA fingerprinting in humans. In the human genome, these represent between 3-6% of the interspersed DNA. Interspersed repetitive DNA has been found in nearly all genomes studied so far. The function of the interspersed repetitive sequences remains largely unknown. It has been proposed that they may play a role in chromosome structure and rearrangement, tandem duplications, differential translation of genes, mRNA stability or transcription termination (Supply *et al.* 1997). Although the roles of these elements are not yet well defined, they may be very useful for a variety of applications, including genetic fingerprinting of mycobacterial strains.

Frotthingham *et al.* detected eleven repeat loci by reviewing published literature and searching cosmid sequences from *M.tuberculosis* H37Rv (Frothingham *et al.* 1998), although it is estimated that there may be as many as 40-50 repetitive sequence loci in the MTB genome (Supply *et al.* 1997). These repeat loci consist of six exact tandem repeat (ETR) loci containing large DNA repeats with identical sequences in adjacent repeats and five major polymorphic tandem repeat (MPTR) loci containing 15-bp repeats with substantial sequence variation in adjacent copies. In this study Frotthingham *et al.* undertook a systematic analysis of the variability of tandem repeat loci by testing 48 strains of the *M.tuberculosis* complex including 23 substrains of *M.bovis* BCG. The VNTR loci of these strains were amplified by PCR and length polymorphisms were identified on agarose gels. Based on the size of the PCR products

the exact number of tandem repeats at each locus in each strain was determined. The results of this study showed that one of the five MPTR loci and all six ETR loci had length polymorphisms corresponding to insertions or deletions of tandem repeats. By assembling a profile based on the number of tandem repeats at each locus a distinct reproducible allele profile can be generated for each strain.

The combined analysis of the seven polymorphic VNTR loci differentiated *M.tuberculosis* complex strains with reasonable power; however, IS*6110* fingerprinting appears to be more discriminatory than VNTR typing in strains with high IS*6110* copy numbers. IS*6110* RFLP was less discriminative than VNTR analysis for the *M.bovis* BCG substrains, all of which had only one or two copies of IS*6110* (Frothingham *et al.* 1998). The stability of VNTR allele profiles in *M.tuberculosis* has not been determined to date. *M.tuberculosis* H37 was isolated in 1905, and the two variants H37Rv (virulent) and H37Ra (avirulent) were identified in the 1930s. These variants showed different IS*6110*-RFLP profiles but still share the same VNTR profile (Bifani *et al.* 2000). The estimated minimum time for a single step transition at a VNTR locus is at least 65 years, which is slower than the predicted rate of change for an IS*6110*-RFLP pattern (Yeh *et al.* 1998). It has been reported that IS*6110*-RFLP fingerprints in documented transmission chains allow a higher degree of stability than that observed for serial patient-derived isolates. Since VNTR allele profiles are stable over long periods of time, it is likely that VNTR profiles of serial patient-derived and epidemiologically related isolates will remain constant over time, unlike IS*6110*-RFLP fingerprints which are reported to vary by one or two bands (Barlow *et al.* 2001, Savine *et al.* 2002).

There are considerable advantages for the development of a PCR based method for the typing of mycobacterial strains independent of the IS*6110*. Firstly, since MTB is a very slow growing organism significant time can be saved if the culture step required for RFLP typing could be reduced or eliminated as is possible with a PCR based method. This is of particular importance in the investigation of outbreaks of MTB especially if multi-drug resistance is a factor. Secondly, PCR based methods are not technically demanding and known controls prepared from heat killed typed strains can be incorporated into each PCR reaction. Thirdly, interlaboratory comparisons of different strains isolated from different countries could be compared on a numerical database, since the PCR method of typing would yield a numerical allele profile number unlike comparisons with IS*6110* RFLP which require complex computer programs for pattern comparisons. Finally, since PCR requires only small amounts of DNA there is the potential for performing VNTR typing directly on clinical samples or for performing VNTR typing on archive material for both epidemiological and evolutionary investigations. As more VNTR loci are detected, they can be applied to further discriminate clusters of identical VNTR allele profiles and, equally importantly, could be used to differentiate between primary, reactivated and new infections with MTB. Since VNTR typing may be a useful epidemiological alternative to RFLP fingerprinting, it is proposed that the five ETR loci (A, B, C, D and E) which have shown the highest degree of polymorphism among the MTB strains should be utilised as the targets for preliminary VNTR typing (Frothingham *et al.* 1998).

1.18 Mycobacterial Interspersed Repetitive Units (MIRUs)

In recent years, additional polymorphic regions have been identified as targets to discriminate unrelated strains of *M.tuberculosis*. Supply *et al.* (1997) reported the identification of minisatellite-like structure in the *Mycobacterium tuberculosis* genome.

These structures are typically of 51-77 bp repetitive sequences called mycobacterial interspersed repetitive units (MIRUs) and have been identified in 41 locations throughout the chromosome of H37Rv (Supply *et al.* 2000). The MIRUs have been classified into three major types. Type 1 sequences contain roughly 77bp. Type 2 and type 3 MIRUs are characterised by a gap of 24 bp and 15 bp corresponding to the 3' and 5' portions of type 1 sequences respectively (Supply et al. 2000). PCR and sequence analysis of 31 different strains of *M. tuberculosis* from distinct geographical regions have shown 12 of the MIRU loci have shown variations in the numbers of repeats. These correspond to MIRU 2,4,10,16,20,23,24,26,,27,31,39,40. Loci 4 and 31 had previously been described as VNTR D and VNTR E respectively (Frothingham *et al.* 1998). MIRU differ from ETR in that they contain repeats with minor sequence variations between them, which consist of a few substitutions, insertions or deletions. The repeat variants are distributed non-randomly in the arrays. ETRs are identical repeats clustered in blocks of contiguous units (Supply *et al.* 2000). In 2001, *Supply et al.* investigated the use of a 12-locus MIRU-VNTR (Mazars *et al.* 2000) approach for high resolution genotyping of *M.tuberculosis*. Analysis of a blinded set of 90 strains from 38 countries (Kremer *et al.* 1999) MIRU-VNTR typing was found to be 100% reproducible, sensitive and specific for *M.tuberculosis* complex isolates, a performance that has not been achieved by any other resolution typing method, including IS*6110* RFLP (Supply *et al.* 2001). In that study, the discrimination power of MIRU-VNTR typing was reported close to that of IS*6110* RFLP (78 compared to 84 patterns respectively). More recently, Cowan *et al.* (2002) studied a set of 180 *M.tuberculosis* and *M.bovis* isolates with low copy numbers of IS*6110* by MIRU-VNTR. The results were compared with IS*6110* RFLP and spoligotyping analysis; MIRU-VNTR proved most discriminatory (Cowan *et al.* 2002). Other VNTR loci, in addition to ETR A-E, have since been identified at Queens University Belfast (Roring *et al.* 2002). It appears

that a combination of of VNTR , MIRU and other polymorphic loci will greatly improve the discriminatory power of DNA repeat polymorphisms.

1.19 Aims of the Study

The aims of the study were to evaluate molecular strategies which could be applied to patient samples, archive material and mycobacterial cultures to expedite tuberculosis diagnosis in patients. Direct nucleic acid detection methods and sample extraction methods are assessed to determine their utility as clinical diagnostic tools and to develop an understanding of their limitations. The study aimed to understand when patient samples should be tested in order to reduce the time for diagnosis of tuberculosis and thereby reduce further transmission of disease. Treating patients with multidrug resistant strains of MTB is difficult and necessitates diagnostic tools to identify such patients. This study aimed to assess the utility of these diagnostic tools.

DNA typing methods were investigated in this study to determine the diversity of strains in distinct geographical regions, to monitor transmission, and to understand what host, environmental or organism factors are at play. The study aimed to determine the "best practice" approach for first line typing of strains of MTB and assess the utility of investigating other VNTR or MIRU targets. In addition, a combination of ETR loci and MIRU loci showing the greatest polymorphisms were applied to investigate their discriminatory power amongst the common clades.

Chapter 2.0 Materials And Methods

2.1 Overview of Specimen processing

Specimens were held at 4°C until processed by standard laboratory procedures. Respiratory specimens were processed within 24 hours by a standard N-acetyl-L-cysteine sodium hydroxide method and were centrifuged at 3,000 x g for 20 minutes. The final pellet was resuspended to 1.5 - 2.0 ml in sterile saline. Fluids from normally sterile sites and tissues were not decontaminated. Tissues were homogenized in a tissue grinder. All procedures were carried out in accordance with established CDC safety recommendations (Kent *et al.* 1985).

2.2 Inoculation of Media

For each specimen, two Lowenstein-Jensen slopes were inoculated with 0.2ml of specimen and a smear was prepared for auramine-phenol staining. Flurochrome staining was performed by standard procedures and all positive smears were counterstained by the ZN stain. Slope cultures were examined weekly for 8 weeks. 0.5 ml of the processed sediment was inoculated into a MB/BacT (Organon Teknika, Durham, N.C.) process bottle containing Middlebrook 7H9 broth, Tween 80 (0.4% w/v), Glycerol (5% w/v), Amaranth (0.002% w/v) and an antibiotic supplement containing Amphotericin B (0.018% w/v), Azlocillin (0.0034% w/v), Nalidixic acid (0.04% w/v), Polymixin B (10,000 units) and Trimethoprim (0.0105% w/v). Liquid cultures were monitored continuously in the MB/BacT 2400 cabinet (Organon Teknika) for up to 8 weeks. The time for culture positivity was recorded in days for MB/Bact bottles and in weeks for solid culture. All positive isolates with positive ZN stain and characteristic morphological appearance were confirmed as *Mycobacterium sp.* isolates.

2.3 Auramine Phenol stain

Smears were heat fixed for 20 min. on a heating block. The slide was flooded with auramine-phenol auramine $10g.1^{-1}$; phenol $30g.1^{-1}$; ethanol 95%v/v) for 10 minutes. Slides were washed with tap water and decolorised with 0.5%v/v HCl in 70%v/v ethanol for 5 min. Slides were counterstained with $0.5gl^{-1}$ potassium permanganate ($KMnO_4$) in distilled water. Smears were examined under fluorescent microscopy using 25x and 40x objectives for apple green fluorescing bacilli.

2.4 Ziehl Neelson (ZN) Stain

0.3g basic fuchsin in 10ml of 95% ethanol,combined with 90ml of a 5% aqueous phenol soln., was filtered and flooded onto a heat fixed smear for 10min. Slides were rinsed with tap water and decolorised with 3%v/v HCl in 95% ethanol. A counterstain of 0. 3 gl^{-1} methylene blue in distilled water was applied for 30 seconds. Smears were examined under light microscopy with an oil immersion 100x objective.

2.5 Amplicor PCR

2.5.1 Sample Preparation

100µl of decontaminated and concentrated sediment was added to 500µl of sputum wash solution and pelleted by centrifugation. Samples were incubated at 60°C for 45 minutes. Following incubation, 100µl of neutralization reagent was added to each sample preparation tube.

2.5.2 Amplification

50µl of prepared specimens and controls were added to each amplification tube containing 50µl of working master mix containing amperase. PCR tubes were loaded into a GeneAmp 2400 thermal cycler (Applied Biosystems). Thermal cycler was programmed as follows:

2min @ 50°C:

2 cycles @ 98°C 20 sec, 62°C 20 sec, 72°C 45 sec;

35 cycles @ 94°C 20 sec, 62°C 20 sec, 72°C 45 sec;

2.5.3 Detection

Denaturation reagent (100µl) was added to each PCR tube and incubated at room temperature for 10 min. Hybridisation buffer (100µl) and denatured amplicons (25µl) were added to each well of a a microwell tray containing the bound amplicon specific oligonucleotide probe and incubated at 37°C for 90 min. Microwell plates were washed 5 times in a microplate washer. A colour reaction was developed in the dark at room temperature following the addition of conjugate and substrate. Optical densitity (OD) was determined at A450 and an OD >0.50 was reported as a positive result.

2.6 Abbott LCx MTB Assay

2.6.1 Sample Preparation Processed Sediments

500µl of decontaminated and concentrated sediment, was added to a LCx-MTB sample preparation tube and pelleted by centrifugation at 1500g for 10 minutes. The supernatant was aspirated and the pellet washed by the the addition of 1000µl of LCx tuberculosis resuspension buffer. The sample tubes were centrifuged for 10 min at 1500g, supernatant was aspirated and a further 500µl of LCx tuberculosis resuspension

buffer was added to each tube. Sample tubes were incubated at 95°C for 20 minutes in a LCx covered dry bath (Abbott LCX Probe System). Sample tubes were allowed to equilibrate to room temperature before loading onto the LCx lysor (Abbott LCx Probe System) for sonication.

2.6.2 Sample Preparation for positive MB/BacT cultures

500μl of positive MB/BacT culture was aspirated using a tuberculin syringe and added to a LCx-MTB sample preparation tube. Samples were then assayed as processed sediments in section 2.6.1.

2.6.3 Amplification

100μl of prepared samples and controls were added to each amplification tube and were loaded into a GeneAmp 2400 thermal cycler (Applied Biosystems). Thermal cycler was programmed as follows:

37 cycles @ 94°C 1sec, 64°C 1 sec, 69°C 40 sec; Hold: 25°C

2.6.4 Detection

Following amplification the amplification tubes were loaded into the LCx analyser (Abbott LCx Probe system) with the LCx detection reagents. By relating the LCx MTB Assay results for the specimen to the cutoff value (CO), the presence or absence of *M. tuberculosis* complex DNA was determined. The CO rate was the mean rate of the positive calibrator controls multiplied by 0.30. The S/CO value was determined by calculating the ratio of the sample rate (S) to the CO. A specimen with an S/CO reading equal to or greater than 1 was considered positive, and a specimen with an S/CO reading less than 1 was negative for the presence of *M. tuberculosis* complex DNA

2.7 Identification by DNA Probe

A loopful of mycobacteria was added to 100µl of Accuprobe (Gen-Probe Accuprobe system, San Deigo Ca.) lysis reagent in a transfer tube. The suspension was sonicated and boiled for 10 minutes to complete cell lysis. 100µl of lysate was added to an Accuprobe tube containing a single stranded DNA probe with a chemiluminescent label complementary to the ribosomal RNA of MTB. Hybridization was achieved by incubating the tube at 60°C in a dry heat block for 10 minutes. Detection was achieved by measuring photometric light units using a luminometer (Gen-Probe).

2.8 Dedicated Laboratory Areas

2.8.1 Sample Preparation

Sample preparation was completed in a dedicated area (Area 1) of the laboratory. Area 1 was used for processing samples (specimens, specimen processing controls, PCR and LCx tuberculosis positive and negative controls), and the addition of processed samples to LCx tuberculosis amplification vials. A Class II biological safety cabinet (BH2000 series, Clyde-Apac) and aerosol contained centrifuge (Megafuge 2.0, Heraeus Instruments) were used until the specimens had been heat-inactivated. All reagents and equipment used in area 1 (such as pipettors, centrifuges, Abbott LCx covered dry bath and LCx lysor) remained in this dedicated area at all times. Area 1 items were never used when working with the amplification product and the amplification product was never brought into this area. Specimens, specimen processing controls, and activated negative and positive controls were stored separately from amplification vials. Pipetting was performed using aerosol barrier tips that were used only once. Amplification and detection of amplification product was completed in the second

dedicated area (Area2). All equipment in Area 2 was dedicated for specific use in that area.

2.8.2 Aerosol Containment

To reduce the risk of DNA contamination due to aerosols formed during pipetting. Pipettors with aerosol barrier tips were used for all pipetting except in the removal of supernatant during specimen preparation where a single-use, plastic disposable pipette was used.

2.8.3 Inactivation of Amplification products.

To reduce the risk of amplification product contamination, at the end of the LCx MTB assay amplification product is automatically inactivated by using a two-reagent, chemical inactivation system. Both reagents (a chelating metal complex and an oxidizing reagent) are delivered into the LCx reaction cells by the LCx analyser after the amplification product has been detected. The ensuing reaction results in the near complete destruction of any nucleic acid present. This effectively reduces the risk of contamination of the laboratory by amplification products.

2.9 LCx-MTB assay for detection of *Mycobacterium tuberculosis* DNA in paraffin embedded tissues

2.9.1 Sample description

One hundred paraffin embedded lymph node biopsies were selected for this study from Histopathology archives at The King Khalid National Guard Hospital.. Each specimen was fixed with formalin, embedded in paraffin, sectioned and stained with hematoxylin-eosin for examination of histopathological changes in addition to staining using the Ziehl-Neelsen procedure for the identification of AFB's.

2.9.2 Sample Population

The biopsy samples were grouped according to histological evaluation.

Group 1 comprised 17 biopsies showing evidence of granulomatous lymphadenitis and demonstrating acid-fast bacilli.

Group 2 comprised 41 biopsies showing evidence of granulomatous lymphadenopathy with negative AFB in sections.

Group 3 comprised 42 lymph node biopsy specimens which had histological diagnoses of reactive hyperplasia, malignancy and normal morphology were used as the control group of specimens.

2.9.3 Amplification and Detection

50μl aliquots of the DNA suspension and 50μl of LCx tuberculosis resuspension buffer containing 27 mmol/ml $MgCl_2$ were added to an LCx MTB amplification vial and tested by the LCR *Mycobacterium tuberculosis* assay (Abbott LC*x* Probe system, Abbott Diagnostics, Chicago, Ill.) according to the manufacturer's directions. Amplification was carried out using a 37-cycle program in a Perkin-Elmer thermal cycler (Abbott LC*x* Probe system). Detection was achieved by loading amplification vials directly into a carousel for automated detection in the LCx analyser (Abbott LC*x* Probe system). Two negative and two positive reagent controls and one saline negative and positive (4000- 6000 cfu/ml MTB) processing control were included in each batch of samples assayed. The cut-off value for the assay was determined at 0.3 times the mean positive reagent control rate. The sample results were calculated as the ratio of the sample rate (S) to the cut-off rate (CO) i.e. (S/CO). Samples in the ratio range S/CO 0.3-1.0 were considered equivocal while ratios greater than 1.0 were considered positive for MTB.

2.9.4 Second round amplification

All equivocal and positive samples were retested by a dual amplification method to confirm the presence of DNA. 50µl of DNA suspension was added to an LCx Amplification vial and loaded in the Thermal cycler as described above. Prior to the detection step, the amplified vials were transferred to a DNA free laboratory and 10µl of the first round amplification vial was transferred to a new LCx amplification vial. In addition, 90µl of LCx resuspension buffer was added to each second round amplification vial to achieve a final volume of 200µl. Detection was achieved by loading both first and second round amplification vials directly into a carousel for automated detection in the LCx analyser (Abbott LC*x* Probe system).

2.10 DNA Extraction from Paraffin Tissue

2.10.1 Phenol Chloroform Method

Paraffin blocks were cooled on ice for 30 minutes prior to sectioning. Disposable microtome blades were used for each sample and microtome, forceps and surrounding bench cleaned with xylene to prevent cross contamination. Five 10µm sections from each tissue block were transferred to a sterile 1.5ml microfuge tube. Sections were deparaffinised with xylene and wash with absolute ethanol. Following centrifugation at 13,000 rpm for 10 minutes the supernatant was removed to discard and the resulting pellets air-dried. 250µl of a digestion buffer containing 1% sodium dodecyl sulphate (Sigma Aldrich), 50mM Tris-HCl (pH8.0), 1mg/ml Proteinase K (Sigma Aldrich) was added to each tube and incubated in a dry heat bath at 56°C for 18-24 hrs. The nucleic acid was extracted by the standard phenol / chloroform method and precipated with ethanol and 3M sodium acetate (pH 5.0) overnight at -20°C. The DNA is pelleted by

centrifugation and resuspended in 250µl of LCx Resuspension buffer. (Abbott LCx Probe system)

2.10.2 Nucleospin column Method (Nucleospin Tissue Kit, Machery Nagel)

Three 12-micron sections were cut each paraffin block with a microtome blade. To prevent carryover tissue contamination of the samples, the microtome blade was cleaned with xylene and 100% ethanol after sectioning each sample. Sections were transferred to a microcentrifuge tube deparaffinised with xylene, washed twice with absolute ethanol and pelleted by centrifugation. The resulting pellet was resuspended in 180µl Lysis Buffer and 25µl of proteinase K stock solution and incubated at 56 °C until all tissue fragments have been digested. Samples were vortexed each 15-30 minutes for 2-4 hours to aid digestion process. 200µl of a binding buffer was added to each tube and incubated at 70°C for 10 minutes. 210µl ethanol was added to each sample tube and transferred to a Nucleospin column unit. Following washing the nucleospin matrix the DNA was eluted in to a clean tube using 100µl of elution buffer.

2.11 DNA Extraction from Processed Specimen Sediments

2.11.1 Nucleospin column Method (Nucleospin Tissue Kit, Machery Nagel)

500µl of processed sediments were transferred to a microfuge tube containing 500µl sterile water and pelleted by centrifugation at 13,000 rpm for 10 minutes and the supernatant discarded. The pellets were resuspended in 180µl of lysis buffer and incubated at 95°C for 10 minutes in a dry heat bath. 25µl of proteinase K stock solution was added and incubated at 56 °C for approximately 15 minutes. 200µl of a binding buffer was added to each tube and incubated at 70°C for 10 minutes. 210µl ethanol was added to each sample tube and transferred to a Nucleospin column unit. Following

washing the nucleospin matrix the DNA was eluted in to a clean tube using 100μl of elution buffer.

2.12 DNA Extraction from Bacterial Cells

2.12.1 Nucleospin column Method (Nucleospin Tissue Kit, Machery Nagel)

A loopful of freshly grown bacterial cells (3 to 5 weeks) were transferred to a microfuge tube containing 500μl sterile water and pelleted by centrifugation at 13,000 rpm for 10 minutes and the supernatant discarded. The pellets were resuspended in 180μl of lysis buffer and incubated at 95°C for 10 minutes in a dry heat bath. 25μl of proteinase K stock solution was added and incubated at 56 °C for approximately 15 minutes. 200μl of a binding buffer was added to each tube and incubated at 70°C for 10 minutes. 210μl ethanol was added to each sample tube and transferred to a Nucleospin column unit. Following washing the nucleospin matrix the DNA was eluted in to a clean tube using 100μl of elution buffer.

2.12.2 Instagene Matrix (BioRad Laboratories)

A colony of *M.tuberculosis* was scraped from a Lownwstein Jensen slope using a disposable loop and resuspend in 1.0ml of sterile water in a microfuge tube. Sample was centrifuged at 10,000 rpm for 10 minutes and the supernatant discarded. 200μl of InstaGene matrix (BioRad Laboratories) was added to the pellet and incubated at 56°C for 30minutes. The suspension were heat treated at 95°C for 20 minutes before a final centrifugation at 10,000 rpm for 3 minutes.

2.13 Quantitation of DNA

Control MTB DNA was prepared by extracting DNA from a loopful of bacterial cells from a four-week-old culture of H37Rv growing on Lowenstein-Jensen medium. DNA was extracted using the mycobacterial cell protocol 2.9.2 Nucleospin tissue kit. The extracted H37Rv DNA was diluted 1:1000 with sterile injection water and quantified by spectrophotometry using a UV Mini 1240 Spectrophotometer (Shimadzu Corporation, Kyoto, Japan). 100µl of a 1:1000 dilution of H37Rv was loaded into a cuvette in the spectrophotometer and using A_{260} quantitated the DNA.

2.14 Gel electrophoresis

2g of DNA grade agarose (Sigma Aldrich) was dissolved in 100ml 1xTAE buffer (Sigma Aldrich) by heating for 90 seconds in a microwave on high. Upon cooling approximately 4µl of 10mg/ml ethidium bromide (BioRad Laboratories) solution was added and the molten agarose was poued into gel casting tray with a gel comb (BioRad Laboratories).

10µl of each contol or PCR product were mixed with 2µl of 6x gel loading dye (BioRad Laboratories) and loaded into wells on the gel. 100bp ladders (MBI Fermentas) were interspersed every 6 wells to size the amplified products. Gel and casting tray were loaded into a Wide Mini Sub Cell GT (Biorad Laboratories) electrophoresis tank and covered with 1x TAE buffer (Sigma Aldrich). Electrophoresis was performed used a BioRad 300 Power Pak for 60 minutes at 100 volts.

Following electrophoresis products were visualised using UV light on a transilluminator (UVTec Limited, Cambridge, England) and photograhed using a (Polaroid Gel Cam) camera using (Polaroid 667) film.

2.15 RT PCR TaqMan assay for the direct detection of MTB-DNA in clinical specimens and paraffin embedded tissues

2.15.1 DNA Extraction

DNA extractions from paraffin tissue, processed sediments and bacterial cells were performed using specific protocols with the Nucleospin tissue kit. (sections 2.10.2, 2.11.1 and 2.12.1 respectively)

2.15.2 Assay Design

Version 1.5 of Primer Express software (Applied Biosystems) was used to design a TaqMan probe and complementary primers to target a region of the insertion sequence *IS6110.*

2.15.3 TaqMan Probe

The TaqMan probe was designed using the following guidelines as recommended by Applied Biosystems:

(12) Avoid probes with a guanine residue at the 5' end of the probe, as a guanine residue adjacent to the reporter dye will quench the reporter fluorescence.

(12) Probes were selected with an estimated melting temperature (Tm) of 68-70°C.

(12) Taqman probe was selected as short as possible not exceeding 30 nucleotides.

(12) Runs of identical nucleotides were avoided. This especially applied to guanine where runs of four or more were avoided.

The internal oligonucleotide probe was labelled with the fluorescent dyes 5-carboxyfluroscein (FAM) on the 5' end and N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA) on the 3' end.

5' -(6-FAM) CCACGCCGCCAACTACGGTGTTTAC

(Tamra)(phosphate)-3' (Genset Pacific, Lismore, Australia). Manufactured as a 210μM concentration

This Taqman probe corresponds to the region of *IS6110* from base 578 to base 602 (bases are numbered as for the sequence with the Genbank accession no. X17348). The probe was 25bp in length with a Tm of 69°C and a 60% GC.

2.15.4 Assay Primers

After selecting the probe for the assay, primers were selected using the following guidelines.

(a) Runs of identical nucleotides were avoided. This especially applied to guanine where runs of four or more were avoided.

(b) Primers were selected with an estimated melting temperature (Tm) of 58-60°C.

(c) Keep the guanine + cytosine content within 20-80%.

(d) Ensure the last five nucleotides at the 3" end contain no more than two guanine + cytosine residues.

(e) Place the forward and reverse primers as close as possible to the probe without overlapping it.

Forward primer: 5' AACTCAAGGAGCACATCAGCC 3' (Genset Pacific, Lismore, Australia).

Manufactured as a 100μM concentration

Reverse primer: 5' CCACACTTTGCGGGCAC 3' (Genset Pacific, Lismore, Australia).

Manufactured as a 100μM concentration

The forward primer corresponds to the region of *IS6110* from base 553 to base 573 (bases are numbered as for the sequence with the Genbank accession no. X17348). The forward primer is 21bp in length with a Tm of 58°C and a 52% GC content.

The reverse primer corresponds to the region of *IS6110* from base 620 to base 604 (bases are numbered as for the sequence with the Genbank accession no. X17348). The reverse primer is 17bp in length with a Tm of 58°C and a 65% GC content.

2.15.5 PCR Reagents

Brilliant Quantitative PCR core reagent kit (Stratagene) containing:

SureStart Taq DNA polymerase 5U/μl

Core PCR Buffer 10x

Magnesium chloride 50mM

dNTP mix 20mM (5mM each of dATP, dTTP, dGTP, and dCTP)

Reference dye (dilute 1:50 with Core PCR Buffer 1x)

Optical tubes and caps (Applied Biosystems)

2.15.6 Optimization of Primers and Probe

Primers were diluted 1:10 with sterile injection water to obtain a working concentration of 10μM. Probe was supplied as a 210μM concentration was diluted 1:84 to obtain a working concentration of 2.5μM. A primer concentration matrix (125, 250, 500 and 1000nmol of each primer) and a probe concentration titration (50, 125, 250 and 500 nmol) were performed to determine the concentrations which provided the smallest Ct value and highest fluorescence for a given target concentration. Final optimised

concentrations for the primers were 500nmol and probe 125nmol in each 50µl PCR reaction.

2.15.7 PCR Reaction mix

Core PCR Buffer 10x	N x 5.0µl
Magnesium chloride 50mM	N x 5.0µl
IS6110 (fwd) primer (500nmol)	N x 2.5µl
IS6110 (rev) primer (500nmol)	N x 2.5µl
IS6110 probe (125nmol)	N x 2.5µl
dNTP mix	N x 2.0µl
Reference dye	N x 0.75µl
SureStart Taq DNA polymerase 5U/µl	N x 0.5µl
DNA template	29.25 µl

2.15.8 Amplification and Detection ABI 7700 (Applied Biosystems)

Amplification occurs using a PCR protocol of 50°C 2 minutes; 95°C 10 minutes 40 Cycles: 95°C 15 seconds, 60°C 1 minutes. During PCR, a multiplexer directs an argon ion laser through fiber optic cables that terminate above each position on the 96-well plate. The light passes through the MicroAmp Optical caps and, for adefault time of 25 milliseconds, the laser excites the fluorescent dyes present in each well. The fibre optic cables then collect the fluorescence emission between 500nm and 660 nm from each of the wells. A system of lenses, filters, and a dichroic mirror focus the fluortescence emission into a spectrograph. The sequence detection application collects the fluorescent signals and applies data analysis algorithms.

2.15.9 Determination of the Limit of Sensitivity of the Assay

H37Rv control DNA was prepared by extracting DNA from a loopful of bacterial cells from a four-week-old culture of H37Rv growing on Lowenstein-Jensen medium. DNA was extracted using the mycobacterial cell protocol of the Nucleospin tissue kit. (section 2.12.1) The extracted H37Rv DNA was diluted 1:1000 with sterile injection water and quantified by spectrophotometry (section 2.12). Once quantified the DNA was subsequently diluted from 10^3 to 10^{11} and assayed in the Taqman assay to determine the Ct for each dilution. The limit of detection of the assay was determined as the as the highest dilution demonstrating a Ct less than 40.0.

2.16 Comparative study: TaqMan Assay versus LCx-MTB assay

2.16.1 Sample population

Five groups of three types of samples (paraffin embedded tissues, processed sediments of clinical samples and mycobacterial cells were analysed:

Group 1: 35 AFB smear positive processed sediments, culture positive for MTB

Group 2: 23 AFB smear negative processed sediments, culture positive for MTB

Group 3: 18 processed sediments, culture positive for NTM

Group 4: 50 AFB smear negative processed sediments, culture negative for mycobacteria.

Group 5: 41 paraffin blocks histologically consistent with mycobacterial infection.

2.17 Protein antigen B PCR for DNA sequencing

2.17.1 DNA Extraction

DNA extractions from bacterial cells were performed using specific protocols with the Nucleospin tissue kit. (sections 2.12.1)

2.17.2 Primer Design

Version 1.5 of Primer Express software (Applied Biosystems) was used to design an additional reverse primers pab4 outside the target a region of the previously described primers MT1 and MT2 (Sjobring *et al.* 1990).

The forward primer MT1 corresponds to the region of pab gene from base 233 to base 252 (bases are numbered as for the sequence with the Genbank accession no. M30046). This primer is 20bp in length with a Tm of 70°C and a 67% GC content.

The reverse primer MT2 corresponds to the region of pab gene from base 649 to base 629 (bases are numbered as for the sequence with the Genbank accession no. M30046). This primer is 21bp in length with a Tm of 66°C and a 67% GC content.

The reverse primer pab4 corresponds to the region of pab gene from base 1476 to base 1458(bases are numbered as for the sequence with the Genbank accession no. M30046). This primer is 19bp in length with a Tm of 65°C and a 65% GC content

2.17.3 Primer sequences(Genset Pacific, Lismore, Australia).

Primer name	Primer sequence
MT1	5'-ACGGCAGGCTGGTGGAGGA-3'
MT2	5'-GATCTGCGGGTCGTCCCAGGT-3'
Pab4	5'-GAGCCTGATCGCACCCATCG

Manufactured as a 100μM concentration

2.17.4 PCR Amplification Reagents (Sigma Aldrich)

MT1 (forward) 100μmol		Nx0.5μl
MT2 (reverse) 100μmol	OR	Nx0.5μl
Pab4 (reverse) 100μmol		
10 x Gene Amp PCR buffer II		Nx5.0μl
$MgCl_2$ (2.0mM)		Nx4.0μl
dNTP mixture 200μmol		Nx4.0μl
JumpStart Taq (2.5units/μl)		Nx0.5μl
Sterile injection water		Nx29.5μl
Template		Nx5.0μl
Total Volume		50μl

Amplification using Perkin Elmer 460 thermalcycler (Applied Biosystems)

12min@94°C; 40cycles 94°C 2min, 60°C 1min, 72°C 2min

2.17.5 Detection

PCR products were detected by 2% agarose gel electrophoresis stained with ethidium bromide as in 2.14.

2.18 DNA sequencing

Double stranded products were purified by nucleospin extract column (Macherey-Nagel, Duren, Germany), the sequencing reaction was performed with the ABI Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) using 3.2pmol of sequencing primers:

MT1 5'-CCACCGAGCGGTTCGCCTGA-3'

pab4 5'-GAGCCTGATCGCACCCATC-3'.

The sequencing products were purified by sodium acetate-ethanol precipitation and the reaction loaded onto the ABI prism 310 Genetic Analyser in accordance with the manufacturers' instructions (Applied Biosystems). The DNA sequencing reaction was performed at Queensland Health Scientific Services, Coopers Plains, Australia. The results of the sequence reaction were analysed using Sequencher Version 3.0 (Gene Codes Corporation, Ann Arbor, MI). Analysis was performed by comparing sequences obtained against Gen Bank Accession M30046.

2.19 Line Probe Assay

2.19.1 Sample population

Twenty rifampicin resistant isolates of *M.tuberculosis* isolated between 1997-1998 at the King Khalid National Guard Hospital in Jeddah, Saudi Arabia and 8 rifampicin susceptible strains including H37Rv were tested by the line probe assay. One water blank was tested as negative controls for the assay

2.19.2 DNA extraction

DNA was extracted from bacterial cells using the Instagene Matrix (BioRad Laboratories) method in 2.12.2.

2.19.3 Primer Sequences (Innogenetics Rif Tb Kit, Ghent, Belgium)

Primer name	Primer sequence
IP1	5'-GGTCGGCATGTCGCGGATGG-3'
IP2	5'-GCACGTCGCGGACCTCCAGC-3'

2.19.4 PCR reaction mixture

Amplification buffer	n x 10µl
Rif Tb primer mix	n x 10µl
25mmol $MgCl_2$	n x 10µl
Jumpstart Taq 2.5Units/µl (Sigma Aldrich)	n x 1 unit
Sterile injection water	n x 18µl
Total Volume	Nx 48µl

Amplification using Perkin Elmer 460 thermalcycler (Applied Biosystems)

min, 72°C 10 min.

2.19.5 Hybridization

10µl of each amplified biotinylated PCR product was added to 10µl of denaturation solution in a test trough, was mixed by gentle pipetting and allowed to stand at room temperature for 5 minutes. 1ml of hybridization solution prewarmed to 62°C was added to each trough. An InnoLipa strip (Innogenetics) was completely submerged in each trough and incubated in a covered twin shaking waterbath (Robbins Scientific, Sunnyvale Ca.) rotating at approximately 80rpm for 30 minutes. Following hybridization the Innolipa strips were washed with a stringent wash solution and incubated with 1ml of wash solution in the covered twin shaking water bath at 62°C for 10 minutes.

2.19.6 Detection

1ml of conjugate (streptavidin labelled with alkaline phosphatase) was added to each trough containing an Innolipa strip and placed on an orbital shaker (Labline Instruments Inc. Melrose Park, Ill.) rotating at approximately 160 rpm for 30 minutes at

room temperature. Each strip was washed twice with a rinse solution and incubated with 1ml of substrate buffer on the orbital shaker for 30 minutes at room temperature. Color development was stopped by washing strips with 1ml of distilled water while agitating troughs on the orbital shaker for 5 minutes. Strips were allowed to air dry and the patterns of purple/brown lines corresponding to the hybridization of the immobilised probes and biotinylated PCR products were recorded.

2.20 VNTR typing

2.20.1 Source of isolates of M.tuberculosis

85 isolates from patients at the King Khalid National Guard Hospital in Jeddah, Saudi Arabia between 1997-1998.

201 isolates from patients at the Queensland Mycobacterium Reference Laboratory, Brisbane, Australia between 1999-2001 were tested.

2.20.2 DNA Extraction

DNA was extracted from bacterial cells using the Instagene Matrix (BioRad Laboratories) method in 2.12.2.

2.20.3 Primer Sequences (Genset Oligos, Lismore, Australia)

Frothingham *et al.* 1998

Locus name	GenBank Accession No	Location in H37Rv	Primer sequence
ETR A	S77045	743-1163	5'-AAA TCG GTC CCA TCA CCT TCT TAT-3'
			5'-CGA AGC CTG GGG TGC CCG CGA TTT-3'
ETR-B	Z70283	7929-8220	5'-GCG AAC ACC AGG ACA GCA TCA TG-3'

			5'-GGC ATG CCG GTG ATC GAG TGG -3'
ETR-C	Z77162	11647-11922	5'-GTG AGT CGC TGC AGA ACC TGC AG-3'
			5'-GGC GTC TTG ACC TCC ACG AGT G-3'
ETR-D	Z77162	14948-15257	5'-CAG GTC ACA ACG AGA GGA GAG C-3'
			5'-GCG GAT CGG CCA GCG ACT CCT C-3'
ETR-E	Z74024	10409-10632	5'-CTT CGG CGT CGA AGA GAG CCT-3'
			5'-CGG AAC GCT GGT CAC CAC CTA AG-3'

2.20.4 Additional VNTR Primer Sequences (Genset Oligos, Lismore, Australia) Mazars *et al* 2001.

Locus name	GenBank Accession No	Location in H37Rv	Primer sequence
ETR F	Z74697	17693-18168	5'-CTC GGT GAT GGT CCG GCC GGT CAC-3'
			5'-GGA AGT GCT CGA CAA CGC CAT GCC-3'
MIRU 10	AL022004	56176-56818	5'-GTT CTTGAC CAA CTGCAG TCG TCC-3'
			5'-GCC ACC TTG GTG ATC AGC TAC CT -3'
MIRU 16	AL021184	4382-5073	5'-TCG GTG ATC GGG TCC AGT CCA AGT A-3'
			5'-CCC GTC GTG CAG CCC TGG TAC-3'
MIRU 40	Z84395	17494-17901	5'-GGG TTG CTG GAT GAC AAC GTG T-3'
			5'-GGG TGA TCT CGG CGA AAT CAG ATA-3'

2.20.5 PCR Amplification Reagents (Sigma Aldrich)

Primer 1 (forward) 100μmol	Nx0.5μl
Primer 2 (reverse) 100μmol	Nx0.5μl
10 x Gene Amp PCR buffer II	Nx2.5μl
25mmol $MgCl_2$	Nx2.0μl
4% DMSO (except ETR-A)	Nx1.0μl
dNTP mixture 200μmol	Nx2.0μl
Sterile injection water	Nx12μl(Nx13μl for ETR-A)
JumpStart Taq (2.5units/μl)	Nx 0.625 units
Total Volume	Nx 22.5μl

Amplification using Perkin Elmer 460 thermalcycler (Applied Biosystems)

12min@95°C; 35cycles 94°C 30sec, 60°C 1min, 72°C 2min

2.20.6 Detection

PCR products were detected by 2% agarose gel electrophoresis stained with ethidium bromide as in 2.14. Interspersed controls of known VNTR alleles were interspersed as controls.

2.20.7 Lineage assignment using UPGMA

Analysis was undertaken using the program S.T.A.R.T. (Sequence type analysis and recombinational tests) available for download from http://outbreak.ceid.ox.ac.uk/software.htm.

Lineage assignment was performed using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) (Sneath *et al.* 1973). The method used a sequential clustering algorithm, in which local homology between operational taxonomic units OTUs were identified in order of similarity, and the tree was built in a stepwise manner. The two OTUs most similar to each other were first determined and then these were treated as a new single 'composite' OTU. Subsequently from among the new group of OTUs (composite and simple), the pair with the highest similarity was identified and clustered. This continued until only two OTUs are left. The algorithm assumes that the two most closely related OTUs are more similar to each other than they are to any other. The algorithm used here utilises a distance matrix constructed from allelic profile data only. Allele sequences are not used, so each allele number difference is treated identically.

Chapter 3. MB/BacT isolation of mycobacteria and MTB culture confirmation using the LCX MTB assay.

3.1 Patient population and clinical specimens.

This study was conducted in the Microbiology Division of the King Khalid National Guard Hospital (KKNGH), Jeddah, Saudi Arabia. KKNGH is a 350-bed general teaching hospital which provides primary and secondary health care to Saudi Arabian National Guardsmen and their dependants with access to all Saudis. The Microbiology Division is a referral centre for specimens from four primary health care clinics in both Jeddah and Taif which investigates approximately 1500 specimens annually for the presence of mycobacteria. Two-thousand nine-hundred consecutive specimens, both pulmonary and extrapulmonary, from patients being investigated for tuberculosis were investigated over a two-year period from January 1996 and January 1998.

3.2 Results

Mean days until detection of a positive culture by MB/Bact The results of the comparison between detection of MTB on Lowenstein-Jensen medium and the MB/BacT are shown in Table 1. From 460 MB/Bact culture bottles culture positive for *Mycobacterium sp.*, 250 samples were culture positive for MTB. Overall, the mean time for detection for these isolates was 18.5 days with 71.2% of isolates being detected in less than 21 days. In the group of patients whose specimens were AFB smear positive on initial examination (58.4%), the time for detection was 16.0 days and 82.2% of these cultures could be detected in less than 21 days. In the AFB smear negative specimens (41.6%), the mean time for detection was 22.1 days with only 50.9% of these cultures being detected in less than 21 days.

The 210 MB/Bact bottles culture positive for NTM were detected on average in 13.7 days with 91.4% being identified in less than 21 days. AFB smear was positive in 36 (17.1%) of the 210 NTM positive cultures and the mean time for detection was 12.0 days with 94.4% detected within 21 days. In the AFB smear negative NTM culture positive group the mean time of detection of these 174 (82.9%) cultures was 14.0 days with 90.8% of these cultures being detected in less than 21 days.

For all the 460 positive cultures for *Mycobacterium* sp. the mean time for detection was 16.3 days with 80.4% of cultures being detected in less than 21 days.

The number and type of *Mycobacterium sp.* isolated from both the pulmonary and extrapulmonary specimens investigated are shown in Table 2. The results show that the sensitivity and specificity of the LCx-MTB for *M. tuberculosis* in detecting and differentiating MTB from NTM in positive MB/Bact cultures was excellent. All 250 MB/Bact bottles culture positive for MTB were detected by the LCX-MTB and all 210 NTM positive MB/Bact cultures gave negative results.

Table 1. Mean days until detection of a positive culture by MB/BacT

	No	Mean time of detection (days)	Minimum time of detection (days)	Maximum time of detection (days)	Standard deviation	Number and percentage of isolates detected under 21 days
MTB Culture positive AFB smear positive	146	16.0	5	46	6.4	82.2% (120)
MTB Culture positive AFB smear negative	104	22.1	11	48	6.9	50.9% (58).
All MTB Culture positive	**250**	**18.5**	**5**	**48**	**7.3**	**71.2% 178**
NTM Culture positive AFB smear positive	36	12.0	4	24	4.5	94.4% 34
NTM Culture positive AFB smear negative	174	14.0	3	56	6.49	90.8% 158
All NTM Culture positive	**210**	**13.7**	**3**	**56**	**6.24**	**91.4% 192**
All Mycobacteria (Including MTB and NTM) AFB smear positive	182	13.2	3	21	4.2	84.6% (154)
All Mycobacteria (Including MTB and NTM) AFB smear negative	278	17.1	4	56	7.7	77.8% (216)
All Mycobacteria (Including MTB and NTM)	**460**	**16.3**	**3**	**56**	**7.2**	**80.4% (370)**

Table 2. *Mycobacterium* sp. isolated according to site with LCx-MTB result from MB/BacT bottle.

Mycobacterial isolate n=460	Sputum/ BAL*	Sterile fluid	Tissue	Abscess	Urine	Percentage of LCR positive results from MB/BacT bottle
M.tuberculosis *n=250*	202 (80.8%)	12 (4.8%)	24 (9.6%)	12 (4.8%)	-	100
M.chelonae *n=9*	9	-	-	-	-	0
M.fortuitum *n=19*	18	-	-	-	1	0
M.gordonae *n=4*	4	-	-	-	-	0
M.kansasii *n=2*	2	-	-	-	-	0
M.malmonese *n=1*	1	-	-	-	-	0
M.simiae *n=41*	38	-	1	-	2	0
M.terrae *n=7*	7	-	-	-	-	0
Mycobacterium avium complex *n=111*	107	-	-	-	4	0
Environmental scotochromogens n=16	16	-	-	-	-	0

* BAL Bronchoalveolar lavage

3.3 Discussion

Following the epidemic of tuberculosis in the United States in the early 1990s the Centres for Disease Control instituted recommendations for diagnostic laboratories designed to improve detection of MTB infection and facilitate prompt isolation and treatment of infected persons. (CDC 1997, Tenover *et al.* 1993). These recommendations stated that results from concentrated acid-fast smears should be reported within 24 hours of collection, culture in liquid medium should be performed in order to detect growth of MTB within 10 to 14 days and that susceptibility tests be performed within 15-30 days following collection. In a commentary by Styrt *et al.* (1997) these recommendations were later relaxed to identification within 21 days.

The aim of the study was to assess the performance and implementation of the MB/BacT liquid culture system to endeavour to achieve the CDC guidelines for rapid turnaround times for mycobacterial detection and identification. The results showed that of the 2900 consecutive samples processed, 460 (15.9%) were positive for mycobacteria and 250 (8.6%) were positive for *M.tuberculosis*. Using the MB/BacT culture 71.2% of MTB isolates were detected within the recommended 21 days with a mean time to detection of 18.5 days. AFB smear positive MTB culture positive samples were grown more rapidly with 82.2% of isolates being detected within 21 days. In 1997, Rohner *et al.* compared the MB/BacT with the BACTEC 460 observed isolation of *M.tuberculosis* with a mean time to detection of 17.5days and 14.3 days respectively.

The mean time to detection of MTB using the MB/BacT was longer in this study and the work of Rohner *et al.* than that observed by Benjamin *et al.* (1998) comparing the MB/BacT system with the BACTEC 460 system. In that study a mean time to detection of 13.7 days was observed using the MB/BacT. A major reason for the longer time to detection observed in this study is that consecutive samples were evaluated and

therefore some samples would have come from patients already on anti-tuberculosis therapy. The CDC guidelines were intended to apply to the first diagnostic specimen from each patient. Subsequent specimens are collected to monitor treatment and often have delayed growth.

The BACTEC 460 TB system has been the benchmark for sensitivity and speed of mycobacterial detection reducing the average detection time of both AFB smear positive and AFB smear negative samples using conventional solid media by nearly 50% (Morgan *et al.* 1983, Roberts *et al.* 1983). The limitations of the BACTEC 460 system have lead to the development of a continuously monitored, nonradiometric system. Despite the 2-3 day increase in the time to detection of *M.tuberculosis* growth in the MB/BacT versus the BACTEC 460 an alternative nonradiometric system was essential in Saudi Arabia as disposal of radioactive waste is restricted (Benjamin *et al.*1998, Rohner et al. 1997). This increase in time to detection is about the maximum increase which is clinically acceptable (Benjamin *et al.*1998). However, specimens positive in the MB/BacT are identified immediately since they are monitored continuously, those positive in the BACTEC 460 would be identified only when the specimens are tested according to the laboratory protocol.

LCx-MTB assay was used to directly identify *M.tuberculosis* from MB/BacT bottle which signalled positive. Although the use of the LCx-MTB assay directly on fluid from positive MB/BacT bottle was not specifically endorsed by the manufacturer, when applied to an aliquot of a positive MB/BacT bottle, a sensitivity and specificity of 100% was obtained for the detection and differentiation of MTB from NTM. Tortoli *et al.* (1998) applied the LCx-MTB assay to positive BACTEC 12B as soon as a positive signal was detected and were able to detect 106 of 108 MTB isolates (Tortoli *et al.* 1998). Other approaches to rapid identification of liquid mycobacterial cultures have used commercial DNA probes to identify the common organisms such as MTB and

MAC. These organisms accounted for 54.3% and 24.1% of isolates in this study respectively. Benjamin *et al.* (1998) reported that although BACTEC 12B cultures may become positive more quickly than MB/BacT cultures DNA probes typically will be negative if tested on a BACTEC vial as soon as it becomes positive. In contrast, MB/BacT cultures can be reliably identified by specific DNA probe on the day the bottle is flagged positive by the instrument. Other approaches have used PCR (Forbes *et al.* 1994), direct DNA sequencing (Rogall *et al.* 1990) or restriction enzyme analysis (Telenti *et al.* 1993c) Although all these methods can provide useful diagnostic information, the key point is which ever method is employed must be able to reliably identify and differentiate MTB from NTM.

In 1996, in Jeddah, Saudi Arabia molecular biology facilities were in a developmental stage and the application of LCx-MTB assay by this means was a rapid cost effective way of screening NTM from MTB. As approx 50% isolates were NTM, it was imperative to distinguish the infectious patients with tuberculosis from the non-infectious patients with NTM. At that time, the implementation of a reliable means of liquid culture confirmation, which could be performed easily within a working day, meant hospital isolation rooms would be properly used for tuberculosis patients. Antituberculous drugs would be administered appropriately and patients with NTM disease would not have to unnecessarily endure any social stigma with respect to infection with TB.

The most effective way to combat the spread of organisms and disease is through rapid diagnosis, thorough contact tracing and prompt initiation of appropriate therapy There is no simple diagnostic solution for the identification *M. tuberculosis* in clinical specimens and combinations of various technologies are required in order to achieve the CDC prevention recommendations (Tenover *et al.* 1993). In the following chapter the

utility of molecular methods to expedite diagnosis of tuberculosis by detecting specific MTB DNA is clinical specimens is investigated.

Chapter 4: Comparison of amplicor PCR and LCx-MT B assay for the detection of DNA in clinical specimens

4.1 Amplicor PCR

The Amplicor *Mycobacterium tuberculosis* Test is based on three major processes. Polymerase Chain Reaction (PCR) target amplification, hybridisation of the amplified product to a specific nucleic acid probe, and detection of the amplified product by colour formation (Saiki *et al.* 1985, Mullis *et al.* 1987).

The development of a PCR assay involves identifying the particular region of the target DNA to be amplified, and synthesising two short biotinylated oligonucleotide primers that are complementary to the regions flanking the target sequence. In the reaction, these biotinylated primers will bind to the target region and then, catalysed by polymerase, extend in the 5' to 3' direction utilising excess deoxynucleotide triphosphates (dNTPs) in the reaction mixture thereby creating a biotinylated, complementary DNA sequence termed an **amplicon.** Oligonucleotide probes, specific for the amplicons, are bound to a microwell plate for capture for the amplicons. The detection molecule used in the assay is an avidin-horseradish peroxidase (Av-HRP) conjugate that binds to the plate-captured biotinylated amplicons. The substrates used for colour formation in the assay are H_20_2 and tetramethylbenzidine (TMB).

4.1.1 PCR Amplification Reaction

Genus-specific primers located in a highly conserved region of the 16S ribosomal RNA (rRNA) gene of *Mycobacterium* are used to amplify a 584 base-pair sequence (Stahl *et al.* 1990, Boddinghaus *et al.* 1990). The DNA-containing sample and reagent mixture are heated to separate the double-stranded helix and expose the primer target sequences. As the mixture cools, the biotinylated primers anneal to their targets. The thermostable

DNA polymerase in the presence of deoxynucleoside triphosphates (dNTPs), including deoxyadenosine, deoxyguanosine, deoxycytidine and deoxyuridine (in place of thymidine) triphosphates, then extends the annealed primers along the target templates to produce amplicons. This process is repeated for a number of cycles, each cycle effectively doubling the amount of target DNA. Because the cycles are exponential in their amplification power, 20 cycles can theoretically result in a 1 million-fold amplification, 30 cycles up to 1 billion-fold, all from a single copy of DNA. For this test, the required number of cycles has been determined to be 37.

4.1.2 Hybridisation Reaction

After the PCR amplification process, the amplicons are chemically denatured to form single strands that are added to a microwell plate (MWP) containing a bound, amplicon specific, oligonucleotide probe. This probe, specific for the *M. tuberculosis* complex, was selected from the hypervariable region of the 16S rRNA gene (Stahl *et al.* 1990, Boddinghaus *et al.* 1990). The biotin-labelled amplicons will then bind (hybridise) to the amplicon specific probe and thus be "captured" onto the plate. This specific hybridisation event increased the overall specificity of the test.

4.1.3 Detection Reaction

After unbound material is removed from the MWP by washing, an Av-HRP conjugate is added to the plate. The avidin binds to the biotin-labelled amplicons captured by the plate-bound probe. After unbound conjugate has been washed off, the bound Av-HRP conjugate is reacted with peroxide and TMB to form a colour complex. The reaction is stopped by the addition of weak acid, the optical density is measured in an automated microwell plate reader and the results are compared to the supplied cut-off value. Positive and negative controls were supplied with the test kit for reference.

4.1.4 Specificity of Reaction

Selective amplification of target DNA from the clinical specimen in the Amplicor *Mycobacterium tuberculosis* Test is achieved by the use of AmpErase. AmpErase contains the enzyme Uracil N-Glycosylase (UNG) (Longo *et al.* 1990), which recognises and catalyses the destruction of deoxyuridine-containing DNA, but not thymidine-containing DNA. Deoxyuridine is not present in microbial DNA, but is always present in amplicons due to the use of deoxyuridine triphosphate (in place of thymidine triphosphate) as one of the dNTPs in the Master Mix reagent; thus, only amplicons will contain deoxyuridine. The presence of deoxyuridine in amplicons renders contaminating amplicons susceptible to destruction by AmpErase prior to the amplification of the target DNA. AmpErase catalyses the cleavage of an oligonucleotide at deoxyuridine residues by opening the deoxyribose chain at the 1 position. The opened chain, when heated in the first thermal cycling step (at the alkaline pH of Master Mix), causes the amplicon's DNA chain to break at the position of the deoxyuridine, thereby rendering the DNA non-amplifiable. The UNG enzyme, which is inactive at temperatures above 55 degrees Celsius (i.e. throughout the thermal cycling steps), is then itself denatured by the addition of the Denaturation Solution immediately after the amplification step is completed, thereby preventing it from destroying any "true" amplified products of the test. The UNG enzyme in the Amplicor *Mycobacterium tuberculosis* Test has been demonstrated to inactivate up to 10 copies of deoxyuridine-containing *M. tuberculosis* amplified DNA.

4.2 Ligase Chain Reaction

4.2.1 Specimen Preparation

Specimen preparation is the first step of the LCx *Mycobacterium tuberculosis* assay. Firstly, potential inhibitors are removed by centrifuging and washing specimens followed by heat inactivating the mycobacteria. Mycobacterial DNA is then released from the *M.tuberculosis* complex by mechanical lysis making it accessible to the enzymes and other components of the LCR reaction.

4.2.2 Amplification

In the DNA amplification step, the prepared sample is added to the LCx MTB reaction mixture consisting of four oligonucleotide probes, thermostable ligase and polymerase, and individual nucleotides in buffer. The four oligonucleotide probes are designed in pairs that hybridise to complimentary single-stranded *M.tuberculosis* target sequences exposed in the sample preparation. During thermal cycling, the temperature is raised above the melting point of the hybridized amplification product causing it to dissociate from the original target sequence. Lowering the temperature allows more of the oligonucleotide probes to hybridise to the targets now available. The temperature continues to be cycled in this manner until sufficient numbers of target amplification product have accumulated in the LCx MTB reaction mixture. The number of targets increases exponentially with each thermal cycle. Thirty-seven thermal cycles are sufficient to achieve up to a billion-fold amplification in the number of target sequences which are then detected by the Microparticle enzyme Immunoassay. (MEIA)

4.2.3 Detection

The two pairs of oligonucleotide probes in the LCx MTB assay are labelled with immunoreactive groups called haptens. Each individual probe has either a capture hapten (recognised by an antibody attached to the MEIA microparticles) or a detection

hapten (recognised by an antibody conjugated to alkaline phosphatase). The probes are labelled such that, when they are joined during the LCR reaction, the amplification product has the capture hapten at one end and the detection hapten at the other. In the LCx analyser, an aliquot of the amplification product is automatically transferred to the reaction well. Here the microparticles coated with anti-capture hapten (rabbit) bind the amplification product as well as any unligated probes carrying the capture hapten. The reaction mixture is then automatically transferred to a glass fibre matrix to which the microparticle complexes bind irreversibly. A wash step removes the unligated probes having only the detection hapten. The bound microparticle complexes are then incubated with anti-detection hapten (rabbit): alkaline phosphatase conjugate which binds to the detection haptens. This antibody conjugate binds only to amplification product. The antibody conjugate can then be detected by the addition of the substrate, 4-methylumbelliferyl phosphate, which is de-phosphorylated by alkaline phosphatase to produce 4-methylumbelliferone, fluorescence of which is measured by the MEIA optical assembly.

4.2.4 Prevention of DNA contamination

Amplification reactions such as LCx MTB are sensitive to accidental introduction of amplification product from previous amplification reactions as well as DNA from processed specimens. False positive results could occur if either the clinical specimens or the LCx MTB reagents used in the amplification reaction step become contaminated by accidental introduction of even a few molecules of amplification product. Measures to prevent DNA contamination in the laboratory include physically separating activities involved in performing LCx MTB, chemically inactivating amplification and complying to good laboratory practice.

4.3 Results

Table 3 summarises the results of the 326 patient samples investigated in this study. In Group 1, of 74 specimens culture positive for MTB, 71 specimens were LCx-MTB positive. The three samples MTB culture positive and LCx-MTB negative were AFB smear negative. Two of these samples gave equivocal S/CO ratios (0.89 and 0.87) and the third sample (S/CO ratio 0.029) was culture positive in liquid media only after 30 days. Sixty-five of seventy-four specimens culture positive for MTB were positive by the Amplicor PCR method (87.8%). Nine specimens (from five patients), 7 of which were AFB smear negative were Amplicor PCR negative. In one of these patients, a previous specimen was Amplicor PCR positive. AFB smear was positive in 46 (62%) of the 74 samples culture positive for MTB. In Group 2, the 68 samples from 46 patients culture positive for NTM were all negative by both LCx-MTB and Amplicor PCR. In group 3, comprising 66 patient samples who had positive AFB smear and no growth on solid media alone, were 64 samples from 28 patients in which *Mycobacterium avium* complex (MAC) had been previously isolated and 2 samples from patients undergoing anti-tuberculous therapy with previous positive cultures for MTB. LCx-MTB was positive only in the two samples with prior isolation of MTB, Amplicor PCR was positive in one of these samples. In group 4, of 118 patient samples AFB smear negative and culture negative, two samples were positive by LCx-MTB with one of these samples also Amplicor PCR positive. This sample was from a known tuberculosis patient on anti-tuberculosis therapy. One sample had a S/CO ratio 1.13 and was from a patient without clinical evidence of tuberculosis and was considered a false positive for LCx-MTB. The sensitivity, specificity, accuracy and predictive values of the molecular methods and AFB smear result in relation to culture are shown in Table 4. The NTM specimens (i.e. Group 2 specimens) were excluded in

the statistical analysis and comparisons were done on the remaining 258 specimens. In smear positive samples, LCx-MTB compared with culture showed a sensitivity of 100% and a specificity of 97.0%. Amplicor PCR versus culture had a sensitivity of 95.6% and specificity of 98.5% in smear positive specimens. In the 28 AFB smear negative culture positive specimens, LCx-MTB and Amplicor PCR had a sensitivity of 89.3% and 75% respectively and a specificity of 98.3% and 99.2% respectively. In all samples, LCx-MTB had the highest sensitivity of 95.9% while AFB smear had the lowest sensitivity of 62.2%. The sensitivity for Amplicor PCR was 87.8%. The specificity of Amplicor PCR was highest (98.9%), that of LCx-MTB second (97.8%) and that of AFB smear lowest at 64.1%. LCx-MTB had the highest accuracy (i.e. the proportion of all specimens that were correctly identified) of 97.3%. The accuracy of Amplicor PCR was 95.7% while that of AFB smear was 63.6 %. The proportion of test positives that were true positives (i.e. positive predictive value) was highest for Amplicor PCR (97%), and was lowest for AFB smear (41.1%). That of LCx-MTB was 94.7%. Similarly, the proportion of test negatives that were true negatives (i.e., negative predictive value) was also highest for Amplicor PCR (98.9%) and was lowest for AFB smear (80.8%). The negative predictive value for LCx-MTB was 98.4%. The results of the statistical significant tests applying the McNemar test to compare each diagnostic test against the others are shown in Table 5. The sensitivity of LCx-MTB was significantly greater than that of Amplicor PCR ($0.025 < P < 0.05$), but there was no significant difference in their specificity ($P > 0.10$). Similarly, there was no difference in their accuracy ($P > 0.05$). However, both LCx-MTB and Amplicor PCR were significantly superior to AFB smear in terms of sensitivity, specificity and accuracy ($P < 0.001$)

Table 3. Comparison of LCx-MTB and Amplicor PCR with mycobacterial culture and microscopy

	Mycobacteria culture and AFB smear result	**No of Samples**	**No. LCx-MTB Positive**	**No. Amplicor PCR Positive**
Group 1	MTB (AFB smear positive)	46	46	44
	MTB (AFB smear negative)	28	25	21
	All MTB positive cultures	**74**	**71**	**65**
Group 2	*M. avium intracellulare*	40	0	0
	scotochromogen	14	0	0
	M.fortuitum	3	0	0
	M.chelonae	5	0	0
	M.terrae	5	0	0
	M.kansasii	1	0	0
	ALL NTM	**68**	**0**	**0**
Group 3	No growth (on solid media alone) (AFB smear positive)	**66**	**2**	**1**
Group 4	No growth (on solid or liquid media) (AFB smear negative)	**118**	**2**	**1**
	Total	**326**	**75**	**67**

Table 4. Sensitivity, specificity, accuracy and predictive values of LCx-MTB, Amplicor PCR and AFB smear in the diagnosis of *Mycobacterium tuberculosis*.

		LCx-MTB (%)	**PCR (%)**	**AFB smear (%)**
Sensitivity	AFB +	100	95.6	-
	AFB -	89.3	75.0	-
	All specimens	**95.9**	**87.8**	**62.2**
Specificity	AFB +	97.0	98.5	-
	AFB -	98.3	99.2	-
	All specimens	**97.8**	**98.9**	**64.1**
Accuracy	AFB +	98.2	97.0	-
	AFB -	96.6	94.5	-
	All specimens	**97.3**	**95.7**	**63.6**
Positive Predictive	AFB +	95.8	97.8	
	AFB -	92.6	95.5	
Value	**All specimens**	**94.7**	**97.0**	**41.1**
Negative Predictive	AFB +	100	97.0	-
	AFB -	97.6	94.4	-
Value	**All specimens**	**98.4**	**98.9**	**80.8**

Table 5. Statistical test* of significance of sensitivity and specificity of LCx-MTB, Amplicor PCR and AFB smear.

		PCR		AFB Smear	
		χ^2	P-value	χ^2	P-value
(a) Sensitivity					
	LCx-MTB	4.1	P < 0.05	23.0	P < 0.001
	PCR	-	-	15.7	P < 0.01
(b) Specificity					
	LCx-MTB	0.5	P > 0.10 (n.s)	56.4	P < 0.001
	PCR	-	-	60.1	P < 0.001
(c) Accuracy					
	LCx-MTB	2.25	P > 0.05 (n.s)	85.0	P < 0.001
	PCR			60.1	P < 0.001

4.3 Discussion

Saudi Arabia still has a high incidence of tuberculosis despite the great decline from 135 per 100,000 in 1980 to 18.6 per 100,000 in 1990. Highest figures have been reported from the Jeddah health region with an incidence rate of 63.4 per 100,000 (Milaat *et al.* 1994). These figures represent an incidence 2-4 times greater than that of developed countries (Al-Kassimi 1994). In this patient population, patients with positive AFB smears and pulmonary symptoms with or without radiological findings are frequently started on anti-tuberculosis therapy pending the results of culture which may take up to 12 weeks. It has been observed in the Southern region of Saudi Arabia that up to 50% of healthy individuals harbour NTM in their mouths and throats (Nsanze *et al.* 1993). The high incidence of NTM complicates the diagnosis of tuberculosis and frequently results in the unnecessary administration of anti-tuberculosis drugs. Therefore tests which can provide a high specificity for MTB in smear positive specimens will prove cost effective in reducing use of anti-tuberculosis drugs, contact investigations and isolation facilities. The sample groups 2 and 3, comprising predominantly_samples with either positive NTM culture or prior history of NTM isolation demonstrate the high specificity of both molecular methods as no false positives results were observed. These samples highlight our experience in NTM culture and supports the report of Hoffner (1988), illustrating that liquid culture supports the growth of mycobacteria far more than Lowenstein Jensen alone, particularly in the isolation of *Mycobacterium avium.*

In this study AFB smear was positive in 62% (46/75) of culture positive specimens for MTB. The sensitivity and specificity of the LCx-MTB assay in the smear positive samples were 100% and 97.0% respectively. In smear positive specimens, Amplicor PCR showed a sensitivity of 95.6% and specificity of 98.5%. The high sensitivity and

specificity of both the Amplicor PCR and LCx-MTB assays in smear positive specimens suggest that both can detect and differentiate MTB from NTM in a single respiratory specimen, hence greatly reducing the time of diagnosis of pulmonary tuberculosis. In contrast, the sensitivity of both assays was diminished in smear negative specimens. The sensitivity of the LCx-MTB and Amplicor PCR assays was reduced to 89.3% and 75.0% respectively in smear negative specimens, however the specificity of both assays remained high at 98.3% and 99.2% respectively. The LCx-MTB and Amplicor PCR positive but culture negative results observed in specimens from two patients, who had previously positive cultures and had anti-tuberculosis therapy support the findings of other investigators that DNA amplification techniques can detect non-viable organisms (D'amato *et al.* 1995, Kolk *et al.* 1992, Carpentier *et al.* 1995). Both Amplicor PCR and LCx-MTB will differentiate equally and accurately between MTB and NTM which increases the reliability of these techniques.

Six extrapulmonary pus samples from patients with abscesses were investigated as part of this study. In three of these samples, MTB was isolated and both PCR and LCx-MTB assays were positive. A sample of tissue from one of these patients was culture positive and negative for both Amplicor PCR and LCx-MTB. Previous studies on discrepant Amplicor PCR and culture results have attributed false negative results to the presence of inhibitors in both pulmonary and extrapulmonary specimens, sample variation, and reduced numbers of organisms (Beavis *et al.* 1995, D'Amato *et al.* 1995, Moore and Curry 1995, Tonjum *et al.* 1996). Moore and Curry in a retrospective and prospective study of 1009 specimens determined that only 7% of false negative PCR specimens were attributed to the presence of inhibitors. The three false negative LCx-MTB specimens were from AFB smear negative samples from three patients and were also negative by Amplicor PCR. Two of these samples which gave equivocal LCx-MTB results, were from patients that had prior positive MTB-LCx specimens. One

culture positive sample but LCx-MTB negative was only culture positive for MTB in liquid media after 30 days of incubation. This suggests that both false negative LCx-MTB and Amplicor PCR results may have been due to reduced numbers of organisms or sample variation. In patients with negative smear and radiological evidence of disease it would be advantageous to test multiple (at least two or three) (D'Amato *et al.* 1995, Tonjum *et al.* 1996) respiratory samples to reduce the risk of a false negative result due to the low numbers of organisms particularly in severely immunocompromised AIDS population where AFB smear has low sensitivity (Bradley *et al.* 1996). The ability of Amplicor PCR to detect MTB is reduced when the number of organisms in the sample is scanty, particularly in smear negative paucibacillary samples (Cartuyvels *et al.* 1996). In a study by Schirm *et al.*, comparing in-house PCR and Amplicor PCR with culture a sensitivity of 90% was obtained with in-house PCR as opposed to 60% with Amplicor PCR in smear negative specimens. The authors correlated the higher sensitivity with the in-house PCR in smear negative samples to the increased sample volume added to the amplification reaction in the in-house PCR (almost 5 times that of Amplicor PCR) (Shirm *et al.* 1995). The larger specimen volume (0.5ml) used in the LCx-MTB assay as recommended by the manufacturer probably contributes to the greater sensitivity observed. However, this may allow for higher concentrations of inhibitors in the reaction as shown by An *et al.* (1995). The use of two wash and centrifugation steps in the sample preparation for the LCx-MTB assay compared with the single sample wash in the Amplicor PCR assay is probably of major importance in the removal of inhibitors. Cartuyvels *et al.* (1996) suggested that the sensitivity of the Amplicor PCR assay may be enhanced by increasing the specimen volume. An increase in the sample volume e.g. from 0.1 ml to 0.5 ml would however necessitate a second wash step in the sample preparation of Amplicor PCR to aid inhibitor removal. Sample preparation in both methods is not technically demanding,

although specimens must be initially processed in a Class 1 Biological Safety Cabinet with strict adherence to the prescribed methodology. In this study, and as previously reported, Amplicor PCR is easily performed and results can be reported within one 8-hour shift (Beavis *et al.* 1995, D'Amato *et al.* 1995, Moore and Curry 1995). Similarly, in the MTB-LCx assay, a batch of 48 specimens including controls can be prepared within 2½ hours. The amplification and detection steps are automated and require approximately one hour for each step. It is therefore possible to incorporate this assay into the normal laboratory workflow and report results within one 8-hour shift.

In this study, the MTB-LCx assay showed greater sensitivity than the Amplicor PCR assay ($P < 0.05$). The high specificity of both assays is of great benefit in differentiating MTB from NTM. In practical terms, the use of DNA amplification techniques allows for rapid and specific diagnosis of MTB, which in turn eliminates unnecessary administration of anti-tuberculosis therapy and isolation measures for NTM patients.

Chapter 5: Detection of *Mycobacterium tuberculosis* DNA in paraffin embedded tissues using the LCx-MTB assay

5.1 Results

The results from 100 paraffin embedded lymph nodes investigated in by PCR are shown in Table 6. Group 1 comprised 17 lymph nodes histologically compatible with tuberculosis in which AFB could be demonstrated and 4/17 (23.5%) samples yielded positive LCx MTB results. Group 2 comprised of 41 lymph nodes with a histological diagnosis of tuberculosis in the absence of AFB, LCx-MTB detected MTB DNA in 5/41(12.2%). In the Group 3 samples which were 42 lymph nodes with histological diagnoses other than tuberculosis, all gave negative results by LCx-MTB. The results of LCx-MTB are given according to the site of lymph node biopsy in Table 7. Of the nine samples in which MTB DNA could be detected by LCx-MTB, six were cervical lymph nodes. The other three lymph nodes positive for LCx-MTB were one axillary node, one abdominal lymph node and a thyroid lymph node. The S/CO rates for both the first round and second round amplifications of samples either initially reactive or equivocal are given in Table 8. All 9 samples yielded high positive S/CO ratios after second round amplification. All control samples remained negative after both first and second round amplifications.

Table 6. First round LCx-MTB amplification results from embedded biopsy material according to histological diagnosis and AFB smear result.

Lymph Nodes	Histological Diagnosis	LCR Positive	LCR Negative	**Total**
Group 1	Tuberculosis AFB smear positive	4	13	**17**
Group 2	Tuberculosis AFB smear negative	5	36	**41**
Group 3	Histologically not tuberculosis	0	42	**42**
				100

Table 7. AFB smear and LCx-MTB results of histologically diagnosed tuberculous lymph nodes according to the site of biopsy.

Lymph Node Biopsy Site	AFB positive	AFB negative	LCR Positive
Cervical (n=33)	13	20	6
Axilla (n=6)	2	4	1
Submandibular (n=5)	0	5	0
Subclavical (n=5)	2	3	0
Abdominal (n=7)	0	7	1
Thyroid (n=1)	0	1	1
Paraaortic (n=1)	0	1	0
Total (n=58)	17	41	9

Table 8. Sample rate to Cutoff value (S/Co) ratios for both first and second round amplification with LCx-MTB for paraffin tissues extracts initially reactive (S/Co>1.0) or equivocal (S/Co >0.3).

Sample No	Lymph node site	AFB smear	Initial S/CO ratio	Reamplified S/CO ratio
10	Cervical	Negative	0.39	5.88
16	Thyroid	Negative	0.44	6.12
18	Cervical	Positive	1.21	5.05
20	Cervical	Positive	2.18	6.12
40	Abdominal	Negative	1.51	4.88
42	Axilla	Positive	0.75	5.02
50	Cervical	Positive	1.07	6.05
51	Cervical	Negative	0.77	4.85
54	Cervical	Negative	0.71	5.12

5.2 Discussion

In pulmonary tuberculosis, the "gold standard" for diagnosis is the culture of *M.tuberculosis* from sputum or bronchoalveolar lavage. In extrapulmonary tuberculosis, a negative culture is not necessarily incompatible with a histological diagnosis of tuberculosis since less than half of the lymph nodes are AFB culture positive (Goel *et al.* 2001). The diagnosis of extrapulmonary tuberculosis is difficult, delayed in many cases and frequently made on circumstantial evidence alone. The clinical manifestations are varied and often mimic those of other diseases. The Zeihl-Neilsen smear is often negative and culture is prohibitively slow although definitive. In a proportion of cases, biopsy is required, particularly in the presence of a single enlarged node if there is no other helpful evidence to give a lead to the correct diagnosis. Culture of material biopsied should always be carried out in order to help in the diagnosis and management, although histological examination plays an important part in the diagnosis in many cases. Failure to make a diagnosis can have serious consequences such as development of meningitis or pericarditis for example. Conversely, blind treatment with anti-tuberculosis agents exposes the patient to the risk of unnecessary toxic effects of the drugs as well as possibly masking other infections which partially respond to the broad antibacterial action of rifampicin. Definitive proof of diagnosis requires culture, although time consuming, is essential for sensitivity testing to determine drug sensitivity or resistance. A negative culture however is not necessarily incompatible with the histological diagnosis as the lymph nodes may not necessarily contain live tubercle bacilli (Dandapat *et al.* 1990).

Various investigators have applied PCR for the detection of *M.tuberculosis* DNA in paraffin embedded tissue samples with varying results and conclusions. Totsch *et al.* (1996) investigated 18 lymph nodes with BCG-induced granulatomatous lesions using three different PCR based assays. Firstly, when using a primer pair to obtain the large 439

base pair sequence of the gene encoding the 65 kd mycobacterial surface antigen common to all mycobacteria (Shinnick 1987), poor sensitivity was observed. No positive results were obtained when a single lymph node section was investigated but when five sections were investigated mycobacterial DNA was detected in only one out of 18 cases (5.6%; Totsch *et al.* 1996). A PCR assay based on the amplification of the 123 base pair IS*6110* insertion DNA sequence has been shown to be highly specific and sensitive for the detection of mycobacterial DNA in sputum specimens culture positive for *M.tuberculosis* (Eisenach *et al.* 1991). Totsch *et al.* (1996) also applied this technique to investigating 18 cases of BCG-induced lymphadenitis and detected a positive result in 12 out of 18 cases (66.7%) when investigating one section per lymph node and could demonstrate mycobacterial DNA in 16 out of 18 (88.9%) cases when five sections were investigated. This suggests there was inadequate DNA material in a single section. When nested (reamplification) PCR was performed on only one section, mycobacterial DNA was detected in 16 out of 18 cases (88.9%). They observed that the amount of amplification product obtained by nested PCR was generally higher than in the other two single round PCR techniques and, furthermore, the sensitivity of the molecular techniques could be increased by the combined use of the 123bp assay and nested PCR. They concluded that the detection of mycobacterial DNA depends on the number of sections examined i.e. the amount of DNA in the sample. This is probably because bacterial foci are unevenly distributed in the routinely processed tissues. In the present study, five sections were used for the investigations. From our results, it appears that five sections did not provide adequate DNA material for amplification.

Diaz *et al.* (1996) set out to develop a PCR targeting the IS*6110* insertion sequence for the detection of *M.tuberculosis* DNA in paraffin-embedded tissues in which granulomas were found in order to evaluate its sensitivity and specificity in the diagnosis of tuberculosis. They obtained a sensitivity of 100% in biopsy material from lymph nodes, liver and lungs

which were culture positive for *M. tuberculosis*, although the number of samples examined was small (n=11). The method of DNA extraction used by Diaz *et al.* was similar to that employed in our study involving incubation with proteinase K and extraction with chloroform isopropyl alcohol and precipitation of DNA with sodium acetate. In their experiments, 0.5 to 2mg of extracted DNA from biopsy samples was used to run the PCR. The amplification step however involved 45 cycles. When they combined the group of tuberculous and possibly tuberculous hepatic granulomas, the sensitivity of the PCR assay was 53%, while the specificity was 96%. They suggested that the sensitivity of mycobacterial culture, regarded as the "gold standard" was low at least in hepatic granulomas and that the reason that some samples from clinically tuberculous patients gave a negative PCR result in hepatic granulomas may be related to the paucity of mycobacteria in the tissue. They concluded that the PCR assay was useful in the diagnosis of tuberculosis from paraffin embedded biopsies, but still far from optimal for the accurate diagnosis of hepatic granuloma of tuberculous origin.

Popper *et al.* (1994) used PCR targeting IS*6110* and could demonstrate *M.tuberculosis* in 7 out of 7 samples (100%) of paraffin embedded tissue with positive AFB stain. In the present study, LCx-MTB was positive in 4/17 (23.5%) of AFB smear positive specimens and 5/41 (12.2%) in AFB smear negative specimens. Overall, in histologically diagnosed cases of tuberculous lymphadenitis, the sensitivity of the LCx-MTB was 15.5% (9/58). However, the specificity of the LCx-MTB was 100% (0/42; Table 6). A reamplification of all samples might have given better results with the LCx-MTB in this study. The benefits of a second amplification step are evident in the results presented in Table 8 as all of the equivocal and low positive results yielded strongly positive rates on reamplification. A reamplification step, however, requires the first round amplification vial to be opened and an aliquot removed. This allows for contamination of the laboratory with target DNA which could also contaminate the second round amplification vials with amplicons causing

false positive results. The results obtained in the present study using LCR were not as favourable as in previous studies (Totsch *et al.* 1996, Diaz *et al.* 1996, Popper *et al.* 1994). There are several possible explanations. Firstly, the 123 base-pair insertion sequence IS*6110* has been shown to be present in *M. tuberculosis* strains from 1-24 copies (Plikaytis *et al.* 1991) as opposed to the gene encoding for protein antigen B which is present in a single copy (Burczak *et al.* 1995). Since the repetitive element IS*6110* is present in multiple copies in most strains of *M.tuberculosis,* this may contribute to the improved sensitivity observed by other investigators. Secondly, the size of the target DNA sequence may also contribute to the sensitivity of a PCR based assay as shown by Totch *et al.*. (1996) when comparing the PCR sensitivity using the relatively large 439bp sequence (sensitivity of 5.6%) and the smaller 123bp insertion sequence (sensitivity 88.9%). This may be of particular importance when archive material is used. Greer *et al.* (1991) reported that the viability of DNA in tissue embedded with paraffin decreases according to the nature and duration of exposure to preservatives. This is of particular importance if the quality of fixation has been sub-optimal or if the archive material has been stored for more than five years (Greer *et al.* 1991). In this study, however, all lymph node tissue used in the study was stored for less than three years. Thirdly, the low positive rates observed after first round amplification may be associated with the DNA extraction protocol. In the DNA extraction procedure employed in this study, both bacterial and cellular DNA would be extracted and precipitated. Due to the paucity of mycobacterial DNA in tissue samples we have effectively a "needle in the haystack" scenario, with a relatively small quantity of bacterial DNA extracted into a pool of cellular DNA. The presence of a large amount of cellular DNA may interfere with the binding of the four oligonucleotide probes to the target template without actually inhibiting the binding. Thus only small amounts of DNA would be amplified which may be below the limit of sensitivity for detection. In this study, this aspect was investigated by spiking AFB smear

positive LCx-MTB negative lymph node extracts with MTB DNA and amplifying the sample. In all cases, the spiked samples yielded positive results. If the presence of cellular DNA does play an interfering role in the amplification of the mycobacterial DNA then a method of purifying the relatively low molecular weight mycobacterial DNA from the high molecular weight cellular DNA may contribute to an improved sensitivity. Silica based mini-columns for cleanup/purification have been developed by several companies including Qiagen Co. (Hilden, Germany) and Machery Nagel (Duren, Germany) if incorporated into the DNA extraction sample preparation methodology, may enhance the extraction process to provide a better yield of bacterial DNA free of inhibitors sample which could be more readily detected with the LCx-MTB probes or by another PCR method ideally targeting IS*6110*.

Despite the reduced sensitivity for detecting *M.tuberculosis* DNA by LCx-MTB in this study, LCx-MTB still has the potential for the detection of mycobacterial DNA in lymph node samples of tuberculous patients and is highly specific. It appears that with the present methodology of LCx-MTB, further studies are still required to improve its sensitivity in paraffin-embedded sections. As observed in previous studies, there is a lack of "gold standard" in the diagnosis of tuberculous lymphadenopathy, as culture is frequently found insensitive in determining the aetiology of the lesion. This will no doubt affect the proper evaluation of molecular techniques.

Chapter 6. Development of RT PCR TaqMan assay for the direct detection of MTB-DNA in clinical specimens and paraffin embedded tissues compared with the Abbott LCx-MTB assay.

6.1 Principle of TaqMan Assay

Figure 1. shows TaqMan probes which are linear DNA probes with a fluorophore, usually at the 5' end of the probe and a quencher, either internal or at the 3' end. As long as the probe is intact, regardless of whether it is hybridised with the target or free in solution, no fluorescence is observed from the fluorophore. During the combined annealing - extension step of PCR, the primers of the TaqMan probe hybridize with the target. The DNA polymerase displaces the TaqMan probe by 3 or 4 nucleotides and the 5' exonuclease activity of the DNA polymerase separates the fluorophore from the quencher (Livak *et al.* 1995). Fluorescence can be detected during each PCR cycle, and fluorescence accumulates during the course of PCR (Heid *et al.* 1996). Both primer and probe must hybridize to the target for amplification and cleavage to occur. The fluorescence signal is generated only if the target sequence for the probe is amplified during PCR. Because of these requirements, non-specific amplification is not detected. During PCR, the multiplexer detects an argon ion laser through fiber optic cables that terminate above each position on the 96-well plate. The light passes through the MicroAmp optical caps and, for 25 milliseconds, the laser excites the fluorescent dyes present in each well. Fibre optic cables collect the fluorescence emission between 500nm and 660nm from each of the wells, with a complete collection of data from all wells every 7 seconds. A system of lenses, filters, and a dichroic mirror focus the fluorescence emission into a spectrograph. The spectograph separates the light (based on wavelength) into a predictably spaced pattern across a charge-coupled device (CCD)

camera. The sequence detection application collects the fluorescent signals from the CCD camera and applies data analysis algorithms. The threshold cycle (Ct) occurs where the sequence detection application begins to detect the increase in signal associated with exponential growth of the PCR product. The Ct is dependant on the starting template copy number, the efficiency of both the DNA amplification and PCR system and the cleavage of the TaqMan fluorogenic probe (Bassam *et al.* 1996).

Figure 2. shows the TaqMan probe and primer binding sites corresponding to the target region IS*6110* (bases are numbered as for the sequence with the Genbank accession no. X17348) and Figure 3 shows a typical printout.

Figure 1. Schematic representation of the TaqMan assay. The TaqMan probe fluoresces when the 5' nuclease activity of the DNA polymerase separates the fluorophore from quencher.

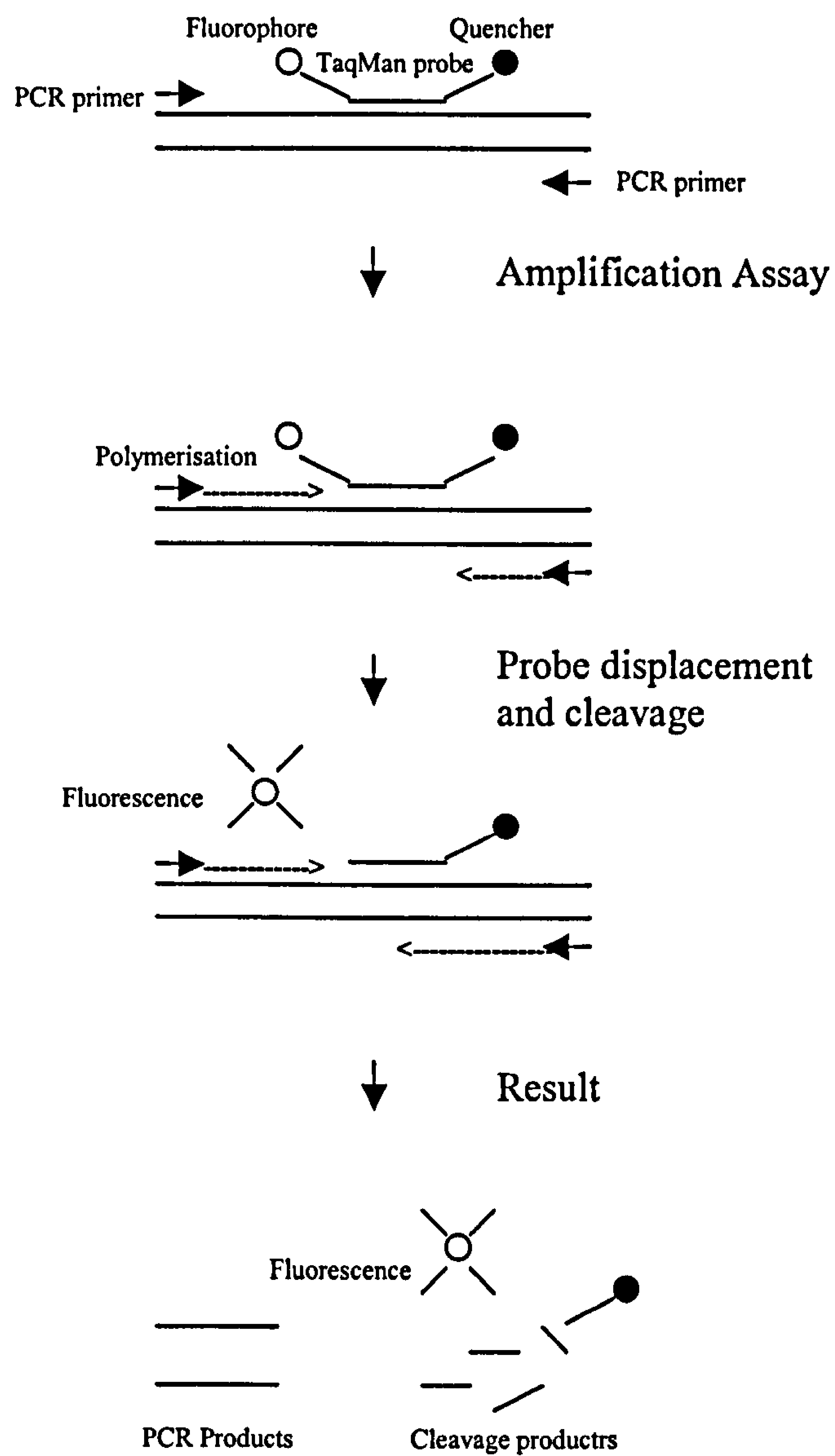
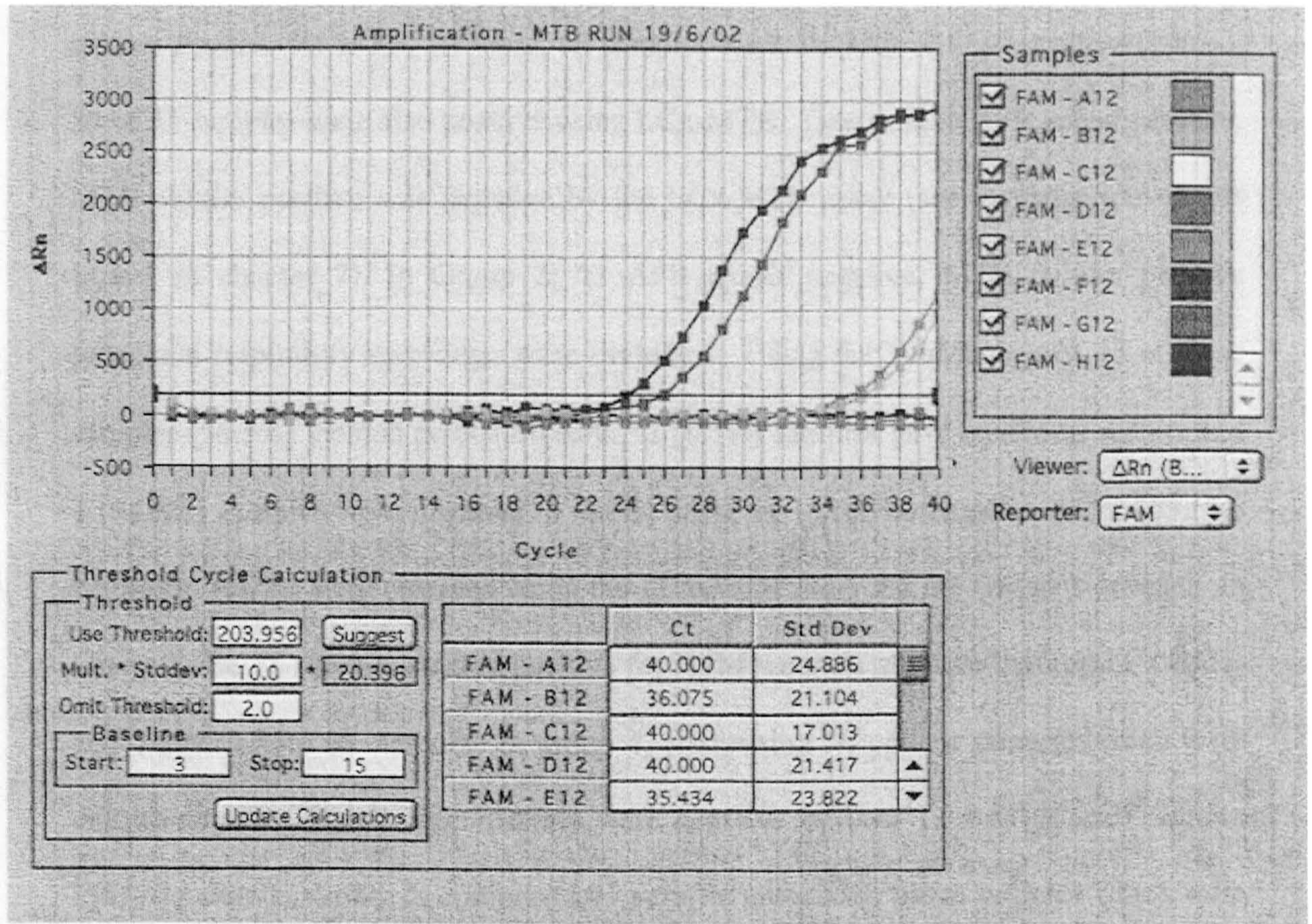


Figure 2. TaqMan probe and primer binding sites correspondings to the target region *IS6110* (bases are numbered as for the sequence with the Genbank accession no. X17348).

```
1 cgatgaaccg ccccggcatg tccggagact ccagttcttg gaaaggatgg ggtcatgtca
61 ggtggttcat cgaggaggta cccgccggag ctgcgtgagc gggcggtgcg gatggtcgca
121 gagatccgcg gtcagcacga ttcggagtgg gcagcgatca gtgaggtcgc ccgtctactt
181 ggtgttggct gcgcggagac ggtgcgtaag tgggtgcgcc aggcgcaggt cgatgccggc
241 gcacggcccg ggaccacgac cgaagaatcc gctgagctga agcgcttagc ggcgggacaa
301 cgccgaattg cgaagggcga acgcgatttt aaagaccgcg tcggctttct tcgcggccga
361 gctcgaccgg ccagcacgct aattaacggt tcatcgccga tcatcagggc caccgcgagg
421 gccccgatgg tttgcggtgg ggtgtcgagt cgatctgcac acagctgacc gagctgggtg
481 tgccgatcgc cccatcgacc tactacgacc acatcaaccg ggagcccagc cgccgcgagc
541 tgcgcgatgg cgaactcaag gagcacatca gccgcgtcca cgccgccaac tacggtgttt
              IS6110 fwd ——▶           IS6110 probe ——▶
601 acggtgcccg caaagtgtgg ctaaccctga accgtgaggg catcgaggtg gccagatgca
         ◀—— IS6110 rev
661 ccgtcgaacg gctgatgacc aaactcggcc tgtccgggac cacccgcggc aaagcccgca
721 ggaccacgat cgctgatccg gccacagccc gtcccgccga tctcgtccag cgccgcttcg
781 gaccaccagc acctaaccgg ctgtgggtag cagacctcac ctatgtgtcg acctgggcag
841 ggttcgccta cgtggccttt gtcaccgacg cctacgtcgc aggatcctgg gctggcgggt
901 cgcttccacg atggccacct ccatggtcct cgacgcgatc gagcaagcca tctggacccg
961 ccaacaagaa ggcgtactcg acctgaaaga cgttatccac catacggata ggggatctca
1021 gtacacatcg atccggttca gcgagcggct cgccgaggca ggcatccaac cgtcggtcgg
1081 agcggtcgga agctcctatg acaatgcact agccgagacg atcaacggcc tatacaagac
1141 cgagctgatc aaacccggca agccctggcg gtccatcgag gatgtcgagt tggccaccgc
1201 gcgctgggtc gactggttca accatcgccg cctctaccag tactgcggcg cgtcccgcc
1261 ggtcgaactc gaggctgcct actacgctca acgccagaga ccagccgccg gctgaggtct
1321 cagatcagag agtctccgga ctcaccgggg cggttcacga
```

Figure 3. Printout of TaqMan assay showing the real time accumulation of fluorescence with subsequent PCR cycles. The Threshold cycle (Ct) is the cycle at which fluorescence can be detected above the baseline.



6.2 Results

Table 9 summarises the results of the 167 patient samples investigated in this comparative study. Group 1, comprised 35 AFB smear positive respiratory specimens culture positive for MTB. Of these, all samples were TaqMan IS*6110* positive (100%). 34 of 35 samples were also positive using LCx-MTB. One sample AFB smear positive, MTB-culture positive was negative by the LCx-MTB assay (see investigation of this isolate in chapter 7). In Group 2, 23 AFB smear negative, MTB culture positive processed respiratory specimens were evaluated. Using the TaqMan assay, 13 of these samples (56.5%) yielded positive results, 2 (8.7%) samples gave equivocal results and 8 (34.8%) samples were negative. 6 (26%) positive, 15 (65%) negative and 2 (43.5%) equivocal results were obtained using the LCx-MTB assay for the Group 2 samples. In Group 3, the 18 samples culture positive for NTM were all negative by both LCx-MTB and TaqMan IS*6110* methods. In group 4, comprising 50 patient samples which were culture negative for all mycobacteria were negative by both LCx-MTB and TaqMan IS*6110* methods. Group 5, comprised 41 paraffin embedded tissue samples which were investigated by both methods. Table 10 gives the nucleic acid amplification results for the 19 samples confirmed culture positive for MTB, 12 samples culture negative for MTB and the 10 paraffin embedded tissues samples for which culture was not performed. In all 19 MTB culture positive paraffin tissue samples, the TaqMan assay yielded a positive result (100%). The LCx-MTB assay gave positive results in 14 of 19 samples (73.7%). Equivocal results were obtained in 4 of 19 samples (21%) and one sample was negative (5.2%) using the LCx-MTB assay. Negative results were obtained for all of the 12 samples (100%) MTB-culture negative, using both the LCx-MTB and TaqMan assays. In the 10 paraffin tissues for which MTB culture was unknown,

positive results were obtained in 5 samples using the TaqMan assay and 4 samples using the LCx-MTB assay.

The limit of sensitivity of the TaqMan assay was determined by quantifying H37Rv control DNA quantified by spectrophotometry (section 12.14.9). 100μl of a 1:1000 dilution of H37Rv was loaded into a cuvette in the spectrophotometer and using A_{260} was quantitated as 620ng/ml of DNA. Table 11 shows the serial dilutions of the H37Rv DNA against TaqMan Threshold cycle (Ct) to determine the limit of sensitivity of the assay as 62 fg/ml.

Table 9. Comparison of TaqMan Assay and Abbott LCx-MTB Assay

	Abbott LCx-MTB			**Taqman Assay**			
	Pos	**Neg**	**Equiv**	**Pos**	**Neg**	**Equiv**	**Total**
Group1: AFB smear pos, Culture pos for MTBC	34	1	0	35	0	0	35
Group 2 : AFB smear neg, Culture pos for MTBC	6	15	2	13	8	2	23
Group 3: Samples culture pos for NTM	0	18	0	0	18	0	18
Group 4: AFB smear neg, Culture neg for mycobacteria	0	50	0	0	50	0	50
Group 5: Paraffin embedded tissue samples	17	20	4	23	18	0	41
Totals							**167**

Table 10. Results of LCx-MTB and TaqMan assays compared with MTB-culture for the Paraffin Embedded Tissue samples in Group 5.

	Abbott LCx-MTB Assay			IS*6110* TaqMan Assay		
Paraffin embedded tissues	Pos	Equiv	Neg	Pos	Neg	Total
Patient tissue samples (MTB culture positive)	14	4	1	19	0	19
Patient tissue samples (MTB culture negative)	0	0	12	0	12	12
Patient tissue samples (MTB culture unknown)	4	0	6	5	5	10
						41

Table 11. Serial dilutions of the H37Rv DNA against TaqMan Threshold cycle (Ct) to determine the limit of sensitivity of the assay.

Dilution of H37Rv extract	Quantity of DNA	TaqMan Assay Ct value
1: 1000 (10^3)	620ng/ml	16.09
1: 10,000 (10^4)	62ng/ml	19.29
1: 100,000 (10^5)	6.2ng/ml	23.36
1: 1,000,000 (10^6)	620pg/ml	26.65
1: 10,000,000 (10^7)	62pg/ml	29.57
1: 100,000,000 (10^8)	6.2pg/ml	32.13
1: 1,000,000,000 (10^9)	620fg/ml	36.57
1: 10,000,000,000 (10^{10})	62fg/ml	39.60
1: 100,000,000,000 (10^{11})	6.2fg/ml	40.0

6.3 Discussion

This investigation has confirmed previous results given in this study that direct nucleic acid methods show high sensitivity and specificity in AFB smear positive samples which are culture positive for MTB. The TaqMan assay, yielded positive results for all AFB smear positive, MTB culture positive samples investigated. The LCx-MTB assay gave similar results except for a single AFB smear positive, MTB culture positive sample in which MTB specific DNA could not be detected. The failure of the LCx-MTB assay to detect MTB DNA in this sample is discussed in detail in Chapter 7.

In contrast, the ability to use nucleic acid testing in samples where AFBs cannot be demonstrated on microscopy is limited. In the 23 AFB smear negative MTB culture positive samples MTB DNA was detected in 56.6% of samples by the TaqMan assay and in only 26% of samples using LCx-MTB assay. Although the TaqMan assay performed better than the LCx-MTB in its ability to detect MTB DNA in smear negative samples, neither method could be recommended as a diagnostic tool because of the low negative predictive values of both tests. The high specificity of both assays is highlighted in the results of Group 3 and Group 4 samples which investigated samples either culture negative for mycobacteria or culture positive for NTM only. Negative results were for all Group 3 and 4 samples using both assays.

Mycobacterial DNA extraction from samples and removal of PCR inhibitors prior to amplification are key components in the development of a sensitive assay. The aim of the extraction method is to capture as much of the mycobacterial DNA as possible from the sample under investigation and endeavour to place as much of the extracted DNA as possible into the amplification tube. Recent studies have shown that the use of silica membranes can aid the removal of sample inhibitors without the need for use of the

toxic substances as employed in the phenol chloroform method (Boddinghaus *et al* 2001).

In the present study a standardised approach to sample preparation was developed so that all samples from processed pulmonary and extrapulmonary sediments, and paraffin tissues could be processed similarly. Lysis was achieved by incubation of the sample material in a proteinase K / SDS solution. Clearly, additional time is required for proteinase K to digest tissue samples than digested respiratory sediments but otherwise the DNA extraction is uniform for all samples. Appropriate conditions for binding of DNA to the silica membrane in the NucleoSpin Tissue columns (Macherey-Nagel, Duren, Germany) were created by the addition of chaotrophic salts and ethanol to the lysate. The binding process is reversible and specific to nucleic acids. Contaminants were removed by subsequent washing with two different buffers. Pure genomic DNA was finally eluted under low ionic strength. This step allows for the extracted DNA to further concentrated by eluting with a small volume of elution buffer. In each extraction 100μl was used as the final elution volume.

The introduction of new fluorescent detection systems, such as molecular beacons (Piatek *et al.* 1998), light cycler methodology (Taylor *et al.* 2001) , and TaqMan probes (Desjardin *et al.* 1998) have been introduced as a simple means of real time quantification of PCR products. These systems rely on quenching fluorescence energy transfer among molecules and have proven to be sensitive, specific and highly discriminative, enabling automated and simultaneous detection of multiple targets in closed tube format (Garcia-Quintanilla *et al* 2002). Quantification of PCR products occurs in real time during the amplification process, with no post amplification handling being necessary. This eliminates potential sources of carryover contamination and reduces the handling time (Desjardin *et al.* 1998).

In this study TaqMan probes were chosen as the platform for the fluorogenic assay best suited for detection of MTB DNA in clinical samples due to the ability to add a large volume of template and thereby increase the sensitivity of the assay. The light cycler methods use a low volume (approx 10μl) capillary tube for the PCR reaction (Taylor *et al.* 2001) which limits the ability to add more than 5μl of extracted DNA template. In contrast, the TaqMan assay can be performed in a 50μl tube format and this allows for the addition of up to 30μl of template for each reaction. This ability is particular important when investigating DNA extracts of paraffin tissues which are often paucibacillary.

Hermans *et al* (1990) suggests that 20 MTB bacteria theoretically correspond to approximately 100fg of chromosomal DNA (Hermans *et al.* 1990). The limit of sensitivity of the Taqman assay in this study was estimated at 62fg. This means that a minimum of 10-20 copies of the extracted DNA needs to present in each amplification tube in order to detect a PCR product. This further highlights the importance of a high yielding DNA extraction method and the ability to add as of the extract to the amplification tube as possible. The target chosen for the TaqMan assay was the transposon IS*6110* which is present in multiple copies in the majority of strains (Fomukong *et al.* 1998)

The main advantage to the use of IS*6110* its presence in multiple copies which serves as a form of "internal amplifier" by providing multiple primer/probe binding sites for each chromosomal copy of DNA. This aspect is probably more relevant in paraffin embedded tissues where the DNA may become degraded or fragmented due to fixation with formalin (Salian *et al* 1998). Hence, a target which is present in multiple copies has a better chance over a single target such as protein antigen B used in the LCx-MTB

assay. This observation alone may have contributed to the improved detection of MTB DNA by the TaqMan assay in the paraffin embedded tissues investigated in Group 5.

The major problem with both the TaqMan and LCx-MTB assays is the inability to detect for inhibition in a single tube. However, the results of this study suggest that inhibition of PCR is not a problem using the TaqMan assay as all of the AFB smear positive, MTB culture positive samples were detected. Similarly all of the paraffin embedded tissues samples for which the culture was known yielded the expected results with the TaqMan assay. Previous work (Chapter 5) did not identify inhibition in extracted paraffin tissues as a problem when assayed using the LCx-MTB. Clearly, inhibition could be monitored by testing samples in duplicate with an aliquot of MTB DNA spiked in a duplicate amplification tube to determine if the added DNA could be amplified. Although providing additional useful information such an inhibition control assay would double the cost of the assay per sample. An alternate approach would be to develop a multiplex assay using two probes labelled with different fluorophores in a single tube with one probe targeting and MTB specific sequence and another targeting a *Mycobacterium* genus specific region. When applied to an AFB smear positive sample a PCR product should be detectable with both probes confirming MTB or with the genus probe alone indicating a NTM. Garcia-Quintanilla *et al.* (2002) developed a multiplex assay targeting single copy sequences in the 16S rDNA gene and only obtained a sensitivity of 90 % in AFB smear positive samples. Any direct nucleic acid amplification method must have a sensitivity and specificity of 100% when being applied to AFB smear positive samples in order to be used a reliable diagnostic tool. Garcia-Quintanilla *et al.* (2002) stated that the selection of different quenching molecules for each probe used reduced the sensitivity of their assay. In addition the use of single copy targets may also have contributed to the reduced sensitivity.

The ideal assay needs to be able to detect and differentiate MTB from NTM in AFB smear positive samples and monitor the reaction for the presence of inhibitors that were not removed by the sample preparation steps. The use of dual tube RT amplification for each sample with a single TaqMan probe in each tube, one targeting the IS*6110* region as tested in this study and, one targeting the 16S rDNA region as proposed by Garcia-Quintanilla *et al.* (2002). In such an assay a PCR product should be detected in at least one of two amplification tubes for each AFB smear positive sample investigated. Failure to detect any PCR products would indicate the presence of inhibition or a sample preparation failure. Detection of a PCR product in both amplification vials would confirm the presence of MTB DNA. Although there are advantages to using IS*6110* as a amplification target in detecting strains of MTB with multiple copies it is important to be aware of occasional strains which do not contain this transposon (Yuen *et al.* 1993). Similarly, users of the commercial systems need to be aware of potential mutations or deletions at primer or probe binding sites which may lead to false negative results. All nucleic acid amplification results for MTB should be qualified with a comment indicating that amplifications tests should be interpreted in conjunction with other available clinical information.

Chapter 7 Failure of the LCx MTB assay to detect MTB specific DNA in an AFB smear positive, MTB culture positive sample .

7.1 Case History

The patient was a 47 year-old Canadian born male who had resided in Australia since the age of four years. He presented to a hospital emergency room where he reported a two-month history of lethargy, fatigue, night sweats and a cough productive of sputum. There was no known contact with tuberculosis. The patient consumed more than 50 standard alcoholic drinks weekly and smoked tobacco heavily. Marked weight loss was apparent. Chest radiograph revealed patchy airspace consolidation and cavitation in the left upper lobe. The patient was admitted to hospital, and three early morning sputum samples were collected on consecutive days.

7.2 Laboratory Investigation

Sputum samples were decontaminated concentrated by centrifugation and inoculated onto Lowenstein Jensen slopes and into liquid medium (as described in sections 2.1 and 2.2). Three concentrated sediments were stained by the Ziehl-Neelson method (section 2.3) which showed 2+ (1-10 bacilli in 10 oil immersion fields) AFB. An LCx-MTB assay was performed according to manufacturers' instructions. By relating the LCx MTB Assay results for the specimen to the cutoff value (CO), the presence or absence of *M.tuberculosis* complex DNA was determined. An S/CO reading of 0.04 was obtained, indicating a negative result.

The strain was isolated after 12 days incubation and direct microscopy revealed AFB showing good serpentine cording. The organism was confirmed as *M.tuberculosis* complex by MTB specific probe (Accuprobe; Gen Probe, San Deigo, Calif.) (section 2.7). MTB complex speciation tests identified the organism as *M.tuberculosis.* The organism accumulated niacin, produced pyrazinamidase, reduced nitrate and was

susceptible to 10μg/ml thiophene-2-carbooxylic acid hydrazide (Wayne 1974, Wayne *et al.* 1976). Susceptibility testing was performed using the BACTEC 460 radiometric assay proportion method (Siddiqi 1996) and the strain was susceptible to rifampicin, isoniazid, ethambutol and streptomycin. A stored aliquot of the sediment was retested by the LCx-MTB assay in parallel with an aliquot of sediment spiked with a control strain of MTB (H37Rv). A negative LCX-MTB result was obtained on retesting the sediment and the spiked sample showed no evidence of inhibition. A sample of the culture was tested by the LCX-MTB assay and a negative result was obtained. The test was repeated with the culture spiked with an aliquot of H37Rv to exclude the possibility of the culture being inhibitory to the assay and a positive result for the assay was observed. It was postulated there was a mutation in one or more of the probe binding sites and the reason for the false negative result was investigated.

7.3 Results

The previously described primers MT1 and MT2 (Sjobring *et al.* 1990) were used to amplify a 419bp product overlapping the 44bp target of the LCx-MTB assay. PCR was performed as previously described (Sjobring *et al.* 1990) and yielded a 419bp product with H37Rv and no product with the LCx-MTB negative strain (Figure 4). An additional primer was designed using Primer Express Version 1.5 (Applied Biosystems, Foster City, Calif.) outside the 419bp region of the protein antigen b gene: pab4 5'-GAGCCTGATCGCACCCATC-3'. PCR was performed using a combination of primers MT1 and pab4 and a product of 719 bp in the patient strain was detected when the expected product size was 1245bp (Figure 4). DNA sequencing was performed on the 719 bp product to determine the region of DNA deletion (section 2.16). Figure 5 shows a schematic representation of the 499 bp deletion in the protein antigen B gene corresponding to bases 328 to 827. Figure 6 indicates the location of the primers used,

the probe binding sites for the LCx probes, and the region of deletion detected in the pab gene (Genbank accession M30046) of this strain. VNTR typing (section 2.18) was performed on this strain and profile of 42453 was obtained.

Figure 4. Ethidium bromide stained agarose gels.
Gel A corresponds to 744 bp PCR products obtained using primers MT1 and PAB4 for the patient strain (PT). No PCR products were obtained for the control strain H37Rv. Gel B shows 419bp PCR product obtained using primers MT1 and MT2 for the control strain H37Rv and the absence of a product with the patient strain (PT)

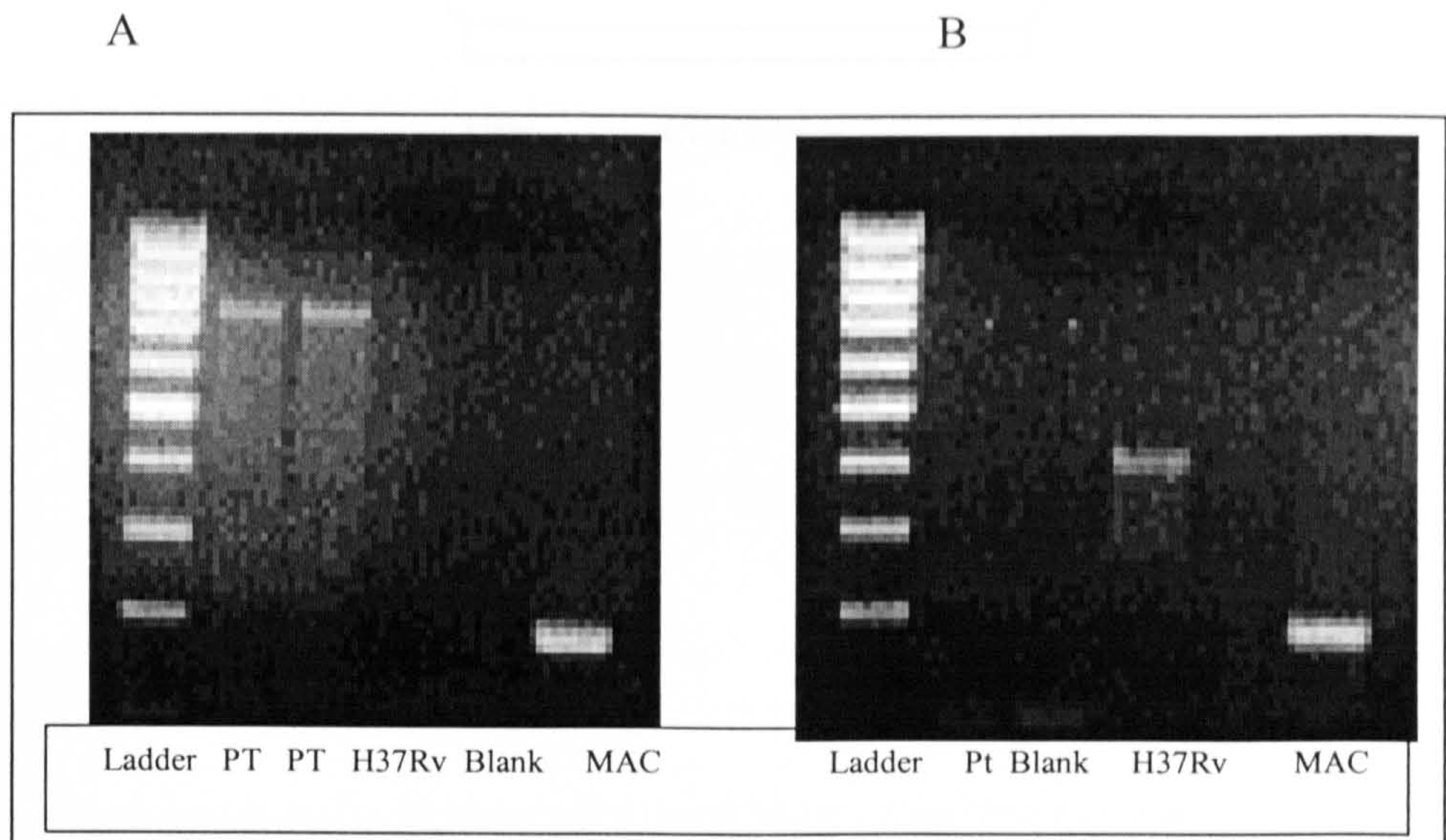


Figure 5. Schematic diagram showing the LCx-MTB assay probe binding sites, primer binding sites (MT1, MT2 and pab4) and the region of deletion in the protein antigen B gene of a strain of *M. tuberculosis.*

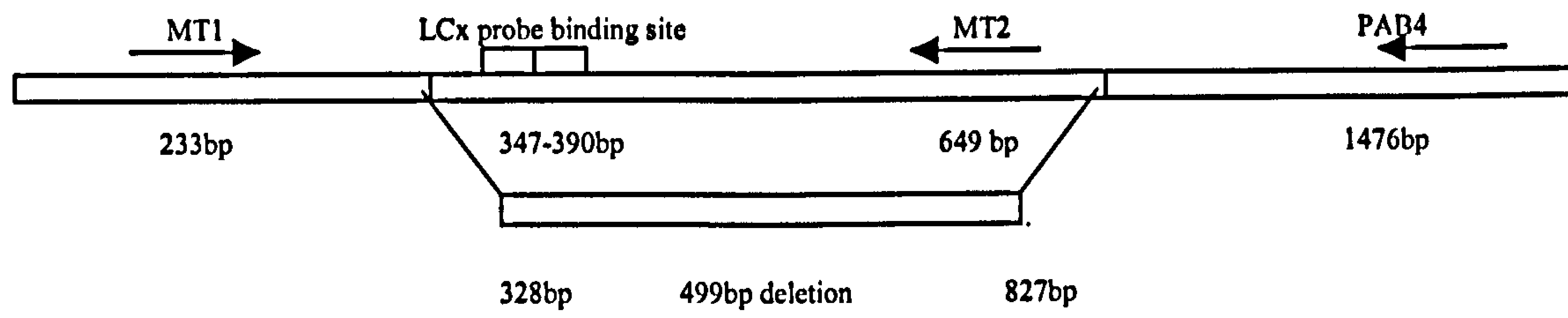


Figure 6. Location of the primers used, the probe binding sites for the LCx probes, and the region of deletion detected in the pab gene (Genbank accession M30046) of a strain of *Mycobacterium tuberculosis*.

1 tgttcttcga cggcaggctg gtggaggaag ggcccaccga acagctgttc tcctcgccga

61 agcatgcgga aaccgcccga tacgtcgccg gactgtcggg ggacgtcaag gacgccaagc

121 gcggaaattg aagagcacag aaaggtatgg cgtgaaaatt cgtttgcata cgctgttggc

181 cgtgttgacc gctgcgccgc tgctgctagc agcggcgggc tgtggctcga aaccaccgag

MT1 →

241 cggttcgcct gaaacgggcg ccggcgccgg tactgtcgcg actacccccg cgtcgtcgcc

301 ggtgacgttg gcggagaccg gtagcacgct gctctacccg ctgttcaacc tgtggggtcc

LCX probe target →

361 ggcctttcac gagaggtatc cgaacgtcac gatcaccgct cagggcaccg gttctggtgc

←LCX probe target

421 cgggatcgcg caggccgccg ccgggacggt caacattggg gcctccgacg cctatctgtc

481 ggaaggtgat atggccgcgc acaaggggct gatgaacatc gcgctagcca tctccgctca

541 gcaggtcaac tacaacctgc ccggagtgag cgagcacctc aagctgaacg gaaaagtcct

601 ggcggccatg taccagggca ccatcaaaac ctgggacgac ccgcagatcg ctgcgctcaa

← MT2

661 ccccggcgtg aacctgcccg gcaccgcggt agttccgctg caccgctccg acgggtccgg

721 tgacaccttc ttgttcaccc agtacctgtc caagcaagat cccgagggct ggggcaagtc

781 gcccggcttc ggcaccaccg tcgacttccc ggcggtgccg ggtgcgctgg gtgagaacgg

841 caacggcggc atggtgaccg gttgcgccga gacaccgggc tgcgtggcct atatcggcat

901 cagcttcctc gaccaggcca gtcaacgggg actcggcgag gcccaactag gcaatagctc

961 tggcaatttc ttgttgcccg acgcgcaaag cattcaggcc gcggcggctg gcttcgcatc

1021 gaaaaccccg gcgaaccagg cgatttcgat gatcgacggg cccgccccgg acggctaccc

1081 gatcatcaac tacgagtacg ccatcgtcaa caaccggcaa aaggacgccg ccaccgcgca

1141 gaccttgcag gcatttctgc actgggcgat caccgacggc aacaaggcct cgttcctcga

1201 ccaggttcat ttccagccgc tgccgcccgc ggtggtgaag ttgtctgacg cgttgatcgc

1261 gacgatttcc agctagcctc gttgaccacc acgcgacagc aacctccgtc gggccatcgg

1321 gctgctttgc ggagcatgct ggcccgtgcc ggtgaagtcg gccgcgctgg cccggccatc

1381 cggtggttgg gtgggatagg tgcggtgatc ccgctgcttg cgctggtctt ggtgctggtg

1441 gtgctggtca tcgaggcgat gggtgcgatc aggctcaacg ggttgcattt cttcaccgcc

← pab4

1501 accgaatgga atccaggcaa cacctacggc gaaaccgttg tcaccgacgc gtcgcccatc

1561 cggtcggcgc ctactacggg gcgttgccgc tgatcgtcgg gacgctggcg acctcggcaa

1621 tcgccctgat catcgcggtg ccggtctctg taggagcggc gctggtgatc gtggaacggc

1681 tgccgaaacg gttggccgag gctgtgggaa tagtcctgga attgctcgcc ggaatcccca

1741 gcgtggtcgt cggtttgtgg ggggcaatga cgttcgggcc gttcatcgct catcacatcg

1801 ctccggtgat cgctcacaac gctcccgatg tgccggtgct gaactacttg cgcggcgacc

1861 cgggcaacgg ggagggcatg ttggtgtccg gtctggtgtt ggcggtgatg gtcgttccca

1921 ttatcgccac caccactcat gacctgttcc ggcaggtgcc ggtgttgccc cgggagggcg

1981 cgatcgggaa ttc

7.4 Discussion

The investigation of this strain of *Mycobacterium tuberculosis* was undertaken to resolve the reason why a AFB smear positive, MTB culture positive sample could not be detected by the LCx-MTB assay in chapter 6. The LCx-MTB assay has been in use in several Australian laboratories for several years and has proved to be a highly reliable test in detecting MTB specific DNA in smear positive respiratory specimens (Lumb *et al.* 1999). It was therefore important to ascertain the reason for the failure and to try an investigate how commonly patients were infected with strains of MTB which could gave false negative results using the LCx-MTB assay. The LCx-MTB assay has been used by several Australian Reference Laboratories as a primary diagnostic tool for the direct detection of MTB DNA in clinical samples.

Although, the patient in this case investigation was Canadian born, he had resided in Australia since he was 4 years of age. He had no history of overseas travel and was therefore presumed to have acquired his tuberculosis locally. It was of public health interest to determine if there were local clones of MTB circulating in Australia which could not be detected by the LCx-MTB assay.

The target nucleic acid sequence for the LCx-MTB assay is found within the single copy chromosomal gene protein antigen b (pab) (Bengard-Andersen *et al.* 1989). The LCx-MTB assay uses four oligonucleotides probes labelled with either a capture or detection hapten to amplify a 44-bp fragment of the pab gene (bases 347 to 390) (Lindbrathen *et al.* 1997). PCR was performed to endeavour to amplify a product outside the LCx-MTB probe binding sites using previously published primers sequences

used by Sjobring *et al.* (1990). When no product could be amplified, an additional primer was designed in order to amplify a larger product. PCR performed with primers MT1 and pab4 yielded a product approximately 500 bp smaller than expected. DNA sequencing and subsequent comparison with the Genbank published sequence for protein antigen B showed a deletion of 499bp of DNA spanning the LCx-MTB probe binding sites. This is the first report of a DNA deletion in the pab gene of MTB. These findings explained the LCx-MTB failure in this instance.

The incidence of such circulating clones among Queensland patients was further investigated by performing VNTR typing. A VNTR profile of 42453 was obtained. Examination of the database of Queensland VNTR profiles (1999 -2001) was undertaken to determine whether there were other MTB strains with the same VNTR profile which could harbour the same deletion. Only one epidemiologically unrelated strain with the same VNTR profile was detected. MTB DNA could be detected in this strain using the LCx -MTB assay indicating that the deletion in the pab gene was not present. This finding laid to rest public health concerns that strains with a deletion in the pab gene were a common finding.

All nucleic acid testing methods have the potential to give false negative results due to mutations which may arise at primer or probe binding sites. This patient was unaffected by the delayed laboratory diagnosis of tuberculosis as treatment for tuberculosis proceeded despite a negative LCx-MTB assay.

At the end of 2002, the LCx-MTB assay was withdrawn from sale in Australia. Abbott Diagnostics cited unresolvable manufacturing problems.

Chapter 8: Utility of Line Probe assay in detection of Rifampicin resistant strains of *Mycobacterium tuberculosis* in Jeddah, Saudi Arabia.

8.1 Assay Background

The INNO LiPA Rif TB assay based on the reverse hybridization principle is a kit used to detect mutations in the *rpoB* gene of *M. tuberculosis* (Cooksey *et al.* 1997). Oligonucleotide probes are immobilised as parallel lines at known locations on a nitrocellulose strip are hybridized under strictly controlled conditions with a 256-bp fragment of *rpo*B biotinylated PCR product. Five partially overlapping probes (S1 through S5) of 19 to 23 bases were designed. These S probes exclusively hybridize to the wild-type sequence. If a mutation is present in one of the five regions, the corresponding probe will be prevented from hybridizing under the stringent hybridization and washing conditions used in the assay (Hirano *et al.* 1999). In addition four R probes (19 or 23 bases) were designed. These R probes, probes R2, R4a, R4b and R5 hybridize with amplicons carrying the following mutations. Asp-516-Val, His-526-Tyr, His-526-Asp and Ser-531-Leu respectively (Hirano *et al.* 1999).

8.2 Results

Figures 7A and 7B. show schematic representations of the Line Probe Assay results on 18 samples of rifampicin resistant *M.tuberculosis*. All of the eighteen rifampicin resistant strains of *M.tuberculosis* tested were multi-drug resistant. Table 12 shows the drug susceptibility pattern for each strain. The mutations detected in the *rpo*B gene for each strain are given in Table 12 with the VNTR profile for each strain. 9 of 18 strains. (50%) carried the mutation Ser-531-Leu. Six isolates (33%) had a His-526-Tyr mutation, two (11.1%) had a Asp-516-Val mutation and one (5.6%) had a His-526-Asp

mutation. VNTR analysis of the rifampicin resistant strains showed clusters of VNTR profiles associated with distinct resistance mutations. Six isolates yielded a VNTR profile of 22431 and all demonstrated the mutation Ser-531-Leu. Five strains with VNTR profile 42235 showed a His-526-Tyr mutation.

Figure 7A and 7B. Schematic representations of the Line Probe Assay results on 18 samples of Rifampicin resistant *M.tuberculosis*.

Figure 7A. Samples 1-10

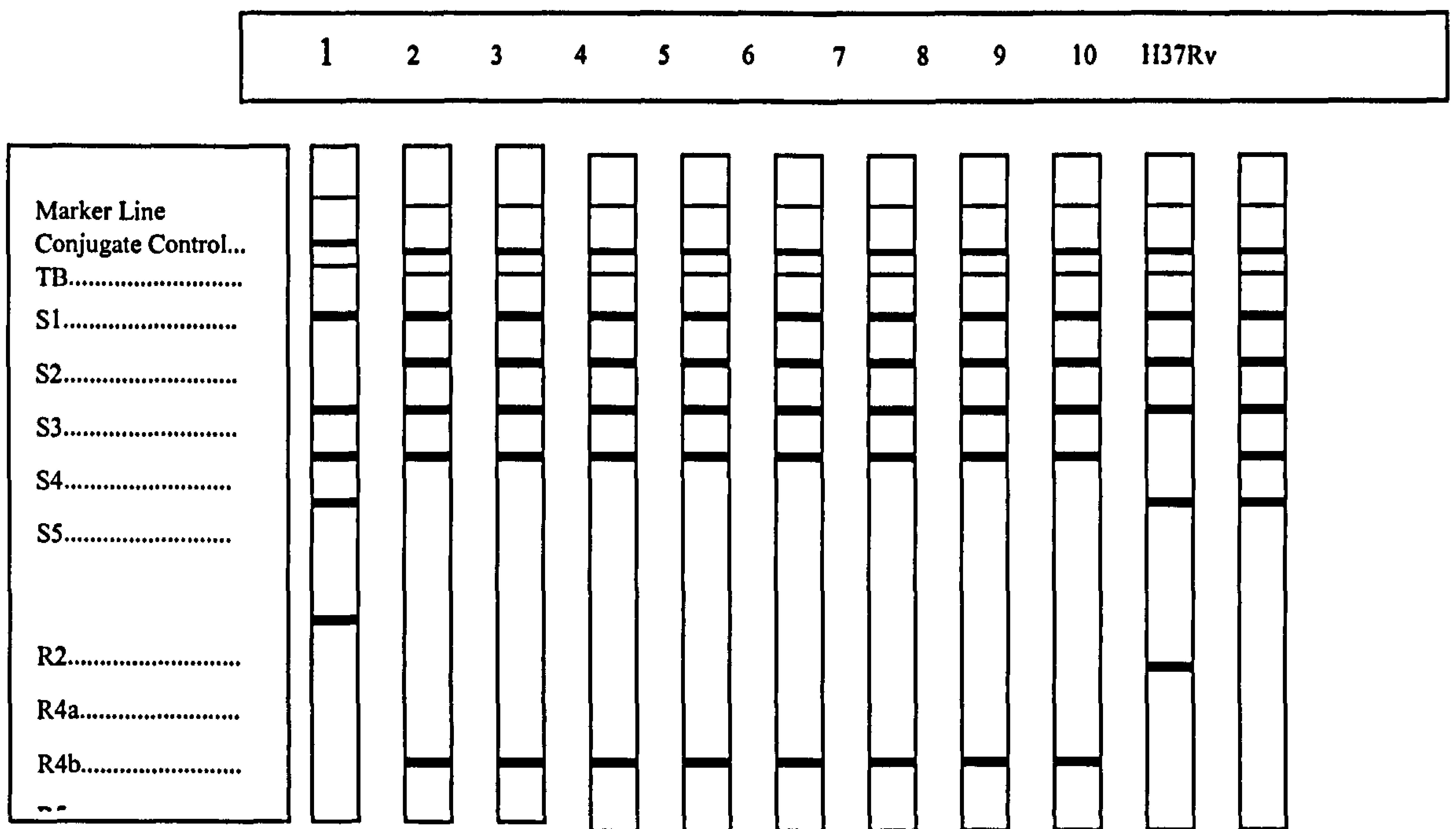


Figure 7B. samples 11-18

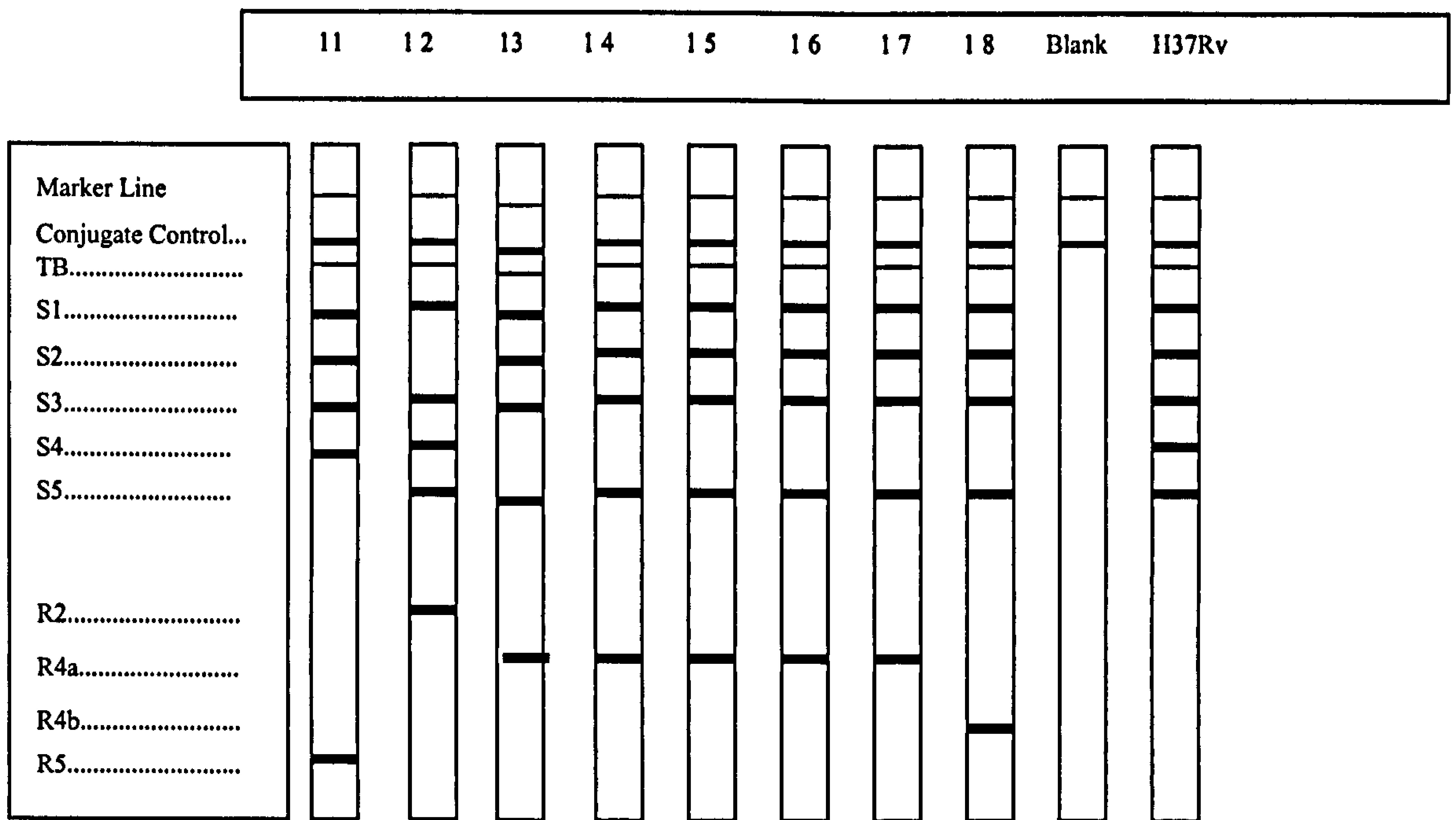


Table12. Table shows VNTR profile, drug susceptibility pattern, and mutations detected using the LiPA Assay on 18 rifampicin resistant strains of *M.tuberculosis.*

Line probe	VNTR	Susceptibility Profile*						Mutation
		RI	I	S	E	Z		
1	21333	R	R	R	S	S	F1034	Asp-516-Val
2	22231	R	R	R	I	I	18205	Ser-531-Leu
3	22431	R	R	S	S	S	16448	Ser-531-Leu
4	22431	R	R	S	S	S	17971	Ser-531-Leu
5	22431	R	R	R	S	I	18569	Ser-531-Leu
6	22431	R	R	R	I	R	17720	Ser-531-Leu
7	22431	R	R	R	R	R	18544	Ser-531-Leu
8	22431	R	R	R	R	S	16522	Ser-531-Leu
9	22434	R	R	R	S	S	19211	His-526-Tyr
10	32233	R	R	S	S	S	F1030	Ser-531-Leu
11	32233	R	R	S	S	S	16488	Ser-531-Leu
12	32433	R	R	R	S	S	F1023	Asp-516-Val
13	42235	R	R	R	R	S	18086	His-526-Tyr
14	42235	R	R	S	S	S	F1012	His-526-Tyr
15	42235	R	R	S	S	S	F1038	His-526-Tyr
16	42235	R	R	R	S	S	19774	His-526-Tyr
17	42235	R	R	R	R	R	17569	His-526-Tyr
18	42435	R	R	R	R	R	18572	His-526-Asp

* Rifampicin (RI), Isoniazid (I), Streptomycin (S), Ethambutol (E), Pyrazinamide (Z)

Resistant (R), Susceptible (S), Intermediate (I)

8.3 Discussion

The use of conventional culturing methods in the diagnosis and susceptibility testing of tuberculosis is protracted, and can take up to 12 weeks before a prediction of a resistance pattern can be made (Pretorius *et al.* 1996). This has negative consequences for the treatment of infected individuals, and can contribute to the spread of multi-drug resistant organisms. Spontaneous mutations that lead to drug resistance occur rarely in *M. tuberculosis*, and multi-drug regimens can prevent the emergence of clinical drug resistance. The problem of resistance results from treatment which is inadequate, often because of irregular drug supply, inappropriate regimens or poor compliance. (Hirano *et al.* 1999). Rifampicin resistance is the most common primary drug resistance observed in Saudi Arabia (Ellis *et al.* 1996) and is also associated with resistance to isoniazid in 90% of cases (Drobniewski *et al.* 1998). As most multi-drug resistant strains are resistant to rifampicin, the rapid detection of mutations, 95% of which occur within an 81bp region of the *rpo*B gene, corresponding to codons 507 to 533, may be used for the presumptive identification of MDR tuberculosis. Different groups from diverse regions of the world have thus far reported 65 substitutions, 12 deletions and 4 insertions in the rifampicin resistance determining region of the *rpo*B gene based on DNA sequence analysis (Mani *et al.* 2001). Therefore, if all of these substitutions occurred with equal frequency, the only useful means of predicting rifampicin resistance would be by DNA sequencing. The most common mutations occurs with codon 531(TCG) Serine to (TTG) Leucine. This mutation accounted for 9 of 18 rifampicin resistant strains (50%) investigated in this study. This mutation has been reported as the most common alteration in the *rpo*B from several geographical regions. It has been reported to be responsible for conferring rifampicin resistance in 53.5% strains isolated in Africa (Rossau *et al.* 1997), 46.8% of 77 Asian strains (Hirano *et al.* 1999), 48.5% strains from

Australia (Yuen *et al.* 1999), 53.7 % strains from Brazil (Valim *et al.* 2000), 56.7% strains from Italy (Piatek *et al.* 1998), 36.1% strains from Germany (Rinder *et al.* 1997), 34.4% of strains in the United States (Williams *et al.* 1994), 47.1% of strains from Greece (Matsiota-Bernard *et al.* 1998), 53% of strains from India (Mani *et al.* 2001), 46.3% of strains from Turkey (Cavusoglu *et al.* 2002) . A transition mutation in codon 526 (CAC) His to (TAC) Tyr was detected in 6 of 18 strains (33.3%). 2 strains (11.1%) showed a transition in codon 516 (GAC) Asp to (GTC) Val. A transition mutation in codon 526 (CAC) His to (GAC) Asp was detected a single strain (5.6%). The mutations observed in this study, corresponding to codons 531, 526 and 516 have been shown to be the most commonly identified from several geographic regions. (Bartfai *et al.* 2001). The InnoLipa kit appears ideal for use in Saudi Arabia as a screen for multi-drug resistance as the resistance probes could detect a mutation in each of the strains investigated in this study, and all strains were resistant to isoniazid in addition to rifampicin. VNTR analysis on each of the rifampicin resistant strains found 3 clusters of VNTR profiles; 22431 (6 strains), 42235 (5 strains) and 32233 (2 strains). In each of these clusters the same mutations in the *rpo*B gene were observed suggesting that there may be primary transmission of multi-drug resistant tuberculosis occurring in Saudi Arabia. A combination of resistance mutation detection and a PCR based typing strategy may together provide a simple approach to detect drug resistance as early as possible. The ability to rapidly identify a potential multi-drug resistant strain based on its VNTR profile may be useful strategy to control the further transmission of drug resistant strains, in countries like Saudi Arabia where resistance is common.

The inability to detect resistance early is one of the major threats to tuberculosis treatment and control programs. It would not be currently feasible to test all tuberculosis cases using Lipa and therefore any strategy which can predict patient

groups with high frequencies of multidrug resistance can contribute to the control of transmission of multi-drug resistant strains. The results of this study suggest, that the application of the Lipa assay could be selectively used when a VNTR profile suggest an epidemiological link with circulating resistant strains. This approach could reduce the time to detect multi-drug resistant tuberculosis from weeks to only a few days. The utility of VNTR as a routine typing strategy is investigated in the following chapter.

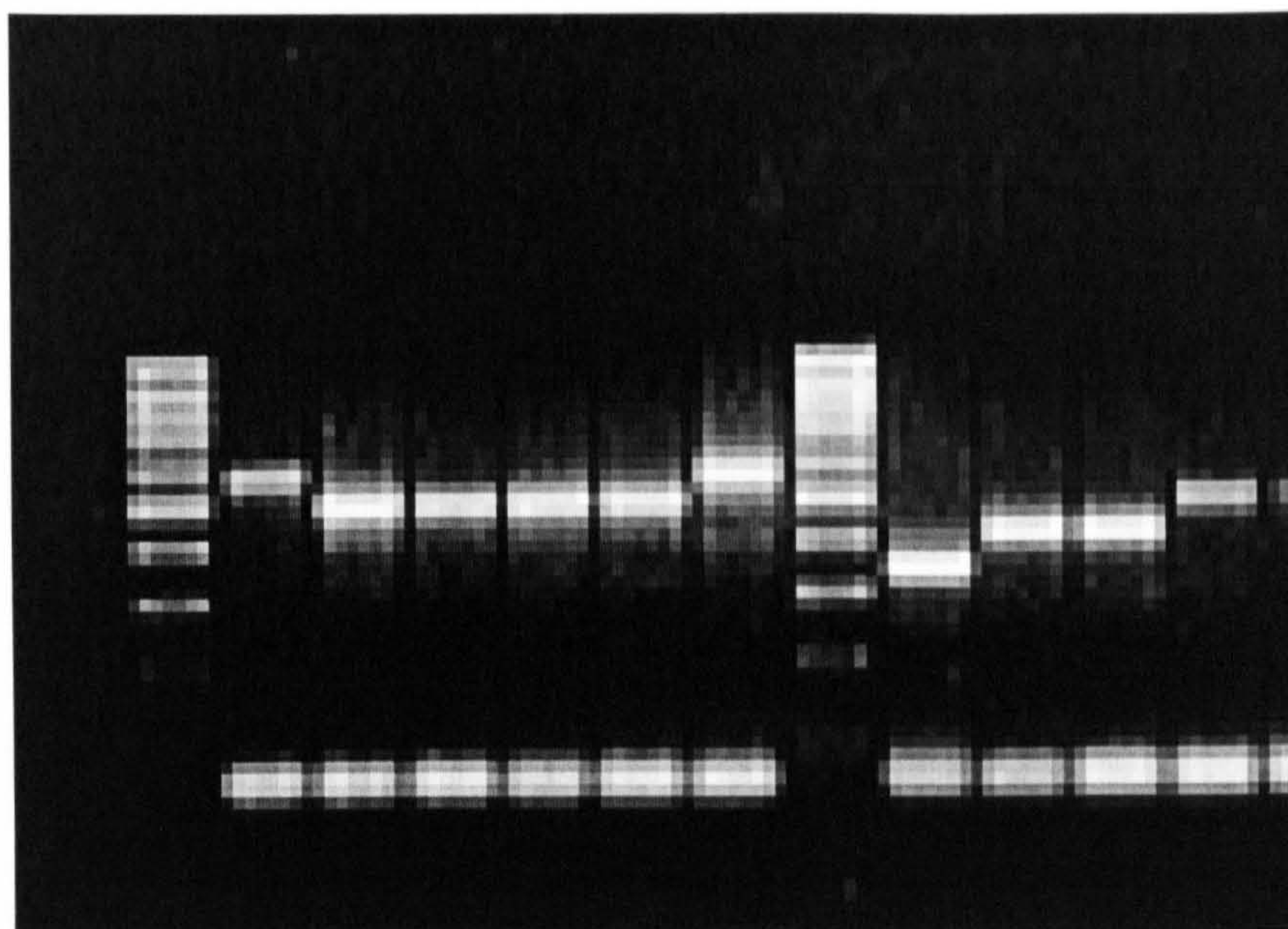
Chapter 9: Diversity of strains of *Mycobacterium tuberculosis* isolated in Queensland, Australia and Jeddah, Saudi Arabia based on Variable numbers of Tandem DNA repeats typing

9.1 Assay Background

Repetitive units of DNA ranging from 40-100bp are often found as tandem repeats dispersed in the intergenic regions of the *M. tuberculosis* genome. The methodology of Frothingham *et al.* (1998) uses PCR to amplify these repetitive sequences to determine length polymorphisms in the five most variable loci. Based on the number of repeat sequences at each locus a numeric profile can be generated for each strain. Thus, the type strain *M. tuberculosis* H37Rv has the profile 33433 using these loci, and *M.bovis* BCG Pasteur has a profile 55623. H37Rv has three copies of a 75bp repeat at locus ETR-A, three copies of 57bp repeat at locus ETR-B, four copies of a 58bp repeat at locus ETR-C, three copies of a 77 bp repeat at ETR-D, and three copies of a 53bp repeat at ETR-E. These regions are difficult to amplify as they have a very high GC content, with lots of repetition and long strings of GC sequence. Loci ETR B, ETR C, ETR D, and ETR E contain some strong hairpin sequences which are more easily amplified when 4% dimethyl sulphoxide (DMSO) is incorporated into the reaction mixture. Stutter bands or ladder bands were observed during the PCR reactions. These stutter bands consist of multiple bands on the gel, corresponding to additions or deletions of one or more tandem repeat units, in addition to the correct band size. These bands are presumed to be due to strand slippage of the DNA polymerase enzyme during PCR. As PCR favours smaller products, once slippage occurs, a single molecule of a smaller size product may catch up or even exceed the correct product. Therefore, if stutter bands are present the darkest band could not be presumed to be the correct product size. A template dilution of 1:1000 with sterile water reduced the incidence of

stutter bands and allowed for a clear visualisation of a single product for each locus. The method has been shown to be highly reproducible but some potential sources of error have been identified. Overreading reactions with stutter bands, clerical errors in sizing bands, and mislabelling errors associated with the DNA preparation, PCR reaction or gel loading steps. In order to improve the accuracy of the method all gels were read blind by three readers. Results were entered individually onto a spreadsheet. Obvious clerical errors were re-read but other discrepant results were retested. Known allele positive controls were incorporated into each PCR run and negative controls were incorporated into each run. ETR-D is a locus described by Supply *et al.* 1997. Although H37Rv has three complete copies of a 77 bp repeat at this locus, most clinical isolates have a 24bp deletion in one of the tandem repeats. This deletion can be detected on agarose gels. Thus, H37Rv has three copies of ETR-D and yields a 310bp product, while other three copy strains yield a 286bp product. This polymorphism may provide some additional discriminative value but has not been utilised in this investigation. Examples of PCR products at each loci are given in Figures 8-12, corresponding to ETR A -E, respectively. Table 13 shows calculations of the number of DNA repeats at each VNTR loci for each of the strains of MTB depicted in Figures 8-12. Table 14 represents the expected PCR product size for the different VNTR polymorphisms – Allele product size in bp (Frothingham *et al.* 1998).

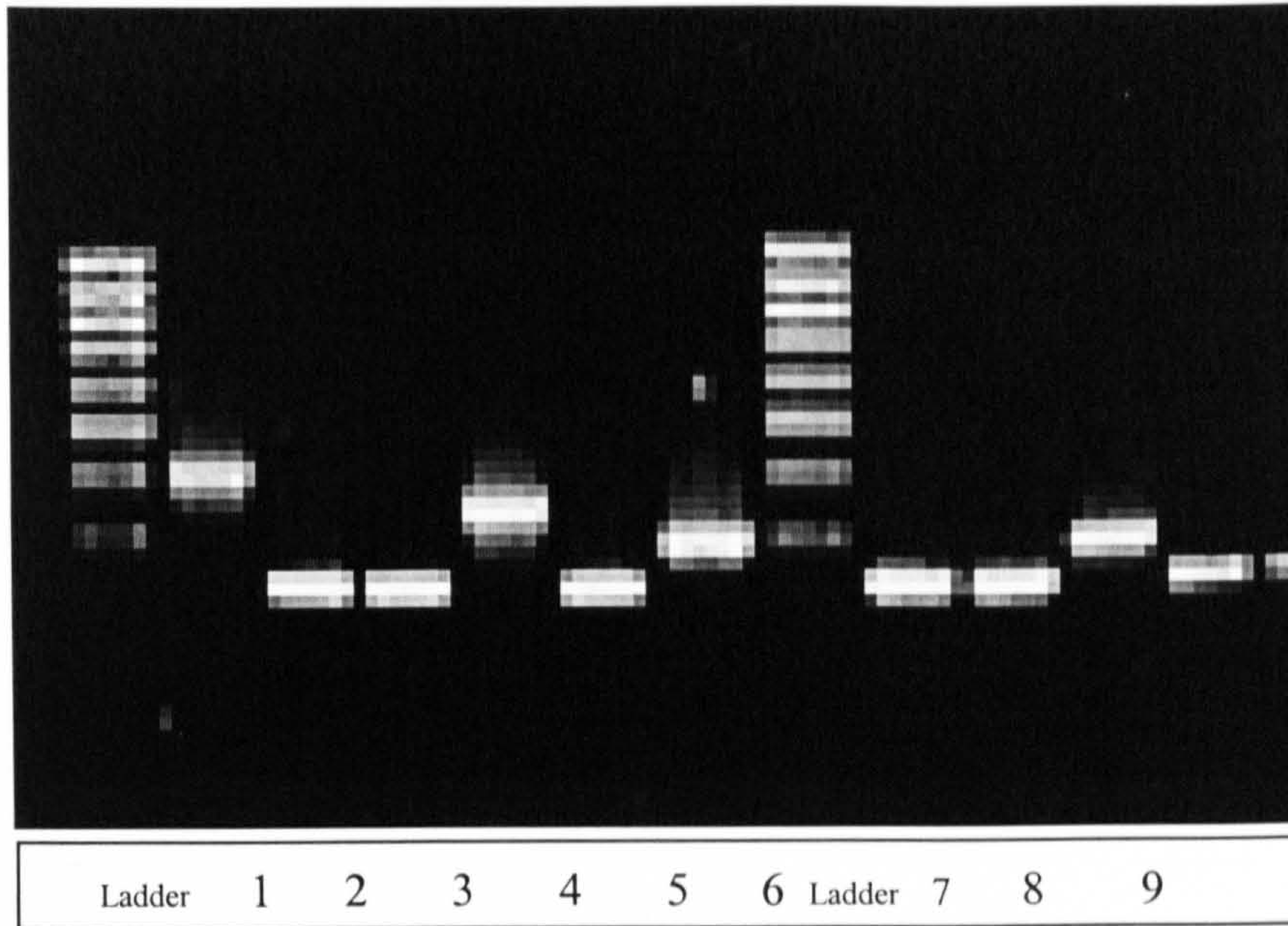
Figure 8. PCR products for ETR-A on 2% agarose gel stained with ethidium bromide. Calculations of the product size and number of repeats at this locus are given.



Ladder 1 2 3 4 5 6 Ladder 7 8 9

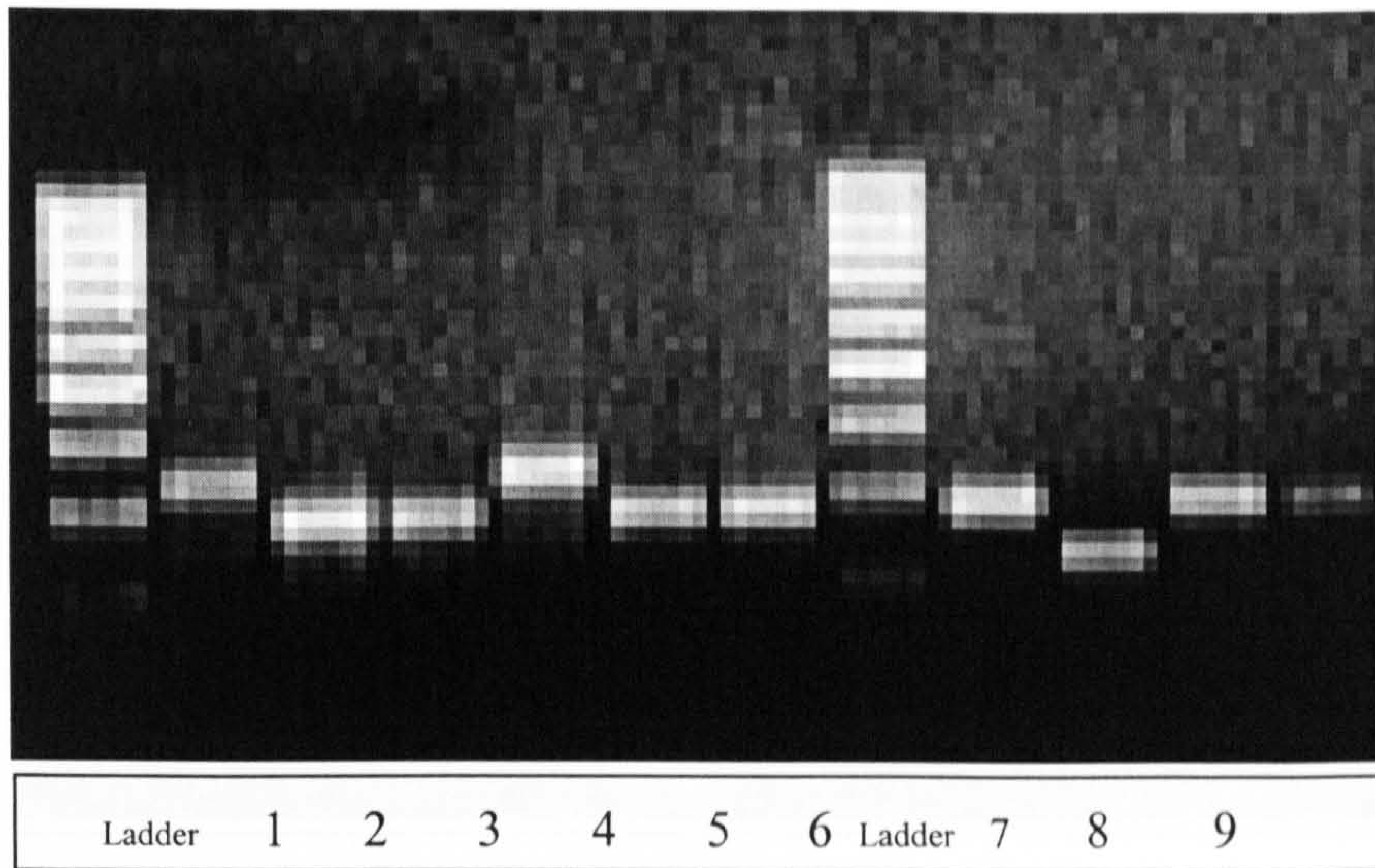
Lane	Product size	No of repeats
1	570	5
2	495	4
3	495	4
4	495	4
5	495	4
6	570	5
7	345	2
8	420	3
9	420	3
10	495	4

Figure 9. PCR products for ETR-B on 2% agarose gel stained with ethidium bromide. Calculations of the product size and number of repeats at this locus are given.



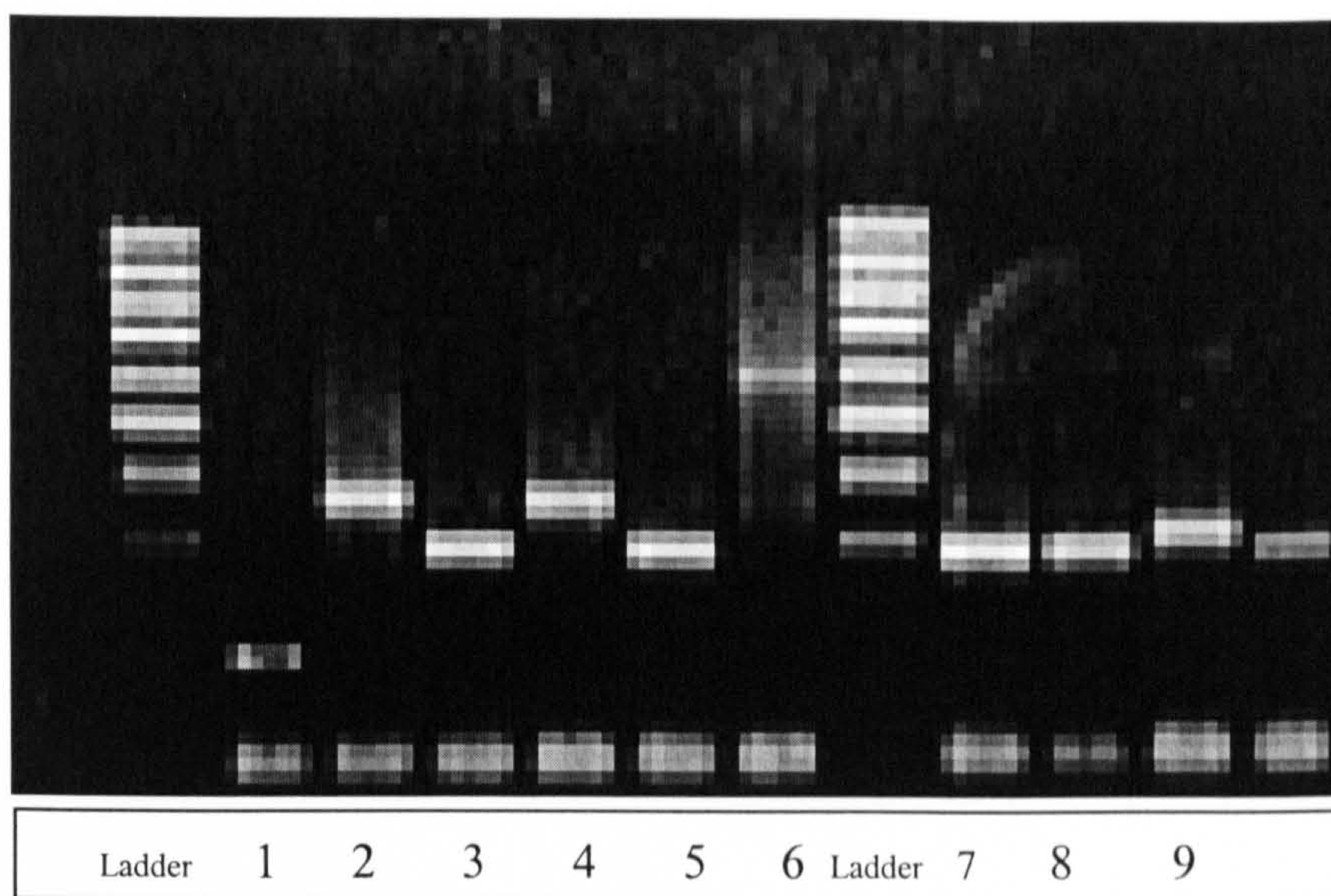
Lane	Product size	No of repeats
1	406	5
2	235	2
3	235	2
4	349	4
5	235	2
6	292	3
7	235	2
8	235	2
9	292	3
10	292	2

Figure 10. PCR products for ETR-C on 2% agarose gel stained with ethidium bromide. Calculations of the product size and number of repeats at this locus are given.



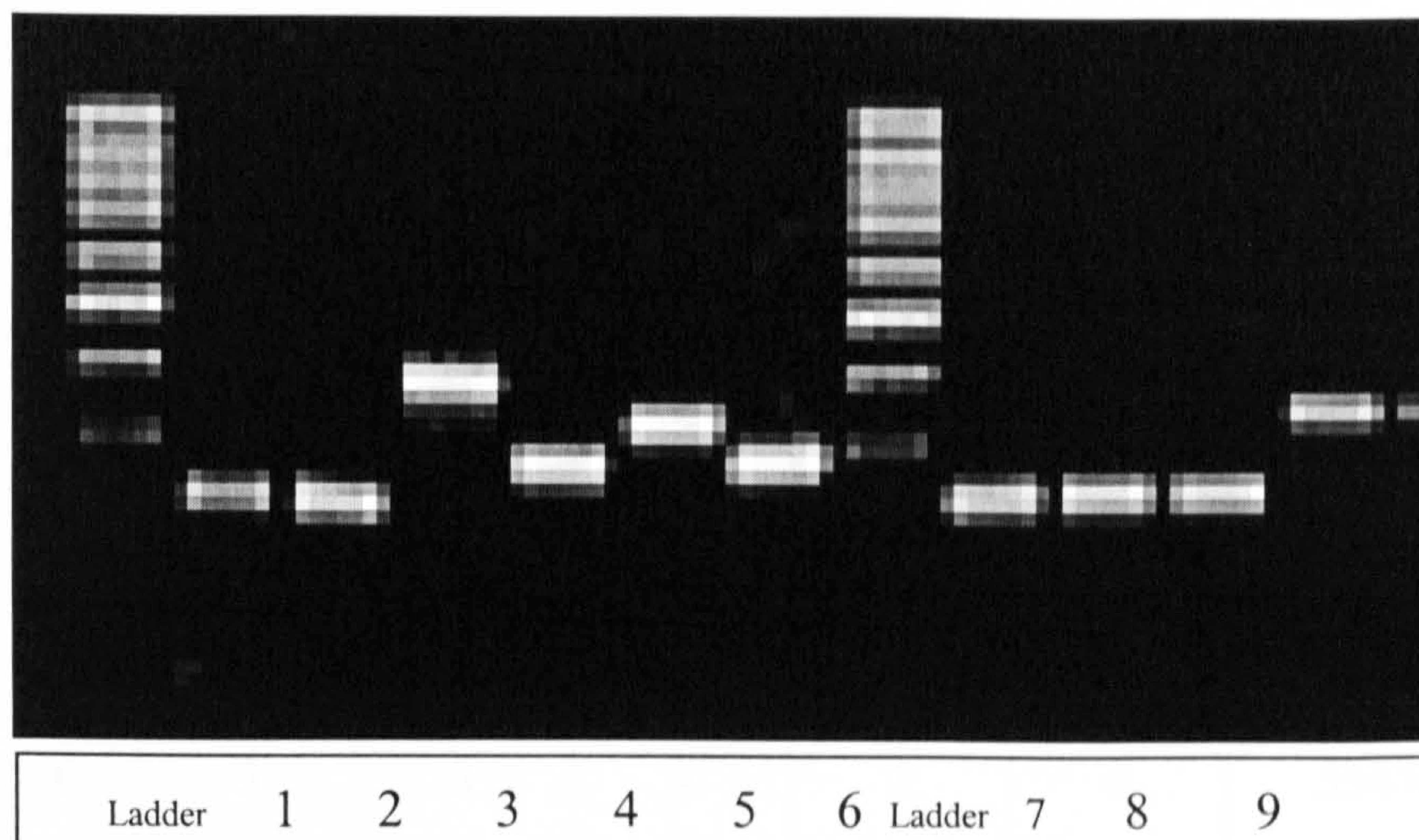
Lane	Product size	No of repeats
1	334	5
2	276	4
3	276	4
4	334	5
5	276	4
6	276	4
7	276	4
8	218	3
9	276	4
10	276	4

Figure 11. PCR products for ETR-D on 2% agarose gel stained with ethidium bromide. Calculations of the product size and number of repeats at this locus are given.



Lane	Product size	No of repeats
1	132	1
2	363	4
3	286	3
4	363	4
5	286	3
6	594	7
7	286	3
8	286	3
9	310	3
10	286	3

Figure 12. PCR products for ETR-E on 2% agarose gel stained with ethidium bromide. Calculations of the product size and number of repeats at this locus are given.



Lane	Product size	No of repeats
1	224	3
2	224	3
3	383	6
4	277	4
5	330	5
6	277	4
7	224	3
8	224	3
9	224	3
10	330	5

Table 13. Examples of VNTR profiles from agarose gels depicted in figures 8-12

No	ETR-A	ETR-B	ETR-C	ETR-D	ETR-E	Profile	Strain type
1	5	5	5	1	3	55513	BCG
2	4	2	4	4	3	42443	
3	4	2	4	3	6	42436	Beijing variant
4	4	4	5	4	4	44544	
5	4	2	4	3	5	42435	Beijing
6	5	3	4	7	4	53474	
7	2	2	4	3	3	22433	
8	3	2	3	3	3	32333	Haarlem
9	3	3	4	3	3	33433	H37Rv
10	4	2	4	3	5	42435	Beijing

Table 14. Table of VNTR Polymorphisms – Allele product size in bp (Frothingham *et al.* 1998)

No of repeats	**ETR A**	**ETR B**	**ETR C**	**ETR D**	**ETR E**
1	270	178	102	156	118
2	345	235	160	233	171
3	420	292	218	310	224
4	495	349	276	387	277
5	570	406	334	464	330
6	645	463	392	541	383
7	720	520	450	618	436
8	795	577	508	695	489

9.2 Results

9.2.1 Saudi Arabian Results

The VNTR method was applied to 85 isolates of *M.tuberculosis* isolated from different Saudi patients at The King Khalid national Guard Hospital, Jeddah, Saudi Arabia as a means of identifying clustering and resulted in 38 distinct profiles. Twenty-four patients yielded unique VNTR profiles. Sixty-one of 85 patients (72%) were found in clusters of two or more strains. The most common profile observed in the Jeddah population was VNTR profile 42235 accounting for 15 of 85 (17.6%) of strains. The Haarlem variant strain accounted for 12 of 85 (14%) of strains. Profile 22431 was unique to the Saudi Arabia study and was associated with 6 multidrug resistant strains of MTB. Only 2 of 85 (2.3%) strains of the Beijing family were found.

9.2.2 Queensland Results

201 *M.tuberculosis* strains isolated between 2000-2001 at the Queensland Mycobacterium Reference Laboratory were characterised by VNTR typing. 11 clusters of 3 or more isolates were identified. A further 8 clusters of two isolates were observed and 46 unique VNTR profiles were observed. Overall, 77% of the strains were found in clusters of two or more strains. Table 15 shows patients country of birth or ethnicity with the number of *M.tuberculosis* strains clustered by VNTR typing with 3 patients or more. The largest cluster observed accounted for 36 (18%) strains with VNTR profile 42435 belonged to the Beijing family. In this cluster of 36 patients, 10 were Caucasians, 14 patients were from SE Asian countries, a further 10 were from Papua New Guinea and two patients were indigenous Australians. A variant of the Beijing family with VNTR profile 42436 (Bifani *et al.*1999) was observed in 11 isolates. Of these eleven isolates, 10 were patients from Papua New Guinea and one patient was an Australian Caucasian. Both of the Beijing family profiles together accounted for 23.4%

of all isolates. A further two clusters 32333 and 32433 accounted for 24% of all strains. Table 16 shows the diversity of VNTR profiles encountered in Queensland. 45 unique VNTR profiles were observed among the 201 strains of *M.tuberculosis* investigated. A further 8 clusters of only two patients were observed.

9.2.3 VNTR profiles common to both study groups.

The Haarlem variant strain was the most common profile observed in both groups and accounted for 26 of 201 (12.9%) of the Queeensland strains and 12 of 85 (14.1%) of the Saudi strains. The most common profile among the Saudi strains was 42235. This strain was isolated in Queensland from 5 of 201 (2.5%) of patients including 3 patients from the Indian sub continent, 1 patient of Middle-East origin and 1 Caucasian.

9.2.4 VNTR analysis using ETR loci A-F and MIRU loci 10,16,and 40

77 strains of *Mycobacterium tuberculois* isolated from patients in Queensland during 2002 were evaluated using a combination of VNTR targeting ETR loci (A-F) and MIRU 10,16,40. Figure 13 is a dendrogram showing the relatedness of strains. 19 (24.8%) Beijing family strains (42435) were identified. The additional loci differentiated these strains into 7 distinct profiles with 424353333 accounting for 9 strains (47%). The 5 Beijing variant strains were further subdivided into 3 clusters. 14 Haarlem family strains (32333) were differentiated into 7 distinct profiles and the 10 Haarlem variant strains (32433) were subdivided into 9 distinct profiles using the combined ETR/MIRU analysis. Overall, 33 of 77 strains (43%) were clustered in groups of two or more strains. 44 of 77 (57%) strains showed unique profiles. Table 17 Shows the allelic profile frequencies of the 77 strains investigated by VNTR combination of ETR and MIRU. 54 different profiles were identified in the dataset.

Table 15. VNTR profiles based on ethnicity. Table shows the relationship of VNTR profiles in clusters of three or more strains observed in either of Queensland population or the Saudi Arabian study samples.

	Ethnicity									
VNTR Profile	No of strains	Caucasian*	Indian sub-continent	SE Asia	Papua New Guinea	Indigenous Australian	Middle East	African	South American	Saudi Arabia
22431	1			1						6
32432	3	1	-	2	-	-	-	-	-	1
52234	-									4
22432	4	3	-	-	1	-	-	-	-	-
42235	5	1	3	-	-	-	1	-	-	15
42433	2	2								4
42453	6	2	-	4	-	-	-	-	-	-
31433	7	1	2	2	-	2	-	-	-	1
22433	8	6	-		1	1	-	-	-	3
46464	11	2	-	6	2	1	-	-	-	-
32333 Haarlem	22	9	-	2	3	5	-	3	-	3
32433 Haarlem variant	26	9	-	1	2	13	-	-	1	12
42435 Beijing	36	10	-	14	10	2	-	-	-	2
42436 Beijing variant	11	1	-	-	10	-	-	-	-	-

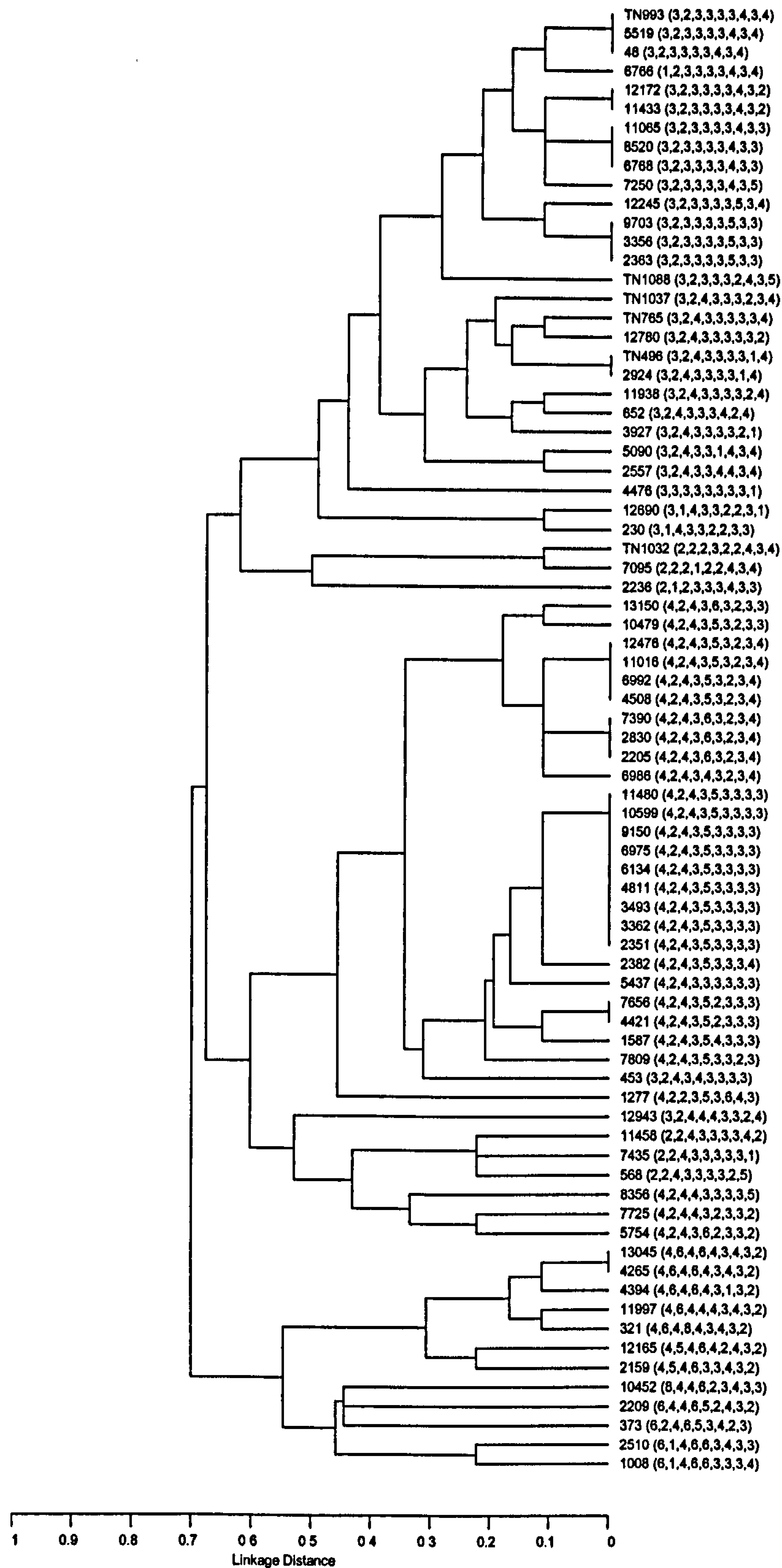
*includes Australian and overseas born

Table 16. Unique and shared VNTR profiles compared with ethnicity. Common profiles observed between Saudi strains and the Queensland strains are highlighted in bold.

Australian profiles	Country of Origin	Saudi Arabian Profiles
		12433
21233	Caucasian	
		21333 (2)
21432	Caucasian	
21433	Caucasian	
		22231
22232 (2 strains)	Australian aboriginal	
22333 (2 strains)	Caucasian	
22431	Chinese	
22434	**Caucasian**	**22434 (2)**
22533	Papua New Guinea	
31333 (2 strains)	Caucasian / Taiwanese	
		31332
		31433
32233	Unknown	**32233 (2)**
		32234
32235	**United Arab Emirates**	**32235 (2)**
		32265
32332	Caucasian	
		32423
		32432
32434	Caucasian	
32443	Caucasian	
33432	Vietnamese	
34435	Vietnamese	
36433	Australian aboriginal	
41433	Caucasian	
		42232
42234	**Somali**	**42234 (2)**
		42243
42323 (2 strains)	Papua New Guinea	
		42333
42423	Iraqi	
42425 (2 strains)	Caucasian / Korean	
		42432
42433 (2strains)	Latvian / Caucasian	
42434 (2 strains)	Burmese / Thai	
		42443
42438	Caucasian	
42445	Korean	
42464	Filipino	
42535	Caucasian	
42735	Somali	
		43235

		43333
		44235 (2)
44435	Papua New Guinea	
44464	Filipino	
45453	Unknown	
45463	Filipino	
45464 ((2 strains)	Thai / Filipino	
46424	Australian aboriginal	
46454	Caucasian	
46484	Filipino	
47464	Filipino	
49464	Filipino	
51464	Sri Lankan	
51643	Caucasian	
52435	Thai	
54445	Vietnamese	
61464	Caucasian	
		62235
		62416
		62433
		62466
64475	Vietnamese	
		64485
64543	Australian aboriginal	
		65416
74455	Indian	
74475	Vietnamese	
74495	Vietnamese	
83475	Vietnamese	
		84233
		84234
		84415
84575	Vietnamese	
94235	Australian aboriginal	
94254	Indian	
94264	Indonesian	

Figure 13. Dendrogram* showing relatedness of 77 strains of MTB evaluated by a combination of ETR and MIRU analysis.



* See Section 2.20.7

Table 17. Allelic profile frequencies of the 77 strains investigated by VNTR combination of ETR and MIRU analysis. 54 different profiles in dataset (displayed in descending order of frequency)

Profile (ETR A, ETR B, ETR C, ETR D, ETR E, ETR F, MIRU 10, MIRU 16, MIRU 40)	Frequency	% of dataset
4, 2, 4, 3, 5, 3, 3, 3, 3	9	11.69
4, 2, 4, 3, 5, 3, 2, 3, 4	4	5.19
3, 2, 3, 3, 3, 3, 4, 3, 4	3	3.90
4, 2, 4, 3, 6, 3, 2, 3, 4	3	3.90
3, 2, 3, 3, 3, 3, 5, 3, 3	3	3.90
3, 2, 3, 3, 3, 3, 4, 3, 3	3	3.90
3, 2, 4, 3, 3, 3, 3, 1, 4	2	2.60
4, 6, 4, 6, 4, 3, 4, 3, 2	2	2.60
4, 2, 4, 3, 5, 2, 3, 3, 3	2	2.60
3, 2, 3, 3, 3, 3, 4, 3, 2	2	2.60
3, 1, 4, 3, 3, 2, 2, 3, 3	1	1.30
4, 6, 4, 8, 4, 3, 4, 3, 2	1	1.30
6, 2, 4, 6, 5, 3, 4, 2, 3	1	1.30
3, 2, 4, 3, 4, 3, 3, 3, 3	1	1.30
2, 2, 4, 3, 3, 3, 3, 2, 5	1	1.30
3, 2, 4, 3, 3, 3, 4, 2, 4	1	1.30
6, 1, 4, 6, 6, 3, 3, 3, 4	1	1.30
4, 2, 2, 3, 5, 3, 6, 4, 3	1	1.30
4, 2, 4, 3, 5, 4, 3, 3, 3	1	1.30
4, 5, 4, 6, 3, 3, 4, 3, 2	1	1.30
6, 4, 4, 6, 5, 2, 4, 3, 2	1	1.30
2, 1, 2, 3, 3, 3, 4, 3, 3	1	1.30
4, 2, 4, 3, 5, 3, 3, 3, 4	1	1.30
6, 1, 4, 6, 6, 3, 4, 3, 3	1	1.30
3, 2, 4, 3, 3, 4, 4, 3, 4	1	1.30
3, 2, 4, 3, 3, 3, 3, 2, 1	1	1.30
4, 6, 4, 6, 4, 3, 1, 3, 2	1	1.30
3, 3, 3, 3, 3, 3, 3, 3, 1	1	1.30
3, 2, 4, 3, 3, 1, 4, 3, 4	1	1.30
4, 2, 4, 3, 3, 3, 3, 3, 3	1	1.30
4, 2, 4, 3, 6, 2, 3, 3, 2	1	1.30
1, 2, 3, 3, 3, 3, 4, 3, 4	1	1.30
4, 2, 4, 3, 4, 3, 2, 3, 4	1	1.30
2, 2, 2, 1, 2, 2, 4, 3, 4	1	1.30
3, 2, 3, 3, 3, 3, 4, 3, 5	1	1.30
2, 2, 4, 3, 3, 3, 3, 3, 1	1	1.30
4, 2, 4, 4, 3, 2, 3, 3, 2	1	1.30

4, 2, 4, 3, 5, 3, 3, 2, 3	1	1.30
4, 2, 4, 4, 3, 3, 3, 3, 5	1	1.30
8, 4, 4, 6, 2, 3, 4, 3, 3	1	1.30
4, 2, 4, 3, 5, 3, 2, 3, 3	1	1.30
2, 2, 4, 3, 3, 3, 3, 4, 2	1	1.30
3, 2, 4, 3, 3, 3, 3, 2, 4	1	1.30
4, 6, 4, 4, 4, 3, 4, 3, 2	1	1.30
4, 5, 4, 6, 4, 2, 4, 3, 2	1	1.30
3, 2, 3, 3, 3, 3, 5, 3, 4	1	1.30
3, 1, 4, 3, 3, 2, 2, 3, 1	1	1.30
3, 2, 4, 3, 3, 3, 3, 3, 2	1	1.30
3, 2, 4, 4, 4, 3, 3, 2, 4	1	1.30
4, 2, 4, 3, 6, 3, 2, 3, 3	1	1.30
3, 2, 4, 3, 3, 3, 3, 3, 4	1	1.30
3, 2, 4, 3, 3, 3, 2, 3, 4	1	1.30
3, 2, 3, 3, 3, 2, 4, 3, 5	1	1.30
2, 2, 2, 3, 2, 2, 4, 3, 4	1	1.30

9.3 Discussion.

9.3.1 VNTR typing in Saudi Arabia

Saudi Arabia, although underdeveloped technically, has a high per capita income, free treatment for active cases and compulsory screening of foreign workers. Saudi Arabia has an unreliable notification system for tuberculosis cases and to date no molecular epidemiological investigations have been undertaken in Saudi Arabia as a means of defining the relationship between mycobacterial strains and to clarify the epidemiology of tuberculosis (El-Kassimi 1994). This study over a two-year period highlighted the need to define the epidemiology of tuberculosis in Jeddah in order to plan strategies to control the disease. By investigating the DNA fingerprints in a population it is possible to gain an improved understanding of the bacterial transmission dynamics. It is hoped that a better understanding of the dissemination of the bacteria in a defined populations will improve the detection of new cases and the control of disease transmission. This approach involved fingerprinting all the mycobacteria in a population, assuming that unique strains are reactivated whereas shared strains were recently transmitted. The VNTR method was applied to 85 isolates of *M.tuberculosis* isolated from different Saudi patients at The King Khalid national Guard Hospital, Jeddah, Saudi Arabia as a means of identifying clustering and resulted in 38 distinct profiles. Twenty-four patients yielded unique VNTR profiles. Sixty-one of 85 patients (72%) were found in clusters of two or more strains. While the unique VNTR profiles clearly reflect different strains, the VNTR clustering amongst the remaining 61 patients must be interpreted with caution. The most common profile observed in the Jeddah population was VNTR profile 42235 accounting for 15 of 85 (17.6%) of strains. This profile was the most common profile accounting for 23 of 118 patients (19.5%) found in a study by Gasgoyne-Binzi *et al.* at the Leeds General Infirmary (LGI), England. The LGI serves

a large immigrant community from the Indian subcontinent with a high incidence of tuberculosis. This profile has previously been detected in a patient from India (Kremer *et al.* 1999) but was not detected in studies from Sicily (Sola *et al.* 2001) or Tanzania (Barlow *et al.* 2001). These studies suggest a strong geographical association of this profile with the Indian subcontinent.

Saudi Arabia imports labour from the Indian Subcontinent for construction, gardening, cleaning and unskilled manual labour. These temporary workers are housed in crowded conditions and have limited access to good health care. In this setting it is likely that Indian subcontinent strains of tuberculosis have been transmitted to Saudi Arabian nationals. Further evidence of the clonal association of this VNTR profile with the Indian subcontinent is evident in the analysis of VNTR profiles from Queensland, Australia. Of the 201 strains typed by VNTR, 5 strains showed the profile 42235. Four of these patients were of Indian subcontinent origin and one patient was Australian born. Another significant cluster of strains accounting for 12 of the 85 (14%) strains investigated gave the profile 32433. A third cluster accounting for 6 of the 85 strains (7%) with VNTR profile 22431 was associated with a group of strains which were all multi-drug resistant. This profile has not been reported in any other studies to date. This finding suggests that this strain may have been associated with transmission of multi-drug resistant tuberculosis. In a setting such as Saudi Arabia where drug resistance is common (Ellis *et al.* 1996, Al-Rubaish *et al.* 2001, Khan *et al.* 2001) VNTR typing can be a useful tool in rapidly identifying circulating clones of multidrug resistant strains of *M.tuberculosis*.

9.3.2 VNTR Typing in Queensland

Queensland has a low tuberculosis incidence rate (2.69 per 100,000 population in 1998) with the highest incidence occurring in migrants from high prevalence countries

followed by indigenous Australian-born people (Ward *et al.* 2001). Among non-indigenous Australians, tuberculosis most often occurs among elderly males, largely due to reactivation of latent tuberculosis. The general impression has been that most cases in Queensland are due to reactivation of latent infection acquired many years earlier, when TB was more prevalent, in Australian-born population or in the country of origin in overseas born people. Transmission within Queensland has usually been considered to occur in micro-epidemics as small clusters, particularly in indigenous communities but also in other social clusters. 201 *M.tuberculosis* strains isolated between 2000-2001 at the Queensland Mycobacterium Reference Laboratory were characterised by VNTR typing. 11 clusters of 3 or more isolates were identified. A further 8 clusters of two isolates were observed and 45 unique VNTR profiles were observed. Overall, over 80% of the strains were found in clusters of two or more strains. The largest cluster observed accounted for 36 (18%) strains with VNTR profile 42435 belonged to the Beijing family. In this cluster of 36 patients, 10 were Caucasians, 14 patients were from SE Asian countries, a further 10 were from Papua New Guinea and two patients were indigenous Australians. A variant of the Beijing family with VNTR profile 42436 (Bifani *et al.* 1999) was observed in 11 isolates. Of these, 10 were from patients from Papua New Guinea and one patient was an Australian Caucasian. Both of the Beijing family profiles together accounted for 23.4% of all isolates. A further two clusters 32333 and 32433 accounted for 24% of all strains.

9.3.3 Haarlem family

Profile 32333 corresponds to the Haarlem family strains which seem to be widespread globally. Strains with this genotype were designated the Haarlem family because the first recognised strain was isolated from a patient living in Haarlem, Netherlands (Kremer *et al.* 1999). In this study, the author refers to profile 32433 as a Haarlem

variant strain as it differs from the Haarlem family by one repeat at ETR-C and like the Haarlem family strains appear to be globally disseminated. Only a few population based epidemiological studies have been carried out using VNTR typing. In Sicily, Sola *et al.* evaluated the genetic diversity of 104 strains of *M.tuberculosis* using both VNTR typing and spoligotyping (Sola *et al.* 2001). This study found 14/104 strains corresponded to the Haarlem family and a further 12/104 strains demonstrated the Haarlem variant VNTR profile. Together these two profile accounted for 25% of strains isolated in Sicily (Sola *et al.* 2001). Similarly a study in England using VNTR typing as means of rapid identification of laboratory contamination with *M. tuberculosis* found the Haarlem and Haarlem variant strains accounted for 17% of all strains investigated (Gascoyne-Binzi *et al.* 2001). VNTR typing was applied to 66 strains of *M.tuberculosis* isolated from the French Caribbean between 1998-1999. Using VNTR typing alone 48 (73%) were grouped into twelve clusters. The most predominant cluster accounting for 36% of strains was VNTR allele 32333 corresponding the Haarlem family clade (Filliol *et al.* 2000). Only 2 of the 66 strains (3%) demonstrated the Haarlem variant profile. Similar results were observed in a study of 93 strains from Tanzania where only 2 strains (2.2%) were typed as Haarlem strains and 9.7% of strains were of the Haarlem variant (Barlow *et al.* 2001). Amongst the 201 Queensland strains typed by the VNTR method 22 (11%) were Haarlem strains and 26 (13%) were Haarlem variant strains. These strains were predominately found in Caucasian and indigenous Australian patients. These studies suggest that there is a predominance of *M.tuberculosis* isolates with similar VNTR profiles within discrete geographical areas and that evolution of these strains is being reflected by the VNTR profiles in the community.

Fillol *et al.* (2000) found VNTR typing poor in discriminating the 24 Haarlem clade strains which could be further subdivided into 10 distinct subtypes by spoligotyping. In

6 out of 12 (50%) cases, VNTR-defined clusters were further subdivided by spoligotyping, whereas 7 out of 18 (39%) spoligotyping defined cases were further subdivided by VNTR typing (Filliol *et al.* 2000). This study shows that a secondary typing method is needed to subdivide this clade. Further studies using additional VNTR loci may allow for more discrimination of strains in this group.

9.3.4 Beijing family

The "Beijing Family" of *Mycobacterium tuberculosis* isolates have shown little genetic diversity by several typing techniques (van Soolingen *et al.* 1995). This group of genetically related strains are the predominant strains in China and are highly prevalent in South-East Asia (Torrea *et al.* 1995, Palittapongarnpim *et al.* 1997). Van Soolingen *et al.* reported that strains of the Beijing family were found to dominate in China and surrounding countries, including Korea, Mongolia and Thailand whereas Beijing strains were rarer in the Middle East, Africa, south America and Europe. The Beijing family of strains was defined as containing only 9 of the 43 spacer sequences by spoligotyping and a 3.6kb *Pvu*II fragment by IS1081 fingerprinting (van Soolingen *et al.* 1995). All Beijing stain families have a deletion that includes spacers 1-34, but they also have multiple *IS6110* insertions (15 to 21) (Beggs *et al.* 2000). In 1999, Kremer *et al.* compared the degree of discriminatory power of several different typing methods in a interlaboratory study of 90 strains of *M.tuberculosis*. The eight "Beijing family" strains investigated as part of this study originated from four Southeast Asian countries and South Africa and were identical when evaluated with six genetic markers. The *IS6110* and spoligotyping patterns of these strains were clearly distinct and the VNTR profiles (Profile number 42435) were identical and distinct from all other strains. It is postulated that these strains, although disseminated globally, have expanded clonally from a recent common ancestor. (Kremer *et al.* 1999). The Beijing strain of *M.*

tuberculosis is reported most commonly in the Beijing area of China, accounting for 92% of strains. These strains have been dominant since the 1950's and have remained so in the 1990's in East Asia (van Soolingen *et al.* 1995, Qian *et al.* 1999). 70% of strains from *M.tuberculosis* isolates in Hong Kong represent the Beijing genotype. (Chan et al. 2001) Anh *et al.* recently reported that 53% of new tuberculosis cases from Vietnam were the Beijing genotype and were associated with patients of a younger age and, in isolates from Ho Chi Minh City, with resistance to isoniazid and streptomycin. (Anh *et al.* 2000). Prodinger *et al.* found the Beijing genotype in 44% of 204 *M.tuberculosis* isolates from Thailand (Prodinger *et al.* 2001). These figures are consistent with the original description of the Beijing family of strains in 1995, where 37% of Thai isolates were Beijing family strains (van Soolingen *et al.* 1995) and a subsequent *IS6110* RFLP analysis of 80/211(38%) isolates from central Thailand collected in 1994-1995 which belonged to the Beijing family (Palittapongarnpim *et al.* 1997) Members of the Beijing genotype have increasingly been found outside of Asia, in particular in South Africa and the United States where they have been implicated in several outbreaks. (van Soolingen 2001). The incidence of the Beijing family strains have been found to varying degrees in other geographical areas.

In Saudi Arabia only 2.3% of strains belonged to the Beijing Family. 5.3% of strains typed as Beijing family in a study of 118 cases from northern England (Gasgoyne-Binzi *et al.* 2001). No Beijing family strains were detected in molecular epidemiological investigations in Tanzania (Barlow *et al.* 2001) or from Sicily (Filliol *et al.* 2001). Among the Queensland strains investigated, 36 (8%) of strains were Beijing family strains. Of these 36 patients, 12 were from South East Asian countries, 10 were from Papua New Guinea, 10 were Caucasians and 2 were indigenous Australians. A variant of the Beijing family which differs by one allele at ETR-E, profile 42436, previously reported from Vietnam (Bifani *et al.*1999) was another clustered strain from the

Queensland study accounting for 5.5% of all strains investigated. This Beijing variant was isolated from 11 patients, 10 patients from Papua New Guinea and from one Australian born patient. . A total of 38 strains of *M.tuberculosis* from Papua New Guinea patients were typed as part of the study and 20 (53%) strains belonged to the Beijing family. The incidence of the Beijing family strains in Papua New Guinea is extremely high and equal to that reported from Vietnam (Anh *et al.* 2000). Le *et al.* found 80% of 168 strains in South Vietnam were the Beijing Genotype and significantly associated with younger aged patients (Le *et al.* 2000). Such evidence suggests that the Beijing genotype is emerging as an important component of the tuberculosis transmission pattern in Vietnam. A recent report of 119 pulmonary isolates of *M.tuberculosis* in the Archangel Oblast, Russia from 1998 and 1999 found 44.5% of strains were of the Beijing Genotype with 43.4% of these strains being multidrug resistant. (Toungoussova *et al.* 2002) In an earlier report of *M.tuberculosis* isolation between 1995-1997 from 74 patients in the Archangel Oblast, only 8.1% of strains were the Beijing genotype suggesting that the present high incidence represents recent transmission. (Toungoussova *et al.* 2002). A study by Caminero *et al.* in the Gran Canaria Island gives further support to the ability of this genotype to rapidly disseminate in a community. In this study from 1993-1996, 566 *M.tuberculosis* isolates were analysis by *IS6110* RFLP. The largest cluster contained 75 cases and was caused by a strain of the Beijing genotype which was introduced in 1993. It was subsequently found increasingly from 5.5% of patients 1993, 8.1% in 1994, 16.4% in 1995 and 27.1% of patients in 1996 (Caminero *et al.* 2001). The "W" strain , which caused a large outbreak of multidrug resistant tuberculosis in New York City and other cities in the United States and which accounted for 25% of all multidrug resistant cases in the 1990s also belongs to the Beijing family (Bifani *et al.*1996, Bifani *et al.*1999). The Beijing genotype has been documented to be associated with drug resistant tuberculosis

in Europe and South Africa (Portaels *et al.* 1999, van Rie *et al.* 1999). These reports and the results from the present study of Queensland strains document the Beijing genotype as the predominant strain found in distinct geographic locations. One potential explanation for this is that these strains have selective advantages over other *M.tuberculosis* genotypes in the ability to gain resistance and to interact with the host immune defence system (Glynn *et al.* 2002). Chance would predict that a few strains would eventually emerge as predominant in any given population, but this cannot explain how a single genotype has become predominant in many different, geographically separate populations. This implies that the Beijing genotype may have some advantage over other strains in its ability to be transmitted and cause disease. Anh *et al.* (2000) reported that Beijing family isolates were more commonly isolated from patients vacinated with BCG suggesting that BCG-induced immunologic protection may not protect against these strains (Anh *et al.* 2000). Other researchers have argued against any selective advantage, as Beijing isolates have spread widely in the United States, where BCG vaccination is not used (Bifani *et al.* 2002). However, BCG vaccination may accelerate the dominance of this family in regions once the strains have been introduced (Drobniewski *et al.* 2002). VNTR analysis will be a useful tool in monitoring further outbreaks of Beijing genotypes or in detecting any increase in the Beijing genotype in a given population.

9.3.5 Other common profiles

A predominant VNTR family of strains of *Mycobacterium tuberculosis* has been reported from South Asian patients. Gascoyne-Binzi *et al.* found profile 42235 to be a heterogeneous group of strains which predominates in patients from India and Pakistan. In this Queensland study, 5 patients were identified with profile 42235. Three of these patients were of Indian subcontinent origin, one patient was Iranian and another was

Australian born. 11 patients in this study shared profile 46464. Five of these patients were from the Philippines, one Malaysian, three from Papua New Guinea and two were Australian born. Of the 201 MTBC isolates investigated as part of this study only four strains were identified as *M.africanum* and all strains shared the profile 46464.

VNTR typing provides a useful platform to monitor distinct geographical populations for evidence of increased transmissions of a particular strain. It is important to ascertain whether the common circulating strains in a population are a result of virulence factors associated with a particular strain, host or environmental factors and more long term studies are required to determine increased rates of transmission amongst circulating strains in a population. Studies using VNTR have proved useful in determining genetic relationships between *M.tuberculosis* strains (Namwat *et al.* 1998, Frothingham *et al.* 1999). VNTR typing is not as discriminatory as the standard IS*6110* RFLP typing (Frothingham *et al* 1998, Kremer *et al.* 1999) but provides for a rapid reproducible typing profile which serves a useful first step approach to strain discrimination and conventional contact tracing. VNTR typing however, is particularly useful for isolates with low copy numbers of IS*6110* (Namwat *et al.* 1998). It is evident that a unique VNTR profile is evidence that transmission is not clustered among patients. In these cases no secondary typing method is warranted. VNTR typing is a useful tool in monitoring the incidence and transmission of clonal strains such as the Beijing family and provides a means of rapidly assessing any increase of transmission within a geographical region associated with this clone. This method has the ability to identify potential multi-drug resistant strains rapidly according to VNTR clusters. In this study almost 50% of strains were clustered amongst the Beijing and Haarlem families and variants by VNTR typing but clearly targeting additional polymorphic loci is needed to improve its discriminatory power. The addition of ETR F and MIRU loci to the VNTR

profile to generate a 9 digit profile showed a significant increase in discriminatory power when applied to 77 strains of MTB isolated in Queensland in 2002. Using the additional loci both Beijing and Haarlem family strains could be readily differentiated into distinct profile clusters. The greatly improved discrimination of common family strains with the ETR/MIRU VNTR analysis reduces the need for secondary typing strategies to confirm clustering of strains.

Chapter 10 General discussion

This study highlights the developments in tuberculosis diagnosis which have enhanced mycobacteriology services in wealthy countries over the last decade. Current diagnostic techniques for tuberculosis in all its forms are too slow, too expensive, and are ill-adapted to conditions in developing countries which bear the highest burden of tuberculosis. The major reasons for the current tuberculosis pandemic are the lack of an effective vaccine, the means to diagnose infection quickly and accurately and drugs specifically targeting the organism in the host. Diagnosis, in the majority of the world still relies on the 100 year old technique of acid-fast microscopy (Perkins 2000). Globally, DOTS has been endorsed by WHO as the preferred model for the monitoring and treatment of tuberculosis. (Bayer *et al.* 1995). The most controversial element of the DOTS model is reliance upon sputum microscopy, not culture for diagnosis. The two major drawbacks of microscopy are its insensitivity and inability to identify drug-resistant strains of TB (Iseman 2002). Sputum microscopy is insensitive, requires multiple visits, performs poorly in many HIV-coinfected patients and is inadequate for paediatric or extrapulmonary tuberculosis (Perkins *et al.* 2002). Microscopy in developing nations is typically performed on unconcentrated sputum using ZN stains. Unfortunately, this system only detects patients with very extensive, typically cavitary lung disease (Iseman 2002). Inability to detect drug resistance is the other drawback of microscopy. In the DOTS model, drug resistance is inferred by failure to respond to treatment, typically after 6 months of therapy. The obvious problems which arise from this approach are progressive damage to the lungs, even death from uncontrolled disease and or ongoing transmission of drug resistant organisms.

One of the most important technical advances for mycobacterial laboratories has been the development of nucleic acid amplification assays for the detection of *M.*

tuberculosis DNA in clinical specimens. However, this study and previous reports have found nucleic acid amplification more sensitive than microscopy but less sensitive than culture (Pfyffer 1999, Roth *et al.* 1997). From a diagnostic point of view, tuberculosis remains a low prevalence condition in developed countries and the fastest way to improve test performance in these countries is to select patients for testing in whom results will have a high predictive value (Perkins 2000).

Since this work was started there have been a large number of reports on the use of molecular methods for identification and typing of MTB. In November 2002 an entire issue of the CDC journal Emerging Infectious Diseases (Volume 8, No 11, November 2002) was devoted to this disease which emerged some 15,000 to 35,000 years ago, in an effort to understand the transmission dynamics (Navin *et al.* 2002). In 1996, CDC established the national tuberculosis and genotyping network in the United States to perform a prospective five year study on the usefulness of genotyping *Mycobacterium tuberculosis* isolates to TB control programmes (Crawford *et al.* 2002). The results of that study have now been published and a major weakness in the programme was the variability in the IS*6110* fingerprinting method and the difficulty of combining and analyzing DNA fingerprint images from multiple laboratories (Bradden *et al.* 2002). Approximately 20 percent of all isolates constituted low copy numbers (i.e. less than six) of IS*6110* strains which are not well discriminated by this method. (Bradden *et al.* 2002). The subjective nature of genotype interpretation and lack of specificity for some isolates may have resulted in some patients being misclassified as clustered or not (Crawford *et al.* 2002). In addition, the stability of IS*6110* RFLP fingerprinting and its adequacy for tracking epidemiological links remains under debate based on studies of the rates of IS*6110* changes in serial patient isolates (Alito *et al.* 1999, Cave *et al.* 1994, de Boer *et al.* 1999, Savine *et al.* 2002). McNabb *et al.* (2002) reported another major

limitation was the time required for obtaining results. In order for DNA fingerprinting to provide value to routine contact investigations, molecular data must be available in a timely manner such that the information can be used by tuberculosis programme managers in the investigation of clusters (Mc Nabb *et al.* 2002).

MIRU-VNTR typing has recently been proposed as a new basis for global analysis of MTB molecular epidemiology and population genetics (Mazars *et al.* 2001). MIRU loci have shown greater stability than IS*6110* RFLP patterns (Savine *et al.* 2002), they have a discriminatory power close to that of IS*6110* RFLP (Supply *et al.* 2001), and perform significantly better than IS*6110* RFLP when strains contain a low copy number of IS*6110* (Supply *et al.* 2000). The stability of these loci may help clarify the definition of exogenous reinfection, which is currently based on observation of changes of at least three or four bands in IS*6110* patterns (Warren *et al.* 2002). This issue is particularly important for accurate estimation of success rates of tuberculosis control programmes and for the evaluation of clinical trials of new therapies (Savine *et al.* 2002) This approach has several advantages over IS*6110* RFLP typing in that it is PCR based and can be performed on mycobacterial colonies without extensive extraction protocols and requires sizing of amplified products only by gel electrophoresis. The method has successfully been automated by combining analysis of multiplex PCRs on a fluorescence based DNA analyser (Supply *et al.* 2001).

MIRU analysis alone shows greater discrimination than VNTR typing targeting ETR A-E (Supply *et al.* 2001). The major problem with MIRU analysis is that it cannot be directly related to other studies on VNTR typing because of the different loci targeted.. The 12 polymorphic loci targeted by MIRU analysis are all not as polymorphic as ETR A-E. Each of the ETR loci contain at least six alleles (Frothingham *et al.* 1998). In

contrast, some of the MIRU loci are reported to demonstrate only 2 or 3 alleles (e.g. MIRU 2, 20, 24, 27). It would appear counter- productive to implement a typing scheme which would selectively exclude the more polymorphic VNTR loci. Cowan *et al.* has suggested that MIRU typing could become more discriminative by the addition of ETR loci A-C (Cowan *et al.*2002). Clearly such an approach would improve the discriminatory power of the method but would require fifteen individual PCR reactions for each strain. Although this approach could provide useful epidemiological information in real-time it would require significant labour and reagent costs. The approach used in this study combined the ETR loci and the more polymorphic MIRU loci in order to use the simplest approach to achieve maximal epidemiological information. In this study, ETR F and three MIRU loci 10,16 and 40 were selected because of their numbers of polymorphic alleles and that the described primer set utilised the same PCR conditions as ETR A-E (Mazars *et al.* 2000). A major advantage of VNTR typing is the ability to readily identify the clonal family strains including the Haarlem and Beijing clades which are identified by characteristic spoligotypes. The MIRU loci used in addition to ETR A-E allowed for good discrimination amongst both Beijing and Haarlem family strains. This strategy provides a useful platform to monitor distinct geographical populations for evidence of increased transmissions of a particular strain. It is important to ascertain whether the common circulating strains in a population are a result of virulence factors associated with a particular strain or host and or environmental factors.

The results of this study support the combined use of ETR and MIRU loci as an efficient alternative to IS*6110* RFLP or MIRU analysis alone as an exclusion/inclusion method for tracking ongoing transmission. In cases where ETR/MIRU VNTR profiles are identical, IS*6110* RFLP could be used as a second line analysis to investigate

potential epidemiological links between isolates. The two-step strategy would be expected to increase the accuracy of outbreak investigations and to considerably accelerate epidemiological studies of *M.tuberculosis* in real time. Whichever method is applied for global epidemiology of tuberculosis it needs to be easily performed by all public health laboratories and consistently over a long period.

10.1 Future Developments

Mycobacterium tuberculosis, is one of the most successful and scientifically challenging pathogens of all time. The complete 4.41Mb genome sequence is available for H37Rv, the paradigm strain for the slow-growing *M.tuberculosis* complex. Bioinformatic analysis led to the identification of approximately 4000 genes in the genome sequence, and provided fresh insight into the biochemistry, physiology, genetic and immunology of the tubercule bacilli (Cole *et al.* 1998). Information obtained is catalysing the conception of new therapeutic measures against tuberculosis, and enhancing our understanding of the biology of the aetiologic agent. Functional genomic approaches are increasingly being utilised to translate this linear sequence information into new therapies and diagnostics. Such strategies include the study of transcriptional regulation using DNA microarrays and differential protein expression using proteomics.

10.2 DNA arrays

DNA arrays are miniaturized checkerboards, each square containing a spot of DNA representing an individual gene and in sum representing the entire bacterial genome. An array that represents the entire genome of *M.tuberculosis* is 20x 20mm, and contains one spot for each of the 3924 genes of *M.tuberculosis*. (Small *et al.* 1999).

This technique allows detection of genetic variation at various genomic sites simultaneously by analysis of hybridization of mycobacterial DNA on high-density

oligonucleotide arrays containing thousands of DNA oligonucleotides on a limited surface. This technology has successfully been applied for monitoring gene expression and screening of mutations and polymorphisms in several human and viral genes. (Kozal *et al.* 1996, Lockhart *et al.* 1996). This approach has been used to examine the genetic variation amongst *M.bovis* BCG vaccine daughter strains in comparison with H37Rv (Gordon *et al.* 1999). Troesch *et al.* has exploited the use of DNA arrays to interrogate the sequence of regions from the 16SrRNA and *rpo*B loci to simultaneously identify and determine rifampicin resistant alleles. (Troesch *et al.* 1999) Other investigators have used mycobacterial DNA arrays for simultaneous genotyping and species identification (Gingeras *et al.* 1998). With these arrays, it would be possible to compare different strains of *M.tuberculosis*. This approach could be used to develop new diagnostics to differentiate individuals who are affected with tuberculosis from those already vaccinated.

10.3 Proteomics

The vast amount of information generated by the various genome sequencing projects has set the framework for experimentation and data analysis at genome-wide scales to fully elucidate the biology of *M.tuberculosis*. Proteomics analyses the complement of proteins expressed by a genome. Unlike genomes, proteomes are dynamic and change with tissue and time. Proteomics is a useful approach to identify immunodominant molecules. Through the use of 2-D protein maps of the subcellular fractions of *M.tuberculosis,* recent publications have demonstrated the feasibility of developing a tuberculosis antigen test in either sputum or serum to detect *M.tuberculosis* infection in a suspected patient and to monitor response to drug treatment.(Landowski *et al.* 2001) Landowski *et al.* suggest that immunodetection of elevated levels of circulating antigen

85 (Ag85) in serum by dot immunoblotting could provide a simple, rapid, and inexpensive diagnostic test for active tuberculosis.

10.3 Future Challenges

These major technical advances using array technology or proteomics may improve our ability to detect *M.tuberculosis* by detecting DNA or measuring protein expression. Additional technical progress is needed to refine, simplify and improve the performance of new TB diagnostics and to adapt them to laboratory conditions in developing countries. However, as none of the recently developed methods are ideal either due to a lack of sensitivity, resistance mechanisms which cannot be determined for all strains, strain discrimination methods which either over predict transmission or lack discriminatory power makes it evident that different tools will need to be applied to diagnose tuberculosis more efficiently in the future.

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Appendix 1: List of publications and awards from this research

Gilpin, C.M., Abdelaal, M., Osoba, A.O., Comparative study of Amplicor PCR and Ligase Chain Reaction for the direct detection of *M.tuberculosis* in clinical specimens. Clin Microbiol and Infect. 1997; 3(2): 158

Poster presented at the 8th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) Lausanne, Switzerland, May 1997

Gilpin, C.M., Abdelaal, M.A., Oni, GA., Osoba A.O. Comparative study of Amplicor polymerase chain reaction and ligase chain reaction for direct detection of *M.tuberculosis* in clinical specimens. Saudi Medical Journal. 1999; 20: 79-84.

Gilpin, C.M., Gascoyne-Binzi, D.M., Barlow, R.E.L., Hawkey, P.M. VNTR typing of *M.tuberculosis* as a rapid means of characterising transmission of multi-drug resistant strains in Saudi Arabia. Micobiological Methods: 1999

Gilpin C M, Dawson DJ, O'Kane G, Armstrong JG, Coulter C. Failure of commercial ligase chain reaction to detect *Mycobacterium tuberculosis* DNA from a patient with smear positive pulmonary tuberculosis due to a deletion of the target region. J Clin Microbiol 2002; 40: (6)

Awards

Scientific Award for publications of Abbott LCx Probe System Mycobacterium tuberculosis assay. **Abstracts: 1st Prize.**

Abstract presented at the 8th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) Lausanne, Switzerland, May 1997