Dedicated to my PARENTS.
Tuberculosis in Malaysia:
Molecular Aspects of Diagnosis and Epidemiology

A thesis submitted in fulfilment for the degree of
Master of Philosophy

by

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Abstract

The multicopy and specific insertion element IS6110 provide a good target for the detection of *M. tuberculosis* complex by amplification techniques such as Polymerase Chain Reaction (PCR). However, the emergence of IS6110-negative strains suggests that false negative results may occur if only IS6110 is used as the target for detection. In this report, a multiplex PCR system was established using primers derived from the insertion sequence IS6110 and a recently discovered IS-like element, designated as B9 (accession number 78639) which has been shown to be potentially useful for the detection of *M. tuberculosis* including IS6110-negative strains (Musa, 1996). The multiplex PCR was then evaluated using adult and paediatric clinical samples. The results shows that the multiplex system is both sensitive (93.1% and 76% in adult and paediatric cases respectively) and specific (89.6% and 72.1% % in adult and paediatric cases respectively) and exceeds that of the conventional methods of microscopy and culture.

The discovery of IS6110 single copy strains has triggered many questions regarding transposability of the IS element in these strains, their relationship to BCG and their epidemiological significance. The lack of transposability in these strains and in BCG are hypothesised to be due to mutation or mutations within this element particularly in the region designated as ORFa which is thought to regulate the expression of the transposase protein. In order to examine this hypothesis, the element in 8 single copy strains, chosen at random from clinical isolates were characterised. The sequence results indicated that the insertion sequence of the single copy strains of *M. tuberculosis* were identical to IS987 which was isolated from a single copy BCG strain. However data of a recently transposed element and a resequence of the original IS986 cloned copy showed that all the sequences were identical (B. Plikaytis and J. Dale, personal communications) and the differences between the published sequences of IS986 and IS987 were due to sequencing errors. This implies that the mobility of the IS elements is dependent on the transcriptional activity of the flanking chromosomal region and not within the IS itself. Further research in the evolutionary relationship of the strains and variants is needed to understand the transposability of IS6110 in these strains.
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Chapter 1
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Introduction

1.1 History of tuberculosis

Tuberculosis, caused by the bacterium *Mycobacterium tuberculosis* (*M. tuberculosis*), has been a plague of humanity for centuries, especially in populations suffering from malnutrition and poor living conditions. The clinical features as well as the communicable nature of this infectious disease have been known since at least 1000 B.C. Examination of a skeleton dating from the fourth millennium B.C., excavated from the Arene Candide Cave in Liguria, Italy, demonstrated evidence of spinal tuberculosis (Formicola et al., 1987). With the advent of the polymerase chain reaction technique, more precise evidence will be obtained from DNA extracted from infected sites in ancient corpses. Both Aristotle and Hippocrates described tuberculosis. Ancient Hindu recognised this dreadful disease as “the king of diseases and the disease of kings” (Collins et al., 1985b). In 1882, Robert Koch said “if the number of victims which a disease claims is the measure of its significance, then all diseases, particularly the most dreaded infectious diseases such as bubonic plague, Asiatic cholera and others, must rank far behind tuberculosis” (Ellner et al., 1993). At the turn of the century, there are still more deaths from tuberculosis than are due to any other single infectious agent. The important landmark in the eradication of tuberculosis was the discovery of bacillus Calmette-Guerin (BCG) vaccine by Calmette and Guerin in 1921. Due to the universal vaccination programme with BCG, together with the availability of specific anti-tuberculous drugs (beginning with streptomycin in 1945 and later isoniazid in 1952), and better living conditions in the Western world, there has been a steady decline in the incidence of tuberculosis in the developed countries. However, it still remains a major health problem in the Third World, especially in countries with poor public health and limited medical facilities (Murray et al., 1990). In the last few years, a revival of}
tuberculosis cases has been reported in the developed countries, prompting fears of a trend of increase in tuberculosis worldwide (Kochi, 1991; Bloom and Murray 1992; Brown 1992; Enarson 1992; Snider and Roper 1992; Collins, 1993; Hart et al., 1996). Three factors were thought to contribute to the increase of tuberculosis cases in the world, i) immigration from endemic countries, ii) reactivation or co-infection of tuberculosis with increasing cases of Human Immune Deficiency Virus (HIV) infection, and iii) outbreak of tuberculosis in congregate facilities such as hospitals, nursing homes, shelter for the homeless, residential facilities for Acquired Immune Deficiency Syndrome (AIDS) patients and correctional institutions (Ellner and Wallis 1989).

Although immigrant populations have long been recognised to be at high risk of developing tuberculosis (Davies, 1995), deteriorating social conditions appear to have a stronger link than migration in the recent increase in United Kingdom (UK) tuberculosis notification (Bhatti et al., 1995 and Mangtani et al., 1995).

In 1991, eight million new cases of tuberculosis were reported. It has been estimated that one third of the world’s population is infected with an annual death count of about three million, particularly in the developing countries (Murray et al., 1990).

In Malaysia, 10,000 to 12,000 cases were registered annually from 1985 to 1995. The data also shows an increasing trend in the number of cases detected beginning in 1984 with the majority of cases detected being pulmonary (Table 1.1) (Annual Report, Ministry of Health Malaysia, 1995).
Table 1.1 Annually registered tuberculosis cases in Malaysia from 1985-1995

<table>
<thead>
<tr>
<th>Year</th>
<th>Number of Tuberculosis cases detected</th>
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<tr>
<td>1985</td>
<td>10,569</td>
</tr>
<tr>
<td>1986</td>
<td>10,735</td>
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<td>1987</td>
<td>11,068</td>
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<td>11,708</td>
</tr>
<tr>
<td>1995</td>
<td>11,778</td>
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1.2 Mycobacteriaceae

The family Mycobacteriaceae, consisting of one genus, *Mycobacterium*, is a name assigned by Lehmann and Newman in 1896, in the Atlas of Bacteriology. The genus originally consisted of two species; *M. tuberculosis* (tubercle bacillus), first described by Robert Koch in 1880, and the leprosy bacillus (*Mycobacterium leprae*), observed by Armauer Hansen in 1874. The name Mycobacterium means fungus-bacterium arising from the characteristic fungus-like pellicle produced by the tubercle bacillus when grown in liquid media. Apart from *M. leprae*, the genus *Mycobacterium* has been dominated by the species *M. tuberculosis* because of the widespread disease it causes. With the emergence of HIV, the pathogenic potential of other mycobacterium species has now been recognised. Mycobacterial species such as *M. kansasii* and *M. avium-intracellulare* cause disseminated infections in immunocompromised patients (Wayne, 1986). This suggests that the atypical mycobacterium can also be clinically significant, which stimulated changes in attitudes and interest towards mycobacterial species other than *M. tuberculosis* (Grange, 1980).
1.3 Properties of Mycobacteriaceae

1.3.1 Acid fastness

The characteristic and most well known property of mycobacteria is its acid fastness (Grange, 1988). This is referred to as the capacity to resist decolorisation by weak mineral acids (such as acid or acid-alcohol), when stained with an arylmethane dye (such as carbol fuchsin). Most of the staining procedures that are used to demonstrate this property are modifications of the methods derived by Ziehl and Neelsen whose name the method now bears (Bishop and Newmann, 1979). Some other micro-organisms such as some strains of *Nocardia*, *Corynebacterium* and *Rhodococcus* can sometimes be weakly acid-fast.

1.3.2 Morphology and cell wall structure

*M. tuberculosis* are classified as Gram-positive non-spore forming rod shape organisms apart from being acid-fast. It is about 1 to 10\(\mu\)m long and 0.5 to 1\(\mu\)m in width. *M. tuberculosis* usually, but not always, forms characteristic serpentine cord structures under the microscope. Mycobacterial species all have a complex lipid rich cell wall. This cell wall structure is probably responsible for the acid fastness. Studies revealed that the sphaeroplasts of *M. tuberculosis* obtained in pure culture seemed to be non pathogenic unless they reverted to the wild type bacilli (Ratnam and Chandrasekhar, 1976), thus indicating the virulence of *M. tuberculosis* is closely associated with the cell wall component. Cellular morphology ranges from rods (*M. tuberculosis*), curved rods (*M. microti*), and coccobacillary (*M. avium*) although ovoid and filamentous forms may also be seen.
1.4 Transmission of tuberculosis

The disease caused by \textit{M. tuberculosis} is normally transmitted via the lung although rare portals of entry, such as through broken skin do exist. Ingestion is the main route of transmission in the case of \textit{M. bovis} infection. Some predisposing factors for the development of the disease include acquired or congenital immunodeficiency, alcoholism, use of immunosuppressive drugs, diabetes, malnutrition, stress and old age. Aerosolised pulmonary secretions are expelled through coughing or sneezing. The bacilli subsequently migrate to the alveoli in the lung. In the alveoli, the bacilli are phagocytosed by macrophages. However, they resist killing by the phagocytes and multiply within the macrophages. The macrophages subsequently lyse resulting in the release of bacilli which may trigger the disease. Milk contaminated with \textit{M. bovis} may lead to infection in the tonsils and intestine. Close contact with a “smear positive” patient poses a maximum risk of infection with 25-50\% of those exposed becoming infected (Steady, 1981) but most of these do not develop the disease. The bacterium can remain dormant for many years; as a consequence, a decline in immunity later in life can allow reactivation of the infection, which then progresses to active tuberculosis. Of those initially infected, the normal lifetime risk of reactivation is estimated to be about 10\% (Styblo, 1991). The advent of AIDS is causing a dramatic change in this figure. The likelihood of patient previously infected with tuberculosis and subsequently infected with HIV developing active tuberculosis is 50\% (Horsburg and Selik, 1989) which would have significant impact in a tuberculosis endemic country such as Malaysia. Not only is tuberculosis the actual cause of death of a high proportion of AIDS patients (especially in the tuberculosis endemic area), but the increasing numbers of active tuberculosis amongst AIDS patients also causes an increased risk for the population as a whole. \textit{M. tuberculosis} could also be a factor in the progression from HIV positive status to AIDS (Selwyn \textit{et al}., 1989).
1.5 Growth Characteristics

*M. tuberculosis* (and probably all other mycobacteria), is a strict aerobe although the growth is enhanced by incubating in an atmosphere of 5-10% carbon dioxide (Beam and Kubica, 1986). The addition of a low percentage of glycerol to the liquid culture media such as 7H9 medium, encourages the growth of *M. tuberculosis* but might inhibit growth of the bovine strains. Tubercle bacilli will grow on top of a liquid medium as a wrinkled pellicle if the inoculum is carefully floated on the surface and the flask left undisturbed. Otherwise they will grow as floccules throughout the medium. Addition of Tween 80 to a concentration of 0.05% in the liquid medium also facilitates a dispersed growth. On solid medium such as Lowenstein Jensen medium (LJ), the colonies are of irregular shape with dry wrinkled or mamellated surface, tough and tenacious consistency with a creamy white colour which later becomes buff.

1.5.1 Conditions of growth

Members of the genus Mycobacterium are commonly classified as slow or fast growers. *M. fortuitum* has a doubling time of 2.5 hours, *M. tuberculosis* is about 18 hours and *M. paratuberculosis* is even slower. *M. leprae* is a unique member of the mycobacteria in that it is an obligate parasite. So far, it only can be propagated on living tissue. It has an estimated generation time of about 12 days with optimum growth at about 30°C. The optimum growth temperature for *M. tuberculosis* is 37°C with pH 6.0-6.5. Some strains of mycobacteria produce pigment in the presence of light (photochromogens) while others produce pigment in the dark (scotochromogens) or do not produce pigment at all. Bovine serum albumen (BSA) is usually added to media for growth of many mycobacterial species. It improves growth by neutralising free fatty acids which might be toxic to the cell. It also serve as a source of amino acids hence benefiting nitrogen
metabolism of the cell. Primary cultures from clinical samples usually yield visible colonies after 2-5 weeks of incubation.

1.6 Mycobacterial Genetics

Mycobacterial species have large genome sizes (3.1x10⁹ to 4.5x10⁹ daltons) compared to most other prokaryotes (Bradley, 1973; Baess and Mansa, 1978; McFadden et al., 1987a), but the pathogenic mycobacterial species seems to have smaller genomes than other mycobacteria (Clark-Curtiss et al., 1985). Genomic sizes of M. tuberculosis, M. leprae and M. bovis BCG are 2.5x10⁹, 2.2x10⁹ and 2.8x 10⁹ respectively (Bradley, 1973). Generally, mycobacterial species have a high GC content which ranged from 64% to 71% (Baess and Mansa, 1978) whereas M. leprae and M. lufu have lower GC contents of 58% and 61% respectively (Clark-Curtiss et al., 1985). DNA from the M. tuberculosis complex were shown to have 78-97% homology to M. tuberculosis H37 chromosomal DNA. Chromosomal DNA of M. fortuitum, M. phlei, M. smegmatis and M. vaccae have homology of 4 to 26% with that of M. tuberculosis (Athwal et al., 1984; Grosskinsky et al., 1989).

1.7 Plasmids in mycobacteria

There have been notable advances in the past few years in understanding the biochemical and genetic basic of antibiotic resistance in M. tuberculosis. Many laboratories have attempted to detect plasmids in M. tuberculosis that could be responsible for antibiotic resistance, but with very little success. An early report by Alberghina, et al., (1973), claimed that aminoglycoside resistance in M. tuberculosis was extrachromosomally mediated and Zainuddin and Dale (1990), showed apparently extrachromosomal bands
in some strains of *M. tuberculosis* by gel electrophoresis, but the data was not conclusive.

The general belief is that *M. tuberculosis* does not naturally contain plasmids. Resistance to several anti-tuberculosis drugs such as isoniazid, rifampicin and streptomycin, is now known to be due to mutation in chromosomal genes and not to the presence of plasmids (Telenti *et al.*, 1993; Zhang, 1993, Banerjee *et al.*, 1994). The reason for this is not clear, especially as plasmids are common in some other mycobacterial species, such as *M. avium* complex. Artificially constructed plasmids have been successfully introduced into *M. tuberculosis* and BCG, by electroporation and are stably maintained (Stover *et al.*, 1991; Norman *et al.*, 1995; McAdam *et al.*, 1995). The most likely hypothesis is that *M. tuberculosis* is normally parasitic and has little contact with other bacteria for it to acquire plasmids from them, whereas *M. avium* is widespread in the environment with the consequent opportunity to acquire plasmids from other bacteria.

In fact, mycobacterial plasmids were first demonstrated in strains of the *M. avium* complex (Crawford and Bates, 1979), and the occurrence of plasmids in *M. avium, M. intracellulare* and *M. scrofulaceum* (MAIS) complex has since been reported (Crawford and Falkinham, 1990). Plasmids have also been reported in isolates of *M. fortuitum* (Labidi *et al.*, 1984 and Hull *et al.*, 1984), including the most thoroughly studied mycobacterial plasmid designated as pAL5000. The sequence of this plasmid has been determined, and it provides the basis for the construction of many shuttle plasmids that are used as cloning vectors for mycobacteria. The presence of plasmids in mycobacteria is significant for several reasons. In other bacteria, plasmids frequently encode drug resistance, virulence factors, antigens, and various functions that promote survival in the environment. Plasmids are excellent markers for epidemiological studies in such bacteria.
1.8  *M. tuberculosis* complex

The *M. tuberculosis* complex is actually a group of very closely related mycobacteria but can be differentiated using a number of biochemical and antigenic markers. The differentiation is distinct enough for division into actual species or variants but for convenience they are usually referred to as the *M. tuberculosis* complex. Some authorities prefer to regard the *M. tuberculosis* complex as a single species, which molecular analysis would support, and the ‘species’ within it as variants. In diagnostic laboratories an acid fast bacillus is identified as *M. tuberculosis* complex or atypical mycobacteria. This information is usually adequate for therapeutic purposes. Further differentiation of *M. tuberculosis* complex into the species level is only done for epidemiological or research purposes. In neonates and immunocompromised patients, isolates identified as *M. tuberculosis* are usually further determined as BCG or otherwise.

The *M. tuberculosis* complex consists of *M. tuberculosis*, *M. africanum*, *M. bovis*, *M. bovis* BCG, and *M. microti*. Collins, et al., (1982) further subdivided the *M. tuberculosis* complex into species and variants. The most common causes of tuberculosis in man are the Classical, Asian and the two African variants. *M. bovis* is an important pathogen causing tuberculosis in cattle but occasionally infects man. BCG is an attenuated vaccine strain used world wide in the control and prevention of tuberculosis (and relatively recently, leprosy). It occasionally causes disease in neonates and immunocompromised hosts. Recent developments in the identification of genetic markers have allowed even further differentiation of members of this complex into specific strains, thus allowing more sensitive epidemiological studies.
1.9 Methods for identifying the *M. tuberculosis* complex

In most bacterial infections, there are substantial numbers of bacteria present, and detection of only a few organisms would not be regarded as significant (cerebral spinal fluid (CSF) and blood excepted), but in the case of tuberculosis infection, a single organism detected can be considered diagnostic.

Many methods are currently used for the detection of the *M. tuberculosis* complex, including microscopy, culture, biochemical assays, immunological assays, gene detection systems, physical identification based on pigmentation and colonial morphology, assessment of growth on nutrient agar and growth at different temperature. Methods for the detection of *M. tuberculosis* can be divided in three parts, namely;

i) Detection methods

ii) Identification methods

iii) Typing methods

1.9.1 Methods for detection

1.9.1.1 Microscopy by staining method

This method demonstrate the presence of tubercle bacilli by direct microscopy. One such technique is the Ziehl-Neelsen staining method, which uses a strongly acting dye (carbol fuchsin), together with heat application to facilitate impregnation; this is followed by decolourization with acid alcohol and then counter staining with methylene blue or malachite green. The tubercle bacillus stains red with a bluish background if methylene blue is used as the counter stain. Most samples can be stained using this method. It is done mostly for samples collected from patients with open pulmonary lesions.
An alternative staining method is by auramine-rhodamine stains, followed by detecting the bacillus under a fluorescence microscope, but this is more expensive compared to Ziehl-Neelsen as the fluorescence microscope is expensive and also requires well trained personnel to interpret the results. This method is therefore not widely used in a developing country like Malaysia.

Staining methods are rapid but a negative result does not exclude tuberculosis infection as at least some $10^4$ bacilli must be present in 1 ml of specimen for a positive microscopic finding. Sensitivity is reported as ranging from 22-78% of culture proven cases (Pfaller, 1994). Although the acid-fast bacilli detected does not distinguish *M. tuberculosis* from other mycobacteria, nevertheless, the rapidity in obtaining results compared to the weeks required for culture makes it the most widely used screening method. In Malaysia, a sample would be reported as AFB positive at this stage and if the clinical picture shows signs and symptoms of tuberculosis infection, treatment would be started without waiting for culture results.

### 1.9.1.2 Tuberculosis culture

Samples are usually cultured on Lowenstein-Jensen (LJ) medium. The LJ medium is an egg-based medium which contains asparagine, glycerol and mineral salts. The dye, malachite green, is also incorporated to serve both as an inhibitory agent to some bacteria and fungi as well as to give a better background against which the colonies can be easily observed.

In order to carry out culturing of the bacilli from a specimen, decontamination procedures have to be carried out (Grange, 1988). This is vital as the presence of contaminants which include fungi and other bacteria will rapidly grow on the medium, hence suppressing the growth of the mycobacterial species. The culture is then incubated
for 6 to 8 weeks before a culture result is dispatched. Samples grown on LJ medium will then be further examined by tests which can differentiate up to the species level.

Culture is recognised as the “gold standard” for the diagnosis of *M. tuberculosis*; however, it has a detection limit of 100 viable bacilli/ml. Subsequent biochemical analysis for species identification requires a further 2-3 weeks. The definitive diagnosis is the identification of the organism in the specimen. The introduction of new culture based methods such as BACTEC radiometric system (Becton Dickenson Diagnostic Instrument Systems, Sparks, Maryland) has reduced these times, but are often too expensive for use in many laboratories and are still considerably slower than conventional bacteriological methods for diagnosis of many other diseases.

1.9.1.3 Immunological detection methods

A variety of serological tests have been developed to detect antibodies against tuberculosis; however most of these tests lack the specificity and sensitivity required to make them clinically useful.

ELISA measures antigen-antibody binding in a solid phase. The antigen used will determine the specificity of the ELISA system developed. Both crude and purified antigen have been employed but as expected highly purified antigens are more specific with sensitivities of between 65-85% for culture positive specimens of extrapulmonary tuberculosis (Daniel and DeBanne, 1987). Mathai *et al.*, (1991), also developed a Dot Enzyme Immunoassay (Dot EIA) for the detection of *M. tuberculosis* antibodies in cerebrospinal fluid using *M. tuberculosis* antigen 5 isolated from *M. tuberculosis* H37Ra. They claimed the Dot EIA has a sensitivity of 70% and a specificity of 100% in detecting tuberculous meningitis patients.
Serological methods are believed to be more useful in detecting extrapulmonary tuberculosis. Despite the sensitivity of serological tests for tuberculosis, there is the problem of specificity to be solved. No single species-specific antigen of *M. tuberculosis* has been shown to be able to elicit significantly elevated levels of antibodies in all cases of the disease. There is also the problem of antigenic cross reactions among mycobacterial species and with species of other genera (Grange, 1989).

1.9.1.4 **BACTEC detection system**

This radiometric method was developed for the detection, identification and drug susceptibility testing of mycobacteria. The procedure involves growing the bacilli in 7H12 broth and growth is detected by measuring the release of $^{14}$CO$_2$ from $^{14}$C-palmitic acid. The positive attribute of this method is that it is faster with an average detection time of 10 days compared to 17 days for conventional culture technique (Anargyros *et al.*, 1990).

1.9.1.5 **The BacT/Alert detection system**

The BactT/Alert System was developed by Organon Teknika Corp, Durham, New York. It is a fully automated, non-radiometric blood culture detection method. Growth is indicated by an increase in CO$_2$ production, and is monitored by the changing colours of the growth media. Preliminary studies using clinical specimens showed that this is a good system for detecting mycobacteria (Wilkins and Thorpe, 1992). The method and the Middlebrook 7H9 broth are being modified to improve mycobacterial detection (Wellstood, 1993)
1.9.1.6 The Septi-Chek AFB

This is a biphasic culture system for the rapid isolation of mycobacteria introduced by Roche Diagnostics. It consists of a bottle of Middlebrook 7H9 broth and a three-sided paddle coated with Middlebrook 7H11 agar, modified egg media and chocolate agar. After adding antibiotic and growth supplements, the broth is inoculated with an aliquot of the specimen. The paddle is then screwed onto the vial, which is inverted to subculture the broth onto the agar paddle. Culture can be examined for up to 8 weeks. Although the sensitivity of the Septi-Chek AFB system is comparable to the BACTEC system (94.2% and 93%, respectively), the detection time for the Septi-Chek system is 19 days compared with 13 days for the BACTEC system (Abe et al 1992). However, the use of a specific DNA probes for *M. tuberculosis* to test the Speti-Chek broth sediment will reduce the time of detection to 8 days (Drummer and Szabo, 1992).

1.9.1.7 Gas Liquid Chromatography detection method

This method detects fatty acid of the mycobacterial cell walls. The type and amount of fatty acids are species-specific (Buesching et al., 1991). The Microbial Identification System (MIS) is a commercially available gas-liquid chromatography system for the rapid identification of mycobacteria and other organisms. The MIS, based on the unique fatty acids profiles of various micro-organisms, consists of a gas chromatograph with capillary columns, a flame ionisation detector, an autosampler, an integrator, a computer and a printer. The extracted sample containing the fatty acid methyl esters are injected into the chromatograph column and as they elute from the column, the fatty acid is detected by a flame ionisation detector which then send a signal to the integrator for processing. It will then be analysed using a computer with databases containing cell wall fatty acid profiles for well characterised species of mycobacteria.
The MIS has a sensitivity of 94% in identification of mycobacteria isolates compared with conventional biochemical methods (Buesching et al., 1991).

1.9.1.8 Gene probe detection systems

Probe methods utilise nucleic acid hybridisation with specific labelled sequences in the test sample. One of the tests was developed by GEN-PROBE and involved the hybridisation of single stranded $^{125}$I-labelled DNA probes with complementary ribosomal RNA giving a DNA-RNA hybrid. The double stranded hybrid is then separated from the single stranded molecules and the amount of radioactivity emitted by the DNA-RNA hybrid is measured. The results are calculated as percentage of input probe that has hybridised. A hybridisation of 10% is regarded as positive.

Another gene probe based detection system (SNAP), developed by Synegene, San Diego, California, utilises an alkaline phosphates-labelled probe that is directed towards a portion of the rRNA gene. The target is immobilised on a membrane and hybridisation is detected by a calorimetric assay.

Probes are available for several mycobacterial species including *M. tuberculosis*, *M. avium*, *M. intracellulare*, *M. kansasii*, and *M. gordonae*. The advantages of gene probe methods includes automation and high specificity for the members of the *M. tuberculosis* complex (>95%). But they do not differentiate between the members of the *M. tuberculosis* complex. The target sequences for these probes are also often too short, in the case where there is an occurrence of a mutation or natural variation, it may lead to failure to identify that organism (McFadden et al., 1990).

The sensitivity of both nucleic acid probes and immunoassay is at about $10^5$ organism per millilitre of sample (Daniel, 1989), whereas the number of bacilli in many clinical
samples is much lower than this. Gene probes therefore are not sufficiently sensitive for the direct detection of mycobacteria in clinical specimens. Nevertheless, gene probe hybridisation is the method of choice for the speciation of mycobacteria after isolation and growth in culture. Despite the success of these detection methods, there are some inherent problems such as high cost of machinery and training of manpower, instability of reagents and low specificity in some cases. It should be noted that some strains of *M. terrae* complex cross react with DNA probes (Syngene, Inc., and Gen-Probe, Inc) for *M. tuberculosis* complex (Ford *et al.*, 1993).

1.9.1.9  **Polymerase Chain Reaction (PCR)**

PCR provides rapid methods for detecting mycobacterial DNA or RNA directly from clinical samples. A number of investigators have reported the usefulness of PCR (De Wit *et al.*, 1990; Brisson-Noel *et al.*, 1991; Kolk *et al.*, 1992; Clarridge *et al.*, 1993; Forbes *et al.*, 1993; Nolte *et al.*, 1993; Kox., 1994; Miller *et al.*, 1994; Pfyffer *et al.*, 1994) but its ability for direct detection of mycobacteria varies according to not only the operational skills of the laboratory, but also the PCR techniques, including lysis methods, the target region of nucleic acid, and the systems employed for detecting the amplified products (Pierre *et al.*, 1991; Clarridge *et al.*, 1993; Kox *et al.*, 1994; Noordhoek *et al.*, 1994). Simplified and standardised amplification systems are, therefore, needed to fit into the work flow of a clinical laboratory. In this thesis, a multiplex PCR system for the direct detection of *M. tuberculosis* in clinical samples was used. The PCR system will be further discussed in the later chapter.
1.9.1.10 Other amplification systems

Other amplification techniques have been developed to avoid the restrictions of the patented PCR process and payment of licence. These amplification techniques include strand displacement amplification (SDA) by Becton Dickenson and transcription mediated amplification (TMA) by Gene Probe Incorporated.

1.9.1.10.1 Strand Displacement Amplification (SDA)

SDA is an isothermal amplification process which utilises the ability of the Klenow fragment of *E. coli* DNA polymerase, starting at the site of a single stranded nick (created by the enzyme HincII) in a double stranded DNA, to extend one strand from the 3' end, and displace the downstream strand of DNA. The process is repeated and it is estimated to produce $10^7$-$10^8$ fold amplification in about 2 hours, which is similar to figures claimed for PCR. Since SDA is an isothermal technique, it has the advantage of not requiring the use of thermal cycler. This method is very sensitive and has been shown to detect as few as one *M. tuberculosis* genome containing 10 copies of IS6110 target sequence (Spargo *et al*., 1993)

1.9.1.10.2 Transcription Mediated Amplification (TMA)

TMA is also an isothermal target based technique. Jones *et al.*, (1993), combined this method with a homogeneous detection method to detect *M. tuberculosis* in clinical samples. This test amplifies rRNA from lysed mycobacteria. The rRNA target sequences are copied into a transcription complex by using reverse transcriptase and RNA polymerase is used to make numerous RNA transcripts of the target sequence from the
transcription complex. The process is then repeated automatically. Three or four hours is needed for the entire assay.

1.9.2 Methods for identification

1.9.2.1 Biochemical Techniques

Biochemical assays for identification of *M. tuberculosis* include tests for niacin production, catalase or peroxidase activity, nitrate reduction, tellurite reduction and arylsulphatase test. The biochemical activities are usually detected as the production of gas or colour. Growth in 5% NaCl or failure to grow in thiopene-2-carboxylic hydrazide (TCH) test is usually recommended for organisms resembling *M. tuberculosis* as some *M. bovis* strains will produce small amounts of niacin and could be mistaken for *M. tuberculosis*. *M. tuberculosis* will grow in 5% NaCl and medium containing TCH while *M. bovis* will not grow. The number of different tests needed for the full confirmation of a species is laborious and expensive.

1.9.2.2 Molecular techniques

As mentioned in the earlier section, gene probes are useful in particular for speciation. PCR is also increasingly used not only for the detection of tuberculosis but also for the identification of mycobacteria generically.
1.9.3 Methods for typing of mycobacterial species

The objectives of the typing scheme are to group isolates according to similarities they share. This typing scheme will generate data which can be applied in fields such as epidemiology and nomenclature. The grouping defined by one typing scheme will usually differ to that defined by another scheme. The implication of the scheme of choice will mostly depend on the specific needs of the researcher.

1.9.3.1 Serotyping

Serotyping has been widely used in characterising members of the *M. avium* complex (Schaefer, 1968). Other more specific typing schemes have shown that some strains classified as *M. intracellulare* are actually *M. avium* (McFadden et al., 1987b). Immunological techniques like enzyme-linked immunosorbant assay (ELISA) (Grange, 1989) and latex agglutination has been employed in serotyping. It involves serodiagnosis of the antigenic components and also detection of the tubercle bacilli in other specimens. However most of these tests lack the specificity and sensitivity required to make them clinically useful.

1.9.3.2 Phage typing

Phage typing relies on the ability of certain bacteriophages to lyse members of the *M. tuberculosis* complex or other mycobacteria. These phages are called mycobacteriophages and they are known to be common in the environment. They differ in host range, morphology, antigenic structure and plaque morphology. For the cell infection to occur, there must be suitable phage receptors on the cell wall for the phages to adhere, and the host cell must lack systems that will restrict the phage DNA. *M.*
Tuberculosis can be divided into 4 phage types, namely A, I, B and C, while BCG form a separate phage type (Grange, 1982). Phage typing is also seen to be related to the geographical origin of the bacilli. For example phage type I is common to Asia. Most of the strains from Hong Kong, Japan and Central Africa are of phage type A. Phage type B is common in Europe and America.

Despite its proven ability to type strains of tuberculosis, phage typing is not widely used due to lack of discriminating power and reproducibility.

1.9.3.3 Genomic typing

This scheme relies on the generation of unique restriction length polymorphism (RFLP) patterns for different strains using a DNA probe. The RFLP will depend on the distribution of the enzyme restriction sites for the endonuclease in the genome. Employing appropriate restriction enzyme will yield characteristic patterns for different strains and species. Collins and De Lissle (1985a) has revealed patterns which distinguish between members of M. tuberculosis complex by digesting total genomic DNA with BstE II enzyme. The Collins’s method does not use any probe, just stained gel. It needs a lot of DNA for the digestion and a very careful standardisation of electrophoresis. So, despite its power (it is still the most successful way of typing M. bovis), it is not widely used.

Cooper et al., (1989), showed that although M. tuberculosis and M. bovis are closely related, they are genetically distinct. Some species harbour unique sequences which are repeated many times in the genome with the number and location of the repeated sequence varying with different isolates, thus providing a suitable target site for generating RFLP.
Some of these repeated sequences have been identified as insertion sequences. The insertion sequences are capable of replicating and inserting at various sites within the genome. They may show species specificity. The ability of these insertion elements to replicate and translocate in the genome varies among strains in the same species, hence creating an ideal situation for generating RFLPs. RFLP's studies have been conducted among members of the *M. tuberculosis* complex (Eisenach et al., 1988; Hermans et al., 1990; McAdam et al., 1990), the *M. paratuberculosis-M. avium* group (McFadden et al., 1987a; McFadden et al., 1987b; Hernandez et al., 1994) and among isolates of *M. leprae* (Clark-Curtiss and Docherty, 1989; Williams et al., 1990). These insertion sequences will be discussed in more detail in the later section.

1.10 Control and treatment of tuberculosis

Control and prevention of tuberculosis in many countries often involves vaccination programmes using *M. bovis* BCG and specific chemotherapy in patients with confirmed tuberculosis.

1.10.1 Vaccination

The vaccine is an attenuated mutant of *M. bovis* developed by two French scientists, Calmette and Guerin. This organism, BCG, is a live vaccine. The vaccine BCG was produced between 1908 and 1921 at the Institute Pasteur by passaging a strain of *M. bovis* through 239 consecutive transfers in potato-glycerol bile medium. Although BCG is the only tuberculosis vaccine currently in use, yet the effectiveness of the vaccine is still a matter of controversy. There have been many trials carried out to determine the effectiveness of the vaccine, but have provided conflicting results. The British MRC trial among school leavers (14-15 years old of age) in industrial areas in England, showed a
protection level of 70-80% during a follow-up period of 15 years (MRC report 1972). Relative protection levels vary from 80% in North American Indians to 0% in Madras, South India. A meta-analysis of data from a number of such trials concluded that BCG showed an overall protective effect of 50% (Colditz et al., 1994). Various reasons may account for the differences including:

i) Variations in the potency and immunogenicity of the BCG strain used.
ii) Regional differences in the tubercle bacilli causing infection.
iii) Difference in host response due to genetic and nutritional factors.
iv) Prior immunisation by strains of M. tuberculosis of low virulence.
v) Prior immunisation by contact with environmental mycobacteria.

It is most likely that the failure of certain trials is due to several factors rather than one factor alone.

1.10.2 Tuberculin skin testing

The tuberculin skin test evaluates a delayed-type hypersensitivity response to mycobacteria using a substance called purified protein derivative (PPD). PPD is a crude mixture prepared from filtrates of heat sterilised cultures of M. tuberculosis. The PPD skin test, normally called the Mantoux test or tuberculin test, is carried out by intradermal injection of 0.1ml of the prepared solution of tuberculin (or 5 tuberculin units of PPD). The exact mechanism of the PPD skin test is not totally known. The PPD injection is thought to cause an influx of sensitised T cells into the areas as well as the release of lymphokines from these T cells. The release of the lymphokines will result in local vasodilatation, oedema, and also stimulates other inflammatory cells migrating to the area. The result is determined from the size of the induration and erythema of the skin area after 48 to 72 hours. The range of the responses are 1 to 5 mm, (negative); 5
to 9 mm, (doubtful); and 10 to 33 mm, (previous or possible current infection). Reports from many parts of the world indicate a relationship between the size of the skin reaction and the risk of developing active tuberculosis: the larger the reaction, the greater the possibility of active disease. Skin reactivity to the introduction of skin-testing material may be suppressed by several factors or conditions. These include advanced age, terminal or severe acute diseases (such as cancer) and immune defects (AIDS and related conditions).

The PPD skin test is neither specific nor sensitive as tuberculin contains many antigens that are shared with non-tuberculous environmental mycobacteria and sensitisation with these organisms can yield a positive skin test in the absence of tuberculosis infection. On the other hand, some recently infected patients and some active tuberculosis patients may show a negative PPD skin test (Ellener and Wallis, 1989).

1.10.3 Chemotherapy

Drugs now commonly used for previously untreated cases are isoniazid (INH), rifampicin, pyrazinamide, streptomycin, ethambutol, thiacetazone and para-amino salicylic acid (PAS). The reserves, or second line drugs, are ethionamide, prothionamide, cycloserine, kanamycin and capreomycin.

Rifampicin, and isoniazid together with either streptomycin, ethambutol or pyrazinamide are used in “short course” regimes, and they are given for 6 to 9 month duration. The rationale behind these multidrug therapies, is based on the experience with the development of anti-tuberculosis regimens, in that the few mutants that emerge which are resistant to a single drug will be killed by the other two components.
With the emergence of MDR strains of *M. tuberculosis*, new drugs were also developed. Among rifamycin derivatives, rifabutin is more effective than rifampicin *in vitro* and in experimental animals (Grassi and Peona, 1995). Rifapentine is more active than rifampin *in vitro* and has a longer half-life, but it is not active against rifampicin resistant strains (Mor *et al.*, 1995). Fluoroquinolones, e.g. Ciprofloxacin, concentrate within macrophages and are effective against *M. tuberculosis*. They also act synergistically with rifampicin and isoniazid (Grassi and Peona, 1995).

Patient non-compliance with drug regimes will lead to partially treated infections re-establishing in patients. These patients may be a source of emergence of the multidrug-resistant strains (MDR) of tuberculosis.

A summary of clinical characteristics of first line anti-tuberculosis agents with their mechanism of action as well as the side effects they may have, is shown in Table 1.2.

1.10.4 Multi Drug Resistant (MDR) Strains of Tuberculosis

The most ominous aspect of the current tuberculosis epidemic is the appearance of MDR *M. tuberculosis* strains. Many MDR cases have been reported in the United States (Beck-Sague *et al.*, 1992; Centres for Disease Control Report 1990; 1991; 1992; Edlin *et al.*, 1992, Pearson *et al.*, 1992). Occurrence of the MDR tuberculosis have also been reported elsewhere (Snider *et al* 1992; Annual Report, Ministry of Health Malaysia, 1995; Carpels *et al.*, 1995; Kritski *et al.*, 1996; Morbidity Mortality Weekly Report 1996). Although they also occur in normal populations, MDR strains are more common in the HIV patients. HIV and tuberculosis are a virulent combination as HIV infected persons are particularly susceptible to infection with MDR strains of *M. tuberculosis* which give rise to a rapidly fatal disseminated disease. In Malaysia, the drug resistance
### Table 1.2 Summary of clinical characteristics of antimycobacterial agents.

<table>
<thead>
<tr>
<th>Agents</th>
<th>Mechanism of action</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoniazid (INH)</td>
<td>Mycobactericidal. Inhibits mycolic acid synthesis</td>
<td>Majority strains still susceptible to INH. Side effects include hepatitis</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>Mycobactericidal. Inhibits DNA-dependent RNA polymerase</td>
<td>Effective when used with INH. Penetrates tissue. Most susceptible. Side effect range from mild gastrointestinal upset, anaemia, thrombocytopenia, leukopenia, to hepatotoxicity.</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>Mycobactericidal. Inhibits protein synthesis.</td>
<td>Most effective against actively replicating cells. Major side effect include ototoxicity, renal toxicity.</td>
</tr>
<tr>
<td>Pyrazinamide</td>
<td>Mycobactericidal. Mechanism of action unknown.</td>
<td>Effective against dividing intracellular organisms. Side effects include hepatitis and nausea.</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>Mycobacteristatic or mycobactericidal, depending on concentration. Inhibits mycolic acid synthesis.</td>
<td>Only effective against replicating cells. Toxicity minimal.</td>
</tr>
</tbody>
</table>

(Adapted from Connie and William 1993)
rate for rifampicin and combination of isoniazid with rifampicin is less than 1%. (Annual Report, Ministry of Health Malaysia, 1995). Malaysia has recently been selected by WHO to be one of the regional centres for MDR surveillance.

1.11 Insertion sequences and transposons

Transposition was first observed by Barbara McClintock in 1956 in an eucaryotic system, namely maize. Transposition, is now known to take place in prokaryotic chromosomes as well. The elements involved in the transposition process are known as insertion sequences (IS) or transposons. One such element known as IS6110, was initially discovered in the genome of *M. tuberculosis* as a repetitive sequence (Thierry *et al.*, 1990a, Thierry *et al.*, 1990b). An almost identical element designated as IS986 was independently identified and characterised by McAdam *et al.*, 1990.

1.11.1 Prokaryotic Transposable elements

Prokaryotic transposable elements are capable of duplicating and inserting as discrete, unchanged segments of DNA at different sites in the genome. Some transposable elements are capable of duplicating and inserting a daughter copy at a new location in the genome, while in others transposition activity result in the deletion of the donor replicon. The enzyme transposase is involved in the insertion of a transposable element at a new site.

Transposable elements can be divided into insertion sequences and transposons.

**Insertion sequences** (IS). IS are the simplest known transposable elements with sizes ranging from 800 to 2500 base pair (bp). They can be found in the genome of many
bacteria varying from a few to more than a hundred copies per genome. Because they encode only functions involved in transposition, they have been branded as selfish genes. They do not carry any selectable markers and preliminary knowledge about an IS usually derived from its effect on surrounding genes. The presence of IS in large numbers in the genome might have a strong influence on the structure and stability of the genome. In addition to their transposition properties, IS can also be detected at the recombination junctions of two replicons, where they are seen to provide homologous sites for recombination (Galas and Chandler, 1989).

**Transposons.** Transposons are larger and more complex IS elements. The simplest type of transposon is very similar in structure to IS in that they have the transposase gene flanked by inverted repeats (IR). However, in contrast to IS, this type of transposon also carries additional genes in addition to the transposase gene. A good example of the transposon is Tn3. Tn3 are similar in structure to an IS, containing a transposase gene and inverted terminal repeats.

More complex transposons are called composite transposons. These consist of a central region containing usually several genes (such as antibiotic resistance genes) and flanked on both sides by IS. An example of composite transposon is Tn10 which consists of a region of DNA carrying a tetracycline resistance genes, flanked at both ends by a copy of IS10.

**1.12 Mechanism of transposition**

There are two group of transposition models: Conservative (cut and paste) and replicative.
1.12.1   Conservative transposition /Non replicative

Conservative transposition is also known as non replicative or cut and paste mechanism. In this process the transposon is excised from its original position by a double strand break at each end. The transposon is then joined to the asymmetrically cut target sequence. The single stranded gap is then repaired, generating a repeat of the target flanking the transposon. In this type of transposition, there will be loss of the transposon at original site and the acquisition of the transposon at a new site.

1.12.2   Replicative transposition

In this mechanism, one copy of the IS element is retained at the original site and a copy is inserted at the second site. In this process, a single stranded cut at each 3’ end of the transposon is made, the loose ends of the transposon are then joined to free DNA ends, at the target sequence, generated by asymmetric cleavage. This intermediate form has 3’ OH ends which are used as primers by DNA polymerase to make new copies of the transposon leading to two DNA molecules combined into what is known as a co-integrate structure. The co-integrate structure is resolved by a homologous recombination process involving two copies of the transposons (or in some cases by site specific recombination by enzyme resolvase, such as in Tn3) resulting in a copy of the transposon at the original site and a second copy at the second site.

Although it might be thought that transposons and IS follow either conservative or replicative pathway, it is likely that they use both mechanisms to different extents.

1.13   Insertion elements in mycobacteria
1.13.1 IS986 and related elements

The insertion sequence IS986 was discovered during an attempt to demonstrate the presence of plasmids in *M. tuberculosis* DNA with probes derived from a *M. fortuitum* plasmid (Zainuddin and Dale 1989). Sequence data (accession number X52471) shows that IS986 is 1358bp in size with a 30bp inverted repeat sequence at the ends.

In addition to IS986, two other nearly identical elements have been published, namely IS6110 (Thierry *et al.*, 1990a, Thierry *et al.*, 1990b) (accession number M29899) and IS987 (Hermans *et al.*, 1991) (accession number X57835). IS986 and IS6110 were isolated from multicopy *M. tuberculosis* strains while IS987 was obtained from a strain of BCG carrying a single copy of the element.

The designation IS6110 was accepted for describing these elements, except where specific copies are referred to (van Embden *et al.*, 1993).

The polymorphic nature of the banding pattern detected with probes derived from this insertion sequence confer advantages in its use as an epidemiological tool (Hermans *et al.*, 1990). Most *M. tuberculosis* strains have multiple copies of IS6110 (from 5 to 20 copies), but there are a significant minority of strains with only one or two copies of IS6110 (Das *et al.*, 1993, Yuen *et al.*, 1993, van Soolingen *et al.*, 1993, Fomukong *et al.*, 1994, Musa *et al.*, 1994) and some IS6110-negative strains have also been reported (Das *et al.*, 1993; van Soolingen *et al.*, 1993; Yuen *et al.*, 1993, Musa *et al.*, 1994).

These IS elements belong to the IS3 family of insertion sequences and show similar organisation of their coding regions. All three elements contain two main open reading frames (ORF) designated as ORFa and ORFb in which the translation of ORFb appears to depend on ribosomal frame shifting in the overlap region between ORFa and ORFb (Fig. 1.1).
Figure 1.1 Structure of IS3 family of insertion sequences. IS3 and related elements, including IS986 and IS6110, have two regions coding for product thought to be involved in transposition, known as ORFa and ORFb. The two overlapping genes are in different reading frames. ORFb is probably not translated independently, but may be expressed by a ribosomal frameshifting event in the region of overlap of ORFa and ORFb, resulting in a fusion protein. ORF: Open reading frame.
Although the published sequences of these three elements are virtually identical, there are several base differences between all three elements particularly in the ORFa region which affects the organisation of ORFa and hence the ribosomal frameshifting from ORFa to ORFb. In IS987, ORFa is continuous (Hermans et al., 1991) whereas in both IS986 and IS6110 ORFa is split into two smaller overlapping ORFs designated as ORFa1 and ORFa2 (McAdam et al., 1990; Thierry et al., 1990b) suggesting that another frame shifting event is required in the latter two elements. However, the structure of ORFa1 and ORFa2 are not identical in IS986 and IS6110.

The mobility of IS986 has been demonstrated in BCG (Fomukong et al., 1993) using artificially constructed composite transposons on a non-replicating vector. However, Mendiola et al., (1992), were unable to demonstrate transposition of IS6110 by essentially the same procedure. Since BCG has only one or two copies of the element it follows that IS987 is also likely to lack transposibility. The variation in transposibility between these three elements has been suggested to be due to the differences in the organisation of ORFa.

However, as will be discussed later, the published sequences of IS986 and IS6110 contain errors, and each is actually identical to IS987, in which ORFa is continuous.

1.13.2 IS900 and related elements

IS900 and other related elements such as IS901, IS902 and IS1110, form another major group of insertion sequences. There is extensive variation among members of the IS900 family, apart from IS901 and IS902, which show 98% identity and are effectively the same element (Green et al., 1989, Kunze et al., 1991, Moss et al., 1992, Hernandaz et al., 1994). IS901 shows greater similarity to IS116 (from Streptomyces clavuligerus)
than to the mycobacterial sequence IS900 (Kunze et al., 1991). This group of insertion sequences is unusual for not having inverted repeat ends.

IS900 has very little polymorphism in human strains of *M. paratuberculosis* (McFadden et al., 1990), but Collins et al., (1990), have shown polymorphism in animal isolates using IS900 as a probe.

IS901 is confined to isolates of *M. avium* from animals and birds, generally, and is rarely found in isolates from AIDS patients or in environmental strains (Kunze et al., 1992)

IS1110 is a further member of the IS900 family characterised by Hernandez et al., (1994), IS1110 was identified because of its exceptional mobility in the strain (*M. avium* LR541) in which it was found. Using an IS1110 derived probe is expected to be useful as a molecular tool in the epidemiological study of *M. avium* infection in AIDS patients.

1.13.3 **IS1081.**

IS1081 was isolated from a library of *M. bovis*, using genomic probes of *M. tuberculosis* and *M. bovis* DNA (Collins and Stephens, 1991). It is present in most strains of the *M. tuberculosis* complex and is capable of distinguishing BCG from other members of *M. tuberculosis* complex. In contrast to IS6110, IS1081 has only a very low degree of polymorphism (van Soolingen et al., 1992; van Soolingen et al., 1993).

1.13.4 **Other IS elements**

A number of other IS elements have been isolated from mycobacteria and are shown in Table 1.3.
Table 1.3: Other IS elements in Mycobacteria

<table>
<thead>
<tr>
<th>IS</th>
<th>Family</th>
<th>Host</th>
<th>Size</th>
<th>Inverted repeat</th>
<th>Direct repeat</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS1096</td>
<td>?</td>
<td>M. smegmatis</td>
<td>2260</td>
<td>25</td>
<td>8</td>
<td>Cirillo et al. 1991</td>
</tr>
<tr>
<td>IS6120</td>
<td>IS256</td>
<td>M. smegmatis</td>
<td>1486</td>
<td>24</td>
<td>9</td>
<td>Guilhot et al. 1992</td>
</tr>
<tr>
<td>IS1652</td>
<td>?</td>
<td>M. kansasii</td>
<td>947</td>
<td>-</td>
<td>3</td>
<td>Yang et al. 1993</td>
</tr>
<tr>
<td>IS6100</td>
<td>IS6</td>
<td>M. fortuitum</td>
<td>880</td>
<td>14</td>
<td>-</td>
<td>Martin et al. 1990</td>
</tr>
<tr>
<td>IS1141</td>
<td>IS3</td>
<td>M. smegmatis</td>
<td>1361</td>
<td>32</td>
<td>3</td>
<td>Falkingham and Crawford 1994</td>
</tr>
<tr>
<td>IS1245</td>
<td>IS256</td>
<td>M. avium</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Guerrero et al. 1995</td>
</tr>
<tr>
<td>Unnamed</td>
<td>IS427</td>
<td>TB complex</td>
<td>969</td>
<td>17</td>
<td>4</td>
<td>Roiz et al. 1995</td>
</tr>
</tbody>
</table>

The table was adapted from Dale (1995) 

IS: Insertion sequence; bp: Base pair
Chapter 2
Materials and Methods

2.1 Materials

2.1.1 Chemicals, Reagents, Kits and other consumables

All chemical used were ANALAR grade unless otherwise stated.

Alpha-Innotech Corporation

Digital Imaging System

BIO-RAD

Power supply for agarose electrophoresis and sequencing

BDH Chemicals Ltd.

Ammonium acetate, boric acid, glycine, sodium acetate anhydrous, phenol, chloroform, acetic acid, ethanol (absolute), isopropanol, ammonium persulphate, calcium chloride, sodium dihydrogen orthophosphate dihydrate, disodium hydrogen orthophosphate, trisodium citrate, Tween 80, sodium hydroxide (pellets)
Boehringer Manheim Biochemica

Taq DNA polymerase, positively charged nylon membrane, digoxigenin (DIG) DNA labelling and non-radioactive detection kit, restriction enzymes and their respective 10X buffers.

Difco

Bacto Middlebrook 7H9 broth (dehydrated), Bacto Middlebrook 7H11 agar (dehydrated), Middlebrook OADC Enrichment, Lowenstein-Jensen Medium Base.

Gibco BRL

100bp DNA ladders, Sequencing gel unit.
restriction enzymes and their respective 10X buffers.

Hoefer Scientific Instruments

Agarose gel units

Invitrogen

TA cloning kit
Kodak Photo Co. Ltd.

X-ray film (Type RX), Polaroid film type 667 (positive/negative) and type 665 (positive only)

Northumbria Biologicals Ltd.

Isopropyl β-D-thiogalactopyranoside (IPTG), 5-bromo-4-chloro-3-indolyl-b-d-galactopyranoside (X-gal)

Oxoid

Bacteriological agar, nutrient broth, nutrient agar, tryptone, yeast extract.

Perkin Elmer

GeneAmp PCR system 9600

Promega

Agarose (ultra-pure), DNA molecular weight markers (λ HindIII, φX174 HaeIII), Urea (ultra-pure), proteinase K, deoxynucleotide triphosphates (dNTPs), restriction enzymes and their respective 10X buffer.
Sigma Chemicals Co.

Ampicillin, ethidium bromide, ethylene diaminotetracetic acid (EDTA), 8-hydroxyquinoline, maleic acid, spermidine, Proteinase K, Tween 20, N,N,N'N'-tetramethylenediamine (TEMED).

United States Biochemicals (USB)

Sequenase version 2.0 DNA sequencing kit.

US Bio-Synthesis

100 bp DNA Ladder.
HPLC purified primers for PCR

USBIO 101 Inc.

Geneclean II kit.

Whatman Laboratories Ltd.

Whatman 3M filter paper.
2.1.2 Culture Media

All media were sterilised by autoclaving at 121°C and 15psi for 15 minute unless otherwise stated. Liquid media were allowed to cool to room temperature before addition of antibiotics or other substances if required and aliquoting into the appropriate containers. Agar-based solid media were cooled to 50-55°C in a water bath before adding the required substances and plating out onto the appropriate containers.

Luria-Bertani (LB) broth

Bacto tryptone 10g
Bacto yeast extract 5g
NaCl 10g
Deionised water to 1 litre

Luria-Bertani (LB) agar

Bacto tryptone 10g
Bacto yeast extract 5g
NaCl 10g
Agar 15g
Deionised water to 1 litre

Middlebrook 7H9 broth

Dehydrated 7H9 powder 4.7 g
Deionised water 900ml
Tween 80 0.9ml
After autoclaving the media was allowed to cool before OADC Enrichment was added to 10%.

**Middlebrook 7H11 agar**

- Dehydrated 7H11 agar: 21 g
- Deionised water: 900 ml

After autoclaving the media was allowed to cool before OADC Enrichment was added to 10%.

**Lowenstein-Jensen Medium (Difco)**

- Dehydrated LJ Base: 37.2g
- Glycerol: 12ml
- Deionised water: 600ml

The mixture was heated to boiling with constant agitation and then autoclaved. A uniform suspension of fresh egg was prepared, added to the sterile base to 1 litre and mixed under aseptic conditions. It was then dispensed into sterile universal bottles. The media were inspissated in a slanting position at 85°C for 45 minutes. The media were tested for sterility in a 37°C incubator overnight and were kept at 4°C prior to use.
2.1.3 General buffers and solutions

3M Sodium acetate pH 5.2

The appropriate amount of sodium acetate was dissolved in deionised water. pH was adjusted with glacial acetic acid and the solution was autoclaved before using.

Phenol/Chloroform solution

Phenol:Chloroform was prepared as a 1:1 (v:v) mixture, saturated with TE buffer and stored in the presence of the antioxidant 8-hydroxyquinoline (0.1% w/v).

10X TE buffer (Tris 100mM, EDTA 10mM)

Tris, pH 8.0  12.1g
EDTA  3.7g

Distilled water was added to 100ml and the solution was autoclaved at 121°C for 15 minutes. The solution was kept at room temperature. For the preparation of 1X TE, the solution was diluted 1:10 with sterile distilled water.

Lysozyme solution (10 mg/ml)

The lysozyme was dissolved in distilled water. The lysozyme solution was used fresh or stored in aliquots at -20°C for not longer than 1 year.
10% SDS

10 gm SDS in 100ml distilled water. The SDS was dissolved by heating at 65°C for 20 minutes. It was stored at room temperature for not longer than 1 month.

Proteinase K (10mg/ml)

Proteinase K 10mg
Distilled water 1ml

Proteinase K was added in 1ml of distilled water. They were then stored in small aliquots at -20°C for not longer than one year.

SDS/Proteinase K mix

Proteinase K (10 mg/ml) 5μl
SDS (10%) 70μl

Both solution were mixed prior to use.

5M Sodium Chloride (NaCl)

NaCl 29.2g
Distilled water to 100ml
The solution was autoclaved and stored at room temperature for not longer than one year.

**CTAB/NaCl solution**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>4.1gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>80ml</td>
</tr>
<tr>
<td>CTAB</td>
<td>10gm</td>
</tr>
</tbody>
</table>

(N-acetyl-N,N,N-trimethyl ammonium bromide)

NaCl was dissolved first with 80ml of distilled water. CTAB was then added while stirring. The solution was heated to 65°C and adjusted to 100ml with distilled water. It was stored at room temperature for not longer than 6 months.

**Chloroform/isoamyl alcohol (24:1)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform</td>
<td>24 volume</td>
</tr>
<tr>
<td>Isoamyl alcohol</td>
<td>1 volume</td>
</tr>
</tbody>
</table>

The solution was mixed and kept at room temperature for not longer than one year.

**Isopropanol (100%)**

The isopropanol solution (Analar Grade) was stored at room temperature for not longer than 1 year.
**Ethanol (70% v/v)**

Ethanol (Analar Grade)  
Distilled water

70ml  
30ml

The solution was kept in room temperature for not longer than one year.

### 2.1.4 Bacterial Species and Strains

All Mycobacterium species and strains were obtained from the National Tuberculosis Centre (NTBC), Kuala Lumpur, Malaysia. All other bacterial species and strains used were obtained from the Diagnostic Microbiology Laboratory, Department of Medical Microbiology and Parasitology, School of Medical Sciences, University Sains Malaysia (USM). The bacterial species and strains used are listed in Table 2.1.

Clinical isolates of *M. tuberculosis* used in this study, which were all from National Tuberculosis Centre, Malaysia, are not listed in Table 2.1.
Table 2.1 Bacterial species and strains used in this study.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. tuberculosis</em> Mt 14323</td>
<td>The Netherlands *</td>
</tr>
<tr>
<td><em>M. simiae</em></td>
<td>NTBC</td>
</tr>
<tr>
<td><em>M. chelonei</em></td>
<td>NTBC</td>
</tr>
<tr>
<td><em>M. phlei</em></td>
<td>NTBC</td>
</tr>
<tr>
<td><em>M. avium</em></td>
<td>NTBC</td>
</tr>
<tr>
<td><em>M. intracellulare</em></td>
<td>NTBC</td>
</tr>
<tr>
<td><em>M. fortuitum</em></td>
<td>NTBC</td>
</tr>
<tr>
<td><em>M. triviale</em></td>
<td>NTBC</td>
</tr>
<tr>
<td><em>M. xenopi</em></td>
<td>NTBC</td>
</tr>
<tr>
<td><em>M. vaccae</em></td>
<td>NTBC</td>
</tr>
<tr>
<td><em>M. kansasii</em></td>
<td>NTBC</td>
</tr>
<tr>
<td><em>M. chelonei</em></td>
<td>NTBC</td>
</tr>
</tbody>
</table>
Table 2.1 Continue...

<table>
<thead>
<tr>
<th><strong>Pseudomonas aeruginosa</strong></th>
<th>USM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Klebsiella pneumonia</strong></td>
<td>USM</td>
</tr>
<tr>
<td><strong>Streptococcus pneumonia</strong></td>
<td>USM</td>
</tr>
<tr>
<td><strong>Streptococcus group B</strong></td>
<td>USM</td>
</tr>
<tr>
<td><strong>Staphylococcus aureus</strong></td>
<td>USM</td>
</tr>
<tr>
<td><strong>Enterobacter species</strong></td>
<td>USM</td>
</tr>
<tr>
<td><strong>Haemophilus influenzae</strong></td>
<td>USM</td>
</tr>
<tr>
<td><strong>Klebsiella aeruginosa</strong></td>
<td>USM</td>
</tr>
<tr>
<td><strong>Escherichia coli</strong></td>
<td>USM</td>
</tr>
<tr>
<td><strong>Candida albicans</strong></td>
<td>USM</td>
</tr>
<tr>
<td><strong>Branhamella catarrhalis</strong></td>
<td>USM</td>
</tr>
</tbody>
</table>

* M. tuberculosis reference strain Mt14323 was obtained from Rijksinstitut voor Volkgezondheden Milieuhygiene, Bilthoven, The Netherlands*
2.2 Methods

2.2.1 Extraction of Nucleic Acid with Phenol-chloroform

Solutions containing nucleic acids was added with an equal volume of phenol-chloroform and mixed. The two phases were separated by centrifugation at 10000g for 5 minutes in a microcentrifuge. The aqueous phase was transferred to a fresh tube. The extraction procedure was repeated until the interphase was clear of debris. A final extraction was performed using an equal volume of chloroform in isoamyl alcohol. The DNA was then precipitated by ethanol or isopropanol.

2.2.2 Precipitation of DNA by ethanol or isopropanol

DNA precipitation was carried out using either method A or B as described below:

A. 3M sodium acetate (pH 5.2) was added to the DNA solution to 0.1 volume followed by 2 volumes of ice-cold absolute ethanol.

B. 3M sodium acetate (pH 5.2) was added to the DNA solution to 0.1 volume followed by 1 volume of ice-cold isopropanol.

After addition of ethanol or isopropanol, the solution was mixed by inversion several times and incubated on ice for 30 minutes (or in the case of isopropanol, at room temperature, 5 minutes) or for shorter time periods at -20°C or -70°C. The DNA was then precipitated by centrifuging at 10000g for 20 minutes. The pellet was washed with 70% (v/v) ethanol and dried in vacuum prior to resuspending in sterile deionised water or TE buffer (pH 8.0).
2.2.3 Quantitation of DNA in aqueous solution

The concentration and purity of nucleic acid was estimated by measuring the U.V. absorbance of the solution at the range of 260-280nm. A pure DNA solution has a 260nm/280nm ratio of >1.8 (Sambrook et al., 1989). The concentration of DNA was calculated by employing the formula $A_{260}$ of $1 = 50 \mu g/ml$ of double stranded DNA or $33\mu g/ml$ of single stranded oligonucleotides (Sambrook et al., 1989).

2.2.4 Agarose-gel Electrophoresis

Buffers used:

1X TAE (Tris-acetate)
- Tris 400mM
- EDTA 2mM
- pH to 8.0 with glacial acetic acid.
- Made as a 50X stock solution which was diluted to 1X prior to use.

0.5X TBE (Tris-borate)
- Tris 89mM
- Boric acid 89mM
- EDTA 2mM
- Made as a 20X stock solution which was diluted to 0.5X before use.

DNA loading buffer (6X)
- Bromophenol blue 0.25% (w/v)
- Xylene cyanol 0.25% (w/v)
- Sucrose 40% (w/v)
The concentration of agarose-gel used ranged between 0.8% to 2%, depending on the size of the DNA to be visualised. Ethidium bromide to 0.5µg/ml was added to the gel before casting. One microlitre of 6X DNA loading buffer was added to each 5µl of sample before loading onto the gel. The gel was run in 0.5X TBE or 1X TAE (according to requirement) at 100 volts for 30-60 minutes depending on the gel dimension and agarose concentration.

2.2.5 Extraction of DNA from Agarose Gel

DNA bands separated on agarose gels were extracted using the Geneclean II kit (BIO 101) according to the manufacturer’s instructions.

2.2.6 Restriction Enzymes Digest

Restriction enzyme digestion were performed according to the manufacturer’s instruction. Restriction digests were performed using the appropriate buffers supplied with the enzyme and approximately 1 microgram of DNA and 1-5 units of enzyme in a volume of 10-20µl.

2.2.7 Cloning of PCR products

The amplified fragment was extracted using Geneclean II kit, ligated to the pCRII™ vector and then transformed into the One Short™ (INVαF) strain provided in the TA Cloning Kit (Invitrogen) according to the manufacturer’s instructions. Recombinant plasmid were characterised by restriction digest, PCR and subsequently by sequencing of the inserts.
2.2.8 Miniprep Plasmid DNA Preparation

1.5ml of an overnight (17 hours) culture, grown in 10ml of L-broth, was centrifuged at 7000g for 5 minutes at room temperature. The supernatant was discarded and pellet was resuspended in 50μl of TEN buffer. Fifty microlitres of Phenol:Chloroform was added, the mixture was vigorously vortexed for 30 seconds and centrifuged at 7000g for 6 minutes at room temperature. Sixty microlitres of the supernatant was carefully collected and 6μl of 5M ammonium acetate was added, followed by 2 volumes of absolute ethanol (-20°C). The solution was well mixed by inversion and incubated on ice for 15 minutes. It was then centrifuged at 7000g for 10 minutes at room temperature. The supernatant was discarded and the pellet was washed with 70% cold ethanol, desiccated under vacuum for 5 minutes, and resuspended in 20μl sterile deionised water or 1X TE. The solution was stored at 4°C (or -20°C for long term storage).

2.2.9 Isolation of Total Genomic Mycobacterial DNA

Mycobacterial total genomic DNA were extracted using a protocol described by van Soolingen et al. 1991.

A loopful of cells from a culture grown on Lowenstein-Jensen was transferred into a microcentrifuge tube containing 500μl of 1X TE. It was then heated for 20 minutes at 80°C to kill the cells and cooled to room temperature. 50μl of 10mg/ml lysozyme was added, vortexed and incubated for 1 hour at 37°C. 75μl of 10% SDS/proteinase K solution was then added, mixed and vortexed briefly, before the mixture was incubated for 10 minutes at 65°C. 100μl of a 5M NaCl solution was added followed by 100μl of CTAB/NaCl solution, which was prewarmed to 65°C. The mixture was vortexed until it turned white (milky) followed by an incubation at 65°C for 10 minutes. An equal volume
(750µl) of chloroform/isoamyl alcohol was added, followed by vortexing for at least 10 seconds, and centrifugation at room temperature for 5 minutes at 12,000g. 180µl of the aqueous supernatant was transferred to a fresh microcentrifuge tube and 180µl of isopropanol was added to precipitate the nucleic acids. After incubating for 30 minutes at -20°C, it was spun for 15 minutes at room temperature in a microcentrifuge at 12,000g. The supernatant was discarded leaving about 20µl above the pellet. 1ml of ice cold 70% ethanol was added and the tube was inverted gently several times. After centrifugation for 5 minutes at room temperature, the supernatant was discarded, leaving about 20µl above the pellet. It was spun again for 1 min in a microcentrifuge and the supernatant was discarded using a micropippettor. The pellet was allowed to dry at room temperature for about 10 minutes. The pellet was re-dissolved in 2µl of 1X TE buffer and stored at 4°C.

2.2.10 Detection of DNA by Southern Blotting and Hybridisation

2.2.10.1 Buffers and solutions used:

Southern blot DNA transfer solution
0.4N Sodium hydroxide (NaOH)

20X SSC
NaCl 3.0M
Tris-sodium citrate 0.3M

Prehybridisation/hybridisation solution
The hybridisation solution for Chemiluminescent contained 5X SSC, 2.5% blocking reagent, 0.1% N-lauroxysarcosine sodium salt, 0.02%
SDS and 100μg/ml herring sperm DNA. The herring sperm DNA was denatured by heating prior to use.

**Wash buffer**
0.3% (w/v) Tween 20 in Buffer 1.

**Buffer 1**
Maleic acid 0.1 mol per litre
NaOH 0.15 mol per litre
pH was adjusted to 7.0

**Buffer 2 (2% blocking solution)**
Made by diluting 10% Blocking stock solution 1:5 with Buffer 1.

**Buffer 3**
Tris-HCL 0.1 mol per litre
NaOH 0.1 mol per litre
Magnesium chloride 50 mol per litre
pH was adjusted to pH 9.0.

**10% Blocking stock solution**
10g of blocking reagent was dissolved in 100ml of Buffer 1, autoclaved and stored at 4°C.

**Antibody conjugate**
Anti-dioxigenin-AP Fab fragment was diluted 1:10,000 in Buffer 2.

**Herring sperm DNA**
A solution of 10 mg/ml of herring sperm DNA was prepared in 1X TE. The DNA was denatured by heating at 95°C before use.
AMPPD [3-(2’spiroadamantane)-4-(3-phosphoryloxy)-phenyl-1,2-
dioxetane]

AMPPD is a chemiluminescent substrate for alkaline phosphotase
which comes as a 10mg/ml stock solution. The working solution of
0.235mM, was derived by diluting the stock solution 1:100 in Buffer

2.2.10.2 Southern Blotting

The DNA to be probed was run on a electrophoresis gel (1% gel) which
was then stained in ethidium bromide (0.5µg/ml) solution for 15 minutes. The DNA on
the gel was transferred onto a nylon membrane (Hybond-N, Amersham) by Southern
blotting technique using 0.4 N NaOH as the transfer buffer and the blotting was carried
out overnight. The membrane was carefully rinsed in 6X SSC for few seconds and it was
then baked at 120°C for 30 minutes to fix the DNA firmly onto the membrane. The
membrane was kept at room temperature before further use.

2.2.10.3 Hybridisation and Non-radioactive Detection

The membrane was placed into a hybridisation bottle sandwiched by
nylon gauzes. The membrane was pre-hybridised using 20ml of hybridisation solution for
at least 1 hour at 68°C.
Probes were labelled with digoxigenin using the DIG kit (Boehringer Mannheim) according to the manufacturer’s instructions. The probe was denatured at 95°C for 10 minutes and immediately placed on ice for 5 minutes before being used.

The pre-hybridisation buffer was replaced with fresh hybridisation solution and the denatured probe was added. The membrane was then incubated at 68°C overnight in a hybridisation oven. The membrane was washed twice for 5 minutes in 2X SSC, 0.1X SDS (100ml) at room temperature, followed by two washings, 15 minutes each time, in pre-warmed 0.1X SSC, 0.1X SDS (100ml) at 68°C.

The hybridisation solution containing the probe was stored in a sterile bottle at -20°C and was re-used for up to 5 times before discarding.

2.2.10.4 Chemiluminescent detection method

The chemiluminescent detection method was carried out at room temperature in the hybridisation bottle. The membrane was washed briefly for 3 minutes in the washing buffer and was then incubated in Buffer 2 for 30 minutes. Buffer 2 was discarded and the membrane was incubated for 30 minutes with Buffer I containing anti-DIG-AP conjugate at 1:10,000. The membrane was then washed twice, 20 minutes each time, with washing buffer. After the last wash, the membrane was equilibrated in 20ml of Buffer 3 for 2 minutes followed by incubation in AMPPD solution for 5 minutes. Excess AMPPD was removed and the damp membrane was then sealed in Saran wrap, incubated at 37°C for 5 minutes and exposed to X-ray film for up to 20 minutes. Further exposures were done when necessary.
2.2.10.5 Removal of probe from membrane for reprobing

The membrane was not allowed to dry out if probe stripping is to be carried out. The membrane was rinsed in distilled water for 3 minutes, followed by washing in a solution containing 0.2M NaOH, 0.1% SDS (w/v) at 37°C for 20 minutes. The membrane was finally washed in 2X SSC for 3 minutes. The membrane was then rehybridised with other probe(s) or were stored air-dried.

2.2.11 Polymerase Chain Reaction (PCR)

All PCR experiments were performed using the Perkin Elmer Cetus (PEC) Thermal Cycler Model 9600. The basic reaction mixtures and initial PCR parameters were based on the recommended methods by PEC and modifications were introduced if and when necessary (and are described in the appropriate sections). Briefly:

The four deoxynucleotide triphosphates (dNTPs) were mixed to a final concentration of 1.25mM. To make up a Master Mix enough for ten PCR reactions, 320μl of sterile deionised water, 80μl of dNTP mix, 50μl 10X buffer containing 1.5mM of Magnesium chloride. The PCR Master Mix was used on the same day or stored at -20°C for up to 2 weeks.

To each 45μl of Master Mix were added 1μl of each primer (100-200ng/μl), 1μl of Taq DNA polymerase enzyme (1 unit/μl), and 2μl of template DNA (1-100 ng/μl) in a 200μl microtube. This was then spun briefly in a microcentrifuge and slotted into the heating block. Oil overlay was not used with the 9600 model.
2.2.11.1 Heating Block Program

Each cycle of thermal cycling comprises of three steps: Denaturation of the template DNA (usually at 94°C), annealing of the primer to the template DNA (temperature used is dependent on the Tm of the primers) and an extension reaction (usually at 72°C). However the actual temperature used, the time that the reaction is held at each step, the number of cycle and other additional steps are determined by the actual primers used and are optimised empirically.

2.2.11.2 Precautionary Procedures in Preparing and Running a PCR Reaction

To prevent contamination by extraneous DNA material from sources which include aerosols, gloves, or contaminated stocks like buffer, primers, deoxyneuclotides, some basic guidelines were observed:

All preparative work was carried out in laminar flow cabinet. The cabinet was regularly cleaned with 70% ethanol and the air flow system was switched on 15 minutes before commencing work. All the manipulation involving stock solutions were carried out to completion before template DNA are handled. Gloves used were changed from time to time during PCR work. Positive and negative control reactions (including extraction controls when clinical samples were processed for PCR) were always included in each run.

2.2.11.3 Primers for PCR

Primers were obtained commercially (US Bio-Synthesis) and were all HPLC purified.
2.2.12 Sequencing

2.2.12.1 Preparation of the sequencing gels

The sequencing gel consisted of the following: Urea (31.5g), 10X Tris-borate buffer (7.5 ml), 50% Long Ranger gel solution (FMC BioProducts) (9 ml) and sterile deionised water to 75ml. The urea was dissolved by heating the mixture in a 37°C water bath, with occasional stirring, for 20 minutes. Just before pouring the gel, 420µl of 10% ammonium persulphate (AMPS), and 75µl of TEMED were added and the gel preparation was mixed with a 60ml syringe and immediately poured.

2.2.12.2 Preparation of sequencing glass plates

Two glass plates were used in the preparation of the sequencing gel. A large glass plate with a sticky surface to which the gel adhered to and a smaller plate with a non adhesive surface. Both plates were thoroughly washed with a non-scratch detergent, rinsed in distilled water followed by a final rinse in ethanol. The gel facing side of the small plate was coated with a commercial product for automobile windscreen called “Rain X” (which contain ethyl alcohol, ethyl sulphate, isopropyl alcohol and siloxanes) and allowed to dry. The plate was then gently wiped with ethanol. The large plate was cleaned with ethanol and air dried. Rain X provided a non-adhesive surface for the gel, hence the gel will only stick to the larger plate. Spacers were placed between both plates, lengthwise. Both plates were then bound together with plastic tapes along their length and bottom. This provided a perfect seal between which the liquid gel mixture was poured. Combs were inserted in the opposite direction and the gel was allowed to set. The sealing tapes were removed and the gel was mounted onto an electrophoresis tank. The combs were then taken out and placed in the right orientation of the gel. The
tank was filled with 1X Tris-borate buffer and the gel was pre-run at 75 watts for 20 minutes before loading. The pre-run was to heat up the gel to the running temperature. Both long run and short run were done for each sample. At the end of the run, the combs and spacers were taken off and the small plate was taken out. The gel was then transferred to a blotting paper and dried using a heated vacuum chamber. Finally, the dried gel was exposed to an X-ray film.

2.2.12.3 Sequencing PCR products (direct DNA sequencing) using Sequenase version 2.0.

The direct sequencing method was adopted from McPherson et. al 1991 and the Sequenase Version 2.0 kit (United States Biochemicals) was used. Double stranded PCR products were sequenced directly using the PCR primers as sequencing primers. DNA was recovered from agarose gels using the Geneclean II kit. 0.2 to 2 μg of PCR DNA recovered from the gel was mixed with 2 pmol of the appropriate primer in a total volume of 8 μl made up with distilled water. The solution was heated to 100°C for 5 minutes and the tubes were then rapidly transferred to a cold Ethanol bath (-70°C) until the solution was frozen (1-2 minutes). 2 μl of 10X sequencing reaction buffer was added for annealing at room temperature for 20 minutes to allow annealing. While annealing, the Labelling reaction was prepared using diluted Labelling Mix (1:5), [35S]dATP, DTT, 0.1M and diluted sequenase polymerase. Sequencing Reaction and preparation of the reaction for loading onto the sequencing gel were then performed according to the manufacturer’s instruction.

In addition to a dilution of 1:5 of the labelling mix, in some cases a dilution of 1:20 was also used to allow sequencing close to the primer. In such cases, 1 μl of Manganese (Mn) buffer (provided by the sequencing kit) would be added to the sequencing reaction. The
addition of Mn buffer to the reactions will decrease the extensions in the termination reactions. Thus will also improves the ability to read the sequence close to the primer.

2.2.13 Method for examination and reporting of a direct smear for AFB

The direct smear examination for AFB was carried out using Ziehl-Neelsen staining according to the method recommended by the National Tuberculosis Centre of Malaysia which was based on the WHO/Tuberculosis/Technical Guide 67.7 (1967).

In this method, after appropriate treatment of the specimen if necessary, a smear was made covering an area equal to about two-thirds of the slide. Ziehl-Neelsen staining was performed according to standard protocols (Collins et al., 1985b). The slide was then examined under the microscope at 100x objective magnification with oil immersion.

The top part of the smear was first examined along the whole length of the smear. Each length of the smear was equal to approximately 100 fields (Collins et al., 1985b). If more than 50 AFB were observed the examination was terminated and the slide was recorded as having >50 AFB/length (L).

If between 10 to 50 bacilli were observed at the end of one length, the actual number of AFB was recorded. For example 36 AFB/L.

If 0 to 10 bacilli were observed at the end of one length, the examination was continued for a further two lengths (giving a total of 3 lengths which was equivalent to approximately 300 fields). Three possibilities can occur:

a. If 50 or more bacilli were observed, the result was recorded as >50 AFB/3L
b. If less than 50 AFB were observed the actual number was recorded for example: 29 AFB/3L or 8 AFB/3 L

c. If no bacillus were observed the result was recorded as 0/3L.
Chapter 3
Chapter 3
Characterisation of IS6110 in Malaysian Strains of M. tuberculosis

3.1 Introduction

IS6110 was originally thought to be present in 10 to 15 copies in M. tuberculosis and one copy in BCG. Studies in University of Surrey have however demonstrated that some BCG strains possess two copies of IS6110 (Fomukong et al., 1992). A collaborative study by University Sains Malaysia and the University of Surrey also found M. tuberculosis isolates carrying only a single copy of IS6110. Single copy strains were also later detected amongst Omani and Tanzanian isolates (See Chapter 1.13.1).

Earlier experimental work carried out in the University of Surrey has shown that IS6110 in these single copy strains appears to be inserted in the first BCG site which had been shown to be a preferred site of integration of IS6110 (Fomukong et al., 1993, Hermans et al., 1991). The variation in band sizes seen when probing these single copy strains with an IS6110-based probe is thought to be due to mutation of the flanking sequence, which could either be deletions or insertions (Fomukong et al., 1993). Further questions have been raised concerning the epidemiological significance of the unusual M. tuberculosis isolates and their relationship to BCG.

3.2 DNA fingerprinting of the M. tuberculosis complex

Analysis of the M. tuberculosis genome was first done by digesting the DNA with restriction enzymes, separating the resultant fragments by agarose electrophoresis and the gel was then stained with ethidium bromide (Collins et al., 1984; Collins et al.,
1985a; Patel et al., 1986). These methods gave results which were difficult to interpret because the numerous fragments generated were not easily differentiated.

Later, cloned species-specific DNA fragments were employed as probes, including repetitive sequences (Eisenach et al., 1988; Reddi et al., 1988; Hermans et al., 1990). The advantage of employing these repetitive sequences is their sensitivity, due to the multiple copy number of the target in the genome. Some repetitive sequences, however, are not highly species specific despite their capability for strain differentiation (Reddi et al., 1988) whereas others show high species specificity such as IS6110 (Hermans et al., 1990; Thierry et al., 1990a).

IS6110 shows highly polymorphic banding patterns with M. tuberculosis isolates. The degree of polymorphism is well suited to epidemiological analysis. A standard protocol allowing comparison of IS6110 fingerprints from different laboratories has been devised (van Embden et al., 1993). This is to enable a central database of banding patterns of strains from different countries to be maintained and analysed using a computer programme such as GELCOMPAR.

Using the standard protocol 476 M. tuberculosis Malaysian isolates were typed using a DNA probe derived from IS6110. The full analysis of the epidemiological data is continuing and will be presented elsewhere. This thesis concentrates on those strains with one or zero copies of IS6110.
3.3 Experimental procedures and results

3.3.1 Extraction of *M. tuberculosis* DNA from clinical isolates

Chromosomal DNA was extracted using the method recommended by van Embden *et al.* (1993) as described in Chapter 2.

3.3.2 *IS6110* derived probe

The probe (BX probe) was derived by PCR of a recombinant plasmid DNA carrying a copy of *IS6110* using primers derived from the restriction enzyme site *Acc* III (designated as AP1 primer) and *Xho* I site (designated as BX2 primer) [Fig 3.1][Table 3.3]. The 376bp PCR product generated by these primers was purified from agarose gels using the Geneclean kit (BIO 101), and labelled with digoxigenin-dUTP as described in Chapter 2.
Figure 3.1 Schematic diagram of IS6110 and location BX probe. BX probe is located at the right-hand end of IS6110 corresponding to the BamHI and XhoI sites. The PCR fragment for cloning and sequencing was amplified using primers derived from the restriction enzyme site Acc III (designated as AP1 primer) and XhoI site (designated as BX2 primer).
3.3.3 The hybridisation

Chromosomal DNA of *M. tuberculosis* was digested with *Pvu* II restriction enzyme and probed with the BX probe. A *M. tuberculosis* reference strain (Mt14323) was included in the digestion as control for correct cleavage by *Pvu* II restriction enzyme (fig. 3.2, lane 6) and also for normalisation of gels by GELCOMPAR. Hybridisation was performed at 68°C for 18-24 hours followed by high stringency washing as described in Chapter 2.

3.3.3.1 Hybridisation Results

Seventy six of the 476 (15.9%) isolates typed were shown to be single copy strains. The majority of these strains presented with a band of approximately 1.45kb in size (IS Type A) (Fig. 3.2, lane 7) while the rest show a band of approximately 5kb in size (IS Type B) (Fig 3.2, lane 8). Ten isolates were found to be IS6110 negative. Two of these IS negative strains are shown in Fig. 3.2, lanes 3 & 4).
Lane 1: US84 (multicopy strain)  
Lane 2: US186 (multicopy strain)  
Lane 3: US15 (IS-negative strain)  
Lane 4: US17 (IS-negative strain)  
Lane 5: US196 (multicopy strain)  
Lane 6: Mt14323 (control strain)  
Lane 7: US 101 (single copy strain)  
Lane 8: US 102 (single copy strain)  
Lane 9: US 201 (multiple copy strain)  
Lane 10: US 202 (multiple copy strain)  
Lane 11: US 203 (multiple copy strain)  

**Figure 3.2:** DNA probing of Malaysian *M. tuberculosis* clinical isolates using BX probe. Number at left indicate the sizes of the standard DNA fragment (lambda *Hind* III) in kilobase pairs.
Isolates which are negative with IS6110 probes present a problem because these isolates could either be genuine *M. tuberculosis* complex or are atypical mycobacteria. In order to resolve this problem, IS6110 negative and single copy strains were hybridised further with two additional probes, B9 and 9kb. The B9 sequence (Genebank accession number U78639) was derived from an EMBL4 recombinant clone designated as EMBL4/A-3, which was previously described by Zainuddin and Dale (1989). Although present only in a single copy in the *M. tuberculosis* genome, the B9 sequence has been shown to be potentially useful for detection of *M. tuberculosis* as it is present in all *M. tuberculosis* strains tested including IS6110-negative strains (Musa, 1996). The 9kb probe is a second sub-fragment from the same EMBL4/A-3 clone, which was shown to give RFLP patterns when used to probe *PvuII* digested chromosomal DNA of *M. tuberculosis* (Zainuddin and Dale, 1989). Figure 3.3(a), 3.3(b) and 3.3(c) show the results of probing an IS negative (lane 4) and IS single copy (lanes 5 and 6) strains with the IS6110, B9 and 9kb probes respectively. One BCG strain (lane 1), 2 atypical Mycobacterium (lanes 2 and 3) and 3 IS multiple copy *M. tuberculosis* strains (lanes 7, 8 and 9) are included in Fig. 3.3 for comparison. IS negative and IS single copy strains are confirmed as *M. tuberculosis* if the strain is positive with B9 and show the typical band pattern with the 9kb probe.
Lane 1: BCG Sweden
Lane 2: *M. fortuitum* (atypical mycobacterium)
Lane 3: AS178 (*Mycobacterium* spp.)
Lane 4: US15 (IS-negative strain)
Lane 5: AS80 (IS single copy strain)

Lane 6: KB29 (IS single copy strain)
Lane 7: NTBC28 (IS multiple copy strain)
Lane 8: NTBC45 (IS multiple copy strain)
Lane 9: NTBC20 (IS multiple copy strain)

**Figure 3.3a:** Results of probing IS negative and single copy *M. tuberculosis* strains with BX probes. One BCG strain, 2 atypical Mycobacterium species and 3 IS multiple copy *M. tuberculosis* strains were included for comparison. Numbers at left indicate sizes of the standard DNA(Lamda Hind III) marker in kilobase pairs.
Figure 3.3b: Results of probing an IS negative and IS single copy *M. tuberculosis* strains with the B9 probe. One BCG strain, 2 atypical Mycobacterium and 3 IS multiple copy *M. tuberculosis* strains were included for comparison. Numbers at left indicate sizes of the standard DNA (Lambda Hind III) fragment in kilobase pairs.
Figure 3.3c: Results of probing an IS negative (lane 4) and IS single copy (lanes 5 and 6) strains with 9kb (3.3c) probes. One BCG strain (lane 1), 2 atypical Mycobacterium (lanes 2 and 3) and 3 IS multiple copy *M. tuberculosis* strains (lanes 7, 8 and 9) are included for comparison. Number at left indicate sizes of standard DNA(Lambda Hind III) fragment in kilobase pairs.
Twenty six single copy strains of IS Type A, four single copy strains of IS Type B and three IS6110-negative strains chosen at random were further subjected to the Spoligotyping assay by Zainuddin Z.F. at Rijkinstitut voor Volkgezondheiden Milieuhygiene, Bilthoven, The Netherlands. The band patterns obtained were scanned and analysed using the GELCOMPAR programme (Schouls et al., 1995 and Mangiapan et al., 1996) and the results (Fig. 3.4) further confirmed the identity of these isolates. Furthermore, the twenty six IS Type A strains and the four IS Type B strains could be further divided into 14 and 3 different Spoligotypes respectively. The three IS6110 negative strains (US15, US17, and US263) were divided into two Spoligotypes as determined by the GELCOMPAR programme. However, a visual analysis of the band patterns (Fig. 3.4) suggests that these three strains should be grouped together under one Spoligotype. This error is likely to be due to the slightly weaker signals of the bands in strain US17. Table 3.1 summarises the results of the strain typing of Malaysian single copy strains using the IS6110 RFLP and the Spoligotyping assays.

The results from the strain typing study show the significant presence of IS6110 single copy and IS negative strains in Malaysia. The differentiation of the IS single copy strains into 2 different IS types which can then be differentiated further into a number of spoligotypes suggests that the single copy strains are fairly heterogeneous. This raises the question of why only one copy of IS6110 is present in these strains. In order to answer this question, it would be necessary to characterise the IS elements, by sequencing, in some of these strains for comparison with each other and with other copies of the element from multicopy strains that have been characterised previously.
Figure 3.4: Spoligotyping band patterns of Malaysian single copy IS6110 \textit{M. tuberculosis} strains obtained by scanning and analysing using the GELCOMPAR programme.
Table 3.1 Summary of strain typing of Malaysian single copy strains using IS6110 RFLP and Spoligotyping assays.

<table>
<thead>
<tr>
<th>Strain number</th>
<th>IS6110 RFLP type</th>
<th>Spoligotype</th>
<th>Strain number</th>
<th>IS6110 RFLP type</th>
<th>Spoligotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>US93</td>
<td>A</td>
<td>1</td>
<td>US101</td>
<td>B</td>
<td>8</td>
</tr>
<tr>
<td>US102</td>
<td>A</td>
<td>1</td>
<td>US496</td>
<td>B</td>
<td>8</td>
</tr>
<tr>
<td>US301</td>
<td>A</td>
<td>1</td>
<td>US323</td>
<td>A</td>
<td>9</td>
</tr>
<tr>
<td>US385</td>
<td>A</td>
<td>1</td>
<td>US124</td>
<td>A</td>
<td>11</td>
</tr>
<tr>
<td>US488</td>
<td>A</td>
<td>1</td>
<td>US756</td>
<td>A</td>
<td>12</td>
</tr>
<tr>
<td>US711</td>
<td>A</td>
<td>1</td>
<td>US315</td>
<td>A</td>
<td>13</td>
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<tr>
<td>US260</td>
<td>A</td>
<td>2</td>
<td>US678</td>
<td>A</td>
<td>14</td>
</tr>
<tr>
<td>US347</td>
<td>B</td>
<td>3</td>
<td>US176</td>
<td>B</td>
<td>15</td>
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<tr>
<td>US343</td>
<td>A</td>
<td>4</td>
<td>US191</td>
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<td>16</td>
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<tr>
<td>US491</td>
<td>A</td>
<td>4</td>
<td>US325</td>
<td>A</td>
<td>19</td>
</tr>
<tr>
<td>US713</td>
<td>A</td>
<td>4</td>
<td>US332</td>
<td>A</td>
<td>19</td>
</tr>
<tr>
<td>US317</td>
<td>A</td>
<td>5</td>
<td>US545</td>
<td>A</td>
<td>19</td>
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<tr>
<td>US481</td>
<td>A</td>
<td>5</td>
<td>US263</td>
<td>IS negative</td>
<td>17</td>
</tr>
<tr>
<td>US773</td>
<td>A</td>
<td>5</td>
<td>US15</td>
<td>IS negative</td>
<td>17</td>
</tr>
<tr>
<td>US772</td>
<td>A</td>
<td>6</td>
<td>US17</td>
<td>IS negative</td>
<td>18</td>
</tr>
<tr>
<td>US470</td>
<td>B</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.3.4 Selection of IS6110 single copy strains for Sequencing Study

Eight IS6110 single copy strains of M. tuberculosis were chosen for this study. The IS6110 type and spoligotype (if available) of the strains are shown in Table 3.2.

Table 3.2 IS6110 single copy strain and the respective spoligotype

<table>
<thead>
<tr>
<th>Single strains</th>
<th>IS6110 RFLP type</th>
<th>Spoligotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>US176</td>
<td>B</td>
<td>15</td>
</tr>
<tr>
<td>US199</td>
<td>B</td>
<td>ND</td>
</tr>
<tr>
<td>US101</td>
<td>B</td>
<td>8</td>
</tr>
<tr>
<td>US278</td>
<td>A</td>
<td>ND</td>
</tr>
<tr>
<td>US102</td>
<td>A</td>
<td>1</td>
</tr>
<tr>
<td>US191</td>
<td>B</td>
<td>16</td>
</tr>
<tr>
<td>US301</td>
<td>A</td>
<td>1</td>
</tr>
<tr>
<td>US774</td>
<td>A</td>
<td>ND</td>
</tr>
</tbody>
</table>

* ND = Not Done

3.3.5 Preparation of PCR product for sequencing and cloning

Analysis of the IS6110/986 sequence suggests that expression of the transposase coded by ORFb is controlled by ribosomal frameshift during translation of ORFa in the ORFa-ORFb overlap region (McAdam et al., 1990). Base changes in the overlap region or in the region coding for the transposase may influence transposability of the element. In
order to study the element from the chosen strains, templates for sequencing were prepared from each strain by PCR employing the AP1 and BX2 primers (see Table 3.3), which gave a product of 1240bp (Fig. 3.5) containing most of the IS6110 element (Fig. 3.1). Amplification was performed using 100ng of each primer and 10-100ng of template. Other parameters are as described in Chapter 2, Section 2.2.11. The PCR product was purified from agarose gels using the Geneclean II kit (BIO101) before sequencing was performed.

Table 3.3 Sequence data of primers derived from IS6110 for PCR and sequencing.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Primers sequence</th>
<th>Position in IS6110 (bp)</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP1</td>
<td>5' GACTCCAGTTCTTGGAAAGG 3'</td>
<td>27-46</td>
<td>PCR and sequencing</td>
</tr>
<tr>
<td>BX2</td>
<td>5' TTGCAGCCGGCGGGACGTC GC 3'</td>
<td>1267-1248</td>
<td>PCR and sequencing</td>
</tr>
<tr>
<td>BX1</td>
<td>5' CTGGCCGCTCGCTCCACGA 3'</td>
<td>891-910</td>
<td>sequencing</td>
</tr>
<tr>
<td>BX3</td>
<td>5' TCGGAGCGGTCAAGCTCC 3'</td>
<td>1077-1096</td>
<td>sequencing</td>
</tr>
<tr>
<td>BX4</td>
<td>5' CCTGCCCTGGGAGGTCGCTC 3'</td>
<td>1062-1043</td>
<td>sequencing</td>
</tr>
<tr>
<td>TB1</td>
<td>5’GGACAACGCCGGAATTCGAAGG3’</td>
<td>295-318</td>
<td>sequencing</td>
</tr>
<tr>
<td>TB2</td>
<td>5’TAGGCCGTCGGTGCAAAAGGGCCA3’</td>
<td>851-826</td>
<td>sequencing</td>
</tr>
<tr>
<td>TB3</td>
<td>5’ACGACCACCATCACCAGGGAG3’</td>
<td>505-524</td>
<td>sequencing</td>
</tr>
<tr>
<td>TB4</td>
<td>5’ CCTGTCGGGACCACCCCGC 3’</td>
<td>689-707</td>
<td>sequencing</td>
</tr>
<tr>
<td>AP2</td>
<td>5’TGTGCAAGATCGACTCGACAC3’</td>
<td>457-438</td>
<td>sequencing</td>
</tr>
<tr>
<td>AP3</td>
<td>5’CTGGCGCACCACCATTTACGCA 3’</td>
<td>222-203</td>
<td>sequencing</td>
</tr>
<tr>
<td>AP4</td>
<td>5’TGGCCGTCAGCAGATTGC 3’</td>
<td>125-144</td>
<td>sequencing</td>
</tr>
</tbody>
</table>

75
Figure 3.5  PCR result of a single copy strain using AP1 and BX2 primers and visualised using a preparative agarose gel. This amplification procedure resulted in a 1,240 bp product. Numbers at left indicate sizes of the standard DNA marker (100bp DNA ladder) fragment.
3.3.5.1 Sequencing the amplification product

The sequencing of the PCR amplification product was based on the method described by McPherson et al., (1991). Sequencing was performed using Sequenase V2.0 (USB) employing either $^{35}$S or $^{32}$P as labels. Both strands were sequenced using primers derived from the IS6110 sequence and are listed in Table 3.3. Sequencing with each primer was performed at least twice to ensure accuracy of the sequence. Ambiguities were resolved by repeating the sequencing procedure until a consensus was achieved. The sequencing strategy is also depicted in Fig. 3.6.

Figure 3.6 Schematic diagram of the position and direction of the derived primers, for PCR and/or sequencing.
3.3.6 Cloning PCR fragment using the Invitrogen TA cloning kit

In addition to direct sequencing of PCR products, the Ap1 BX2 product from two strains, US301 and US774, were also cloned.

3.3.6.1 Ligation of the purified PCR amplified product

The extracted product was ligated to the pCR™ II vector provided by the TA Cloning kit (Invitrogen). One to two µl of PCR product (corresponding to approximately 20 ng of DNA) was ligated with 50 ng (20 fmole) of pCR™ II vector. A map of the pCRII™ vector (Invitrogen) is shown in figure 3.9. Each ligation reaction was set up as follows:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh PCR product</td>
<td>1-2 µl</td>
</tr>
<tr>
<td>10X ligation buffer</td>
<td>1 µl</td>
</tr>
<tr>
<td>pCRTM II vector (25ng/µl)</td>
<td>2 µl</td>
</tr>
<tr>
<td>Sterile water</td>
<td>to 9 µl</td>
</tr>
<tr>
<td>T4 DNA ligase (4 Weiss units)</td>
<td>1 µl</td>
</tr>
</tbody>
</table>

The ligation reaction was incubated at 14°C overnight.
Figure 3.7 The map of the PCR™ II vector (Invitrogen). The site of insertion of the PCR product is shown in the multiple cloning site.
3.3.6.2 Transformation of the ligation reaction

Transformation was carried out using the competent cells provided by the manufacturer (One Shot™ INVαF' competent cells). The transformation process was started by thawing a vial of 0.5 M β-mercaptoethanol (β-ME) (provided by the manufacturer) and one 50μl vial of frozen One Shot™ INVαF' competent cells. Two microlitres of 0.5 M β-mercaptoethanol was pipetted into each vial of the competent cells and mixed by gently stirring with the pipette tip. Two microlitres of each ligation reaction was pipetted directly into the competent cells and mixed by stirring gently with the pipette tip. The vials were then incubated on ice for 30 minutes. The vials were heat shocked for exactly 30 seconds in a 42°C waterbath without mixing or shaking. Four hundred and fifty microlitres of SOC medium (provided by the manufacturer) was added at room temperature. The vials were then shaken at 37°C for 1 hour at 225 rpm in a rotary shaking incubator. Fifty microlitres and 200μl from each transformation were spread on separate, labelled LB agar plates containing 50μg/ml of ampicillin and X-Gal (40μl of 20mg/ml X-Gal solution overlaid on agar plates, and let the agar plate dried in incubator before use). The plates were then incubated in a 37°C incubator over night. The plates were then transferred to 4°C for 2 to 3 hours to allow for colour development.

3.3.6.3 Analysis of the recombinant clone

Colonies most likely to carry the desired insert (white colonies) were picked from the LB plates and plasmid isolation was performed (plasmid extraction protocol in Chapter 2). The plasmid extracted were digested with EcoR1 restriction enzyme and were run on a 0.8% TBE gel. Plasmids showing inserts of approximately 1.2kb (Fig. 3.8) were subjected to PCR using the API and BX2 primers to confirm that the plasmids carry the correct insert. Two clones, designated pTT301 (Fig. 3.8, lane 3) and pTT774 (Fig. 3.8, lane 4) carrying the PCR product from strains US301 and US774 respectively, were selected for sequencing.
Lane 1: ØX174HaeIII DNA marker
Lane 2: Uncut pTT301
Lane 3: pTT301
Lane 4: pTT774
Lane 5: pTT301/1
Lane 6: pTT774/1
Lane 7: pTT301/2

Figure 3.8 Digestion of recombinant plasmids with EcoR I restriction enzyme and electrophoresis on a 0.8% TBE gel. Number at left indicate the sizes of standard DNA marker (ØX174 Hae III) fragment in base pairs.
3.3.7 Sequencing of the insert

Two approaches were carried out to determine the sequence of the recombinant clone. First, the recombinant clones were sequenced using the standard manual method employing Sequenase V2.0 and the same set of primers used for direct sequencing of PCR products as described previously. In addition, pTT301 was independently sequenced by automated sequencing (ABI Model 373) by Susan Wall and Steve Dyer at the University of Surrey.

3.3.8 Sequence results and assembly

Sequences obtained from each template were assembled using the Microgenie program (Beckman). The final sequences, of which eight were produced by direct sequencing of PCR products of the strains listed above (Table 3.2) and two by sequencing of the recombinant plasmids pTT301 and pTT774, were aligned using the same program and found to be identical. The insert sequence of pTT301 obtained by using automated sequencing independently performed at the University of Surrey was also in complete agreement with the results of this study. The consensus sequence, which will be referred to as TH101 was then compared to the published sequences of IS986, IS6110 and IS987.

3.3.9 Comparison of sequences of TH101, IS986, IS6110 and IS987

Sequences of IS986, IS987 and IS6110 were obtained from the EMBL/GenBank/DDBJ Nucleotide Sequence Databases under the accession numbers X52471, X57835 and M29899 respectively.
TH101 is 1234 bp in length. A multiple alignment of the corresponding regions of the four sequences was performed using the Microgenie program. The results of this alignment is shown in Fig. 3.9. The significance of this result will be discussed further.
**Figure 3.9** Sequence alignment of TH101, IS987, IS986 and IS6110 showing that the sequence of TH101 is identical to that of IS987.

Region showing base differences are marked by bold characters or ( - ) symbol.

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CATCGCCGAT CATCACGGCCC ACCGGCGGAG ACCGGCGGAGG CCCCGATGTT TGCGCTGAGTC IS6110
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CATCAACCACG GAGCCCGACCC CCGCCGAGCT GCCGGATGCC GAACCTCAAGG AAGGACATCC IS6110
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CGAGCGATGC GAGCAAGGCA TCTGGACCAG CCACACAAGAA GGCGTACTCG ACCTGAAAGA IS986
CGAGCGATGC GAGCAAGGCA TCTGGACCAG CCACACAAGAA GGCGTACTCG ACCTGAAAGA IS6110
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CGTTATCCAC GATACGGATA AAGGGATCTCA GTACACATCG ATCCGGTCTA GCAGGACGGCT IS986
CGTTATCCAC GATACGGATA AAGGGATCTCA GTACACATCG ATCCGGTCTA GCAGGACGGCT IS6110
******************************************************************************
The sequences shown in Figure 3.9 span the region between the AccIII and XhoI sites of the insertion sequence (corresponding to position 31 to 1264 of the insertion sequence IS6110).

2. Bases 1 to 15 and 1218 to 1234 shown in Figure 3.9 are actually derived from the primers used to prepare the sequencing template.
3.4 Discussion

Several studies (including this one) have shown that a significant minority of *M. tuberculosis* strains has only one or two copies of IS6110 (Fomukong et al., 1994, Musa et al., 1994). Analysis of a selection of single copy strains has established that in most strains the insertion sequence is located at the same position on the chromosome, which corresponds to the site of insertion in *M. bovis* BCG (referred as the BCG1 site by Fomukong et al., 1994). A logical explanation of this phenomenon is that in these organisms the element is defective in transposition and that the loss of transposability may have occurred in a strain at an early stage in the evolution of the *M. tuberculosis* complex and the single-copy *M. tuberculosis* strains are the direct descendants of this "ancient strain".

Studies have shown that some BCG strains have two copies of this element while the majority have one copy (Fomukong et al., 1992). Identical results were obtained from BCG strains of various sources and lineages (van Soolingen et al., 1991; Cave et al., 1992; Musa et al., 1994) suggesting that the element in BCG is defective in transposition. Consequently the identical nature of the sequence of the element in IS6110 single copy *M. tuberculosis* strains and that of IS987 tend to suggest that the element in these strains are also defective in transposition.

Comparison of the published sequences of IS987 and IS986 (which has been shown to be transposable, Fomukong and Dale, 1993) appeared to show that the fundamental difference between the two is that in IS986 the ORFa is split into two reading frames, designated as ORFa1 and ORFa2 (McAdam et al., 1990) whereas ORFa is in a single reading frame in IS987 (Hermans et al., 1991). It is possible that proper expression of ORFa (which is thought to regulate expression of the transposase coded by ORFb) require translational frameshifting as seen in IS986 and a mutation which results in realignment of ORFa1 and ORFa2 into a single reading frame such as seen in IS987 (and
TH101) would result in lost of transposibility. This hypothesis is indirectly supported by the fact that IS6110 also differs from IS986 in the ORFa region and has also been shown to be non-transposable (Mendiola et al. 1992).

However, this hypothesis may not be correct in the light of results obtained from another study. In that study a copy of the insertion sequence (from a variant of the IS6110-multicopy W strain, Pablo et al., 1996) which was shown to have recently transposed, were sequenced and also found to be identical to IS987 (B. Plikaytis, personal communication) and thus to TH101.

This result therefore suggests that IS987 and the elements in the IS single copy strains used in this study actually are capable of transposition. This raises the question that the difference in the ORFa region between IS987 and IS986 may be due to sequencing error. In the light of this study, a cloned copy of the original IS986 element was re-sequenced at the University of Surrey and confirms that the sequence of IS986 and IS987 are identical (J. Dale, personal communication).

A cloned copy of IS6110 has not been available for re-sequencing; however, this originated from the M. tuberculosis strain H37Rv, which is also the source of DNA for the M. tuberculosis genome sequencing project. One of the cosmids (Y339) has been sequenced and shown to contain an IS with sequence that is identical to IS987 (Badcock, K., Churcher, C.M., Barrell, B.G., Rajandream, M.A., Walsh, S.V.,(1996); unpublished; accession number Z77163) indicating that the differences in the published sequence of IS6110 are also due to sequencing errors, and that all copies of this element in the M. tuberculosis complex are identical.

These results therefore suggest that the element in most if not all low copy number strains of the M. tuberculosis complex are fully capable of transposing and the low copy number in these strains requires a different explanation.
ORFa in IS986 has been shown to contain a potential initiation codon preceded by a region showing a good match with the consensus Shine-Dalgarno sequence. However, examination of the sequence further upstream does not show the presence of a potential promoter region suggesting that transcription of ORFa is driven exogenously. The mobility of the element therefore will be dependent on the transcriptional activity of the flanking chromosomal region.

A study by Fomukong et al (1994) indicated that in most single copy *M. tuberculosis* strains, the element is located in the region designated as the DR region by Herman et al (1991). The structure of the DR region suggests that it is unlikely to be transcribed, and hence any IS inserted into this region will be of low mobility. The ancestral strain of the *M. tuberculosis* complex may have originally acquired IS986 into this region before diverging into the various variants of the *M. tuberculosis* complex hence resulting in the presence of this element in the same site within the DR region in almost all strains and variants that have been studied. The multicopy strains may have arisen by a rare event which resulted in the insertion of a copy of IS986 from the DR region into a transcriptionally active site. Alternatively, the multicopy strains may have arisen from acquisition of a second copy of IS986 directly into a transcriptionally active site.

The DR region is postulated to be a “hot spot” for integration of IS986 (Herman et al 1991) and regardless of the original site of integration almost all strains would eventually have a copy of IS986 in this region. It is then possible that the low copy number strains may have evolved by the loss of the elements from multicopy strains except the copy inserted into the DR region which as a consequence of its low mobility is also of greater stability and is not easily lost.

The evolutionary relationships of the strains and variants of the *M. tuberculosis* complex requires further research. However the data generated from this study may provide some clues towards the final answer and at the very least have resulted in a re-examination and
correction of the basic structure of IS986 which has been and continues to be an important tool in mycobacterial research and diagnosis.
Chapter 4
Chapter 4

Establishment and Evaluation of a Multiplex PCR for the Rapid Diagnosis of *M. tuberculosis*

4.1 General Introduction

The use of PCR for diagnosis of tuberculosis is not new. Many PCR assays have been developed for detection of mycobacterial DNA which uses different strategies and target sequences. Some of the targets used are able to detect mycobacteria in general (Hance *et al.*, 1989; Böddinghaus *et al.*, 1990; Patel *et al.*, 1990), while others are more specific such as for *M. tuberculosis* (Shankar *et al.*, 1991; Cousins *et al.*, 1992) or *M. leprae* (Williams *et al.*, 1990). Table 4.1 lists some examples of the targets commonly used for the detection of mycobacteria by PCR. Some primers are designed to amplify species-specific target, for example, primers derived from the *mtp40* protein gene (Table 4.1) for the detection of *M. tuberculosis*, whereas some primers were derived for multiple species amplification which will be further followed by a second amplification or by a DNA probe step to detect the specific species. An example of this is the detection of the 65kDa protein gene (Table 4.1), in which the primers for the first PCR amplification allow detection of the mycobacteria genus, followed by a nested PCR or a DNA probe using internal sequences for identification to species level. Recently, multiplex PCR has also been designed to amplify two or more target sites using two or more pairs of primers. Portillo *et al.*, (1996), described a multiplex PCR for the detection and differentiation of tuberculosis and non-tuberculosis infection using three pairs of primers. The first set of primers amplifies a 506bp fragment of the 32kDa antigen gene, which is present in most mycobacterium species. The second set of primers amplifies a 984bp fragment from IS6110 which belongs to the *M. tuberculosis*...
complex. The third primer pair, derived from an *M. tuberculosis* species-specific sequence named *mtp40*, amplifies a 396bp fragment. It should be noted however that a recent paper suggests that the *mtp40* gene may not be present in some *M. tuberculosis* strains (Weil *et al.*, 1996). Other multiplex PCR methods for the detection of *M. tuberculosis* were also reported by Sinclair *et al.*, (1995) and Tötsch *et al.*, (1994).
Table 4.1 Some common PCR targets and primers for the detection of Mycobacterium species.

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<td><em>M. tuberculosis</em></td>
<td>Portillo <em>et al</em> 1991</td>
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<td>Repetitive element</td>
<td><strong>Primers</strong>&lt;br&gt;MtBa: GGTCGGCTGACTCCCGGGGCT(sense)&lt;br&gt;MtBb: CG TGAGGACCGGGCCGCT(antisense) &lt;br&gt;<strong>Primers</strong>&lt;br&gt;MtBc: TACGGATTCCGTCCTCCGGTCGACCTGATTCCGATTCGTTGAGTCCGACTCCGAGCTCCCGGGGCT(antisense)&lt;br&gt;MtBd: CACGCCCGGAAACTCCGACCCGCGGCGGCTCAGGCTCCGAGCTCCCGGGGCT(antisense) &lt;br&gt;<strong>Primers</strong>&lt;br&gt;5'GCCAGGGCGAGCTGTTGAGTCCGACTCCGAGCTCCCGGGGCT(antisense)&lt;br&gt;5'GCCAGGGCGAGCTGTTGAGTCCGACTCCGAGCTCCCGGGGCT(antisense)</td>
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<td>MPB 64 protein</td>
<td><strong>Primers</strong>&lt;br&gt;1: 5'TCCGCTGCCCAGCTGCTTCCGCTCCGAGCTCCGACTCCGAGCTCCCGGGGCT(antisense)&lt;br&gt;2: 5'GTCCTGCAGGATCTAGGCCATTCCGACTCCGAGCTCCCGGGGCT(antisense)</td>
<td><em>M. tuberculosis complex</em></td>
<td>Shankar <em>et al</em> 1991</td>
</tr>
<tr>
<td>38 kDa protein</td>
<td><strong>Primers</strong>&lt;br&gt;MT1: ACCACCCGAGGAGGCTCCGCTGA(sense)&lt;br&gt;MT2: GATCTGCGGAGGCTCCGCTGAGGT(antisense)</td>
<td><em>M. tuberculosis complex</em></td>
<td>Sjöbring <em>et al</em> 1990</td>
</tr>
<tr>
<td>MPB 70 antigen</td>
<td><strong>Primers</strong>&lt;br&gt;TB1A: 5'GAACAATCCCGAGGTCCGACAA3' &lt;br&gt;TB1B: 5'ACACGCTGCTCAATCATGTA3'</td>
<td><em>M. tuberculosis complex</em></td>
<td>Cousins <em>et al</em> 1992</td>
</tr>
</tbody>
</table>
Table 4.1 continue......

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primers</th>
<th>Specificity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>18kDa protein</td>
<td>Primers&lt;br&gt;ATTGTCGAGCGTTTGTTGCTGAGCT (sense)&lt;br&gt;TGACAACAAACGTTGCTGAGCT (antisense)</td>
<td>M. leprae</td>
<td>Williams et al 1990</td>
</tr>
<tr>
<td>16S rRNA</td>
<td>Primers&lt;br&gt;246: AGAGTTTGATTCCTGCTAG (sense)&lt;br&gt;264: TGCACACAGGGCAAGGGA (antisense)</td>
<td>Genus</td>
<td>Boddinghaus et al 1990</td>
</tr>
<tr>
<td></td>
<td>Primers&lt;br&gt;246: AGAGTTTGATTCCTGCTAG (sense)&lt;br&gt;260: GTTCTGTGGATGTAAGGCA (antisense)</td>
<td>Genus</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Primers&lt;br&gt;246: AGAGTTTGATTCCTGCTAG (sense)&lt;br&gt;266: CACGCCCCAGTTAGCTGT (antisense)</td>
<td>Genus</td>
<td></td>
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<tr>
<td></td>
<td>Probes&lt;br&gt;5’TTCAGAAAGACGAGACAC3’&lt;br&gt;5’ACCACAGAAAGACGAGACAC3’&lt;br&gt;5’ACCAAGAAAGACGAGACAC3’&lt;br&gt;5’ACCTAAAAGACGAGACAC3’</td>
<td>Genus&lt;br&gt;M. tuberculosis&lt;br&gt;M. avium/paratuberculosis&lt;br&gt;M. intracellulare</td>
<td></td>
</tr>
<tr>
<td>IS6110</td>
<td>Primers&lt;br&gt;TB294 5’GGACAAACCGGGAAATTTCGGCAGG3’&lt;br&gt;TB850 5’AGAGCGTGCCTGACAAAGCCAG3’</td>
<td>M. tuberculosis complex</td>
<td>Wilson et al 1993</td>
</tr>
<tr>
<td></td>
<td>Nested Primers&lt;br&gt;TB505 5’ACACCAATCAGCACC3’&lt;br&gt;TB670 5’AGTTCGGTCATCGCC3’</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4.2 Development of a multiplex PCR for detection of *M. tuberculosis* in clinical specimens.

4.2.1 Choice of targets for amplification.

Among the targets used in amplification, primers derived from IS6110 have been the most commonly used for PCR (Clarridge *et al.*, 1993; Wilson *et al.*, 1993, Kox *et al.*, 1994; Noordhoek *et al.*, 1994). IS6110 has been shown to be a powerful tool in epidemiological studies of *M. tuberculosis* (Hermans *et al.*, 1990; Otal *et al.*, 1991; van Soolingen *et al.*, 1991). It was originally thought that all strains of *M. tuberculosis* contain multiple copies of IS6110 providing a good target site for PCR. Figure 4.1 shows a schematic representation of IS6110 indicating the regions commonly targeted for amplification. Table 4.2 shows the sequences of the primers used to amplify the regions depicted in Figure 4.1.

However, it was subsequently shown that there are some strains of *M. tuberculosis* that possess only a single copy of IS6110, while others do not carry any copy of IS6110 (IS6110-negative strains). Yuen *et al.*, (1993) reported four IS6110 negative strains from Vietnamese patients, van Soolingen *et al.*, (1993) reported one IS6110-negative strain from India and Das *et al.*, (1993) reported three IS6110-negative strain in Hong Kong. In the recent restriction length polymorphism (RFLP) epidemiological study in Malaysia, using IS6110 as a probe, 491 *M. tuberculosis* isolates were screened and out of that, five IS6110-negative strains were also detected (unpublished data). The distribution of these IS6110-negative strains seems to be geographically restricted, to some extent to the Asian region. The data suggest that false-negative results may occur when only IS6110 is used as the target for detection particularly when screening tuberculosis samples from the Asian region.
Figure 4.1 Schematic diagram showing IS6110 regions commonly targeted for amplification.

P: *Pvu*II enzyme restriction site

B: *Bam*H1 restriction enzyme and primer BX1 site

X: *Xho*1 restriction enzyme and primer BX2 site
### Table 4.2: Primer sequences and expected amplification product sizes with reference to Figure. 4.1

<table>
<thead>
<tr>
<th>References</th>
<th>Primers sequences based on IS6110/IS986</th>
<th>position(bp) IS6110/IS986 5' to 3'</th>
<th>size (bp)</th>
</tr>
</thead>
</table>
| Eisenach et al 1990 (IS6110) | CCTGCGAGCGTAGGCCTCGG  
CTCGTCCAGCGGCTTTGCG | 865-883  
762-781 | 123 |
| Kolk et al 1992 (IS986) | INS1 CGTGAGGGCATCGAGGTGGC  
INS2 GCGTAGGCGTCGGTGACAAA | 641-660  
866-885 | 245 |
| Shawar et al 1993 (IS6110) | 41: CCTGCGAGCGTAGGCGTCGG  
43: TCAGCCGCGTCCACGCGCCA | 865-883  
568-588 | 317 |
| Wilson et al 1993 (IS6110) | TB1 GGACAACGCAGGATTGCGAAGGCG  
TB2 TAGGCCTCGGTACAAAAAGGCACG  
TB3 ACGACCACATCAACC  
TB4 AGTTTGCTACATCGCC | 295-318  
51-874  
505-519  
669-685 | 586  
165  
165  
165 |
| Portillo et al 1996 (IS6110) | IS5 CGGAGACCGTGCCTGAAATGGG  
IS6 GATGGACCGCCAGGGCTCTGC | 194-213  
1158-1177 | 984  
541 |
| Kox et al 1994 (IS6110) | PT8 GTGCGGATGCTCGCAGAGAT  
PT9 CTCGATCCCTACGTTCA | 106-125  
627-647 | 541  
541 |
Most *M. tuberculosis* strains contain multiple copies of IS6110 which makes this target still an excellent choice for detection of PCR because the multiple targets would make the test less vulnerable to mutations that might affect PCR targeting single copy genes. However, the existence of IS6110-negative and IS6110 single copy strains also suggest that it would be prudent to include a second different target to avoid missing such strains due to the absence of the target or in the case of the single copy strains mismatches at the priming sites that could have arisen from mutations.

For reasons already described above, the first choice as target is the insertion element IS6110. The selected region in IS6110 was amplified using primers designated as BX1 and BX2 (see Fig. 4.1). The second target is another insertion sequence-like element designated as B9 (Musa *et al*., 1994). The B9 sequence, was derived from the A3/2 fragment (Fig. 4.2) previously described by Zainuddin and Dale (1989). Although present only in single copy in the *M. tuberculosis* genome, the B9 sequence has been shown to be potentially useful in for detection of *M. tuberculosis* as it is present in all *M. tuberculosis* strains tested including in IS6110-negative strains (Musa, 1996). The primers used to amplify a region within the B9 sequence were designated as MM1 and MM2.
Figure 4.2 Schematic diagram of the A3/2 fragment (Zainuddin and Dale, 1989). The position of the B9 sequence and the region that is amplified using the MM primers.
4.2.2 Choice of Primers

The following criteria were taken into account in selecting primers for multiplex PCR:

a) The sizes of the amplified products should be different enough to allow easy discrimination in agarose gels.

b) The primers should not have potential priming sites with each other.

c) The optimal parameters for PCR for both pairs of primers should be equal or nearly equal.

In this study the IS6110 primers used were derived from the region of IS6110 between the BamH1 and XhoI restriction sites (see Fig. 3.1, Chapter 3) designated as the BX region. The BX region has previously been used as a probe to generate RFLP from M. tuberculosis complex isolates (Fomukong et al., 1992). The BX primers used in this study are:

BX1: 5' CTG GCG GGT CGC TTC CAC GA 3' (891-910)
BX2: 5' TTC GAC CGG CGG GAC GTC GC 3' (1266-1247)

The number in brackets refer to the base pair position according to the IS986 sequence previously reported by McAdam et. al., (1990). Amplification with the BX primers (both 20bp in length) will give a 376bp amplification product.

Primers for amplification of the B9 element are based on the sequence of this element previously reported by Musa et al., 1994. The sequence of the primers, designated as MM1 and MM2 are:

MM1: 5’ GGC CTG GAC TCC CGT AGC CT 3’ (1007-988)
MM2: 5’ TCT CGT GGC GAC TGT TAT GAC A 3’ (830-851)
The numbers in the brackets refer to the base position according to the sequence reported by Musa, (1996). The MM1 and MM2 primers are 20bp and 21bp in length respectively. Amplification with the MM primers will give a 178bp amplification product.

4.3 Optimisation of the multiplex PCR reaction condition

4.3.1 Annealing temperature

To begin optimisation of the PCR test, the initial amplification parameters adopted were as follows:

Template DNA = 10ng
dNTPs (each) = 200µmol
MgCl2 = 1.5 mmol
Taq = 1.25U
Primers = 200ng each

The reaction was done in a total volume of 50µl.

The amplification parameters used in the initial set up were as follows:

1. One cycle consisting of an initial denaturing step of 95°C for 2 minutes.
2. Twenty nine cycles consisting of denaturation at 95°C for 1 minute, annealing at 68°C for 1 minute and extension at 72°C for 1 minute.
3. One cycle consisting of 95°C for 1 minute, annealing at 68°C for 1 minute and extension at 72°C for 2 minutes.
4. The reaction was then held at 4°C before further analysis.
The BX primers were previously used at an annealing temperature of 65°C by Fomukong and Dale (1993) whereas the MM primers were previously shown to work well at 68°C by studies done at this laboratory (Musa, 1996). Since the higher temperature would minimise the chances of non-specific binding and amplification, it was decided to begin the optimisation process by using 68°C as the initial annealing temperature. It was subsequently shown that both pairs of primers worked well at this annealing temperature (see results shown later).

The Tm of each primer, calculated using a simple formula (Sambrook et. al., 1989), \[ Tm = 2(A+T) + 4(G+C) \] shows that the Tm of the primers used are 68°C (BX1), 70°C (BX2), 68°C (MM1) and 66°C (MM2). Although the annealing temperature that was used is actually 2°C above the Tm of MM2, the experimental results obtained (see below) proved that it is suitable for this PCR test. This annealing temperature and the thermal cycling programme described above were maintained throughout this study.

4.3.2 Optimisation of primer

Primer concentration is a critical parameter for successful multiplex PCR and should be determined for each type of reaction. To establish the optimal concentration of each primer multiplex PCR was carried out using a fixed amount of template DNA (10ng) and variable amounts of BX and MM primers. Other PCR parameters were as described above. The results in Figure 4.3 shows that the best results were obtained using 100ng and 200ng of each BX and MM primer respectively (lane 3). These primer amount were used as parameters for optimisation of other PCR.
Figure 4.3: Optimisation of PCR using different combinations of BX and MM primers amount. The template DNA was 10ng of chromosomal DNA of an IS6110 single copy strain.
4.3.3 Optimisation of MgCl₂ concentration

A number of publications describes the effect of various MgCl₂ concentration (Erlich, 1989; Saiki, 1989; McPherson et al., 1991; Mullis et al., 1994) on PCR. The MgCl₂ concentration is important as excess MgCl₂ will result in the accumulation of non-specific amplification product and insufficient MgCl₂ will reduce the yield. However, the exact concentration of MgCl₂ has to be empirically determined as the optimal concentration has been found to vary from primer to primer. Figure 4.4a shows the results of amplification using BX primers at various MgCl₂ concentration. The results show that the best MgCl₂ concentration is in the range of 1 to 6mM. The MgCl₂ range for the MM primers are from 1.5mM to 8mM [Fig. 4.4b]. The results suggest that the optimum MgCl₂ concentration is in the range of 1.5-6mM. To determine the optimum MgCl₂ concentration when the primers are used together in one tube, multiplex PCR was then performed using MgCl₂ in the range of 0.5-10mM (Fig. 4.5a). The results shows that 1.5mM of MgCl₂ in the multiple PCR gave the best yields of both products. Specificity of the amplified fragment was verified using BX and MM probes (results not shown).
Figure 4.4: Results of amplification using BX primers (a) and MM primers (b) at various MgCl₂ concentration. The template DNA was 10ng of chromosomal DNA of an IS6110 single copy strain.

<table>
<thead>
<tr>
<th>Lane</th>
<th>MgCl₂ concentration used</th>
<th>MgCl₂ concentration used</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.5 mM</td>
<td>3 mM</td>
</tr>
<tr>
<td>3</td>
<td>1.0 mM</td>
<td>4 mM</td>
</tr>
<tr>
<td>4</td>
<td>1.5 mM</td>
<td>6 mM</td>
</tr>
<tr>
<td>5</td>
<td>2.0 mM</td>
<td>8 mM</td>
</tr>
<tr>
<td>6</td>
<td>2.5 mM</td>
<td>10 mM</td>
</tr>
</tbody>
</table>

Lane 1: 100bp ladder DNA marker
Figure 4.5: Multiplex PCR using various concentration of MgCl\(_2\). The template DNA was 10ng of chromosomal DNA of an IS6110 single copy strain.
4.3.4 Optimisation of Taq polymerase enzyme

The most commonly used DNA polymerase in PCR is Taq DNA polymerase isolated from *Thermus aquaticus*. Alternative heat stable enzymes are also available such as *Thermus thermophilus* (United State Biochemicals), *Bacillus stearothermophilus* (Bio-Rad) and *Thermococcus litoralis* which is also referred to as the “Vent” polymerase (New England Biolabs).

Taq DNA Polymerase was the only heat stable enzyme used in this study. To ensure consistency of results Taq polymerase from only one manufacturer (Boehringer Mannheim) was used throughout this study. To determine the optimum amount of enzyme required, varying amounts of Taq DNA polymerase were tested using purified chromosomal DNA from an IS6110 single copy MTB strain in a total volume of 50μl reaction. The results (Fig. 4.6) show that an enzyme amount as low as 0.25 units gave sufficient yield of products for observation on agarose gels. 1 unit of Taq per 50μl reaction was chosen as standard throughout this study. This amount of enzyme should ensure that the enzyme is slightly but not grossly in excess while at the same time is sufficiently economical for large scale usage. Consistent results were obtained in a number of preliminary tests using 1.0 unit of enzyme for each 50μl PCR reaction (results not shown)
Lane 1: 100bp ladder DNA markers

<table>
<thead>
<tr>
<th>Amount of Taq used (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lane 2: 0.25</td>
</tr>
<tr>
<td>Lane 3: 1</td>
</tr>
<tr>
<td>Lane 4: 2</td>
</tr>
<tr>
<td>Lane 5: 4</td>
</tr>
<tr>
<td>Lane 6: 8</td>
</tr>
<tr>
<td>Lane 7: 10</td>
</tr>
</tbody>
</table>

Note: The template DNA was 10ng of chromosomal DNA of an IS6110 single copy strain

**Figure 4.6**: Optimisation of Taq DNA polymerase enzyme.
4.3.5 Final optimised PCR parameters

Based on the results of the optimisation procedures described above further work using multiplex PCR were performed using the following protocol:

Template DNA = 10ng or 16μl of processed clinical sample (see below)
dNTPs (each) = 200μmol
MgCl₂ = 1.5 mmol
Taq DNA polymerase = 1 U
BX 1 primer = 100ng
BX 2 primer = 100ng
MM 1 primer = 200ng
MM 2 primer = 200ng

The PCR reaction was done in a total volume of 50μl.
The thermal cycling programme described in Section 4.3.1 was used throughout the rest of this study.

4.4 Sensitivity and specificity of the multiplex PCR test

To determine the sensitivity of the test, multiplex PCR was performed using different amounts of template DNA (IS6110 single copy MTB strain) ranging from 100ng to 1fg and the results are shown in Fig. 4.7. The results indicated that the limit of detection of this PCR test is 1pg of template DNA. Although the detectable level is lower than that reported by some workers (Manjunath et al., 1991; Shawar et al., 1993) this sensitivity level is comparable to that reported by others (Andersen et al., 1993; Portillo et al., 1996). The PCR products, after running on agarose gels, were then transferred to a
nylon membrane and probed consecutively with the BX and MM probes as previously described (Chapter 3). The results shown in Fig. 4.7 (a) and (b) confirms that the respective targets were correctly amplified.

The next step in the developmental process is to determine the specificity of this multiplex PCR. Recently, Kent et al., (1995), demonstrated that the IS6110-derived primers reported by Wilson et al., (1993), were able to amplify DNA from other mycobacterium species such as M. fortuitum, M. kansasii, M. chelonei, M. xenopi, M. avium-intracellulare complex and M. malmoense. This is believed to be due to the presence of other elements of the IS3 family in atypical mycobacteria, and that these primers were derived from a conserved region of the IS3 family (Kent et al., 1995). The data suggested that false-positive results may be obtained if primers derived from this region (which is thought to code for the transposase protein of IS6110) are used, and should be avoided.

The IS6110 primers used in this multiplex PCR were derived from an area which is not within the region reported by Kent et al. (1995) (Fig. 4.1). To determine the specificity of this test, PCR were performed using 10 ng of chromosomal DNA from various atypical species of mycobacteria as templates. Fig 4.8a shows that the BX and MM primers did not amplify any of the atypical mycobacteria tested. This was then verified by Southern hybridisation using both BX and MM probes (Fig. 4.8b). The results show that no products were seen in the lanes where atypical mycobacterial DNA were used as templates.
Lane M: 100bp ladder DNA marker

<table>
<thead>
<tr>
<th>Amount of template</th>
<th>Amount of template</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lane 1: 100ng</td>
<td>Lane 6: 1pg</td>
</tr>
<tr>
<td>Lane 2: 10ng</td>
<td>Lane 7: 100fg</td>
</tr>
<tr>
<td>Lane 3: 1ng</td>
<td>Lane 8: 10fg</td>
</tr>
<tr>
<td>Lane 4: 100pg</td>
<td>Lane 9: 1fg</td>
</tr>
<tr>
<td>Lane 5: 10pg</td>
<td>Lane 10: negative control</td>
</tr>
</tbody>
</table>

Note: The template DNA was chromosomal DNA of an IS6110 single copy strain

Figure 4.7(a) Multiplex PCR result with amounts of template DNA.
Lane M: 100bp ladder DNA marker

<table>
<thead>
<tr>
<th>Lane</th>
<th>Amount of template</th>
<th>Amount of template</th>
</tr>
</thead>
<tbody>
<tr>
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<td>5</td>
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<tr>
<td>9</td>
<td></td>
<td></td>
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<tr>
<td>10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 4.7(b)** Hybridisation results of multiplex PCR (Fig. 4.7a) with BX and MM probes.
Lane 1: 100bp ladder DNA marker  
Lane 2: *M. xenopi*  
Lane 3: *M. vaccae*  
Lane 4: *M. triviale*  
Lane 5: *M. phlei*  
Lane 6: *M. intracellulare*  
Lane 7: *M. kansasii*  
Lane 8: *M. simiae*  
Lane 9: *M. avium*  
Lane 10: *M. fortuitum*  
Lane 11: Negative control  
Lane 12: *M. tuberculosis* (positive control)

**Figure 4.8(a)** Multiplex PCR results using atypical mycobacteria chromosomal DNA.
Lane 1: 100bp ladder DNA marker
Lane 2: *M. xenopi*
Lane 3: *M. vaccae*
Lane 4: *M. triviale*
Lane 5: *M. phlei*
Lane 6: *M. intracellulare*
Lane 7: *M. kansasii*
Lane 8: *M. simiae*
Lane 9: *M. avium*
Lane 10: *M. fortuitum*
Lane 11: Negative control
Lane 12: *M. tuberculosis* (positive control)

**Figure 4.8(b)** Hybridisation result of multiplex PCR (Fig. 4.8a) using BX and MM probes.
The PCR were then tested with various normal flora and pathogenic organisms of the respiratory tract. The PCR results (Fig. 4.9a) were also verified by DNA probes (Fig 4.9b). The results obtained with atypical mycobacteria and other bacteria confirmed the specificity of the BX and MM primers to *M. tuberculosis* complex.

Finally, to determine that the test that has been developed will actually work with clinical specimens, PCR was carried out using sputum specimens containing high, medium and low numbers of AFB. For the purpose of this study, the definitions of high, medium and low numbers of organism are >50 AFB/L, 20-30 AFB/3L and <10 AFB/3L respectively (the method for scoring the bacterial counts is described in Section 2.2.13). These definitions were chosen deliberately to include the centre and extreme ends of the range.

The available specimens were screened and the ones with the appropriate AFB loads were chosen. Specimens from non-TB cases were also included in the study. The results shown in Fig. 4.10 (a) suggest that the test was equally efficient in detecting the organism in all three types of specimens. Note that on one of seven specimens with microscopy results of 0/3 length was positive by PCR (Fig. 4.10a, lane 12) which was also verified by hybridisation (Fig. 4.10b, lane 12). This specimen was later shown to be AFB positive by culture. The other 6 AFB negative specimens (by both microscopy and culture)(Lane 3, 4, 9, 10, 11, 14) came from patients not suspected as having tuberculosis.

During this initial preparatory period it was also noticed that occasionally, bands with different sizes (other than the BX and MM amplification products) were observed on the agarose gel. However these bands did not hybridise with either the BX or MM probe (results not shown).
Lane 2: *Pseudomonas aeruginosa*  
Lane 3: *Streptococcus gp. B*  
Lane 4: *Haemophilus influenzae*  
Lane 5: *Enterobacter species*  
Lane 6: *Klebsiella aeruginosa*  
Lane 7: *Staphylococcus aureus*  
Lane 8: *Escherichia coli*  
Lane 9: *Streptococcus pneumoniae*  
Lane 10: *Neisseria meningitides*  
Lane 11: *Candida albicans*  
Lane 12: *Neisseria catarrhalis*  
Lane 13: *Extraction Negative Control*  
Lane 14: Reagent control  
Lane 15: *M. tuberculosis* KB29  
Lane 16: *M. tuberculosis* AS80  
Lane 17: 100bp ladder DNA marker

**Figure 4.9(a)** Multiplex PCR using 10ng of chromosomal DNA from various normal and pathogenic organisms of the respiratory tract.
Lane 2: *Pseudomonas aeruginosa*  
Lane 3: *Streptococcus gp. B*  
Lane 4: *Haemophilus influenzae*  
Lane 5: *Enterobacter species*  
Lane 6: *Klebsiella aeruginosa*  
Lane 7: *Staphylococcus aureus*  
Lane 8: *Escherichia coli*  
Lane 9: *Streptococcus pneumoniae*  
Lane 10: *Neisseria meningitides*  
Lane 11: *Candida albicans*  
Lane 12: *Neisseria catarrhalis*  
Lane 13: *Extraction Negative Control*  
Lane 14: Reagent control  
Lane 15: *M. tuberculosis KB29*  
Lane 16: *M. tuberculosis AS80*  
Lane 17: 100bp ladder DNA marker

**Figure 4.9(b)** Hybridisation of multiplex PCR (Fig. 4.9a) using MM and BX probes.
<table>
<thead>
<tr>
<th>Lane</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>0X174HaeIII DNA marker</td>
</tr>
<tr>
<td>1</td>
<td>15 /3L Length (L)</td>
</tr>
<tr>
<td>2</td>
<td>30/3L</td>
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<tr>
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<td>17</td>
<td>PCR reagent negative control</td>
</tr>
<tr>
<td>18</td>
<td>PCR reagent positive control</td>
</tr>
</tbody>
</table>

**Figure 4.10(a)** Multiplex PCR of AFB positive and AFB negative.
Lane M: ØX174HaeIII DNA marker  Lane 10: 0/3L
Lane 1: 15 /3Length (L)         Lane 11: 0/3L
Lane 2: 30/3L                   Lane 12: 0/3L
Lane 3: 0/3L                    Lane 13: 3/3L
Lane 4: 0/3L                    Lane 14: 0/3L
Lane 5: >50 /L                  Lane 15: Positive extraction control
Lane 6: >50/L                   Lane 16: Extraction reagent control
Lane 7: >50/L                   Lane 17: PCR reagent negative control
lane 8: 45/3L                   Lane 18: PCR reagent positive control
lane 9: 0/3L

**Figure 4.10(b)** Hybridisation of multiplex PCR (Fig. 4.10a) with MM and BX probe.
4.5 Controls for the Multiplex PCR

Four controls were ran for each set of PCR reaction: 1). extraction positive (using AFB-positive sputum specimens); 2). extraction buffer negative (extraction buffers and reagents run in parallel with actual test specimens), 3). PCR positive (using 10ng of purified DNA from an IS6110 single copy MTB strain) and 4). PCR negative controls.

4.6 Evaluation of the Multiplex PCR test Using Clinical Specimens

Finally, a large scale evaluation study was conducted in order to determine the accuracy and reliability of the PCR test that has been developed using clinical specimens obtained from Hospital Universiti Sains Malaysia (HUSM) and Hospital Kota Bharu (HKB). The samples were collected and transported to the laboratory in sterile universal bottles. The clinicians involved in this collaborative study were requested to send specimens from cases with high and low index of suspicion for tuberculosis. The following criteria were used to assess the likelihood of tuberculosis in a patient:

i) Chest X-ray showing abnormal changes suggestive of tuberculosis
ii) A Mantoux reaction of >10mm in diameter to 10 TU of PPD
iii) Prolonged coughing (>3 weeks in duration)
iv) Haemophthysis
v) Prolonged low grade fever (> 3 weeks in duration)
vi) Weight loss
vii) Night sweats
viii) History of contact with TB patient(s)
ix) Signs and symptoms suggestive of pneumonia but with lack of response to a full course of antibiotics.
The initial diagnosis of TB was confirmed if the patient showed clinical improvement in response to anti-tuberculous therapy.

However, to ensure impartiality in interpreting the PCR results, none of these data were given with the specimens and the final clinical diagnoses were only obtained at the end of the study.

4.6.1 Sample Processing

Upon receipt, the samples were either processed directly on the same day or stored at 4°C for less than one week. DNA was extracted from these samples using a modification of the method described by Wilson et al., (1993). Sputum, urine, pus, bronchial wash and gastric lavage samples were homogenised and decontaminated with an equal volume of 0.5M NaOH, 0.05M sodium citrate at room temperature for 15 minutes, followed by centrifugation at 3500g for 15 minutes. The pellets were washed once with 10ml of phosphate buffer (pH6.8). Samples were taken, using wire loops, from each pellet for Ziehl-Neelsen (ZN) staining and culture onto Lowenstein-Jensen (LJ) media.

The remainder of the pellet was re-suspended in 1ml of 50 mM Tris pH8.3, inactivated by incubating in a boiling waterbath for 15 minutes and centrifuged at 12,000g for 5 minutes. The supernatant was removed and the pellet was vortexed for 1 minute in 100μl of chloroform. Distilled water (100μl) was then added and this was vortexed again for at least 1 minute to emulsify with the chloroform phase. The mix was transferred to a sterile microfuge tube and the phases were separated by centrifugation for 5 minutes in a microfuge. Sixteen microlitres of the aqueous phase was used for PCR performed according to the optimised protocol described in Section 4.4.5 and the products were analysed by running 20μl of the reaction mix at the end of the amplification process in a 1.5% agarose gel.
Cerebrospinal fluid (CSF) and pleural fluid (PF) samples were treated slightly differently from that described above. CSF and PF were first spun down at 3500g for 10 minutes and the supernatant was removed. After taking samples from each pellet for culture and microscopy as described above, the pellet was resuspended with 1ml of Tris pH8.3 before proceeding with the inactivation and the rest of the extraction process.

Each specimen was tested once only. PCR was repeated only if any of the 4 controls did not give the expected result.

4.6.2 Interpretation of Results

Each PCR test reaction was scored as positive if:

a) both or either one of the BX and MM product were observed on the gel, and

b) all 4 controls gave the expected results.

Similarly, negative results (no bands or bands of unexpected sizes) were only accepted if all controls gave the expected results. Although almost all positive results showed the presence of both bands, the presence of either one was considered sufficient to be scored as positive since it is possible that the BX primer may not give a product if the strain is IS\textit{6110} negative or as the result of priming site mismatches in IS\textit{6110} single copy strain. Although no B9 negative strain has yet been found (Musa, 1996), it is a single copy element and priming site mismatches can occur.

This evaluation study was done to assess the accuracy and performance of the test as a rapid and relatively cheap confirmatory assay. Hence, Southern hybridisation was only
done occasionally as a quality control because it would add considerably to the cost and the processing time of the test.

4.7 Evaluation of PCR in Adult Tuberculosis

4.7.1 Clinical samples for multiplex PCR

A total of 346 samples were included in this study. 121 samples were collected from the Chest Clinic of HKB, 31 from various other clinics and wards of HKB, 162 were from various wards and clinics of HUSM and 32 samples were collected from normal healthy individuals. The number of each type of samples collected for this study are listed in Table 4.3.

4.7.2 PCR Results

The results of the PCR and bacteriological tests in comparison to the clinical diagnosis are shown in Table 4.4. The final clinical diagnosis were arrived at by the physician involved by taking into account, in addition to the information available at the initial point of admission, other data that became available later such as response to chemotherapy and results from further clinical, bacteriological and other laboratory examinations. It should also be noted that in some cases the latter data were not available because the patient absconded or failed to keep their follow-up appointments and the diagnosis is very much dependent on the skill and experience of the attending physician.
Table 4.3 Types of samples collected for evaluation of PCR in adult patients.

<table>
<thead>
<tr>
<th>Type of sample</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum</td>
<td>283</td>
</tr>
<tr>
<td>Bronchial wash</td>
<td>19</td>
</tr>
<tr>
<td>Pleural fluid</td>
<td>18</td>
</tr>
<tr>
<td>Urine</td>
<td>9</td>
</tr>
<tr>
<td>CSF</td>
<td>7</td>
</tr>
<tr>
<td>Pus</td>
<td>6</td>
</tr>
<tr>
<td>Gastric lavage</td>
<td>4</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>346</strong></td>
</tr>
</tbody>
</table>
Table 4.4. Results of PCR, Culture and AFB Smear in Clinically Diagnosed TB and Non-TB Adult individuals

<table>
<thead>
<tr>
<th>Group</th>
<th>Final Clinical Diagnosis</th>
<th>Culture</th>
<th>AFB Smear</th>
<th>No. of cases</th>
<th>PCR positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TB</td>
<td>Positive</td>
<td>Positive</td>
<td>86</td>
<td>86</td>
</tr>
<tr>
<td>2</td>
<td>TB</td>
<td>Positive</td>
<td>Negative</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>TB</td>
<td>Negative</td>
<td>Positive</td>
<td>26</td>
<td>24</td>
</tr>
<tr>
<td>4</td>
<td>TB</td>
<td>Negative</td>
<td>Negative</td>
<td>34</td>
<td>25</td>
</tr>
<tr>
<td>5</td>
<td>TB</td>
<td>Contaminated</td>
<td>Positive</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>6</td>
<td>TB</td>
<td>Contaminated</td>
<td>Negative</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>Non-TB (Healthy individuals)</td>
<td>Negative</td>
<td>Negative</td>
<td>32</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>Non-TB (patients)</td>
<td>Negative</td>
<td>Negative</td>
<td>125</td>
<td>9</td>
</tr>
<tr>
<td>9</td>
<td>Non-TB (patients)</td>
<td>Contaminated</td>
<td>Negative</td>
<td>14</td>
<td>8</td>
</tr>
<tr>
<td>10</td>
<td>Non-TB (patients)</td>
<td>Positive</td>
<td>Negative</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>
4.7.3 Sensitivity, Specificity and Predictive Values of PCR, Culture and AFB Smear.

Analysis of the sensitivity and specificity of the PCR test can be done in several different ways depending on which factor is taken as the "gold standard". In many diseases culture has always been accepted as the gold standard. However, in diseases such as tuberculosis bacteriological methods such as culture and microscopy have many flaws. Culture for example takes a long time and contamination is often a problem particularly in specimens likely to contain normal flora such as sputum. Microscopy is rapid but suffers from the problem of low sensitivity and the bacteriological load in many specimens such as CSF is often too low for successful detection. In real life situation therefore clinical examination plays a crucial role in diagnosis.

A clinical diagnosis is not 100% reliable of course but nevertheless it is often based on several different lines of investigations and coupled with the clinical skill and experience of the physician can be remarkably accurate.

In this study therefore, the final clinical diagnosis is taken as the gold standard. The data from Table 4.4 are reorganised as shown in Table 4.5.

In order to perform the necessary statistical analyses the following definitions (Park, 1988) were used:

\[ a = \text{the total number of true positives that were also positive with the respective laboratory test.} \]

\[ b = \text{the total number of true positives that were negative with the respective laboratory test.} \]
c = the total number of true negatives that were positive with the respective laboratory test.

d = the total number of true negatives that were also negative with the respective laboratory test.

The following definitions and formulae (Park, 1988) were then used:

**Sensitivity**

Sensitivity is defined as the proportion of true positives which are test positive and is calculated using the formula:

Sensitivity = \[
\frac{a}{a+b} \times 100\%
\]

**Specificity**

Specificity is defined as the proportion of true negative which are test negative and is calculated using the formula:

Specificity = \[
\frac{d}{c+d} \times 100\%
\]

**Positive Predictive Value (PPV)**

PPV is defined as the likelihood of a positive test result being a genuine positive and is calculated using the formula:

PPV = \[
\frac{a}{a+c} \times 100\%
\]
**Negative Predictive Value (NPV)**

NPV is defined as the likelihood of a negative test result being a genuine negative and is calculated using the formula:

\[
NPV = \frac{d}{b+d} \times 100\%
\]

The sensitivity, specificity and predictive values of each test, calculated using the above formulae, are shown in Table 4.6
Table 4.5 Comparison of PCR, Culture and AFB smear results to True Positive and True Negative.

<table>
<thead>
<tr>
<th></th>
<th>PCR +ve</th>
<th>PCR -ve</th>
<th>Culture +ve</th>
<th>Culture -ve or contam.</th>
<th>Smear +ve</th>
<th>Smear -ve</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>True Positive</strong> (n=173)</td>
<td>161</td>
<td>12</td>
<td>95</td>
<td>78</td>
<td>120</td>
<td>53</td>
</tr>
<tr>
<td><strong>True Negative</strong> (n=173)</td>
<td>18</td>
<td>155</td>
<td>2</td>
<td>171</td>
<td>0</td>
<td>173</td>
</tr>
</tbody>
</table>

Contam = Contaminated, +ve = positive, -ve = negative

**Note:**

True positives and negatives are cases where the final clinical diagnosis are TB and non-TB respectively.

Table 4.6: Sensitivity, Specificity, PPV and NPV of PCR, Culture and AFB Smear.

<table>
<thead>
<tr>
<th></th>
<th>PCR</th>
<th>Culture</th>
<th>Smear</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sensitivity</strong></td>
<td>93.1%</td>
<td>54.9%</td>
<td>69.4%</td>
</tr>
<tr>
<td><strong>Specificity</strong></td>
<td>89.6%</td>
<td>98.8%</td>
<td>100%</td>
</tr>
<tr>
<td><strong>PPV</strong></td>
<td>89.9%</td>
<td>97.9%</td>
<td>100%</td>
</tr>
<tr>
<td><strong>NPV</strong></td>
<td>92.8%</td>
<td>68.7%</td>
<td>76.6%</td>
</tr>
</tbody>
</table>
4.7.4 Comparison of Sensitivity of PCR, Smear and Culture

Sensitivity of PCR

It should be noted that in cases where the clinical diagnosis, culture and smear are in complete agreement (Group 1, see Table 4.4), the PCR detection rate was 100%. Although this gives a good indication of the reliability of the PCR test that has been developed, there were a significant number of cases where such agreement was not seen.

The results presented in Table 4.6 show that PCR has the highest sensitivity followed by smear and culture respectively. The high sensitivity of PCR is expected and the rate of 93.1% is comparable to many previous studies (Forbes et. al., 1993; Wilson et. al., 1993; Tötsch et. al., 1994; Beige et. al., 1995).

The difference in sensitivity between PCR and microscopy of AFB smear, the most rapid conventional direct laboratory test currently available, is significant. The results suggest that addition of the PCR test would significantly increase the detection rate of the organism allowing rapid commencement of the appropriate treatment.

It should also be noted that PCR was able to provide confirmation of tuberculosis in 35 out of 44 cases (79.6%) where both conventional bacteriological methods failed to do so (Groups 4 and 6) and the diagnoses were made solely on clinical grounds. In addition, PCR was able to provide rapid confirmation in 8 out of 9 cases (88.9%) where conventional confirmation was provided only by culture which in contrast would have taken more than a week for the results to become available.

The difference in sensitivity between PCR and culture is even more significant compared to PCR and smear. The sensitivity of culture in actual fact is unexpectedly low but this could be explained by the fact that many of the patients may already be on anti-
tuberculous treatment. The negative culture results obtained were probably due to the fact that most of the organisms were already dead.

In addition, the negative culture results in these cases could also be due to a logistical problem. All samples from HKB could only be collected once a week. Thus samples from HKB were kept at 4°C before collection and some will have been stored for more than 2 days before culture was performed. This may have resulted in the negative culture results seen in Group 3 and probably also account for the relatively high numbers of contaminated cultures seen in this study (Groups 5, 6, and 9).

**PCR False Negative**

Twelve out of the 173 cases clinically diagnosed as TB were PCR negative giving a false negative rate of 6.9%.

In Group 2 (Table 4.4), 1 out of the 9 samples was negative by PCR while the culture was positive but the smear was negative. The negative PCR result may be due to the fact that the number of organisms in the sample (pleural fluid in this case) was too low to be detectable even by PCR. It should be noted that although the sensitivity of this PCR test was determined to be 1pg of DNA, it was done using purified DNA. In a real clinical specimen, other biological materials are present which may be inhibitory to the extraction process and reduce the sensitivity of the test. Alternatively, the negative result may be due to the presence of PCR inhibitors in this patient’s sample or that the infection was due to atypical mycobacteria.

In Group 3, 2 out of 26 samples were PCR negative. In one of these cases the sputum sample received was heavily blood stained and the PCR could have been inhibited by heme which is a potent inhibitor of PCR (Innis et al., 1990, Mercier et al., 1990, Kolk et al., 1994).
In the second case, a second sputum specimen was received but the results obtained were identical to the first specimen. Thus the negative result may be due to the presence of PCR inhibitors in this patient's samples or that the infection was due to atypical mycobacteria.

In Group 4, 9 samples were PCR negative. In one case, the sample received (bronchial wash) was heavily contaminated with blood but a second sample sent later from this patient was PCR positive suggesting that the negative result obtained in the first sample could have been due to inhibition. This patient was also a confirmed case of TB reactivation.

Review of the data available for the 12 cases showed that the clinical presentations of these cases were consistent with tuberculosis and the PCR results are genuine false negatives. The most likely cause of the false negative results is inhibition of the PCR reaction (although the possibility of infection due to atypical mycobacteria may account for some of these cases).

Detection of inhibition was not incorporated into the PCR test because of several reasons such as the potential problem of PCR competition which may reduce the sensitivity of the test. The results obtained here suggest that the inhibition rate is low enough such that detection of inhibition need not be built into the test.

Additional samples was obtained in only 2 of the 12 false negative cases and one was PCR positive. Testing 2 or more samples from a single patient may help resolve discrepant results particularly if the patient has a high index of suspicion of tuberculosis as has been suggested by Clarridge et al., (1993). Indeed, the value of multiple samples was also seen in this study. For example, multiple samples were obtained from most of the PCR positive cases in Groups 4 and 6 where the clinical diagnoses were based solely
on clinical grounds. The positive PCR results obtained with the additional samples added to the confidence in the PCR test and also the clinical diagnosis.

Alternatively, the use of a more effective method of DNA extraction and removal of inhibitors may also resolve this problem.

4.7.5 Comparison of Specificity of PCR, Smear and Culture

Specificity of PCR

The PCR results obtained in Group 7 (all negative) in which the sputum samples were taken from 32 healthy individuals gave a good indication of the specificity of the PCR test and is consistent with data obtained from studies during the development of the multiplex PCR. However, 18 PCR positive results were obtained in the other categories of non-TB cases giving an actual specificity of 89.6%.

The specificity of PCR obtained in this study is comparable to those reported in other studies which ranged from 74% to 100% (Eisenach et al., 1990; Hermans et al., 1990; Brisson-Noel et al., 1991; Forbes et al., 1993; Jones et al., 1993) Although the specificity of 89.6% is not as high as that obtained by culture and smear, this result should be seen in the context of the much higher sensitivity of PCR in comparison to the other two tests.

PCR False Positive

PCR false positives were detected in 10.4% (18 out of 173) of cases. The false positive rate found in this study is reasonably low and compares favourably with other studies (Noordhoek et al., 1993; Noordhoek et al., 1994; Doucet-Populaire et al., 1996).
Given the extensive evaluation of the specificity of IS6110 (Clarridge et al., 1993, Forbes et al., 1993; Wilson et al., 1993) and B9 sequences (Musa et al., 1994; Musa, 1996) as targets for PCR and the multiplex PCR test during this study, the false positive results that occurred are likely to be due to contamination of the samples. Contamination of samples particularly by amplicons from previous rounds of amplification is a well known problem (Persing, 1991). Although controls were included in each run of amplification to detect such contamination and precautions were taken to reduce it, measures taken are not completely foolproof. In future developments of the test, contamination may be reduced by further improvement on various technical aspects to eliminate cross-over contamination and by incorporating the use of uracil N-glycosylase (UNG) in the test (Kolk et al., 1992; Abe et al., 1993; Kox et al., 1994).

In addition to contamination, some of the 18 false positive cases may actually be due to misdiagnoses. The clinical and laboratory data of these 18 cases were reviewed.

Nine out of 125 cases in Group 8 (Table 4.4) were PCR positive. A second specimen was obtained in only 1 of the 9 false positive cases in this group but PCR was also positive with the second sample and it seems likely that this case was a misdiagnosis. No further information were available for the other 8 cases.

In Group 9 (Table 4.4), 8 out of 14 cases in this group were PCR positive. Additional samples were not obtained for most of the 8 cases but a review of the clinical data yielded some useful information for 5 of these cases.

One patient was a confirmed case of pulmonary tuberculosis who was supposed to have completed his treatment and thus the PCR may either have detected dead MTB or the treatment was ineffective. In the second case, the 75 year old patient had a Mantoux reaction of 15mm and an ESR result of 115. In the third case, the patient was diagnosed as having bronchial asthma but a second specimen received from this patient was also
PCR positive. The fourth case was a 65 year old patient with collapsed right lung and an ESR of 120. The fifth patient was a 69 year old man with productive cough of 3 months duration. Therefore the actual diagnoses, clinical history or the observed clinical manifestations do not rule out the possibility of tuberculosis in 5 of the 8 cases of PCR false positives in Group 9.

Lastly, the two cases in Group 10 (Table 4.4) appear likely to be misdiagnoses as cultures were positive and PCR for one of these cases was also positive.

Review of the 18 false positive cases suggests that some appear to be obvious misdiagnoses whereas the possibility of tuberculosis cannot be ruled out in some others. If such cases were taken into account the specificity of the PCR test would actually be higher than the calculated figure described above.

4.7.6 Comparison of Predictive Values of PCR, Smear and Culture

Predictive values are dependent on the ratio of true positives to true negatives (unlike the sensitivity and specificity values). Thus the values obtained will depend on the level of TB in the specimens examined, which in a real-life situation will depend on the level of TB in the population and on the way the clinician choose the specimens to be analysed.

The PPV of PCR of 89.9% compares favourably with those previously reported in the literature (D’Amato et. al., 1995). Although PPV of PCR is less than that obtained for culture and smear, the figure is fairly high and seen in the context of its high sensitivity suggest that this test would be a useful addition to TB diagnostic methods. PPV, in similarity to specificity, is affected by false positive results. Thus in a similar manner to
specificity, PPV of PCR would be higher if the misdiagnosed cases were taken into account.

In contrast to PPV, NPV of PCR is the highest compared to culture and smear and is expected given the high sensitivity of PCR. Thus, the high PPV and NPV obtained with PCR suggest that this test would be useful in a TB endemic country such as Malaysia and would greatly assist in resolving cases which are difficult to diagnose especially when the presenting signs and symptoms are unclear.

In addition, the sensitivity and rapidity of the test (which could perhaps be improved upon) may assist the clinician to detect tuberculosis during the first visit allowing the appropriate treatment to be instituted as soon as possible and that the necessary information are obtained for the health authorities to ensure patient compliance. This would help to reduce the number of cases lost through failure of the patients to attend their follow-up appointments.

4.8 Evaluation of PCR In Paediatric Tuberculosis

The recent increase of tuberculosis world-wide both in industrialised and developing countries has stimulated interest in childhood tuberculosis. In the United States alone, it has been reported that there was a 35% increase in notification cases in children less than 15 years old from 1985 to 1992 (CDC report 1993). In the developing countries, the incidence of child tuberculosis is often difficult to estimate and the only guide is the number of children referred to tuberculosis centre for treatment.

In Malaysia, a study of childhood tuberculosis by the Ministry of Health showed that the overall number of incidence have not shown an increasing trend from 1987 to 1995
The risk of tuberculosis in exposed children is quoted as up to 42% in those less than 1 year old, 24% in children aged between 1-5 years and 15% in adolescents (age 11-15 years old) compared to 5-10% of immunologically normal adults (Starke et al., 1992). Tuberculosis in children are more often extrapulmonary with a high mortality rate due to disseminated disease and meningitis (Styblo and Sutherland, 1982). In contrast to adult tuberculosis, bacteriological tests in childhood tuberculosis are often negative. Confirmation by sputum smear or culture is uncommon except in older children, and examination of gastric aspirates is usually reserved for problem cases, for example, HIV-infected children with negative tuberculin tests or where drug resistance is suspected. In developing countries, immunosuppression associated with other infections and malnutrition make the diagnosis in children much more difficult to assess. Previous BCG vaccination, and poor quality chest X-rays also added to the difficulty in interpretation of the disease (Hart et al., 1996). Thus paediatricians often have to resort to a trial of chemotherapy (Hart et al., 1996).

The difficulty in establishing rapid diagnosis of tuberculosis in general and in the paediatric population in particular, has led to considerable interest in the development of rapid tests for TB. The PCR test that was developed was therefore evaluated using samples from paediatric cases.
Table 4.7 Childhood tuberculosis cases in Malaysia from 1970 to 1995

<table>
<thead>
<tr>
<th>Year</th>
<th>West Malaysia Peninsular Malaysia</th>
<th>East Malaysia Sabah</th>
<th>Sarawak</th>
<th>Total in Malaysia</th>
</tr>
</thead>
<tbody>
<tr>
<td>1970</td>
<td>104</td>
<td>111</td>
<td>18</td>
<td>232</td>
</tr>
<tr>
<td>1975</td>
<td>100</td>
<td>74</td>
<td>56</td>
<td>230</td>
</tr>
<tr>
<td>1980</td>
<td>75</td>
<td>125</td>
<td>60</td>
<td>260</td>
</tr>
<tr>
<td>1985</td>
<td>76</td>
<td>145</td>
<td>181</td>
<td>402</td>
</tr>
<tr>
<td>1986</td>
<td>81</td>
<td>140</td>
<td>183</td>
<td>404</td>
</tr>
<tr>
<td>1987</td>
<td>73</td>
<td>186</td>
<td>51</td>
<td>310</td>
</tr>
<tr>
<td>1988</td>
<td>94</td>
<td>165</td>
<td>62</td>
<td>321</td>
</tr>
<tr>
<td>1989</td>
<td>111</td>
<td>135</td>
<td>70</td>
<td>316</td>
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<td>1990</td>
<td>98</td>
<td>140</td>
<td>63</td>
<td>301</td>
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<td>1991</td>
<td>113</td>
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<td>1992</td>
<td>89</td>
<td>148</td>
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<td>1993</td>
<td>98</td>
<td>148</td>
<td>44</td>
<td>288</td>
</tr>
<tr>
<td>1994</td>
<td>84</td>
<td>184</td>
<td>43</td>
<td>275</td>
</tr>
<tr>
<td>1995</td>
<td>84</td>
<td>161</td>
<td>42</td>
<td>287</td>
</tr>
</tbody>
</table>
4.8.1 Samples from Paediatric Cases

The format and technical procedures used in this evaluation study were identical to that used for the adult cases although naturally the proportion of each type of sample sent for PCR differ somewhat mostly due to the difficulty in obtaining sputum samples from paediatric patients. Table 4.8 shows the numbers received for each type of sample.

A total of 68 paediatric cases were used in this evaluation study of which 25 cases were clinically diagnosed as TB while 43 were cases thought to be non-TB.

Table 4.8: Types and numbers of samples collected for the evaluation of PCR in paediatric tuberculosis.

<table>
<thead>
<tr>
<th>Type of sample</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum</td>
<td>17</td>
</tr>
<tr>
<td>Bronchial wash</td>
<td>1</td>
</tr>
<tr>
<td>Pleural fluid</td>
<td>3</td>
</tr>
<tr>
<td>Urine</td>
<td>8</td>
</tr>
<tr>
<td>CSF</td>
<td>2</td>
</tr>
<tr>
<td>Gastric lavage</td>
<td>37</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>68</strong></td>
</tr>
</tbody>
</table>
4.8.2 Test Results

The results of the PCR and bacteriological tests obtained in this study are shown in Table 4.9.

4.8.3 Sensitivity, Specificity and Predictive Values of PCR, Culture and AFB Smear.

The data from Table 4.9 are reorganised as shown in Table 4.10.

Based on the figures in Table 4.10 the sensitivity, specificity and predictive values of each test were calculated and are shown in Table 4.11.
Table 4.9 Results of clinical diagnosis, bacteriological and PCR tests in childhood tuberculosis

<table>
<thead>
<tr>
<th>Group</th>
<th>Final Clinical Diagnosis</th>
<th>Culture</th>
<th>AFB Smear</th>
<th>No. of cases</th>
<th>PCR positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TB</td>
<td>Positive</td>
<td>Positive</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>TB</td>
<td>Positive</td>
<td>Negative</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>TB</td>
<td>Negative</td>
<td>Negative</td>
<td>12</td>
<td>7</td>
</tr>
<tr>
<td>4</td>
<td>TB</td>
<td>Contaminated</td>
<td>Positive</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>TB</td>
<td>Contaminated</td>
<td>Negative</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>Non-TB (patients)</td>
<td>Negative</td>
<td>Negative</td>
<td>41</td>
<td>11</td>
</tr>
<tr>
<td>7</td>
<td>Non-TB (patients)</td>
<td>Contaminated</td>
<td>Negative</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 4.10: True positive and True negative value

<table>
<thead>
<tr>
<th></th>
<th>PCR +ve</th>
<th>PCR -ve</th>
<th>Culture +ve</th>
<th>Culture -ve or contam.</th>
<th>Smear +ve</th>
<th>Smear -ve</th>
</tr>
</thead>
<tbody>
<tr>
<td>True Positive (n=25)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>6</td>
<td>9</td>
<td>16</td>
<td>3</td>
<td>22</td>
</tr>
<tr>
<td>True Negative (n=43)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>31</td>
<td>0</td>
<td>43</td>
<td>0</td>
<td>43</td>
</tr>
</tbody>
</table>

Contam = Contaminated, +ve= positive, -ve = negative
Table 4.11: Sensitivity, Specificity and Predictive Values of PCR, Culture and AFB Smear calculated based on figures in Table 4.10.

<table>
<thead>
<tr>
<th></th>
<th>PCR</th>
<th>Culture</th>
<th>Smear</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>76%</td>
<td>36%</td>
<td>12%</td>
</tr>
<tr>
<td>Specificity</td>
<td>72.1%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>PPV</td>
<td>61.3%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>NPV</td>
<td>83.8%</td>
<td>72.9%</td>
<td>66.2%</td>
</tr>
</tbody>
</table>
4.8.4 Comparison of Sensitivity of PCR, Smear and Culture in Paediatric cases

Sensitivity of PCR

Table 4.9 shows that PCR was positive in 9 out of 10 cases (90%) where the clinical diagnoses of TB were supported by one or both bacteriological tests (Groups 1, 2, and 4 respectively). A review of the clinical records show that the organism isolated from the one patient in which PCR was negative (Table 4.9, Group 2) was identified as an atypical *Mycobacterium*. Thus, if this particular case is excluded, the actual agreement between the PCR and bacteriological tests is 100%.

These 10 cases also illustrate the point that in contrast to adult cases, conventional laboratory confirmation of tuberculosis often relies more on culture (9 out of 10 cases) rather than smear examination (3 out of 10) which is consistent with other reports in the literature (Fauville-Dafaux *et al.*, 1996). The excellent agreement between PCR and culture highlights the utility of PCR in diagnosis of TB in paediatric patients particular as it is a much more rapid test than culture.

The sensitivity of PCR in paediatric cases is lower than that found in adults. However the number of paediatric cases are much lower than in adults which may affect the accuracy of the result. On the other hand the differences in sensitivity of PCR when compared to culture and smear are even more significant.

PCR False Negative

False negative PCR occurred in 24% of cases diagnosed as TB. The false negative PCR result obtained in one of the cases in Group 2 has already been explained. However, 5
cases (out of 12) in Group 3 were also negative by PCR. The following data (Table 4.12) were obtained following a review of the clinical and additional laboratory records:

Three patients in this group (Nos. 1, 2 and 3 respectively) had 5 samples sent for analysis and all were consistently negative except patient 1 who had one sample positive with PCR. Thus the samples from these 3 patients appear to consistently contain PCR inhibitors or the bacterial load is too low even for PCR or the pathogens involved are atypical mycobacteria. Patient 4 had one additional sample which was PCR positive. The clinical record from the last patient in this group did not contain further details.

The data seem to suggest that multiple samples may not necessarily improve the sensitivity rate. However, it should be noted that sensitivity may be higher if a more appropriate specimen was sent for PCR. For example, in cases of lymphadenopathy, the number of organisms in a lymph biopsy might be higher than in a gastric lavage (the most common specimen sent regardless of the major site of infection) and thus easier for PCR to detect.
Table 4.12 Review of the clinical and additional laboratory records of 5 cases (out of 12) in Group 3.

<table>
<thead>
<tr>
<th>No</th>
<th>Patient</th>
<th>Clinical data</th>
<th>Additional laboratory results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>KKS</td>
<td>Lymphadenopathy, calcified cavity on chest X-ray, Mantoux reaction of 14mm diameter</td>
<td>5 additional samples sent; all were smear and culture negative but one was PCR positive</td>
</tr>
<tr>
<td>2</td>
<td>LCF</td>
<td>Fever, breathlessness, abnormality seen on chest X-ray, Mantoux reaction of 18mm</td>
<td>5 additional samples sent; all were culture, smear and PCR negative</td>
</tr>
<tr>
<td>3</td>
<td>N</td>
<td>Prolonged fever, Hillary lymphadenopathy, Mantoux reaction of 20mm in diameter and history of contact with TB patient</td>
<td>5 additional samples sent; all were culture, smear and PCR negative.</td>
</tr>
<tr>
<td>4</td>
<td>WNI</td>
<td>Lymphadenopathy, fever, cough, night sweats, history of TB contact and Mantoux reaction of 15mm</td>
<td>1 additional sample sent; culture and smear were negative but PCR was positive</td>
</tr>
<tr>
<td>5</td>
<td>MA</td>
<td>“TB”. No other additional data</td>
<td>None</td>
</tr>
</tbody>
</table>
Comparison of Specificity of PCR, Smear and Culture

Specificity of PCR

The specificity of PCR obtained in this study (72.1%) compares quite well with some of the studies previously reported in the literature which ranges from 74% to 100% (Brisson-Noel et al., 1989; Eisenach et al., 1990; Hermans et al., 1990; Forbes et al., 1993; Jones et al., 1993).

The specificity of PCR though is lower when compared to both culture and smear (both 100%). However, as in the case of adult TB, this result needs to be seen in the context of the sensitivity of PCR which is much higher than both culture and sensitivity.

PCR False Positive

PCR was positive in 12 out of 43 cases which were clinically diagnosed as non-TB (Table 4.10, Groups 6 and 7) giving a false positive rate of 27.9%. The result is much higher than 7% reported by Doucet-Populaire et al., (1996). The false positive rate obtained in this evaluation study is also not consistent with the much lower rates obtained during the development of the test and in the adult study. The data from patients with PCR false positive results were reviewed and are shown in Table 4.13.

Three cases appear to be obvious cases of false positive PCR even with repeat samples (Patients 9, 10 and 11 respectively). These appear to be cases of non-TB since the patients responded to normal antibiotic treatment (patients 9 and 11 respectively) or recovered without any anti-microbial therapy (patient 10). A review of the laboratory record shows that in each respective case, the multiple samples from the patient were
received, processed and tested at the same time. Thus contamination could have occurred at an early stage during processing resulting in the false positive result in multiple samples from each patient. These cases also suggest that multiple samples from a single patient should not be processed at the same time.

Patient 4 had no additional samples but this appear to be a genuine case of PCR false positive since the patient responded to a normal course of antibiotics.

Patient 7 also clearly appear to be a case of genuine PCR false positive (first sample) as Cryptococcus was isolated and the patient responded to anti-fungal treatment. Two additional samples (taken and processed at different times from the first) were PCR negative which highlights the value of repeated samples.

However, 4 cases had additional samples that were also PCR positive and their clinical presentations do not rule out TB (patients 3, 5, 6, and 8). In the case of Patient 5 one of the additional samples was also culture positive. Unfortunately, no further details regarding treatment and response could be elicited from the records. No further details were available from the other three patients (patient 1, 2 and 12 respectively).

The data described above show that in a number of cases the possibility of TB cannot be ruled out. Thus if these cases were taken into account the specificity of the PCR test in paediatric cases would actually be higher than the calculated figure.
Table 4.13 Review of the data from patients with PCR false positive.

<table>
<thead>
<tr>
<th>No</th>
<th>Patient</th>
<th>Clinical data</th>
<th>Additional laboratory results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MI</td>
<td>&quot;Non-TB&quot;</td>
<td>None</td>
</tr>
<tr>
<td>2</td>
<td>SNH</td>
<td>&quot;Non-TB&quot;</td>
<td>None</td>
</tr>
<tr>
<td>3</td>
<td>KBH</td>
<td>Pneumonia, prolonged cough, fever, cerebral palsy, abnormality seen in chest X-ray</td>
<td>2 additional samples sent were also PCR positive</td>
</tr>
<tr>
<td>4</td>
<td>RS</td>
<td>Confirmed pneumonia. Patient responded to standard course of antibiotics</td>
<td>None</td>
</tr>
<tr>
<td>5</td>
<td>MIQ</td>
<td>Subdural effusion</td>
<td>2 additional samples sent; PCR was positive in both second and third samples, culture was positive in the third sample</td>
</tr>
<tr>
<td>6</td>
<td>CS</td>
<td>Bronchopneumonia, fever, cough, shortness of breath, cerebral paralysis</td>
<td>2 additional samples sent; both culture and smear negative but PCR were positive</td>
</tr>
<tr>
<td>7</td>
<td>NM</td>
<td>Pneumonia, no history of TB, chest X-ray normal, Cryptococcus was isolated</td>
<td>2 additional samples sent; both culture, smear and PCR negative</td>
</tr>
<tr>
<td>8</td>
<td>AB</td>
<td>Broncho asthma</td>
<td>2 additional samples sent; both culture and smear negative but PCR were positive</td>
</tr>
<tr>
<td>9</td>
<td>MF</td>
<td>Pleural effusion, fever, cough, <em>Staphylococcus aureus</em> isolated. Patient improved on normal antibiotics.</td>
<td>2 additional samples sent; both culture and smear negative but PCR were positive</td>
</tr>
<tr>
<td>10</td>
<td>SF</td>
<td>Viral fever, malnourished, patient well on follow up after 3 months</td>
<td>1 additional sample sent; culture and smear negative but PCR was positive</td>
</tr>
<tr>
<td>11</td>
<td>NH</td>
<td>Acute laryngo trachitis, resolved on antibiotic therapy</td>
<td>2 additional samples sent; both culture and smear negative but PCR were positive</td>
</tr>
<tr>
<td>12</td>
<td>MS</td>
<td>&quot;Non-TB&quot;</td>
<td>None</td>
</tr>
</tbody>
</table>
4.8.6 Comparison of Predictive Values of PCR, Smear and Culture

The relatively high PCR false positive cases naturally affected the PPV of PCR (61.3%). However, this should also be seen in the context of the higher sensitivity of PCR compared to the other tests. Furthermore the PPV would be higher if cases of possible misdiagnoses were taken into account.

NPV of PCR however is highest amongst the three tests compared. This suggests that PCR would greatly help the clinician to reduce the risk of unnecessary anti-tuberculous amongst paediatric patients particularly if multiple samples and the use of more rigorous procedures to eliminate cross contamination and reduce inhibition of PCR are incorporated into future developments of the test. This would likely result in increased sensitivity and specificity and thus better predictive values.

4.9 Discussion

Sensitivity

The results obtained indicated that the multiplex PCR developed in this study show good sensitivity in both adult (93.1%) and paediatric cases (76 %). The results obtained in this study are consistent with other reports in the literature (Altamirano et al., 1992, Wilson et al., 1993, Tötsch et al., 1994, Fauville-Dufaux et al., 1996). The sensitivities in both types of cases would be higher in fact if the cases where obvious misdiagnoses (such as cases diagnosed as non-TB but with subsequent culture positive results or where PCR were consistently positive in multiple specimens from the same patient) have occurred are taken into account.
The lower sensitivity obtained in paediatric cases is to a certain extent affected by the much smaller number of cases used for the analysis. Inappropriate samples sent for PCR may also play an important role. Three of the 4 genuine false positive paediatric cases (Table 4.12) for example had lymphadenopathy but multiple samples of gastric lavage were sent, most of which were PCR negative. Material from biopsy of the affected lymph node in each case may have yielded positive results.

Inhibition of PCR is a recognised problem (Brisson-Noel et al., 1991; Nolte et al., 1993; Clarridge et al., 1993; Hawkey, 1994). The inhibition may account for some of the false negative PCR results found in this study. The addition of an inhibition detection system was not included in the study for four reasons. First, such a system which would entail spiking the sample with a target amplifiable by the same set of primers, would result in competition that may affect sensitivity. Second, the detection of inhibition does not actually resolve the question of whether the specimen contained M. tuberculosis or not. Third, the additional step would add to the chances of introducing contamination. Fourth, in cases where TB are strongly suspected, additional samples can be easily collected and tested. The best solution is actually to develop or use DNA extraction methods that can remove inhibitors with a high degree of efficiency with minimal addition to cost.

One other possible reason for false negative results are low numbers of organisms in the specimens or infection caused by atypical mycobacterium which will not be detected by the primers used in this study. This needs to be addressed in future developments of the PCR assay.

An important point gained from this evaluation study was that multiple samples from each patient are often useful in resolving discrepant results and is consistent with the findings of Clarridge et al. (1993) and Devallois et al., (1996). Thus multiple samples (3
or more) should be tested if the assay is to be incorporated as part of routine laboratory diagnosis.

Addition of a hybridisation step may improve sensitivity but Manjunath et al (1991), reported that hybridisation after PCR neither increased the sensitivity nor the specificity of their assay over agarose gel electrophoresis detection of the amplified product. This suggestion is contrary to most other reports (Nolte et al., 1993; Shawar et al., 1993 ) which found increased sensitivity with hybridisation but the relatively high level of sensitivity found with the multiplex PCR used in this study perhaps do not warrant additional steps that would increase the cost and processing time of the assay.

Other methods to increase sensitivity such as nested PCR (Miyazaki et al., 1993), re-amplification of negative samples (Pierre et al., 1991), reverse hybridisation (Mangiapan et al., 1996), calorimetric detection (Wilson et al., 1993), more efficient DNA extraction methods and removal of inhibitors (Buck et al., 1992, Kocagöz et al 1993, Perera et al., 1994, Amicosante et al., 1995) and many others were not tested but could be incorporated in future developments of this multiplex assay.

The sensitivity of the multiplex PCR assay in both adult and paediatric cases were higher than that of either culture or smear. The difference is particularly marked in paediatric cases which are well known to be difficult to confirm by conventional laboratory methods. Thus, this assay will be a very useful addition to clinicians in the future in the diagnosis of tuberculosis particularly if primers to detect some of the clinically important atypical mycobacteria are included.

Specificity

The specificity of IS6110-based amplification assays has recently been questioned (Kent et. al., 1995, Hellyer et. al., 1996). Primers from IS6110 used in this multiplex system
was carefully designed to avoid the regions reported earlier. By using known cultures from 9 species of mycobacteria other than *M. tuberculosis*, there were no false positives indicating the high specificity of the PCR assay described here (section 4.4, fig. 4.11a,b). However, in the clinical comparison trial, the specificity of the multiplex PCR was found to be 89.6% and 72.1% in adult and paediatric cases respectively compares well with those obtained in other studies, which are between 74% to 100% (Brisson-Noel et al., 1989; Eisenach et al., 1990; Hermans et al., 1990; Forbes et al., 1993; Jones et al., 1993).

Much higher specificity's were expected based on the results of many studies regarding the specificity of IS6110 (Clarridge et al., 1993; Forbes et al., 1993; Wilson et al., 1993) and B9 (Musa, 1996) sequences and primers (determined by laboratory studies using purified DNA of numerous species of micro-organisms as templates). A specificity of 100% was indeed seen when samples from 32 healthy individuals (Table 4.4) were tested.

However, false positive results were observed in both adult and paediatric cases. This however is not unexpected and false positive PCR have been reported in many studies (Brisson-Noel et al., 1989; Eisenach et al., 1990; Hermans et al., 1990; Pao et al., 1990; Persing, 1991; Cousins et al., 1992; Forbes et al., 1993).

Given the high specificities of the targets to members of the *M. tuberculosis* complex and the use of very stringent amplification parameters, false positive results must be due to contamination of samples or reagents by chromosomal DNA or more likely by amplicons from previous amplifications (Persing, 1991).

In addition to adhering to procedures to minimise cross contamination, many laboratories use an amplicon digestion system by using incorporation of dUTP instead of dTTP and digestion of the UTP containing amplicon by uracil-N-glucosylase (Kolk et
al, 1992; Abe et al, 1993; Kox et al, 1994). However, Noordhoek et al., (1994 and 1996 respectively) reported that in their blind studies conducted among several laboratories, high levels of false positivity were found even in laboratories using this system.

Good laboratory practice, standardisation of reagents, monitoring of the performance of the whole assay therefore play very crucial central roles in ensuring high specificity of the test while other methods of contamination prevention should be used only to complement them. Additionally, testing of 3 or more samples of the appropriate type from each patient should also ensure accuracy of the results and reduce the chances of false positivity.

Predictive Values

The relatively high predictive values (PPV 89.9%, NPV 92.8% in adults and PPV 61.3%, NPV 83.8% in children respectively) suggests that this multiplex PCR assay will be a useful addition to routine laboratory diagnosis of tuberculosis even in endemic countries. However, further technical and procedural improvements need to be done to increase sensitivity and particularly specificity if the test is to be included as part of routine diagnostics.
Chapter 5
Chapter 5
Conclusion and Future Work

5.1. Characterisation of IS6110 in Malaysian single copy strains of *M. tuberculosis*

Earlier studies have shown that IS6110 in most single copy strains is located in the same position on the chromosome (Fomukong *et al.*, 1994). It was thought that the IS element in these organisms is defective in transposition which occurred in a strain at the very early stage in the evaluation process of the *M. tuberculosis* complex and the single copy strain are actually the direct descendents of this “ancient strain”. Comparison of published sequence of IS986 and IS987 shows that ORFa is the fundamental region of the IS. The aim of this study was to characterise the sequence of the ORFa region in Malaysian *M. tuberculosis* single copy strain. The results from this study suggest :-

1. The insertion sequence on the Malaysian single copy strains *M. tuberculosis* were identical to IS987.

2. A comparison of this sequence and a sequence from a recently active copy of this element showed that the sequences were identical. This led to a re-examination of the original cloned copy of IS986 and showed that the sequences of all the elements were identical and that the differences between the published sequences of IS986 and IS987 were due to sequencing errors.

3. These results suggest that the elements in BCG and in single copy strains are actually fully capable of transposing and the mobility of the IS element depends on the transcriptional activity of the flanking chromosomal region and not within the IS itself.
5.2. Multiplex PCR system

A multi-primer PCR system permitting the amplification of two different mycobacterial sequences, IS6110 and the B9 sequences, was developed and evaluated in these studies. The multiplex PCR was shown to be highly efficient in detecting DNA of *M. tuberculosis* from clinical samples. Potential advantages of the multiplex PCR system merit mention:

1. The IS6110 is present in one to twenty copies in *M. tuberculosis* strains although strains lacking IS6110 have been reported. In contrast, the B9 sequence appears to be present in all strains of *M. tuberculosis* tested and strains not containing this sequence have not yet been found. The multicopy nature of IS6110 provides a good target for amplification and the B9 sequence provides a good backup for the detection of the unusual IS6110-negative strains.

2. Combination of B9 and IS6110 derived primers in the multiplex PCR provide a high degree of confidence in the results as both are highly specific for MTB complex.

3. Evaluation study of the multiplex PCR using clinical samples showed that this system is sensitive (93.1% in adult cases and 76% in paediatric cases) and specific (89.6% in adult cases and 72.1% in paediatric cases)

4. The experience gained from this study suggests that at least 3 or more specimens should be sent for PCR to reduce the rate of false positivity and false negativity.

5. The specificity of IS6110-based amplification assays has recently been questioned (Kent *et. al.*, 1995, Hellyer *et. al.*, 1996). Primers from IS6110 used in this multiplex system were carefully designed to avoid the regions reported earlier. Further PCR
with various species outside the *M. tuberculosis* complex were tested and the primers shown to be specific.

In conclusion, this multiplex PCR assay provides an efficient strategy to detect and identify *M. tuberculosis* from clinical samples and performs well in comparison with culture.

5.3. **Future work**

1. To study the flanking regions of IS6110 and the postulated frameshift region of ORFa-ORFb in order to determine the factors which regulate transposability of IS6110.

2. To improve the sensitivity and specificity of the multiplex PCR test by incorporating more efficient methods or procedure for extracting DNA, removal of inhibitors from clinical specimens and prevent contamination.
Chapter 6
Chapter 6

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