STUDIES OF CHILDHOOD TUBERCULOSIS

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Dedication

My parents Mike and Hazel have dedicated their life to their children. In return, I hope that in some small way, the dedication of this work to them may reflect their unfailing love and support. My beloved wife and friend, Liesl, has transformed my life in so many ways and I share in this achievement with her.

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Abstract

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Background
Childhood tuberculosis presents particular clinical challenges, two of which form the foci of this thesis. Firstly, establishing a diagnosis is frequently difficult as children are unable to expectorate, conventional microbiology is insensitive and the clinical presentation non-specific. Consequently there has been interest in the potential diagnostic utility of novel interferon-gamma release assays, such as the enzyme-linked immunospot (ELISpot) assay.

Secondly, the outcome of tuberculosis infection in children is diverse, with some infections progressing to severe, extra-pulmonary disease. The bacterial factors which influence outcome are poorly understood, however there is increasing evidence of strain-specific variation in virulence, with W-Beijing strains thought to be particularly virulent.

Methods
Part A. Two studies were conducted to investigate the potential utility of ELISpot for the diagnosis of active childhood tuberculosis. In the first study, children presenting to a tertiary care hospital with suspected tuberculosis were tested using an 'in-house' ELISpot assay. In a subset, tests were repeated at 1, 3 and 6 months. In the second study, the commercial T-SPOT.*TB* assay was used to test children at high risk for active tuberculosis in a community setting.

Part B. All strains of *Mycobacterium tuberculosis* isolated from children presenting to Red Cross Children's Hospital from 2000-2003 were genotyped and the genotype was linked to clinical outcome. In addition, in order to investigate whether W-Beijing strains of tuberculosis were emerging in Cape Town, we genotyped tuberculosis strains in archived histological material from a 76-year period.

Significant Findings and Conclusions
Part A. The sensitivity of ELISpot was 83% for the diagnosis of culture-confirmed tuberculosis in children presenting to hospital. ELISpot values increased during early therapy and then declined. In the community, ELISpot was less sensitive than tuberculin-skin testing, particularly in very young children. ELISpot may be useful as an adjunct to clinical diagnosis in children presenting to hospital, but should not be used to exclude disease in very young children.

Part B. There was no clear relationship between broad strain lineage and clinical outcome however there was an association between the major sub-lineages of W-Beijing strains and tuberculous meningitis. There was, however clear evidence from both paediatric and histological studies that W-Beijing strains have recently emerged in Cape Town and are rapidly expanding.

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Statement regarding the specific contributions of others

This work represents the combined efforts of a large team of dedicated individuals. Whilst these studies were conceived, initiated and analysed primarily by the author, the specific contributions of others to each chapter are detailed below.

Chapter 3: Recruitment of children for this study was performed by a team of clinical nurses (Rene Goliath, Nomsa Yekiso and Asa Barendse). ELISpot assays were performed by a laboratory team including Kathy Wood (who also assisted with assay optimisation), Sandy Pienaar and Liezel Swarts. The candidate was responsible for study design, assay set-up and optimisation, conducting assays, analysis and write-up.

Chapter 4: Recruitment of children was performed by the SATVI clinical staff at Worcester. ELISpot assays were performed by Kathy Wood and Sandy Pienaar. Mary-Ann Davies assisted with managing the study and performed the initial analysis. The candidate was responsible for study design, assay set-up and optimisation, analysis and write-up

Chapter 6: Bradley February assisted with performing genotyping of strains and curating the strain database. The candidate was responsible for study design, set-up and optimisation of assays, performing genotyping, analysis and write-up.

Chapter 7. The author supervised Donna Cowley, a visiting MSc student from Melbourne, who selected autopsy samples, performed DNA extraction and strain genotyping of histological samples. Donna also contributed significantly to the analysis of data from the histological samples. The component of this chapter dealing with histological samples was submitted in partial fulfilment of the requirements for an MSc at the Royal Melbourne Institute of Technology. The candidate was responsible for study design, overseeing optimisation of assays, performing genotyping (paediatric samples), analysis and write-up.

Declaration

I, Mark Patrick Nicol, do hereby declare that the experiments presented in this thesis were conceived and executed by myself, except where otherwise indicated.

Neither the substance nor any part of this thesis has been submitted in the past, or is being, or is to be submitted for a degree in the University or any other University. This thesis is presented in fulfilment of the requirements for the degree of PhD.

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Signed: _____

Date: _____

Publications arising from work related to this thesis

Mark P Nicol, Beate Kampmann, Patricia Lawrence, Kathy Wood, Sandy Pienaar, David Pienaar, Brian Eley, Michael Levin, David Beatty, Suzanne T.B. Anderson. Enhanced anti-mycobacterial immunity in children with erythema nodosum and a positive tuberculin skin test. J Invest Derm. 2007 Sep;127(9):2152-7

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Under review: Donna Cowley, Dhirendra Govender, Bradley February, Mary Wolfe, Lafras Steyn, Robert J Wilkinson, **Mark P Nicol**. Recent and rapid emergence of a virulent lineage of *Mycobacterium tuberculosis* in Cape Town, South Africa

Under review: **Mark P. Nicol**, Mary-Ann Davies, Kathy Wood, Mark Hatherill, Lesley Workman, Anthony Hawkridge, Monique Hanslo, Brian Eley, Katalin Wilkinson, Robert J Wilkinson, Willem A. Hanekom, David Beatty, Gregory Hussey. T-SPOT.TB offers no advantage over tuberculin skin testing for diagnosis of tuberculosis in young children.

Chapter 1. Background to the thesis and scope

Tuberculosis was responsible for 1.6 million deaths in 2005(1). Of the 8.8 million cases that occurred worldwide, 7.4 million were in sub-Saharan Africa and Asia alone. Whilst global rates of tuberculosis are levelling off, the epidemic continues to escalate in Africa, driven largely by increasing rates of HIV co-infection. The emergence of epidemic multi-drug resistant and extensively drug-resistant strains has raised worldwide concern(2). With disease rates up to sixty times higher than those in the USA or Western Europe and double those observed in most other developing countries, South Africa faces one of the worst tuberculosis epidemics in the world(3). The Western Cape region is especially affected, with an annual incidence of 1037/100000 in 2005(3) and particularly high rates of childhood tuberculosis and tuberculous meningitis(4).

Tuberculosis in children remains a neglected area of research, despite causing considerable morbidity and mortality, particularly in sub-Saharan Africa(3). In high burden areas, children may account for up to 40% of the total tuberculosis case load(5). Cases of tuberculosis amongst children accounted for 12-15% of the total case burden in Cape Town from 2000-2004(5;6). For the past several years, my interest has focused on two issues in childhood tuberculosis which are of particular concern for clinicians, and which relate directly to my role as a clinical microbiologist. The first of these is the difficulty in confirming the diagnosis of tuberculosis in children and the second, the high rate of severe, extrapulmonary infection, particularly tuberculous meningitis, in young children.

The lack of accurate, cost-effective diagnostic methods is a major obstacle facing health care workers involved in the treatment and control of tuberculosis. The diagnosis of tuberculosis in children is challenging at several levels. Firstly, the clinical presentation of tuberculosis in young children is often non-specific(7) and the radiographic features of disease equivocal. Particularly poor inter-observer correlation is shown in interpreting chest radiographs(8). Secondly, appropriate specimens for microbiological investigation are often difficult to obtain, since young children are unable to expectorate. Where samples can be obtained, acid-fast microscopy is seldom positive due to the paucibacillary nature of primary tuberculosis(9). Culture remains the gold-standard for diagnosis, but lacks sensitivity in children(10) and requires prolonged incubation.

Delayed diagnosis of childhood tuberculosis may have serious consequences, with a high proportion of cases progressing to complicated infection(11). Since microbiological evidence is frequently lacking in children, clinicians rely on the clinical and radiographic features of disease together with indirect evidence of infection such as a positive tuberculin skin test or a history of contact with a patient with active tuberculosis.

The introduction of new techniques for the diagnosis of childhood tuberculosis is a priority. The enzyme linked immunospot (ELISpot) assay relies on the detection of interferon-γ production by circulating sensitized T cells following *in vitro* re-stimulation with *Mycobacterium tuberculosis*-specific antigens. Chapters 2-4 describe work which we conducted to evaluate this novel immunodiagnostic method for childhood tuberculosis. The study detailed in Chapter 3 was one of the first descriptions of the use

of the ELISpot assay for the diagnosis of active tuberculosis in hospitalised children(12), whilst a further evaluation of this assay in a community setting is described in Chapter 4. This work will be important in defining the role and performance of these assays in the diagnosis of childhood tuberculosis.

The second focus of this thesis is the role of strain variation in influencing the outcome of infection with *Mycobacterium tuberculosis* (*Mtb*). Childhood tuberculosis is characterised by a wide range of clinical presentations, ranging from limited pulmonary infections to life-threatening, extra-pulmonary or disseminated disease(13). The determinants of these diverse outcomes remain largely unknown, but epidemiological studies point to influence of both host and bacillary factors(14). Until recently, most attention has focused on the role of host factors. There is, however, increasing interest in the bacterial determinants that influence the outcome of infection(15;16). This is a nascent area of research, but there are clear indications that clinical strains of *Mtb* differ in their behaviour *in vitro* and *in vivo*(17).

Animal models clearly demonstrate increased virulence of selected strains(18;19) but there is no compelling evidence from human studies that certain strains or strain families are more virulent than others. This may be in part due to difficulties in identifying clear differences in outcome in adult tuberculosis. An important manner in which strains may differ is their ability to disseminate and cause extra-pulmonary tuberculosis. Since children more frequently develop extra-pulmonary disease, childhood tuberculosis may be a useful model for exploring strain variation.

Strains of *Mtb* from children in Cape Town are of interest for a number of reasons. Firstly, the Western Cape region represents an area of particularly intense tuberculosis transmission, with incidence rates approaching 1% per annum(3;5). The reasons for this are as yet unclear and may relate to host or bacterial determinants. The area has lower prevalence rates of HIV infection than much of the country and yet a higher burden of tuberculosis(3). Since tuberculosis in children is classically primary disease, following soon after exposure to an adult infectious source case, strains causing tuberculosis in young children will tend to reflect those transmitted by adults in the same community. Secondly, since extra-pulmonary disease is frequent amongst children hospitalised with tuberculosis in Cape Town, this represents an important opportunity to study potential associations between strains and their ability to cause extra-pulmonary disease. Chapters 5-7 detail a series of studies which were conducted to evaluate the association between strain genotype and clinical phenotype in childhood tuberculosis.

Part A. Studies of the use of interferon-γ release assays for the diagnosis of tuberculosis in children

Chapter 2. The use of interferon-γ release assays for the diagnosis of tuberculosis in children: a review

2.1. Limitations of current diagnostic tests for tuberculosis

The difficulties involved in establishing a diagnosis of tuberculosis in children were highlighted in the Introduction. These remain a major obstacle to the effective care of young children presenting with respiratory illness. Since many cases of tuberculosis in young children will progress to severe, disseminated or extra-pulmonary disease, early diagnosis is critical(11;20), but seldom confirmed. The first step in diagnosis is obtaining a suitable specimen for microbiological investigation. This is challenging, since young children are unable to effectively expectorate. Aspiration of early morning gastric lavage fluid containing swallowed sputum has, for many years, been the favoured approach, with improved sensitivity over bronchoalveolar lavage(9;21). However gastric lavage is invasive, unpleasant and requires overnight hospitalisation. The induction of sputum production using nebulized hypertonic saline may offer significant advantages. Hospitalisation is not required and the culture yield of one induced sputum sample has been shown to be equivalent to that of 3 gastric lavage samples(22).

When appropriate specimens are obtainable, early diagnosis depends largely on smear microscopy for acid fast bacilli. Since a positive smear requires a high concentration of organisms in the sample and since young children with primary tuberculosis seldom develop cavitary disease(20), smear microscopy is less sensitive than in adult disease(23;24). Culture remains the gold-standard for diagnosis but is insensitive in children(10) and seldom provides information early enough to guide therapy. Molecular tools, such as polymerase chain reaction (PCR) for the amplification of *Mtb*-specific

DNA or RNA have met with variable success(25-28), but appear to be highly operator dependent and prone to false-positive results due to cross-contamination(29).

In most cases therefore, the diagnosis of tuberculosis is based the clinical and radiographic features of disease. However since the radiographic features of primary tuberculosis, such as hilar lymphadenopathy, may be subtle, radiography is unreliable(30) and subject to considerable inter-observer variability(31). Similarly, the clinical features of tuberculosis in children are non-specific(7).

Since young children are at high risk of developing active tuberculosis in the period early following infection, a history of recent exposure to a case of active tuberculosis is important supporting evidence. The tuberculin skin test (TST) has, for decades, been used as a marker of infection with *Mtb*. The TST is performed by intradermal injection of purified protein derivative (PPD) of *Mtb*(32;33). The presence of delayed hypersensitivity to PPD is detected by the development of induration 48-72 hours following infection(34). Such a reaction is thought to correlate with sensitization to, and hence infection with, *Mtb*(34). PPD is a complex mixture of proteins extracted from the whole tubercle bacillus. These proteins include many which are found in other members of the *Mtb* complex (including the vaccine strain *Mycobacterium bovis* BCG) as well as environmental mycobacteria(35;36). It is therefore likely that previous BCG vaccination or exposure to environmental mycobacteria may cross-sensitize to the PPD reagent(36), leading to false positive results and reduced specificity(35;37). Moreover, the ability to mount a delayed hypersensitivity reaction may be impaired in children with immune

defects, such as those associated with malnutrition, HIV infection or severe tuberculosis(38;39), reducing the sensitivity of TST.

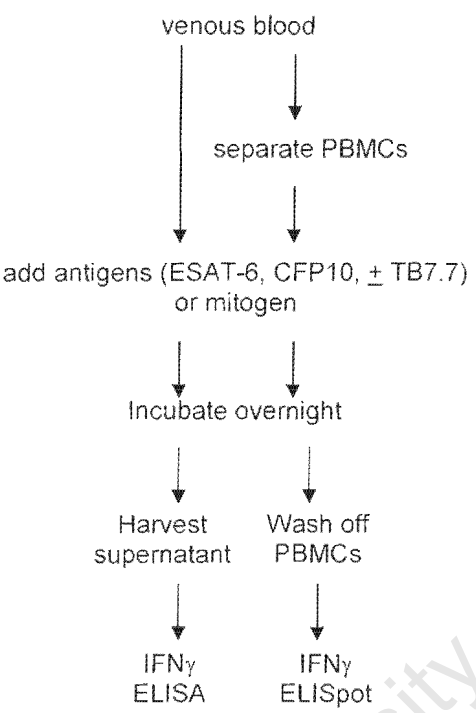
Clinicians are therefore unable to rely solely on the clinical or radiographic features of disease or a positive TST for the diagnosis of TB in children. As a result, a number of scoring systems have been developed that use a combination of these methods in an attempt to improve diagnostic accuracy(40-43). The sensitivity and specificity of these systems varies widely. The introduction of new diagnostic tests for the diagnosis of childhood tuberculosis is a priority.

2.2. In vitro assays to determine sensitization to tuberculosis-specific antigens

An important development has been the identification of a number of protein antigens, encoded by the RD1 locus of *Mtb*, which are absent from *M. bovis* BCG and most environmental mycobacteria(44). During the process of attenuation of *M. bovis* BCG, the RD1 region was deleted from the chromosome. Two key T-cell antigens have been identified within this locus, early secreted antigenic target-6 (ESAT-6) and culture filtrate protein 10 (CFP10)(45;46). These antigens form the basis of several *in vitro* interferon-γ (IFN-γ) release assays (IGRA). These assays rely on the detection of IFN-γ production by circulating sensitized T cells following *in vitro* re-stimulation with *Mtb*-specific antigens. The production of IFN-γ is detected in whole blood cultures using an enzyme-

linked immunosorbent assay (ELISA)(47) or in peripheral blood mononuclear cell cultures using an enzyme-linked immunospot (ELISpot) assay(48) (Figure 2.1).

Figure 2.1 Outline of ELISpot and ELISA methods for IGRA



Commercial assays currently in use are based on detection of IFN-γ production by either ELISA (QuantiFERON-TB Gold, and QuantiFERON-TB Gold *In tube*, Cellestis)(49;50) or ELISpot (T-SPOT.*TB*, Oxford Immunotec)(50). The first generation of QuantiFERON assays incorporated PPD as a stimulant and offered little improvement over TST. The newer generation QuantiFERON-TB Gold assays as well as the T-SPOT.*TB* assay utilize recombinant ESAT-6 and CFP10 and/or overlapping peptides derived from these antigens. Since the antigens used in these assays are not present in

BCG or in most environmental mycobacteria (exceptions include *M.marinum*, *M. szulgai* and *M. kansasii*(51)), they are proposed to offer improved specificity over TST. The most recent form of the Quantiferon assay, QuantiFERON-TB Gold *In tube* includes a third antigen TB7.7 which is encoded by a prophage and absent from BCG.

In the QuantiFERON-TB Gold *In tube* method, these antigens are used to coat a blood collection tube, so that blood is directly drawn into the tube containing the antigens. The tube can then be immediately incubated thus overcoming concerns regarding any effect of delayed incubation on *in vitro* responses. Such whole-blood based IGRAs are technically simple (relying on a standard ELISA technique) and rapidly performed. This is of particular importance in under-resourced, high burden areas. In contrast, the ELISpot assay relies on separation and enumeration of peripheral blood mononuclear cells (PBMCs), a technically demanding and time consuming task.

There are a number of potential roles for these assays in the diagnosis of tuberculosis infection. Firstly, they may be used to determine the presence of latent tuberculosis infection (LTBI) in individuals at high risk of progression to active tuberculosis(48), such as young children. Secondly, they may be used to determine infection with *Mtb* in patients with suspected active tuberculosis(12). However, since IGRAs detect sensitization by *Mtb*, they are unable to distinguish between active and latent infection. Their role in the diagnosis of active tuberculosis in adults in highly endemic areas, such as South Africa, where the majority of healthy adults show evidence of infection with Mtb, is therefore limited(52), except possibly in HIV-infected individuals(53). In

children, however, even in high burden regions of South Africa, the annual risk of infection, as determined by skin test surveys, is 3%(4), implying that most young children remain uninfected. ELISpot may therefore provide valuable supporting evidence for the diagnosis of active tuberculosis in young children by confirming the presence of infection. In the following two chapters, we describe studies which we conducted to evaluate the potential utility of the ELISpot assay for the diagnosis of tuberculosis in children. At the time of conducting the study described in Chapter 3, the use of these assays for the diagnosis of active tuberculosis in children had not been investigated. The discussion of our findings, at the end of Chapter 3, will therefore be in this context. Subsequently, however, further work has been conducted in this field, which is reviewed below.

2.2.1. The use of IGRA for the diagnosis of tuberculosis infection and disease in children

Since IGRA have been proposed to be a more sensitive and specific alternative to TST for the diagnosis of tuberculosis infection, most studies have evaluated their performance in comparison with TST. Unfortunately, there are no widely accepted standards for the interpretation of a positive TST in children. Most studies evaluating IGRA have used the Mantoux test as the comparator, whilst others have used the Heaf test. An important consideration is the cut-off value which is used to define a positive test. Different cut-off values may be appropriate in different settings. In general, a Mantoux diameter of $\geq$10mm in children is regarded as signifying infection, irrespective of prior BCG vaccination(33;54;55), although this has not been subjected to rigorous evaluation. Some

would consider a diameter of ≥5mm as positive in children who have been exposed to a case of active tuberculosis(56) (this conservative approach is used to avoid 'missing' cases of *Mtb* infection in children who are at high risk of developing progressive illness). There is general agreement that more conservative values (such as ≥5mm) are appropriate in HIV-infected children(39), although, even at this level, the test lacks sensitivity(57). Most of the studies summarised here have used a cut off value of ≥10mm for HIV-uninfected children, but this is specified in each case. Cut-off values for IGRA generally follow those established during studies of adult TB. Cut-off points used for ELISpot assays vary. Early reports(52;58) used cut-off values of either 5 or 10 spots per well (after correction for the number of spots in the unstimulated well) which is equivalent to 33 spots per million PBMC. The commercial T-SPOT.*TB* assay uses a cut-off of 6 spots per well, corrected for the number in the unstimulated well(50;59). A QuantiFERON test is considered positive if the concentration of IFN-γ in the tuberculosis-antigen tube (or well) less the value in the unstimulated tube (or well) is ≥0.35 IU/ml(59-61). These values appear to have been empirically derived from observations of individuals at high and low risk of tuberculosis infection.

2.2.1.1. The use of IGRA for the diagnosis of LTBI in children

2.2.1.1.1. The use of ELISpot for the diagnosis of LTBI in children

A fundamental problem when evaluating novel diagnostics for latent tuberculosis infection is the lack of a gold-standard test for the diagnosis of LTBI. Since this is unavoidable, studies rely on surrogate markers for the likelihood of infection, such as a history of exposure to a case of active tuberculosis. One may obtain some idea of the

performance of a novel assay by stratifying individuals into different exposure categories, and comparing the proportion of individuals with a positive TST or IGRA in each exposure category.

Four studies have applied this strategy to evaluate the use of ELISpot for the diagnosis of LTBI in children (Table 1). Ewer and colleagues compared the relationship between ELISpot or TST (Heaf test grade 3 or 4 [all cases] or grade 2 [BCG-unvaccinated]) and intensity of exposure amongst 535 children exposed to a source case during a school outbreak of TB(62). This study showed a strong correlation between ELISpot and TST (89%, $p<0.0001$). The nature of the school setting afforded the opportunity to precisely determine the degree of contact of each child with the source case. ELISpot correlated more closely than TST with intensity of exposure as determined by duration ($p<0.007$) or proximity ($p<0.03$) of contact with the source case. A positive TST, but not ELISpot was associated with a history of prior BCG vaccination.

A second study compared ELISpot with TST in 979 Turkish children who were household contacts of adults with TB(63). In this study, both ELISpot and TST ($\geq$10mm) correlated well with intensity of household exposure to TB. The performance of both tests was similar in this study, although a positive ELISpot was intriguingly associated with the absence of a BCG scar, suggesting that BCG may in some way protect against *Mtb* infection (rather than disease only, as generally understood).

Richeldi and colleagues(64) used ELISpot and TST to evaluate transmission of *Mtb* to hospital contacts (neonates and their mothers) of a mother admitted to a maternity ward with undiagnosed multi-drug resistant TB. TST was positive (≥5mm) in 4/88, and ELISpot in 17/88 contacts. The odds of a positive ELISpot increased by 1.05 for each hour of exposure to the source case. The same authors(65) describe a case of an infant exposed to a mother with multi-drug resistant tuberculosis. The infant tested positive at 6 months of age on the ELISpot assay and, despite having a persistently negative TST, developed active TB by the age of 18 months. This case study illustrates how targeted use of the ELISpot assay may be useful in high risk individuals.

By contrast, Hill and colleagues compared ELISpot with TST (≥10mm) amongst the childhood contacts of 287 Gambian adults with TB(66). The agreement between tests was 83%. Both tests showed a correlation with the exposure gradient, however this was more marked with TST. The authors concluded that ELISpot was less sensitive than TST for the diagnosis of *Mtb* infection arising from recent exposure. Neither test appeared to be confounded by BCG vaccination.

In summary, 2 of the 4 studies concluded that the ELISpot assay correlated better than TST with degree of exposure to tuberculosis, whilst one showed similar performance and the last, impaired sensitivity of ELISpot. There are a number of potential reasons for the conflicting findings. All of the studies described here used an 'in house' ELISpot assay, since the commercial T-SPOT.*TB* assay (Oxford Immunotec) was not available at the time of study. It is difficult to directly compare these results, since the choice of antigen

(recombinant ESAT-6 and CFP10 and/or peptide pools), the comparator TST (Mantoux or Heaf) and the cut-off value for TST used differ between studies (Table 1). Moreover, in the absence of a gold-standard test, the use of exposure gradients to determine performance of the assays allows considerable room for interpretation. The data regarding the sensitivity and specificity of ELISpot assays for the diagnosis of LTBI in children is therefore conflicting, however it appears that, on balance, ELISpot may be marginally more sensitive and specific than TST.

Table 1. Studies evaluating the use of ELISpot for the diagnosis of LTBI in children

Study	Number of participants	Antigen used in ELISpot	TST (antigen)	Cut-off for TST	Major finding
Ewer et al(62)	535 (children)	rESAT-6*, peptides†	Heaf test (Evan's PPD)	Grade or 4 (Grade 2 if no BCG)	ELISpot correlated better with intensity of exposure; Heaf test confounded by BCG
Hill et al(66)	718 (children)	Peptides	Mantoux (2 TU‡ RT23)	≥ 10mm	ELISpot less sensitive than TST, neither confounded by BCG
Richeldi et al(64)	88 (adults and infants)	rESAT-6, rCFP10§, peptides	Mantoux (5 IU PPD-Siebert)	≥ 5mm	ELISpot more sensitive and correlated better with exposure
Soysal et al(63)	979 (children)	Peptides	Mantoux (2 TU RT23)	≥ 10mm	Similar performance of ELISpot and TST

*recombinant ESAT-6, †sequential peptide pools, ‡Tuberculin units, §recombinant CFP10,

2.2.1.1.2. The use of whole blood-based assays for the diagnosis of LTBI in children

A number of studies have compared various forms of the QuantiFERON assay with TST for the diagnosis of LTBI (or recent infection) in children (Table 2). Brock et al(49) evaluated the use of the QuantiFERON whole-blood assay for the detection of LTBI in children exposed during an outbreak of TB at a Danish high school. The first generation QuantiFERON assay, which used PPD as an antigen was significantly confounded by prior BCG vaccination, whilst the newer version, using recombinant ESAT-6 and CFP10, now marketed as QuantiFERON-TB Gold) was unaffected by BCG. There was excellent agreement between TST (>10mm) and QuantiFERON-TB Gold, with discordance in only 5/85 (6%) of subjects. TST did not therefore appear to be significantly confounded by prior BCG vaccination in this study.

By contrast, in their evaluation of QuantiFERON-TB Gold, Connell et al(67) showed a relatively poor correlation between the *in vitro* assay and TST (agreement 69%, kappa 0.3) in 106 high risk children. 70% of children with a positive TST (defined as >5mm in all contacts, >15mm in BCG-vaccinated, >10mm in all others) had a negative QuantiFERON-TB Gold test. The authors suggest that the *in vitro* assay may have impaired sensitivity in children.

The third generation of QuantiFERON assays, the QuantiFERON-TB Gold *In Tube* (QFGIT) may offer an improved formulation. Nakaoka(68) and colleagues investigated 207 children in Nigeria who were stratified according to their history of exposure to TB. There was 74% agreement (kappa=0.5 for high risk children) between QFGIT and TST

(>10mm). The majority of the discrepancies occurred in TST-negative, QFGIT-positive exposed children and in TST-positive, QFGIT-negative non-exposed children, suggesting that QFGIT may be more sensitive and specific than TST for the diagnosis of LTBI. Tsiouris et al(69) compared QFGIT with TST amongst 184 South African school children. TST was more frequently positive (≥10mm) than QFGIT (44% vs 33%, agreement of 78.8%, kappa=0.56), although whether this was due to impaired specificity of TST or impaired sensitivity of QFGIT is unclear.

In summary, agreement between TST and QuantiFERON-TB Gold (and QFGIT) assays ranges between 69% and 94% for the diagnosis of LTBI or recent infection in children, with studies confined to children showing agreement between 69 and 83%. Only one study(68) has suggested that QuantiFERON correlated better than TST with the degree of exposure to tuberculosis. The performance of the assay may be suboptimal in children.

In general, the evidence to support the use of IGRA for the diagnosis of LTBI in children is therefore equivocal. ELISpot appears to correlate better with TST than do the whole blood-based assays in children. There is limited data to suggest that both ELISpot and whole blood assays correlate better with intensity of exposure to TB than does the TST, however the differences are subtle and by no means clear. This issue can only be resolved by prospective study of the development of active TB in exposed individuals however ethical issues will preclude the possibility of conducting such investigation. It is likely that these assays will be increasingly applied for the diagnosis of LTBI in children in the absence of firm evidence to support their use.

Table 2. Studies evaluating the use of QuantiFERON assays for the diagnosis of LTBI in children

Study	Number of participants	Assay	TST (antigen)	Cut-off for TST	Major findings
Brock et al(49)	125 children and adults	QuantiFERON-RD1 (equivalent to QuantiFERON-TB Gold	Mantoux (2TU RT23)	>10mm	94% agreement (κ 0.87) PPD affected by prior BCG vaccination, RD1 not affected
Connell et al(67)	106 children	QuantiFERON-TB Gold	Mantoux (10TU, Cellestis)	>15mm BCG vaccinated >5mm if known contact >10mm all others	69% agreement (κ 0.3) High rate of failed assays
Nakaoka et al(68)	207 children	QFGIT	Mantoux (10U [~5TU], Evans)	≥10mm	74% agreement (κ 0.5 in high risk cases). QFGIT correlated better with exposure risk
Tsiouris et al(69)	184 children	QFGIT	Mantoux (2TU RT23)	≥10mm, or ≥15mm	≥10mm TST: agreement 78.8% (κ 0.56); ≥15mm TST: agreement 82.6% (κ 0.62)

2.2.1.2. The use of IGRA for the diagnosis of active TB in children

2.2.1.2.1. The use of ELISpot for the diagnosis of active TB in children

Three studies, two of which were conducted in South Africa, have evaluated the use of ELISpot for the diagnosis of active TB in children. One of these studies is the subject of the following chapter of this thesis. In the other South African study, Liebeschuetz and colleagues(70) performed ELISpot on 293 children presenting to hospital with suspected TB in KwaZulu-Natal. The final diagnosis was determined by the use of a clinical algorithm. ELISpot was positive in 81% of children with microbiologically confirmed TB (compared with 37% for TST ≥10mm) and 83% of children with confirmed and highly probable TB (compared with 65% for TST). Importantly, the sensitivity of ELISpot was unaffected by HIV status, whilst TST was more likely to be negative in HIV-infected children. The use of a 5mm cut-off for the TST failed to improve the sensitivity of skin testing. Spyridis et al report a case of an HIV-infected child with confirmed tuberculosis, a negative TST and a positive ELISpot assay(71).

Detjen et al(50) compared the performance of T-SPOT.*TB*, QFGIT and TST in a cohort of 73 children with culture-confirmed tuberculosis, culture-confirmed non-tuberculous mycobacterial infection or non-mycobacterial respiratory tract infections in a low incidence setting. The sensitivity of all three tests was comparable (93% for QFGIT and T-SPOT-*TB* and 100% for TST), however TST was less specific (58%) than the IGRAs (100% for QFGIT and 98% for T-SPOT.*TB*). All of the false-positive TST occurred in children with non-tuberculous mycobacterial infection. This study differed from the first two studies in that children were not tested on admission, but rather once microbiological

confirmation of the diagnosis had been obtained. This delay in testing may be one reason for the particularly good performance of the *in vitro* assay in this study, as will be discussed in Chapter 4.

In the case report documented by Richeldi and colleagues above(65), the ELISpot result in a child exposed to an adult with MDR TB increased 10-fold prior to the child developing active disease.

2.2.1.2.2. The use of whole blood-based assays for the diagnosis of active TB in children

Only one study has prospectively addressed the use of QuantiFERON assays for the diagnosis of active tuberculosis in children. Dogra and colleagues(61) evaluated 105 children with suspected TB or a history of TB exposure who were admitted to a rural Indian hospital. There was strong concordance (95%) between TST (≥10mm) and QFGIT. Neither TST nor QFGIT were affected by prior BCG vaccination. There were too few children with bacteriologically confirmed TB to determine the sensitivity of the two tests. Connell et al describe two cases of children with culture-confirmed perinatal TB. In both cases the TST was negative but QuantiFERON-TB Gold positive.

2.3. Conclusion

There is a clear need for further studies addressing the role of IGRA for the diagnosis of both active and latent tuberculosis in children. Case reports, whilst of interest, are likely to reflect publication bias in favour of the newer assays and prospective studies are limited by the number of cases with culture-confirmed disease and by lack of data regarding the specificity of these assays in highly endemic areas. Important issues that need to be addressed in subsequent evaluations, include the use of standardised methodology and interpretative criteria for both TST and ELISpot.

The following two chapters describe the contribution of the author and other members of the team at the University of Cape Town towards understanding the role of IGRA in the diagnosis of TB in children. Chapter 3 describes a hospital-based study of the ELISpot assay in children presenting to Red Cross Children's Hospital with suspected TB. This study was conducted prior to the availability of any data regarding the performance of ELISpot assays for the diagnosis of active TB in children and published(12) shortly after that of Liebeschuetz et al(70). Chapter 4 describes a community-based study which evaluated the ELISpot assay for the diagnosis of active TB in children presenting to their local health care facility with symptoms suggestive of TB or with a history of contact with an adult with active TB. The aim of this study was to determine the performance of the ELISpot assay in less severely ill children in a community setting.

Chapter 3. The enzyme-linked immunospot assay for the diagnosis of active tuberculosis in hospitalised children

3.1. Introduction

The difficulties in confirming the diagnosis of tuberculosis in children have been described in Chapter 2. The ability to detect the presence of circulating tuberculosis-specific lymphocytes in an interferon-γ release assay (IGRA) may represent an alternative strategy for diagnosing tuberculosis as well as monitoring therapeutic efficacy. We wished to determine the potential utility of IGRA for the diagnosis of active tuberculosis in childhood.

At the time of conducting this study, the potential use of whole blood and PBMC-based IGRA had recently been described for diagnosis of latent tuberculosis infection in adults(48;72). ELISpot responses to ESAT-6 and CFP-10 appeared to be more specific than TST when evaluating infection with *Mtb* amongst adult tuberculosis contacts(48;73). ELISpot using ESAT-6 or CFP10, but not the TST, distinguished between tuberculosis and prior vaccination with BCG. In addition, early data suggested that ELISpot might be useful for the diagnosis of active tuberculosis in adults, with positive ELISpot responses in 45 of 47 adult patients with bacteriologically confirmed tuberculosis(58). Little was known regarding tuberculosis-specific cellular immune responses in childhood tuberculosis at this time. ELISpot had been used to track transmission of tuberculosis infection amongst school children in a low-incidence setting(62), but at the time of conducting this study there were no data evaluating ELISpot responses in children with active tuberculosis.

In order to document the presence and kinetics of specific cellular responses amongst children with tuberculosis, we conducted a study to evaluate ELISpot responses to ESAT-6 and CFP10 amongst children admitted to hospital with a clinical diagnosis of tuberculosis in a tuberculosis-endemic area. In sub-groups of subjects we repeated the assay after 1, 3 and 6 months of treatment in order to assess the evolution of responses. We also examined responses of 26 healthy children who were household contacts of adults with active tuberculosis.

3.2. Methods

3.2.1. Selection and Description of Participants

All children under the age of 14 years presenting to the Red Cross Children's Hospital, Cape Town from January 2002 to August 2004 with an admission diagnosis of tuberculosis were eligible for inclusion. Children were considered for inclusion if the admitting clinician placed the child on anti-tuberculous chemotherapy and if a parent or legal guardian was available and consented to enrolment. The cohort of healthy childhood contacts was recruited by tracing the household contacts of adults presenting with newly diagnosed tuberculosis at one urban clinic in the Cape Town Metropole. Ethics approval was obtained from the Research Ethics Committee of the University of Cape Town (Rec Ref 327/2001). All participating children were tested for HIV infection by ELISA following pre-test counselling. Children found to be HIV ELISA positive were excluded from participation and referred to the hospital HIV clinic.

A sequential subgroup of children was enrolled for evaluation of the time course of responses at 1, 3 and 6 months after commencing therapy. In order to evaluate responses after 1 month of therapy, a sequential sub-group of 42 had repeat ELISpot assays after 1 month. Of this sub-group, 25 children in whom follow-up was possible were retested at 3 months and 10 at 6 months. Of the 70 children, 58 received tuberculin skin testing (TST) with 2 TU of PPD RT23 (Statens Serum Institut, Copenhagen, Denmark). Responses were read at 48-72 hours.

Diagnostic investigations, including radiography, tuberculin skin testing and microbiology were at physician discretion. Once the results of this testing were available, records and chest X-rays were reviewed in a blinded fashion by a study clinician with extensive experience in the diagnosis of childhood tuberculosis, and children were categorised as having definite, probable or possible tuberculosis (Table 1).

An additional 26 children (median age 24 months) who were healthy contacts of adults with tuberculosis were recruited. Active tuberculosis was excluded on X-ray and clinical examination and TST was performed as above. Children with positive TST were referred for chemoprophylaxis according to national guidelines.

Table 1. Clinical Classification of Cases

Definite Case	(n=12) Isolation of *Mtb* in culture (sputum, gastric aspirate or cerebrospinal fluid)
Probable Case	Must fulfil all criteria in A, B, C or D below
	A. (n=15) Symptoms consistent with TB Known close contact with TB patient Mantoux skin test ≥10 mm AND abnormal Chest Radiograph
	B. (n=20) Symptoms consistent with TB No known close contact with TB patient Mantoux skin test ≥10 mm AND abnormal Chest Radiograph
	C. (n=6) Symptoms consistent with TB Known close contact with TB patient Mantoux skin test ≥10 mm OR abnormal Chest Radiograph
	D. (n=6) Symptoms consistent with TB AND ONE OF: Gastric aspirate smear positive (n=1) Cerebrospinal fluid and cranial CT consistent with tuberculous meningitis (n=4) Abdominal ultrasound and CT suggestive of TB abdomen and response to therapy (n=1)
Possible Case	(n=11) Symptoms consistent with TB No known close contact with TB patient Mantoux skin test ≥10 mm OR abnormal Chest Radiograph

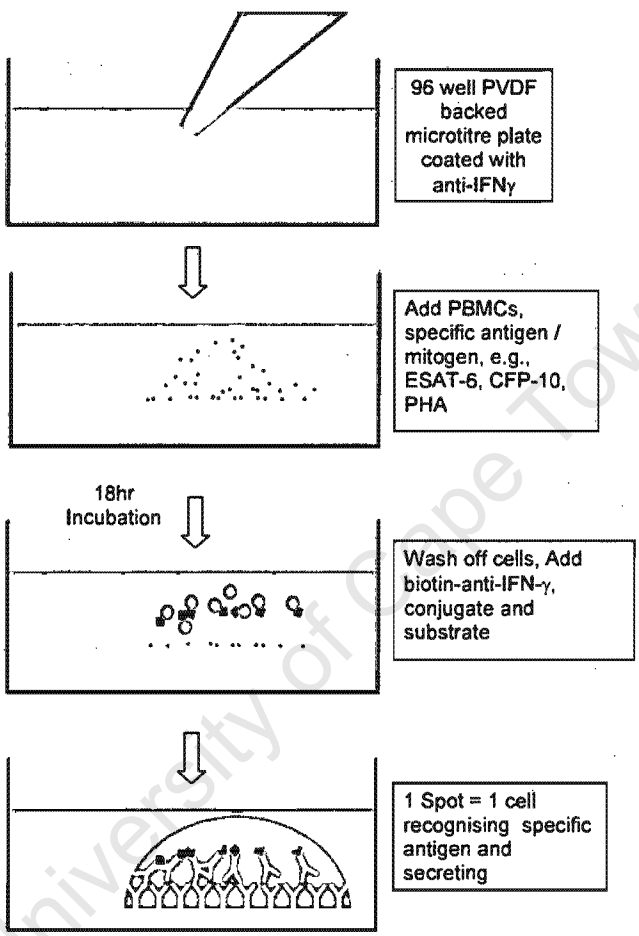
3.2.2. ELISpot Assays

After obtaining informed consent, 5ml of blood was collected by peripheral venipuncture and processed within 2 hours. ELISpot (Figure 3.1) was performed as described in Appendix A(58). Spots were enumerated independently by two observers using a stereomicroscope. The mean values of the two observers (mean inter-observer coefficient of variation = 8%) and both duplicate wells were used in all calculations. The number of spots in the background control wells was subtracted from that in the test wells and a response considered positive if $\geq$ 10 spots/well (representing 33 spots/million PBMC) and at least twice the value of the background control wells. The use of this value to discriminate a positive response was based on previous reports by Lalvani et al(52;58). This value also corresponded with more than 2 standard deviations above the mean of the unstimulated control wells (mean=6.6 spots/million PBMC, SD=8.5).

3.2.3. Statistical analysis

Comparisons between ELISpot results in clinical categories were made using the Kruskal-Wallis test. The chi-square test for trend was used to compare the proportion of positive results by clinical category. The two-tailed Mann-Whitney test was used to compare unpaired results (single time point) and the Wilcoxon signed rank test for paired observations (acute and 1 month). Friedman's test was used for evaluation of responses over 6 months.

Figure 3.1 The ELISpot Method



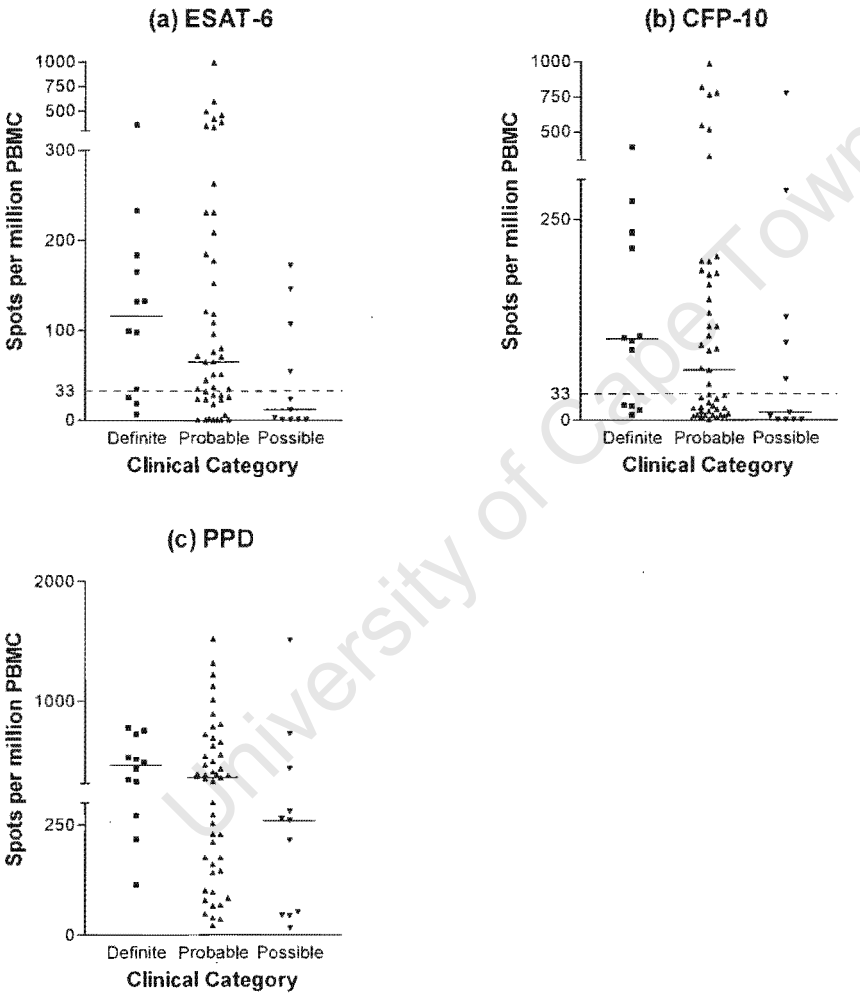
3.3. Results

A total of 70 children (median age 32, range 3-154 months) with a diagnosis of tuberculosis were recruited. After record review, 12 children were assigned as definite cases of tuberculosis, 47 as probable and 11 as possible cases (Table 1). A sequential sub-group of 42 had repeat ELISpot assays after 1 month. Of this sub-group, 25 children in whom follow-up was possible were retested at 3 months and 10 at 6 months. Of the healthy children who were contacts of adults with tuberculosis, 14/26 had a non-reactive TST (0mm) and the remaining 12 had TST ≥15mm. None had a TST between 0mm and 15mm.

3.3.1. Responses to ESAT-6, CFP-10 and PPD at diagnosis

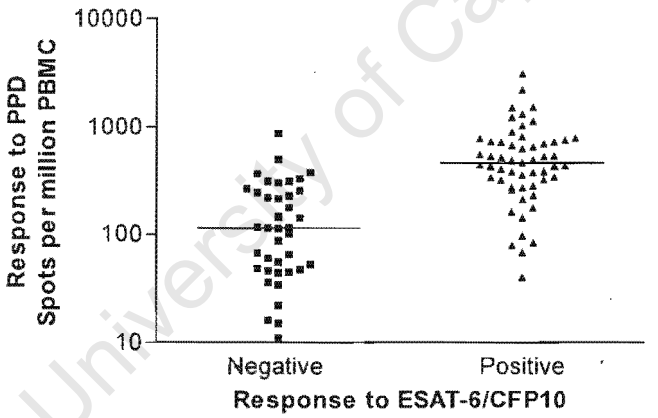
The distribution of responses to ESAT-6, CFP-10 and PPD by diagnostic category at diagnosis is shown in Figure 3.2 There was a non-significant trend for median ESAT-6 (p=0.0631) and CFP10 (p=0.1968) responses to be lower in the probable and possible groups. Positive responses to either ESAT-6 or CFP10 were found in 10/12 (83.3%) definite, 34/47 (72.3%) probable and 5/11 (45.5%) possible cases (chi-square 3.77 p=0.05). There was no significant difference between the median ages of those with positive and negative responses. There was no correlation between response to PPD and TST result (Spearman r = 0.1587).

Figure 3.2 Response to ESAT-6 (a), CFP10 (b) and PPD (c) by clinical category at diagnosis. Results are means of two independent observers, given as spots per million PBMC, corrected for background. A horizontal line is included to show the cut-off for a positive response (33 spots per million) for ESAT-6 and CFP-10. Median is shown.



Responses to PPD were significantly higher in those with positive responses to ESAT-6 or CFP10 (median = 446 spots per million) than those with negative responses to both (median = 144 spots per million, $p<0.0001$, Figure 3.3). There was no correlation between response to any antigen and weight for age Z score (PPD r=-0.070 p=0.55; ESAT-6 r=-0.1718 p=0.138; CFP10 r=-0.1721 p=0.1372).

Figure 3.3 Responses to PPD at diagnosis amongst those with negative compared with positive responses to ESAT-6/CFP10. Results are means of two independent observers, given as spots per million PBMC, corrected for background. Median is shown.



3.3.2. Agreement between ELISpot and TST in children with a clinical diagnosis of tuberculosis

Since TST formed part of the diagnostic algorithm, it was not possible to compare the utility of TST and ELISpot as a diagnostic tool, except in those children with culture-confirmed tuberculosis. In this group, TST was positive in 6/7 children tested (compared with 10/12 children for ELISpot). Overall, there was relatively poor agreement between TST and ELISpot (69%, kappa 0.04), with a high proportion of children with a positive TST having a negative ELISpot (Table 2).

Table 2. Comparison of TST and ELISpot in children with clinically defined tuberculosis

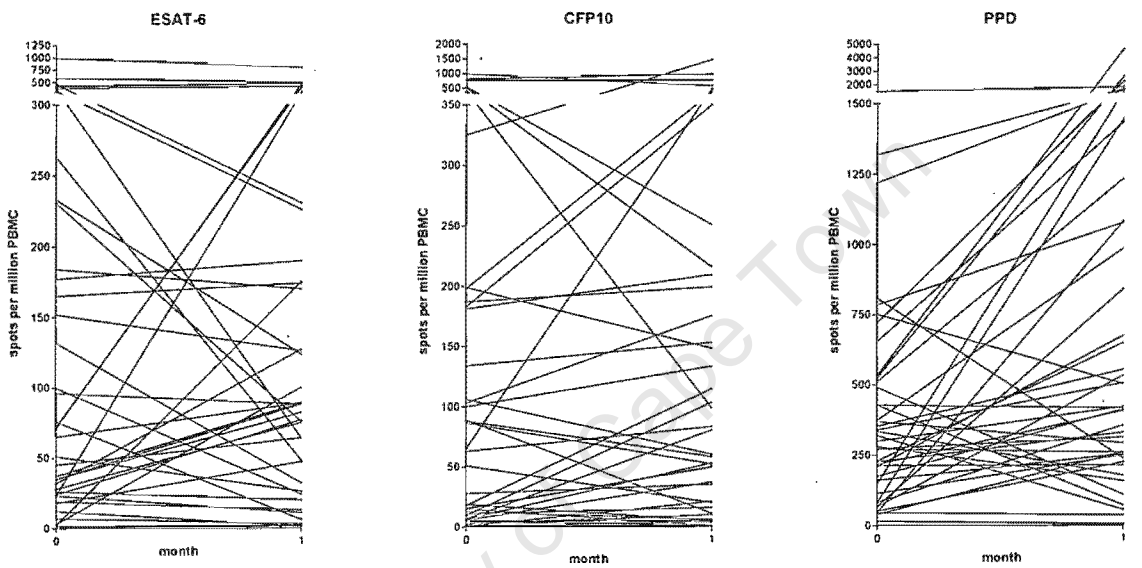
		TST (Mantoux ≥10mm)		
		Positive	**Negative**	**Total**
ELISpot	**Positive**	39	1	40
	Negative	17	1	18
	Total	56	2	58

3.3.3. Responses to ESAT-6, CFP-10 and PPD after 1 month of treatment

Repeat ELISpot testing was performed at 1 month in 42 subjects. Nine cases were assigned as definite, 6 as probable and 27 as possible tuberculosis. The acute and 1 month responses are shown in Figure 3.4. The median PPD response was significantly higher at 1 month (419 spots per million) than diagnosis (190 spots per million, p=0.0004). There was no significant difference between the acute and 1 month visit in median response to ESAT-6 (58 and 85 spots per million) or CFP10 (57 and 59 spots per million). Acute and 1 month results were highly correlated for CFP-10 (Spearman r = 0.827, p<0.0001) and ESAT-6 (Spearman r = 0.6289, p<0.0001).

After 1 month of treatment, median responses to ESAT-6, CFP10 and PPD were different between diagnostic groups (Kruskal-Wallis p=0.0133, p=0.0118 and p=0.0079 respectively) with significantly higher responses amongst probable when compared with possible cases. There was a trend for responses in the definite group to be higher than those in the possible group, but this failed to reach statistical significance.

Figure 3.4 Responses to ESAT-6, CFP10 and PPD at diagnosis and after 1 month of therapy. Results are means of two independent observers, given as spots per million PBMC, corrected for background.

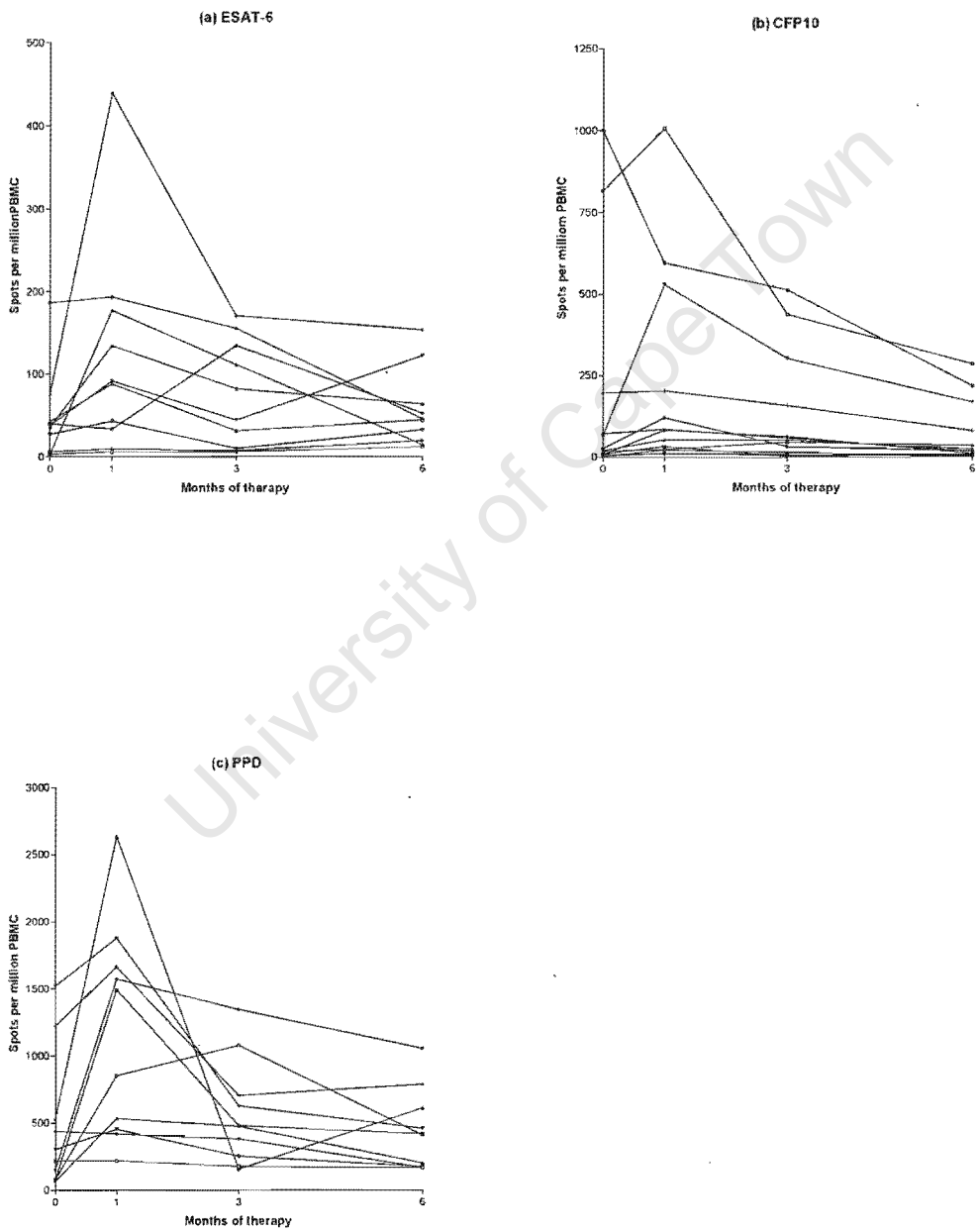


3.3.4. Responses to ESAT-6, CFP-10 and PPD after 3 and 6 months of treatment

Further responses were evaluated in a consecutive subset of 10 patients. The evolution of responses to PPD, ESAT-6 and CFP10 is shown in Figure 3.5 An initial rise in response to these antigens at 1 month was followed by a decline at 3 and 6 months (Friedman test PPD: p=0.002, ESAT: p=0.011, CFP10: p=0.002).

Responses to ESAT-6 were significantly higher amongst probable than possible cases at 3 months (Kruskal-Wallis p=0.0406). The trend for definite and probable cases to have higher responses to CFP10 and PPD than possible cases was still present at 3 months but non-significant (Kruskal-Wallis p=0.1097 for CFP10, p=0.0797 for PPD).

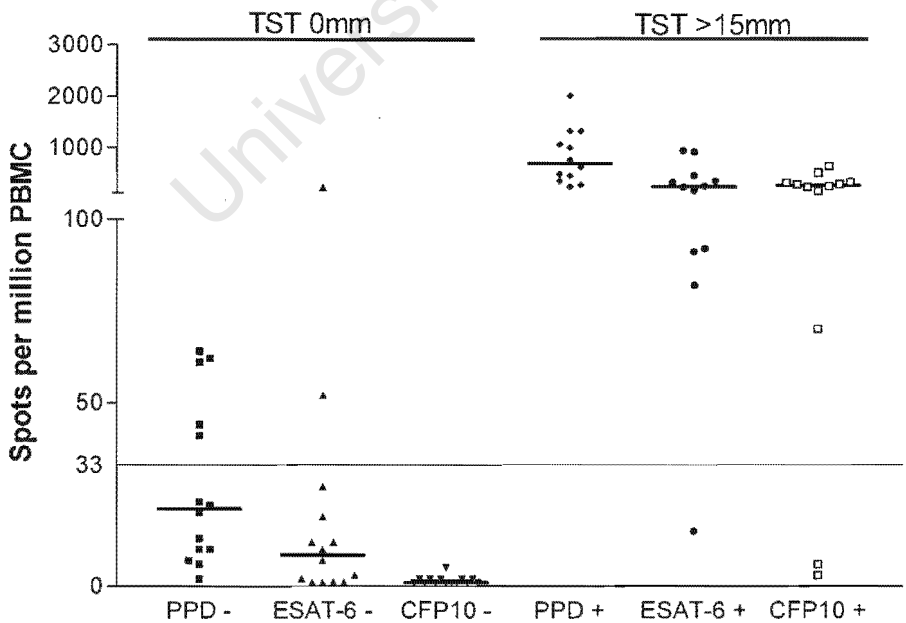
Figure 3.5 Responses to ESAT-6, CFP10 and PPD during the course of therapy. Results are means of two independent observers, given as spots per million PBMC, corrected for background.



3.3.5. Responses amongst healthy childhood contacts

Responses to ESAT-6, CFP10 and PPD (Figure 3.6) were strikingly lower amongst children with negative skin tests ($p<0.0001$ for each antigen). Only two children with a negative skin test had a positive response to ESAT-6 and none to CFP10. A cut-off level of 100 spots per million to PPD discriminated between TST-positive and negative individuals in all cases. Only 1 child with a TST>15mm had a negative response to ESAT-6 and 2 had negative responses to CFP10.

Figure 3.6 Responses to PPD, ESAT-6 and CFP10 amongst healthy childhood contacts of adults with tuberculosis. Results are means of two independent observers, given as spots per million PBMC, corrected for background. Lines represent median responses. Responses from those children with TST of 0mm are on the left and those with TST of >15mm on the right.



3.4. Discussion

This study and that of Liebeschuetz et al(70) were the first to explore the potential role of IGRA in the diagnosis of active tuberculosis in childhood. Several studies had suggested that ELISpot and whole-blood based assays detecting responses to RD1-coded antigens were more specific than PPD skin testing in diagnosing *latent* infection in both adults and children(48;62). ELISpot had been shown to be sensitive in detecting *active* tuberculosis infection in small groups of adults(72). Sequential monitoring of ELISpot responses had been proposed to be useful in monitoring response to anti-tuberculous chemotherapy(74). The potential utility of ELISpot for the diagnosis and monitoring of acute tuberculosis in children had not been reported at the time of this study. The discussion that follows is in this context.

In this study, we have defined IFN-γ ELISpot responses to ESAT-6 and CFP10 in a group of children with a clinical diagnosis of tuberculosis. The findings of this study have a number of implications for the potential use of RD1-based ELISpot for the diagnosis and monitoring of tuberculosis in childhood.

3.4.1. Sensitivity of ELISpot for diagnosis of clinically-defined tuberculosis

It is clear that a proportion of children with a clinical diagnosis of tuberculosis have negative ELISpot responses to ESAT-6 and CFP-10 (21/70, 30%) at diagnosis. This may be due to impaired sensitivity of the ELISpot assay in this setting or poor specificity of

the clinical diagnosis of tuberculosis in children, or both. Of note, positive responses at diagnosis were found amongst fewer possible (45.5%) than probable (72.3%) or definite (83.3%) cases. The sensitivity documented for ELISpot for the diagnosis of culture-confirmed tuberculosis (83%) in this study coincides closely with that found by Liebeschuetz et al (81%)(70). The observation that those children in the 'possible' group with negative responses to ESAT-6 and CFP10 at diagnosis had persistently negative responses at 1, 3 and 6 months (despite positive responses to PPD) suggests that this subset may well represent children with a diagnosis other than tuberculosis. The clinical diagnosis of tuberculosis may therefore lack specificity in our setting. Over-diagnosis of tuberculosis is important, given the expense, prolonged duration and potential for adverse effects of therapy. This hypothesis will remain difficult to prove, given the difficulty in confirming tuberculosis in children, as no appropriate 'gold-standard' sensitive and specific comparator is available.

Since there were 2 ELISpot-negative cases with culture-confirmed tuberculosis, we re-tested 42 consecutive subjects after 1 month in order to evaluate whether repeating the ELISpot test would improve the diagnostic yield. There was no significant difference in the proportion who responded to either antigen at diagnosis compared with 1 month. However 6 children had a positive response to at least one antigen at the one month visit, but not at diagnosis, including one child with culture-confirmed tuberculosis. Only 1 of the 9 children with culture proven disease had persistently negative responses at 1 month. The strategy of repeating borderline or negative tests at 1 month might be a useful approach. The 1 month data may however be confounded by the potential for boosting of

ELISpot responses by TST testing, since PPD will contain small amounts of ESAT-6 and CFP10.

3.4.2. Comparison of TST and ELISpot responses

Agreement between ELISpot and TST was poor, with a large group of children having negative ELISpot responses with a positive TST. There are two possible interpretations for this finding. Firstly, this may be due to increased sensitivity of TST in the diagnosis of childhood tuberculosis. This is difficult to assess in this cohort, since the group of children with culture-confirmed tuberculosis is small. Alternatively, TST may possess impaired specificity, with false-positives resulting from prior BCG vaccination or sensitization by environmental mycobacteria. Since this study did not include a large group of children with a confirmed alternative diagnosis, we are unable to exclude this possibility. The following chapter will address this issue further.

3.4.3. Change in the magnitude of ELISpot responses during therapy

There was no significant difference between the magnitudes of the responses to either CFP-10 or ESAT-6 at diagnosis compared with one month. In contrast, PPD responses were significantly higher at 1 month. A number of potential mechanisms could underlie this finding.

Firstly, TST might prime the ELISpot response to PPD. The effect of TST on responses to ESAT-6 and CFP-10 is not yet clear. Mawa et al(75) have documented a transient increase in interferon-γ production in response to culture filtrate proteins of *Mtb* following TST of HIV-infected persons. In contrast, Richeldi et al(76) have shown no effect of repeat TST on ELISpot responses to ESAT-6 and CFP10.

Secondly, Wilkinson et al(77) have shown that the ratio of ESAT-6/PPD responsive cells in central compartments is higher than that in blood. Such sequestration during active infection may explain the paucity of specific cells found by ELISpot in some subjects. Recirculation of these cells during therapy might account for the subsequent increase in ELISpot responses.

Finally, severe TB has been associated with decreased levels of IFN-γ production by PBMC, in a proportion of TB patients(78). The recovery of antigen-specific responses during therapy has previously been shown to be antigen dependent(79), which could account for the differences between PPD and the RD1-encoded antigens in this regard.

It has been previously shown that ELISpot responses to PPD are unable to adequately distinguish between prior BCG vaccination and exposure to tuberculosis(35). The median responses to PPD at diagnosis were significantly higher in that group of children who responded to either of the specific antigens when compared with those who did not. PPD responses were positive in almost all children however, and there was considerable overlap in PPD responses between children with positive and negative RD1 responses.

No single threshold value for PPD corresponded with a positive response to the specific antigens.

Carrara(74) has demonstrated the disappearance of ELISpot responses in patients who responded to three months of therapy, but persistent responses in those who failed therapy. Sequential testing has thus been advocated as a biomarker of successful chemotherapy. In our study, an increase in ELISpot responses at the 1 month visit was documented in 45% (ESAT-6), 40% (CFP-10) and 69% (PPD) of definite and probable cases. This cautions against the use of an early decline in ELISpot responses as a surrogate for successful treatment. Results amongst the 10 cases in whom 3 and 6 month data were available suggest that an early rise in responses to PPD and ESAT-6 at 1 month is followed by a decline in responses by 3 and 6 months to a level similar to that seen at presentation.

3.4.4. Responses amongst healthy contacts

ELISpot responses to PPD discriminated between TST-positive and negative children. Healthy TST-positive children had similar responses to children with active tuberculosis. TST-negative (and presumably uninfected) healthy children had negative responses to the specific antigens in 12/14 cases. Therefore, ELISpot appears to be useful in identifying children infected with tuberculosis, but is unable to discriminate between active and latent or sub-clinical infection.

3.4.5. Limitations of this study

This study was designed to document ELISpot responses in children with a clinical diagnosis of tuberculosis, whose diagnosis is, at best, imperfect. We acknowledge that a number of the children included in this study, particularly those in the 'possible' group, may have an alternative diagnosis. Indeed, the likelihood of over-diagnosis of childhood tuberculosis is an important conclusion of this study. We did not intend to conduct an evaluation of ELISpot as a diagnostic tool, but rather, to document responses in children with a clinical diagnosis of tuberculosis. We believe that these data suggest that ELISpot merits formal evaluation as a diagnostic tool for childhood tuberculosis, ideally with a repeat test at 1 month, and that early decline in tuberculosis-specific immune responses can not be used as a marker of successful therapy. The profound effect of HIV infection on cellular immunity may affect the sensitivity of the ELISpot assay, although results from adults(53;72), and a study including small numbers of HIV-infected children(70) are encouraging.

3.5. Conclusion

ESAT-6 or CFP10-specific interferon-γ-producing lymphocytes were detectable at diagnosis in two thirds of children presenting with a clinical diagnosis of tuberculosis, however responses were more frequently positive in those with culture-proven disease. Repeat testing after 1 month showed a similar pattern, however median responses to PPD were significantly higher at the second visit. Responses tended to increase during the first month and then decline by 3-6 months. ELISpot is a promising tool for the clinical evaluation of children presenting to hospital with suspected tuberculosis where it may provide supportive evidence of TB infection.

Chapter 4. The utility of T-SPOT.*TB* for the diagnosis of active tuberculosis in very young children

4.1. Introduction

Since neither IGRA nor TST are able to distinguish between active and latent infection, in practice a positive result in a child with suspected tuberculosis is non-specific for active disease and may at best lend supporting evidence to the diagnosis. However, should a positive IGRA result be a highly sensitive marker for the presence of tuberculosis infection, a negative result would be very useful in excluding the diagnosis.

Three prospective studies, including our own, detailed in the previous chapter, (12;50;70) have specifically examined the utility of ELISpot assays for the diagnosis of active tuberculosis in children and documented sensitivities ranging from 81%-93% for the diagnosis of culture-confirmed tuberculosis. A test with a sensitivity of 80% is unlikely to be widely acceptable to exclude tuberculosis infection in a child presenting with suspected tuberculosis. However, since IFN-γ responses have previously been shown to be depressed in severe illness(80) and since previous studies were predominately hospital-based (suggesting that severely ill patients were enrolled) it is feasible that the sensitivity of ELISpot may be higher in children with milder illness. We therefore wished to determine the sensitivity of ELISpot in detecting active tuberculosis in a community setting in children who presented to their local health care facility with symptoms suggestive of tuberculosis or a history of recent contact with a case of tuberculosis.

4.2. Methods

4.2.1. Participants

This study was performed in the context of a large phase IV trial conducted by the South African Tuberculosis Vaccine Initiative which compared percutaneous and intradermal routes of BCG vaccination (manuscript in preparation). The study was based in Worcester in the Western Cape province of South Africa. This is a stable, semi-rural population with a high case notification rate (tuberculosis smear positive case detection rate: 602/100 000 in 2004) and a relatively low HIV prevalence (prevalence amongst antenatal clinic attendees: 8.1% in 2005).

During the course of the study, 11680 BCG-vaccinated children were followed up from birth for at least 2 years. Surveillance for incident illnesses that could be tuberculosis was active over the first 3 months (children were screened during their routine vaccination visits at 6, 10 and 14 weeks of age) and passive thereafter. Over this period, the following categories of children were admitted to a case verification ward at the regional tuberculosis referral hospital: (a) children who presented to any of the referral hospitals or community clinics in the region with symptoms suggestive of tuberculosis (loss of weight or failure to gain weight, unexplained fever or prolonged cough); (b) children who had chest radiographs performed for respiratory symptoms at any of the referral hospitals; (c) children who lived at the same address as any adult case of tuberculosis diagnosed over the period. All children presenting to the case verification ward between February and December 2005 were included in the study. Written informed consent was obtained

from parents or guardians of all participants and the study was approved by the Research Ethics Committee of the University of Cape Town (REC Ref 271/2000).

The degree of exposure to tuberculosis amongst all participants was quantified by a history of contact with a patient with active tuberculosis. The non-exclusive exposure categories were stratified as follows: no contact, any contact with a patient with active tuberculosis, contact with an adult currently receiving anti-tuberculous therapy and contact with a household member with tuberculosis.

All children were thoroughly investigated for tuberculosis including clinical examination, chest radiograph, acid fast microscopy and mycobacterial culture of 2 gastric aspirates and 2 induced sputa samples and a Mantoux skin test (2 tuberculin units of PPD RT23, Statens Serum Institut, Copenhagen, read at 48 hours). A final diagnostic classification was assigned using an automated diagnostic algorithm (which did not include either TST or ELISpot result) drawn up by an expert clinical panel (Table 1). This algorithm is different from that used in the previous chapter, firstly since TST is specifically excluded (so that we could perform a direct comparison with T-SPOT.*TB*) and secondly since this cohort did not include any children with suspected extra-pulmonary tuberculosis. Chest radiographs were independently reviewed by 3 expert reviewers who were blind to clinical details, with a minimum of 2/3 agreement required. According to the algorithm, children were classified as having definite, probable or possible tuberculosis, or not having tuberculosis. Maternal HIV exposure was diagnosed by antibody ELISA on collected blood, following pre-test counseling of the parent/guardian.

Table 1. Diagnostic algorithm for the classification of tuberculosis cases

Definite TB*	Positive culture for *Mtb*	
Probable TB	Chest X-Ray suggestive	PLUS 1 or more additional features†: Cough > 2 weeks Failure to thrive OR recent loss of weight Known TB contact
Possible TB	Chest X-Ray suggestive	PLUS No additional features (above)
	OR Chest X-Ray not suggestive, Diagnosed as TB by treating clinician	
	OR Chest X-Ray not suggestive, Not diagnosed as TB by treating clinician	PLUS 2 or more additional features (above)
Not TB	Chest X-Ray not suggestive. Not diagnosed as TB by treating clinician.	One or no additional features

*tuberculosis

† Mantoux >= 10mm or positive T-SPOT.*TB* included as an additional feature only when evaluating the effect of addition of these tests to the diagnostic algorithm.

4.2.2. T.SPOT-*TB* Assay

At the time of admission, blood was collected by peripheral venepuncture for T-SPOT.*TB*, which was performed according to the manufacturer's guidelines (Appendix B). Briefly, peripheral blood mononuclear cell (PBMC) preparation tubes (CPT tubes, Becton Dickinson) were used to collect 3-4 ml of blood from each participant. Tubes were transported to the laboratory 100km away in insulated boxes and processed if received within 5 hours of collection. PBMCs were separated by centrifugation, washed, resuspended and counted. Cells (2.5×10^5) were added to each of 4 wells of the microtiter plate containing ESAT-6 and CFP-10 (Panel A solution or Panel B solution), mitogen or media only, After 16-20 hours of incubation, plates were washed, conjugate reagent containing anti-IFN-γ mAb added to each well and the plate re-incubated for 60 minutes. After additional washing, substrate solution was added and incubated for a further 7 minutes. Plates were then washed and dried before enumeration of spots by 2 observers using a stereomicroscope. The mean spot count of the observers was used for interpretation. A T-SPOT.*TB* assay was reported positive if either panel of peptides was positive, following the manufacturer's recommendations. T-SPOT.*TB* results were excluded if samples were received more than 5 hours after collection, if the positive control failed or if there were insufficient cells to perform the assay.

4.2.3. Statistical considerations

The primary outcomes were the result of the T-SPOT.*TB* and the size of the TST reaction in relation to the assigned clinical categorization. For T-SPOT.*TB*, the cut off point for a

positive test was as defined by the manufacturer. For the Mantoux test, a transverse diameter of induration greater or equal to 10mm was considered positive. The Chi-square test for trend was used to test for association between the clinical classification and T-SPOT.*TB* and TST as well as between degree of exposure and T-SPOT.*TB* and TST. The approximation method was used to calculate confidence intervals for proportions. The Mann Whitney test was used to compare ages between clinical categories as well as time to sample processing with T-SPOT.*TB* result. Chi-square was used to compare the proportion of positive T-SPOT.*TB* tests by hours to sample processing. Fisher's exact test was used to determine the association between TST or T-SPOT.*TB* and degree of exposure. The kappa statistic was used to summarize agreement between TST and T-SPOT.*TB*. The Spearman test was used to determine the correlation between size of TST or number of spots on T-SPOT.*TB* (expressed as continuous variables) and age.

4.3. Results

A total of 243 BCG-vaccinated children were evaluated, of whom 214 had interpretable T-SPOT.*TB* results (in 15 cases the T-SPOT.*TB* positive control failed and in 14 cases insufficient blood was obtained or the specimen was rejected after being received in the laboratory more than 5 hours after collection, Figure 4.1). The median age of the children was 18 months (IQR 14-24 months). The majority of children (61%) were between 13 and 24 months of age (Table 2).

Figure 4.1 Flow diagram of patient numbers and diagnostic classification

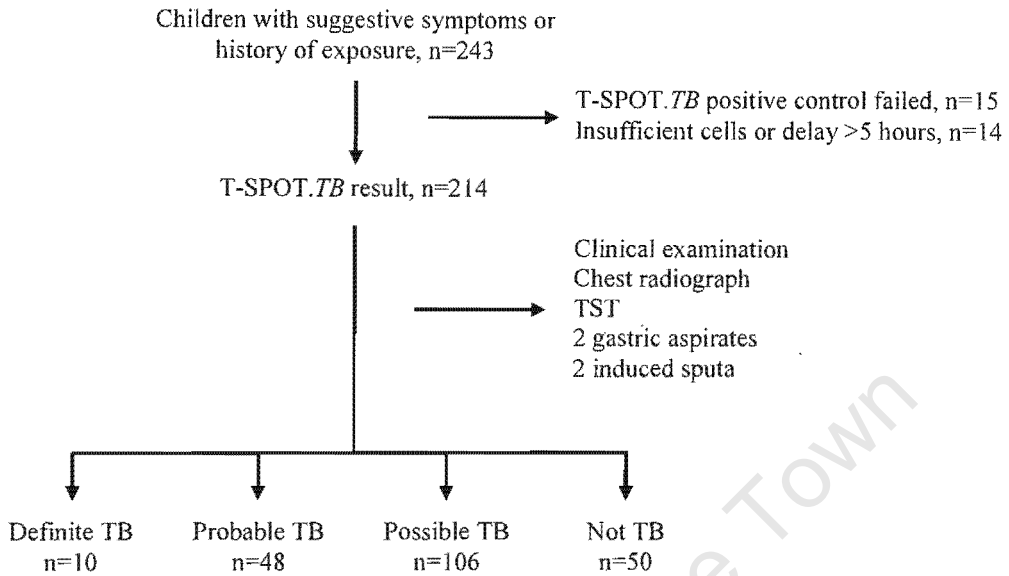


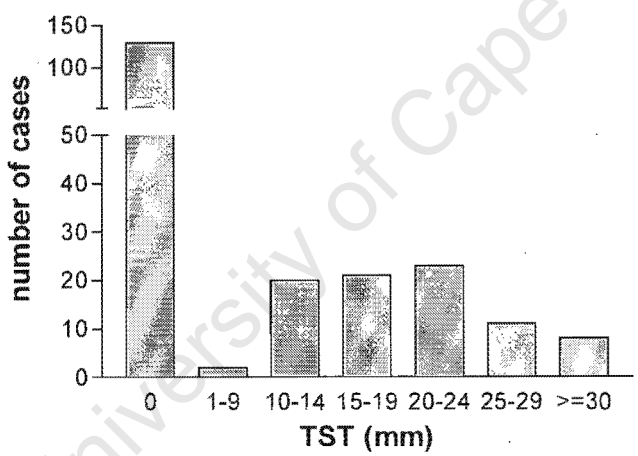
Table 2. Demographic data and test results by age

Age Category (months)	Total number	Gender Female (%)	Clinical Categorization				TST positive* (%)	T.SPOT.*TB* positive (%)
			Definite	Probable	Possible	Not TB		
0-12	30	9 (30)	1	3	19	7	12 (40)	1 (3)
13-24	131	56 (43)	7	37	59	28	48 (37)	32 (24)
25-36	43	22 (51)	1	6	24	12	18 (42)	15 (35)
>36	10	5 (50)	1	2	4	3	5 (50)	6 (60)
Total	**214**	**92 (43)**	**10**	**48**	**106**	**50**	**83 (38)**	**54 (25)**

* TST: tuberculin skin test (Mantoux >=10mm)

Only one child had a positive HIV ELISA and this child was included in the analysis (the child had probable tuberculosis with both TST and T-SPOT.*TB* negative). The distribution of TST responses is shown in Figure 4.2.

Figure 4.2 Frequency histogram of TST responses (all cases). The distribution of TST results (Mantoux) is shown. This follows a bimodal distribution with few children having a TST result between 1 and 10mm in size. (TST: tuberculin skin test)



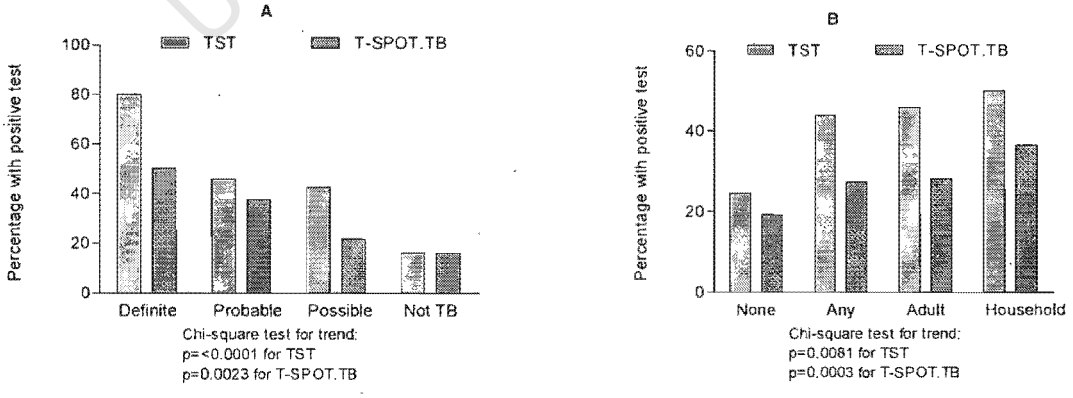
4.3.1. T-SPOT.TB, TST and the clinical diagnosis of active tuberculosis

Both a positive T-SPOT.*TB* and a positive TST response were strongly associated with increasing likelihood of tuberculosis (p=0.0023 for T-SPOT.*TB* and p<0.0001 for TST, Figure 4.3A).

Figure 4.3

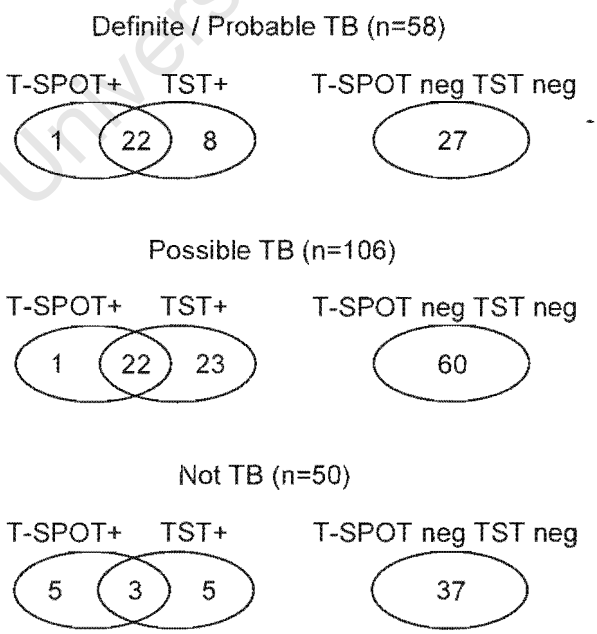
A. Proportion of cases with a positive TST (Mantoux≥10mm) or T-SPOT.TB test by clinical category. Both T-SPOT.*TB* and TST were more likely to be positive with increasing clinical certainty of the diagnosis of tuberculosis.

B. Proportion of cases with a positive T-SPOT.*TB* or TST by degree of exposure to a case of tuberculosis. Both T-SPOT.*TB* and TST were more frequently positive with increasing exposure. (any: any contact with a case of tuberculosis; adult: contact with an adult with tuberculosis; household: a contact with active tuberculosis in the same household)



T-SPOT.*TB* and TST responses were positive in 5/10 (50%, 95% CI 24-76%) and 8/10 (80%, 95% CI 49-94%) children with culture-confirmed tuberculosis respectively. The sensitivity of T-SPOT.*TB* for clinically-defined tuberculosis (using the combined categories of definite and probable tuberculosis) was 40% (95% CI 27-53%) whilst that of TST was 52% (95% CI 38-65%). The specificity of both tests, when evaluated using the clinical category "Not tuberculosis," was 84% (95% CI 71-93%). The numbers of children with positive TST and T-SPOT.*TB* assays in each diagnostic category are detailed in Figure 4.4. Of note, only 2 of the 113 children classified as having definite, probable or possible tuberculosis had a negative TST but a positive T-SPOT.*TB*.

Figure 4.4 Number of patients with a positive TST or positive T-SPOT.*TB* result according to clinical classification. The number of patients with positive responses is shown by diagnostic category. A Mantoux test of ≥10mm was regarded as a positive TST. (TST: tuberculin skin test)



In order to determine whether the use of T-SPOT.*TB* or TST would have substantially affected the diagnostic categorization of children, we compared the number of children in each diagnostic category when either TST or T-SPOT.*TB* was added as an additional feature to the diagnostic algorithm (Table 2, see footnote). There was excellent agreement between categorization of children using TST and T-SPOT.*TB* (93% agreement, kappa=0.902, p<0.00001). The inclusion of either T-SPOT.*TB* or TST in the diagnostic algorithm would have resulted in the reclassification of 7 children from "Not tuberculosis" to possible tuberculosis in each case, however not all of the same children were reclassified using the two tests, so that including a positive result on either test would have resulted in 11 children being reclassified. Neither test would have altered the diagnostic categorization of any of the children classified as having probable (or definite) tuberculosis.

There was no significant association between anthropometric measures (z-scores for weight for age or height for age) and a positive T-SPOT.*TB* amongst children with definite and probable tuberculosis (data not shown).

4.3.2. Association between the degree of exposure to a case of active tuberculosis and a positive T-SPOT.TB or TST test

There was an increasing likelihood for T-SPOT.*TB* and TST to become positive with increasing exposure (p=0.0003 for T-SPOT.*TB* and p=0.0081 for TST; Figure 4.3B). Similar numbers of children with no history of exposure had a positive TST (14/57, 24.6%) or T-SPOT.*TB* (11/57, 19.3%). A history of any contact with a case of

tuberculosis was associated with significantly increased odds of a positive TST (OR 2.4, 95% CI 1.2-4.8) but not a positive T-SPOT.*TB* (OR 1.6, 95% CI 0.7-3.3). Both a positive TST and a positive T-SPOT.*TB* were significantly associated with having a household contact with tuberculosis (OR 2.4 for both, 95% CI 1.4-4.2 for TST and 1.3-4.6 for T-SPOT.*TB*).

4.3.3. Association between age and T-SPOT.TB or TST

Young children (≤12 months) were more likely to have a positive TST (40%, 95% CI 25-58%) than a positive T-SPOT.*TB* result (3%, 95% CI <0.0001-18%). When considering only children with a history of exposure to tuberculosis, age did not appear to affect TST response but did affect T-SPOT.*TB* (children ≥12 months of age were no more likely than younger children to have a positive TST [OR=0.83, 95% CI 0.34-2.02] but were more likely to have a negative T-SPOT.*TB* result [OR=10.04, 95% CI 1.31-77.05]).

The proportion of children having a positive T-SPOT.*TB* result increased with age (Chi-square test for trend p<0.0001) whilst there was no such trend with TST (p=0.4). There was a weak but significant correlation between age and the total number of spot-forming cells in T-SPOT.*TB* (Spearman r=0.22, p=0.0065) but not age and TST diameter (Spearman r=0.13, p=0.1).

4.3.4. Effect of time to sample processing on results

The time between collecting and processing samples did not affect T-SPOT.*TB* results, with no significant difference in the proportion of positive results when categorized by number of hours to processing (p=0.5276). The median time to processing between T-SPOT.*TB*-positive (3.5 hours) and negative (3.6 hours) assays was similar (p=0.319). Further, there was no difference in median time to processing between assays with a failed positive control (3.11 hours) and those without (3.4 hours, p=0.15).

4.4. Discussion

The most important finding of this study was that neither T-SPOT.*TB* nor TST could be used to exclude the diagnosis of tuberculosis in young children in a community setting. Our results, which showed that T-SPOT.*TB* detected only 5/10 definite cases (compared with 8/10 for TST) and 40% of definite or probable cases (compared with 52% for TST) contrasted with those of Liebeschuetz et al who described a sensitivity of 85% for ELISpot and 70% for TST amongst HIV-negative children admitted to hospital with confirmed or highly probable tuberculosis at another study site in South Africa(70). A second study, conducted in a hospital in a low prevalence country reported a sensitivity of 93% for the diagnosis of tuberculosis in children with established culture-proven disease(50). Whilst the clinical algorithm used in this study may have over-diagnosed tuberculosis amongst probable cases, the proportion of positive T-SPOT.*TB* responses in the definite (50%) and probable (37.5%) categories was similar, suggesting that T-SPOT.*TB* misses a substantial proportion of true cases in both of these categories.

The inclusion of either T-SPOT.*TB* or TST (or both) in the diagnostic algorithm used in this study would not have changed the diagnostic categorization of any of the children categorized as having definite or probable tuberculosis. Only 2/164 children classified as having definite, probable or possible tuberculosis had a negative TST and a positive T-SPOT.*TB*, so the use of T-SPOT.*TB* in place of or in addition to TST would not have improved the diagnostic yield.

T-SPOT.*TB* did not therefore appear to offer any improvement in sensitivity over TST for the diagnosis of active tuberculosis. What are the possible reasons for the relatively poor sensitivity of T-SPOT.*TB* in this study, when compared to previously published work?

The first potential explanation relates to the patients studied. Previous studies have examined children presenting to hospital with suspected tuberculosis, whereas we also recruited children who attended their local clinic with symptoms suggestive of tuberculosis or with a history of contact with a patient with tuberculosis. These children may have had milder or earlier illness than in previous studies. It is feasible that very early presentation and testing might precede the development of adaptive immune responses (which are detected by the T-SPOT.*TB* assay). However the TST was positive in 3 of the 5 cases of culture confirmed tuberculosis who had a negative T-SPOT.*TB* assay, suggesting that, at least in these 3 children, specific responses had developed. This difference between the tests may arise because perinatal BCG vaccination primes a cross-reactive TST response to *Mtb* in those children who subsequently become infected. BCG

will not prime a recall response to ESAT-6 and CFP-10 in the same way as these antigens are absent from BCG.

Secondly, we used the commercial T-SPOT.*TB* assay in this study. In the previous chapter, we demonstrated that an in-house ELISpot assay using recombinant ESAT-6 and CFP-10 proteins had a sensitivity of 83% for the diagnosis of culture-confirmed tuberculosis in children(12). The other prospective study examining children with suspected tuberculosis used both recombinant ESAT-6 as well as overlapping peptides derived from ESAT-6 and CFP10(70). In contrast, the commercial T-SPOT.*TB* assay uses only pools of overlapping peptides derived from these proteins. The differences in antigen processing and presentation associated with peptide and recombinant antigens may influence responses, however, since peptides do not require processing this ought perhaps to favour the commercial assay. On the other hand, possible extracellular recognition of free antigen(81), may favour the recombinant antigen

The final explanation relates to the possible age dependency of T-SPOT.*TB* but not TST responses. The median age of children in this study (18 months) was markedly lower than in the three previous studies (32-39 months). Whilst Liebschuetz and colleagues(70) did not find an association between age and ELISpot responses in their cohort, this may be due to small numbers in the younger age group (the median age amongst those with confirmed and highly probable tuberculosis was 50 months, IQR 24-84 months). In our study, there was a clear trend for the proportion of positive T-SPOT.*TB* responses to increase with age. There was no such association between age and a positive TST,

therefore this is unlikely to simply represent an increase in cumulative exposure to tuberculosis with age. The association was particularly striking when considering children of 12 months of age and younger who had been exposed to a tuberculosis case. A number of studies have described an association between age and IFN-γ production in response to mitogen and mycobacterial antigens in the whole blood model, suggesting that age may play a role(59;67;82).

It may be argued that the cross-sensitization by BCG was responsible for the relatively high proportion of young children with a positive TST when compared with T-SPOT.*TB*. This is a difficult issue to resolve, however it appears from the strongly bimodal distribution of TST responses, with few TST responses between 0 and 10mm, that if BCG does prime TST responses in this community, that these responses are seldom in the range commonly described following BCG vaccination of infants(83). It seems more plausible that the strongly positive TST responses which were seen were the result of true exposure to *Mtb*. In addition, the specificities of T-SPOT.*TB* and TST were identical (84%) in the group of children clinically classified as not having tuberculosis, suggesting that BCG did not play a major role in priming a TST response.

Whilst not the primary outcome of this study, it is interesting to note that TST and T-SPOT.*TB* both correlated with the degree of exposure to tuberculosis. A positive TST (but not T-SPOT.*TB*) was associated with a history of any exposure to tuberculosis, whilst both a positive TST and positive T-SPOT.*TB* were associated with a history of exposure to a household member with tuberculosis. This may indicate that TST is a more

sensitive marker of infection with tuberculosis in young children, or alternatively that T-SPOT.*TB* is more specific. This result is in keeping with the findings of Hill and colleagues who concluded that ELISpot was less sensitive than TST for the diagnosis of recent tuberculosis infection amongst children in the Gambia(66).

4.5. Conclusion

We have shown that a substantial number of young children with clinical and/or microbiological evidence of tuberculosis have a negative T-SPOT.*TB* response at presentation. The addition of T-SPOT.*TB* to a clinical diagnostic algorithm would not have improved the sensitivity of this algorithm. The test should be interpreted with caution in young children in whom it should not be used to exclude active TB or recent infection.

Part B. Studies of the clinical implications of strain diversity in Mycobacterium tuberculosis

Chapter 5. The clinical implications of strain diversity in *Mycobacterium tuberculosis*: a review

The previous three chapters have described work conducted to address one important clinical problem in childhood tuberculosis, namely the difficulty in confirming the diagnosis in young children. The second part of this thesis will explore a separate issue, but one which is of similar concern for clinicians who treat children with tuberculosis, namely the high incidence of severe forms of tuberculosis, including disseminated and extra-pulmonary disease amongst children. Whilst age clearly plays an important role in influencing the outcome of infection, there is renewed interest in the potential role of strain variation in this regard. This topic will be reviewed below, followed by two chapters describing work we have conducted to investigate the clinical implications of strain diversity in *Mtb*.

5.1. Introduction

Mtb is an extraordinarily successful pathogen. One third of the world's population is thought to be infected, with up to 9 million cases of active disease worldwide every year(1). A hallmark of the natural history of tuberculosis is the diverse outcome of infection with *Mtb*. Following exposure to a case of tuberculosis, not all of those exposed will show immunological evidence of infection(84). This may relate to the duration and intensity of exposure, the sputum bacillary load of the source case and the possibility of innate pre-immune clearance of bacilli. Of those who do become infected (thought to number 2 billion people worldwide), it is estimated that, in the absence of immunosuppression, only 10% will ever develop active disease. The manifestations of disease itself are varied, ranging from self-limited pulmonary infection to localized extra-pulmonary infection and disseminated disease. The factors which determine these

outcomes remain incompletely understood but may relate to variation in both the host and the pathogen. An appreciation of the inherited and acquired host factors that predispose to mycobacterial infection has advanced our understanding of mechanisms of protective immunity. Key examples of such host factors include single gene defects affecting the interferon-γ / interleukin-12 axis which predispose to severe non-tuberculous and tuberculous mycobacterial infection(85-87), reactivation of latent tuberculosis associated with therapeutic blockade of tumour necrosis factor by monoclonal antibodies used for treating rheumatoid arthritis and Crohn's disease(88) and the profound increase in the frequency of reactivation and new infection associated with the cellular defects consequent from HIV infection(89). It remains unclear however, to what extent more subtle host immunological deficiencies may play a role in mediating susceptibility to tuberculosis in the general population(90).

There is increasing evidence that genetic variation in the bacillus may also play a role. Indeed, in many other infectious conditions, genetic variation in the pathogen is a key determinant of outcome. The presence of toxin-encoding genes in staphylococci and streptococci, pathogenicity islands in enteric pathogens and capsular variation in *Haemophilus influenzae* and *Streptococcus pneumoniae* are important examples. In many cases, differential virulence amongst bacterial strains relates to the presence of transmissible genetic elements encoding virulence determinants. In contrast, whilst the ancestor of *Mtb* is thought to have been an environmental organism that was able to exchange genetic material(91), one of the distinctive features of the lifestyle of modern

strains of *Mtb* is the inability of these strains to undergo lateral gene transfer(92), although evidence suggesting rare recombination events has recently been described(93). Since exchange of genetic material is rare, *Mtb* evolves primarily by deletion and duplication events. One important consequence of this solitary lifestyle is a strongly clonal pattern of evolution(94) with the emergence of separate lineages of strains, each descended from a common ancestor and with no evidence of lateral exchange of genetic material between lineages. A natural consequence of such divergence might be the acquisition of differential pathogenic characteristics amongst these lineages.

5.2. Defining strain diversity in *Mycobacterium tuberculosis*

Any understanding of the implications of strain diversity in *Mtb* must start with an appreciation of the nature of such diversity. Strains and strain lineages need to be clearly defined in order to compare their behaviour.

The inability of *Mtb* to exchange genetic material might be predicted to result in restricted genetic diversity. Initial reports suggested that this was indeed the case, with a low frequency of silent single nucleotide polymorphisms (SNPs) amongst 26 structural genes in a representative collection of strains(95). However, subsequently evidence has emerged of considerable genetic diversity within *Mtb*. This diversity takes the form of SNPs as well as deletion, duplication and insertion events. Filliol et al(96) describe high levels of SNP diversity amongst a global collection of strains, with 96% of SNP locus

pairs in complete linkage disequilibrium (some SNP alleles occur together more frequently than would be expected by chance), supporting the clonal nature of *Mtb* evolution. Their analysis defined 6 phylogenetically distinct lineages, with strong associations between lineage and geographic origin of the strain.

The insertion sequence IS6110 has been shown to be an important contributor to the generation of genetic diversity in *Mtb*. IS6110 may insert into genes, disrupting the coding sequence, generate genomic deletions though recombination events and affect gene expression through its intrinsic promoter activity(97).

For many years, our appreciation of the phylogenetic relationships between strains of *Mtb* has been restricted by the use of limited measures of genetic variation. The standard genotyping instrument for *Mtb* has been IS6110 restriction fragment length polymorphism (RFLP) analysis. This is an excellent tool for epidemiological investigations since it is highly discriminatory, but is not necessarily best suited for phylogenetic analysis. The insertion sequence has irregular rates of transposition and a predilection for insertion at favoured sites(97;98). Moreover, the difficulty in standardizing methodology and interpretation of the fingerprints obtained has lead to a confusing proliferation of nomenclature compounded by a lack of communication between centres.

More recently, typing methods such as spoligotyping(99) and multiple interspersed repetitive unit (MIRU)(100;101) analysis have offered a more easily standardized approach, albeit not necessarily more suited to phylogenetic analysis.

Spoligotyping is based on polymorphism in the direct repeat region of the *Mtb* chromosome. At this locus, there is a series of repeat 36 base pair sequences which are interspersed by non-repetitive spacer elements each 35 to 41 base pairs in length. The presence or absence of each of 43 of these spacer regions is detected using a reverse dot blot technique. Briefly, the spacer regions are amplified using primers directed at the common flanking direct repeat elements. The amplified fragments are then hybridized to a membrane which has probes for each of the spacer elements. The membrane is washed and the presence of bound fragments detected by chemiluminescence. This technique is technically simple, reproducible and easily coded and has, as a result, allowed the development of an international database and introduced a degree of consistency to the naming of the major lineages of *Mtb*(102;103). Moreover, there is a high degree of consistency in the identification of the major lineages of Mtb between spoligotyping, large sequence polymorphism and single nucleotide polymorphism analysis(96). However, since major changes in spoligotype may occur due to single deletion events, it is not always useful for defining the relationship between strains, nor is it sufficiently discriminatory to differentiate closely related strains(104). It is therefore less useful for epidemiological investigations where it is important to differentiate between closely related but different strains.

The genome of Mtb contains repetitive 40-100 base pair sequence elements known as 'mycobacterial interspersed repetitive units' (MIRU) which are found as tandem repeats at multiple intergenic loci throughout the chromosome(100). These loci demonstrate hypervariability in the number of repeats, leading to the use of the term "variable number of tandem repeats" (VNTR).

The typing method MIRU-VNTR is based on the detection of the number of tandem repeats present at several different loci. The most commonly used method examines 12 of these loci. MIRU-VNTR is performed by amplifying each locus and determining the size of the amplicon and hence the number of repeats at each locus(100). These are then combined into a multi-digit code. This technique is more discriminatory than spoligotyping(105), and, since many independent loci are assessed, may be more appropriate for phylogenetic analysis. However it is less visually intuitive than spoligotyping, more labour intensive and does not always concord well with SNP-based phylogenetic analysis(96). This may relate to the potential for gain or loss of repeats at each locus, leading to the possibility of convergent evolution (broadly different strains acquiring similar numbers of repeats at a particular locus). More recently, an updated 15-locus MIRU-VNTR protocol has been proposed and shown to be as discriminatory as IS6110 RFLP(106;107).

One of the important mechanisms by which genetic diversity arises in *Mtb* is through large chromosomal deletions. Such deletions, termed large sequence polymorphisms (LSP), have been used to classify strains of *Mtb* into distinct lineages(108;109). Since

recombination is rare, all progeny of a parent strain will carry the same deletion. The presence or absence of these deletions is used to define major lineages within *Mtb*. This approach infers a basic structure to the evolutionary tree by assuming the sequential acquisition of deletions. It is also useful for establishing clonal relationships amongst strains with different spoligotype or RFLP patterns. This technique has been used to show that those strains of *Mtb* which have been sequenced cluster in two related 'modern' lineages and do not represent the global diversity of strains(94).

The most robust method, however, for defining the global phylogeny of *Mtb*, is the use of SNP analysis and whole genome sequencing. SNP analysis, in a restricted form, was used to classify *Mtb* strains into 3 principal genetic groups, based on SNPs in codon 463 of the katG gene and codon 95 of the gyrA gene(110). This 3-branched tree defined the broad phylogeny of *Mtb* for many years. The results of recent more extensive SNP analysis will advance this understanding. Gagneux and colleagues(111) have sequenced 90 genes in 104 globally representative strains to define the relationships between the major lineages of *Mtb*. This is an important step forward in defining the nature of diversity in *Mtb* and offers an ideal starting point for evaluating the clinical implications of such diversity. The application of relatively low cost, high throughput sequencing technology to multiple representative strains of *Mtb* may significantly advance our understanding of the extent of genetic diversity in tuberculosis as well as the relationships between strains. Figure 5.1 and Table 1 summarize the major methods used for genotyping *Mtb* strains.

Finally, the region of the *Mtb* chromosome containing the so-called PPE/PE gene families has been shown to be an important source of genetic variation(17). In particular, it has been suggested that this region may be partly responsible for antigenic variation in *Mtb*, which could feasibly be a mechanism for immune evasion.

Figure 5.1 Schematic representation of the *M. tuberculosis* genome, indicating the genetic basis of genotyping techniques. The circular chromosome of the reference strain H37Rv is shown together with examples of the major genetic elements used for strain genotyping. For clarity only one MIRU (mycobacterial interspersed repetitive unit) locus, one IS*6110* (insertion sequence), one region of difference (RD) and one single nucleotide polymorphism are shown.

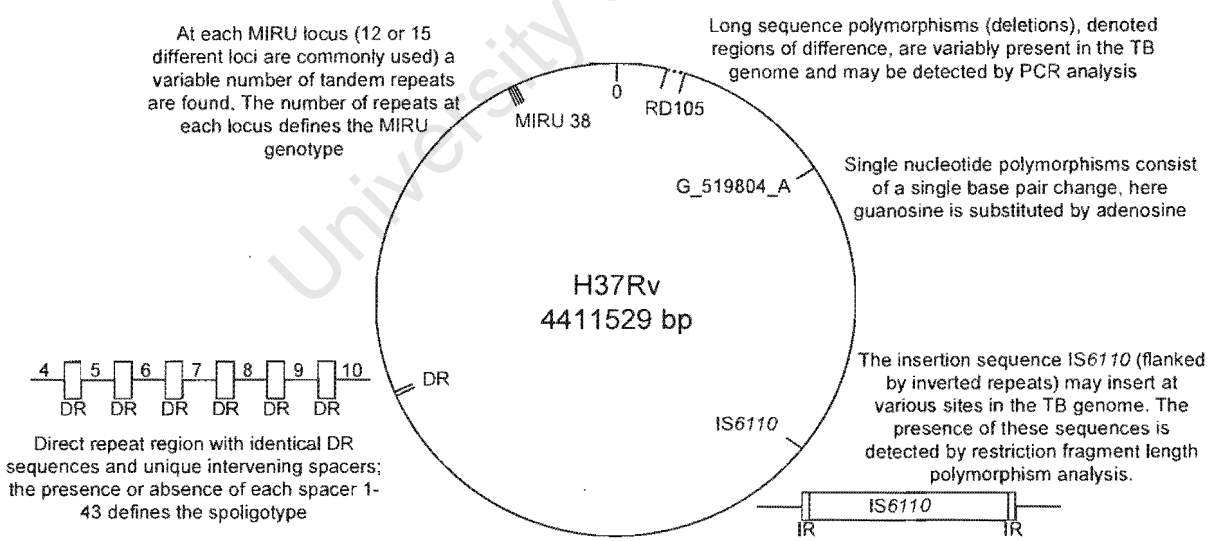


Table 1. Comparison of techniques for molecular typing or evaluating genetic diversity within *Mtb*

	IS6110 RFLP*	Spoligotyping	MIRU-VNTR†	LSP analysis‡	SNP§ analysis
Discriminatory power	Excellent	Fair to poor	Good to excellent (newer protocols)	Poor	Poor, likely to improve with increased SNP identification
Ease of use	Time consuming and technically demanding; requires extracted chromosomal DNA	Rapid and simple; can be performed directly on specimens or heat-killed cultures.	Rapid and fairly simple; automation requires access to sophisticated equipment; can be performed on heat-killed cultures.	Simple and robust	Rapid and fairly simple; high throughput analysis requires access to sophisticated equipment
Interpretation	Simple, but not easily standardized	Simple visual interpretation	Simple visual interpretation	Straightforward	Straightforward
Data sharing	Complex, lack of standardized nomenclature	Straightforward, standardized binary or octal coding	Straightforward, standardized numerical coding	Straightforward, standardized nomenclature	Straightforward, standardized nomenclature
Utility for epidemiological investigations	Excellent due to high discriminatory power	Useful for rapid cluster identification but requires secondary confirmation	Excellent and rapid with newer protocols	Poor due to low discriminatory power	Poor at present due to low discriminatory power
Utility for phylogenetic analysis	May be limited by irregular rates of transposition and favoured sites.	Fairly good correlation with SNP-based phylogeny. Large deletions may bias analysis	Relatively poor correlation with SNP-based phylogeny	Useful for evolutionary history (sequential deletions) and for identifying major lineages	Gold-standard: low rate of SNP in *Mtb* necessitates large-scale sequencing to identify informative SNPs

Table footnote:

* restriction fragment length polymorphism

† mycobacterial interspersed repetitive unit-variable number of tandem repeat

‡ large sequence polymorphism

§ single nucleotide polymorphism

5.3. Defining clinical outcomes

Just as any attempt to link strain diversity and clinical outcome relies on a robust definition of strain genotype, it also relies on the ability to clearly define a spectrum of clinical outcomes. The complex natural clinical history of tuberculosis has lead to the use of a variety of measures of virulence, including the number of secondary infections caused by an index case(112), the ratio of active to latent infections(15), the propensity to cause cavitary disease(113) and the ability to disseminate or to cause extra-pulmonary infection(114). Many of these measures are time-dependent, and may be affected by the early institution of appropriate therapy. Due to the difficulties in defining appropriate clinical endpoints and the influence of non-strain factors (such as host immunity and the effect of time) on outcome, much of the literature evaluating the relevance strain variation in tuberculosis is based on experimental animal models or the use of laboratory mutants of *Mtb*. Whilst these studies have done much to advance our understanding of the pathogenesis of tuberculosis, they have provided limited insight into the clinical consequences of strain variation in human infection, which is the focus of this review.

5.4. Lessons from outbreak strains

Our understanding of the implications of strain diversity derives largely from work describing strains of *Mtb* responsible for outbreaks in the United States and the United Kingdom. The first well-described outbreak strain, CDC1551, caused a large number of tuberculosis infections, including 21 cases of active tuberculosis in a small rural community between 1994 and 1996(115). The high rate of transmission to contacts (72% of contacts had a positive tuberculin skin test) was considered unusual. The initial report also described enhanced virulence in mice, with more rapid early growth than a comparator laboratory strain (Erdman). Subsequent reports, however, suggested that this strain was no more virulent in mice than other comparator strains, including the laboratory reference strain H37Rv as well as two clinical strains(116), but induced a rapid and robust pro-inflammatory immune response in mononuclear phagocytes that may be related to the high rate of tuberculin skin test conversion amongst contacts. In contrast, Anderson and colleagues describe an outbreak in the United Kingdom, due to a strain of *Mtb* that appeared to inhibit the development of a positive skin test amongst those infected, possibly due to the suppression of proinflammatory tumour necrosis factor and interleukin-12p40 production(117). The mechanisms whereby these strains differentially induce pro-inflammatory responses remain unknown as does the mechanism relating the tuberculin skin test to the magnitude of *in vitro* cytokine responses.

A third strain, HN878 (a member of the W-Beijing lineage of strains), caused several outbreaks of tuberculosis in Houston, Texas between 1995 and 1998 (although the clinical circumstances have never been fully reported)(19). This strain was subsequently

shown to cause rapid death in immune competent mice(18) as well as increased dissemination, more severe clinical manifestations and higher bacillary loads in a rabbit meningitis model(19). The increased virulence of HN878 was subsequently attributed to the production of a phenolic glycolipid (PGL) which is able to suppress pro-inflammatory cytokines such as tumour necrosis factor and interleukins 6 and 12(16). PGL production is dependent on the presence of a polyketide synthase gene, which is intact in the majority of W-Beijing strains, but interrupted by a deletion in many other strain families. Disruption of PGL synthesis resulted in attenuation of virulence. More recently, HN878 was shown to induce a strong early Th1 response that was rapidly down-regulated by the late emergence of an interleukin-10 producing population of regulatory T cells(118).

In the final example, strain CH caused a large outbreak of tuberculosis in Leicester, United Kingdom(15). This outbreak was characterized by a high proportion (23%) of infected cases progressing to active infection within one year. Paradoxically, this strain was actually less resistant than comparator strains to acid and oxidative stress in axenic culture but unimpaired in its ability to replicate in human monocyte-derived macrophages. Such infected macrophages expressed and secreted less protective interleukin-12p40 and more regulatory interleukin-10. This ability to subvert the innate immune response was ascribed to a deletion affecting *Rv1519*. This deletion characterizes the broad East-African-Indian lineage of tuberculosis strains. It will be important to confirm that the pattern of cytokine induction observed in the case of strain CH is widely conserved amongst strains from this lineage.

The common pattern emerging from these studies is of variable induction of host inflammatory responses by clinical strains from different genetic backgrounds. In particular, the ability to subvert Th1 responses (here interleukin-10 production is commonly ascribed a central role) appears to be an important determinant, at least in animal and *in vitro* models. The extent to which this is reflected in human tuberculosis remains essentially speculative. A question which remains largely unanswered is whether these strain-specific characteristics are features of individual strains only, or whether they operate also at the broader level of strain lineages.

5.5. Do *Mycobacterium tuberculosis* strain lineages differ in their pathogenic potential?

Perhaps the first clues that there may be lineage-specific patterns of virulence emerged from the work of Mitchison and colleagues, who compared large numbers of isolates from South Indian and British patients in the guinea pig model(119-121). South Indian isolates were attenuated in a number of measures, including mortality, semi-quantitative assessment of bacterial burden and rate of progression. Interestingly, approximately one third of the Indian cultures studied were as virulent as the British cultures and one third were markedly attenuated, causing limited local disease only. Since modern genotyping tools were not available at the time, it is unclear whether these early observations were related to specific strain lineages. These large-scale experiments have never been repeated using strains of known genotype, however a number of investigators have

shown that strains of differing genotypes have variable virulence in mice(122-125) and rabbit(19;126) models.

One of the most widely described strain lineages worldwide is the W-Beijing lineage(127;128) whose members have easily recognizable IS6110 and spoligotype patterns. As the name suggests, these strains are endemic in many parts of East Asia, where they have been the predominant strain lineage for decades(129), are predominately drug-sensitive and account for the majority of cases of tuberculosis(129;130). In contrast, epidemic spread of W-Beijing strains, as described in New York ('strain W')(131), Russia(132;133), Latvia(134), Vietnam(135) and South Africa(136;137) is frequently associated with multidrug- resistance. There is clear evidence from animal models that W-Beijing strains are highly virulent, causing higher bacillary load, increased dissemination(19) and early death(18), however evidence from human disease is less clear.

Strain HN878, described above, is the best characterised member of the W-Beijing lineage. The extent to which the particular immunologic characteristics of HN878 are shared amongst other W-Beijing strains remains unclear since the production of phenolic glycolipid is not universal amongst W-Beijing strains(138). Reed et al have recently shown that a wide spectrum of W-Beijing strains all constitutively over-express genes which are members of the DosR-controlled regulon, including triacylglyceride synthase(138). One consequence is the accumulation of large quantities of triglyceride during *in vitro* aerobic culture in W-Beijing strains. In contrast, in non W-Beijing strains

DosR genes are not constitutively expressed, but are inducible by nitric oxide and low oxygen tension(139) and are thought to contribute to bacterial persistence during latent infection. The authors hypothesize that the accumulation of triglycerides may provide an energy source during periods of nutrient starvation, such as during transmission and in the face of potent host immune responses. This important finding is the first clear example of an *in vitro* phenotypic characteristic which is broadly shared by the members of a particular strain lineage.

There are now a number of reports describing an association between infection with W-Beijing strains and aspects of clinical tuberculosis. Kong and colleagues found that patients with extra-pulmonary tuberculosis were three times more likely to be infected with W-Beijing strains, after correcting for potential confounders(114), although in children these was no evidence of such an association(140). In Vietnam, W-Beijing strains have been associated with treatment failure and relapse(141) and HIV infection(142). In another study, W-Beijing strains were associated with fever early during treatment(143). This raises the possibility of a strain-specific association with the paradoxical deterioration occasionally seen during tuberculosis treatment, particularly in patients on antiretroviral therapy(144;145). The association between W-Beijing lineage and drug-resistance remains contentious, but is increasingly described(136;146). The apparent predilection of these strains to acquire resistance was ascribed to a series of mutations in the genes responsible for DNA repair(147); however this has subsequently been challenged(148). An intriguing question is whether W-Beijing strains are emerging or increasing in frequency, with several studies suggesting that this may be the

case(135;149-151). One possible explanation for the emergence of these strains relates to the variable efficacy of BCG vaccine, which, in mice, protects poorly against challenge with a W-Beijing strain(152) and in rabbits confers poor protection against central nervous system infection with a W-Beijing strain(153;154). The widespread use of BCG vaccine might therefore feasibly select for the spread of W-Beijing strains(153).

More general evidence has emerged that strain lineages may possess specific pathogenic characteristics that have arisen from long-standing interactions between particular lineages and human populations. Gagneux and colleagues used LSP analysis to define six broad lineages of *Mtb* which appeared to be associated with specific host populations. In San Francisco, strains were more likely to spread amongst humans whose geographic origins corresponded with that of the strain(155). The authors suggest that mycobacterial lineages have adapted to particular host populations. The same group has shown that strain genetic background may influence the acquisition of particular resistance determinants as well as the relative fitness of drug-resistant strains(156;157).

5.6. Does clinical outcome correlate with a particular *in vitro* or genotypic characteristic of *Mycobacterium tuberculosis* strains?

Whilst there is emerging evidence that W-Beijing strains are distinct from many other strain lineages in a number of important respects and that these differences may relate to

differences in clinical outcome, there are as yet no convincing data to support the notion that other strain lineages may also have developed their own unique but conserved virulence determinants. This may however simply reflect the paucity of research in this area.

An alternative approach to the identification of strain-specific virulence mechanisms in *Mtb* is to identify strains causing similar clinical outcomes and then attempt to define common *in vitro* characteristics amongst those strains. For example, strains which have been identified as causing clusters of cases (suggesting recent transmission) have been shown to grow more rapidly than unique strains in an *in vitro* macrophage model(158;159) Similarly, strains from households where there was co-prevalent tuberculosis (disease amongst more than one household member) grew more rapidly in macrophages than non-transmitted control isolates(112). The ability to replicate rapidly within macrophages would intuitively associate with increased transmissibility. Talarico et al have described an association between clustering and the presence of deletions in the PE_PGRS33 gene, a member of a gene subfamily of unknown function but possibly a source of antigenic variation(160). It remains to be seen whether these *in vitro* and genotypic characteristics are found broadly amongst unrelated strains or whether they are associated with strains belonging to particular lineages.

A number of investigators have compared the characteristics of strains responsible for extra-pulmonary and pulmonary infection. Strains causing extra-pulmonary disease have been shown to infect macrophages more efficiently(161), have frequent interruptions in

the phospholipase-C gene D(162) and belong to certain 'superfamilies,' based on RFLP analysis(163). Early reports that strains able to cause cavitary disease had fewer genomic deletions than strains causing non-cavitary disease(113) have however not been confirmed.

In summary, the evidence that strain genetic variation may influence the outcome of infection with *Mtb* remains fragmented and inconclusive. Isolated outbreak strains have been well characterised and some appear to share similar immunosuppressive phenotypes. To what extent these phenotypes are shared by strains belonging to broader phylogenetic lineages, or indeed, to clinical strains of *Mtb* as a whole, is unknown. There is increasing evidence to support the hypothesis that strains belonging to the emerging W-Beijing lineage may be characterised by enhanced virulence. Recent attempts to better define the phylogenetic relationship between strain lineages will increase our ability to conduct rational studies to determine the association between outcome and strain lineage. These studies will ultimately provide a platform for a better appreciation of the evolving relationship between *Mtb* and the human host.

In the following two chapters we describe work which we have conducted in Cape Town, using strains of *Mtb* isolated from children in order to improve our understanding of the relationship between strain genotype and clinical phenotype. Chapter 6 focuses on the relationship between strain lineage and extra-pulmonary infection, whilst in Chapter 7 we describe evidence that indicates that W-Beijing strains are rapidly emerging in Cape Town and we suggest several possible explanations for this phenomenon.

Chapter 6. Distribution of strain families of *Mycobacterium tuberculosis* causing pulmonary and extra-pulmonary disease in hospitalized children in Cape Town

6.1. Introduction

One of the characteristic features of tuberculosis in children is the extraordinarily diverse range of clinical presentations, ranging from limited pulmonary or nodal disease to severe extra-pulmonary or disseminated disease(164). As discussed in Chapter 5, the determinants of these diverse outcomes are largely unknown. Host factors such as immaturity of the immune system and genetic predisposition to infection are likely to play a role, however disease is the result of a complex interplay between host resistance and bacillary virulence determinants. There is increasing evidence that strains of *Mtb* differ with regard to their pathogenic potential *in vitro* and *in vivo*(17). These differences may be important in influencing the outcome of infection in children.

Despite the fact that animal models clearly demonstrate increased virulence of selected strains, such as those of the W-Beijing family(18;116), there is no clear evidence from human studies that certain strains or strain families are more virulent than others. This may be partly due to difficulties in assessing virulence in adult tuberculosis. A commonly used measure of virulence is the ratio of active cases to latent infection(112). A more tangible and easily measured outcome is the proportion of cases of extrapulmonary infection caused by a particular strain or strain lineage. Since children more frequently develop extra-pulmonary disease, childhood tuberculosis may be a useful model for exploring strain variation.

We reviewed records and typed all strains of *Mtb* from 285 children presenting to Red Cross Children's Hospital, Cape Town over a period of 3 years in order to correlate strain

lineage with the ability to cause extra-pulmonary disease. Spoligotyping, 12-locus mycobacterial interspersed repetitive unit variable number tandem repeat (MIRU-VNTR) typing and large sequence polymorphism (LSP) analysis were performed since these typing methods clearly delineate the major strain lineages and facilitate classification of strains into recognised strain families using an international database(103).

6.2. Methods

This study was conducted at Red Cross War Memorial Children's Hospital, a paediatric referral hospital serving a large part of metropolitan Cape Town. Children with suspected tuberculosis are referred to this hospital from peripheral clinics and general practitioners in order to confirm the diagnosis. The number of culture-confirmed cases represents a minority of the total number of cases of paediatric tuberculosis in the region. This is, in part, due to the difficulty in obtaining suitable culture specimens from children (induced sputum or gastric lavage are required). At present, these procedures are confined to hospitalized children. Therefore almost all of the culture-confirmed cases of paediatric tuberculosis in the region are made at one of two academic centers. The strain collection that we describe includes all of the strains from one of these two centers.

We collected all isolates of *Mtb* from children presenting to this hospital over the period December 2000 to December 2003. Culture confirmation of childhood tuberculosis presents difficulties in primary care and this culture collection therefore represents a large proportion of the isolates of *Mtb* from children in the region served by this hospital. We reviewed records to obtain comprehensive clinical details from all children from

whom an isolate was available. Ethical approval for this study was granted by the Research Ethics Committee of the University of Cape Town (Ref 320/2002).

6.2.1. Genotyping

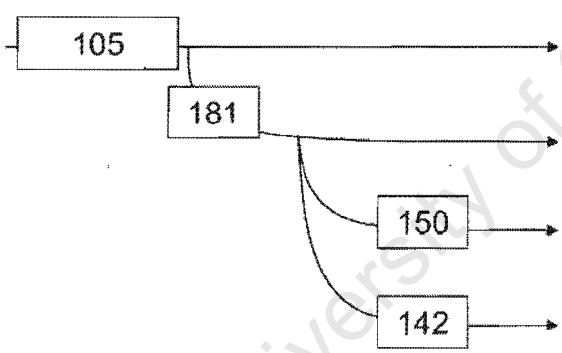
Genotyping was performed using three PCR-based techniques, namely spoligotyping, 12-locus MIRU-VNTR and large sequence polymorphism (LSP) analysis. Spoligotyping was performed as previously described (Appendix C)(99). MIRU-VNTR was performed by a modification of the semi-automated method described by Supply et al(101) on an ABI3100 analyzer (Applied Biosystems, Foster City, CA). A series of multiplex PCR reactions, each amplifying 3 MIRU loci, were performed using fluorescently-labeled primers. The size of the fragments was then detected using an automated capillary sequencer. The modification to the technique of Supply et al was as follows. For each multiplex reaction, 2μl of 1 in 70 diluted product, 0.2μl Genescan-2500 Rox size standard (Applied Biosystems) or MapMarker1000 size standard (Bioventures) and 8μl HiDi formamide (Applied Biosystems) were denatured for 2 minutes at 95°C and then run for 40 minutes on the ABI3100 using dye set D. Analysis was performed using GeneScan software (Applied Biosystems). The full protocol including details of optimization is documented in Appendix D.

LSP analysis was performed on strains identified on the basis of spoligotyping as being members of the two major strain lineages represented in the Western Cape, namely the W-Beijing family and the LAM3 (Latin-American-Mediterranean)/F11 lineage. We used LSP typing firstly to classify the W-Beijing lineage into sub-lineages and secondly to

confirm that the LAM3/F11 family (as identified by spoligotyping) is indeed monophyletic (derived from a single lineage).

Tsolaki et al(109) have previously shown that three LSPs entitled Region of Difference (RD)142, RD150 and RD181 may be used to identify the major sub-lineages within W-Beijing (Figure 6.1).

Figure 6.1 The sequential acquisition of deletions within the W-Beijing lineage. All W-Beijing strains are characterized by the RD105 deletion, whilst the other deletions define sub-lineages as indicated (from Tsolaki et al(109)).



In order to detect RD142 and RD150, we used a real-time PCR method as previously described(109;109) and detailed in Appendix F. Briefly, for each of RD142 and RD150, two pairs of primers were designed to amplify firstly a short fragment within the relevant deleted region and secondly a conserved region of DNA encoding the 16S ribosomal subunit. Short probes were designed to bind specifically to the amplified regions. The probes were labelled with different fluorescent dyes allowing separate but simultaneous

detection of each product by real time PCR on an Applied Biosystems 7000 sequence detection system. The fluorescence of each probe is quenched by the presence of a quencher molecule also attached to the probe sequence. If specific product is present the probe will hybridize to the product and cleavage of the fluorescent molecule from the probe occurs, resulting in the emission of fluorescence which is automatically detected. For each strain of *Mtb* tested, amplification of the 16S fragment acted as a positive amplification control, whilst the presence or absence of the deleted region was detected by fluorescence in a second channel, corresponding to the probe directed at the deleted region.

For RD181 we used conventional PCR with primers flanking the region of interest followed by agarose gel electrophoresis. The presence of a 1712bp product indicated that the RD181 had not been deleted, whilst a 100bp product confirmed the presence of the deletion (Appendix F).

Similarly, we designed an additional set of primers (Appendix F) to amplify the region of difference 761 (RD761) in all strains. Our collaborator (Sebastien Gagneux, Institute for Systems Biology, Seattle) had previously used Affymetrix GeneChip analysis(155) to identify the presence of this RD in two representative LAM3/F11 strains which we had supplied. We wished to confirm that the presence of this deletion was unique to LAM3/F11 strains. A 1371bp product indicated the absence of the deletion, whilst a 277bp product indicated the presence of the deletion.

6.2.1.1. Acronyms and Nomenclature

Spoligotypes were represented in the Octal format as defined by Dale et al(165). The former ST (shared-type) designation in the spoligotype database SpolDB3(166) has now been renamed SIT (spoligo-international-type). Typing data (MIRU and spoligo) was entered into an Excel spreadsheet file and received an SIT (Spoligo-international-type) and VIT (MIRU-VNTR international-type) designation according to cluster assignment, after processing by SpolDB4. SpolDB4 was a fourth update of an international spoligotyping database project coordinated by the Institute Pasteur of Guadeloupe. SpolDB4 is available for public interrogation at http://www.cdc.gov/ncidod/eid/vol8no11/02-0125.htm(167).

Phylogenetic reconstruction was performed both by manual parsimonious analysis and using Bionumerics software (version 3.5, Applied Maths, St Maarten-Latems, Belgium). Using a model that allows one step transition of either spoligotyping or MIRU alleles, we first constructed for each family a Maximum Parsimony (MP) genetic network that simulates evolution of genotypes. This was performed by linking spoligo-MIRU alleles differing by a single change. The network was constructed by first ordering spoligotype changes, since the rate of evolution of spoligotyping appears to be slower than that of MIRUs(168). Intermediate alleles required to create a monophyletic network within a given family were also created when necessary. The visual display was created using BioLayout(169). In addition, eBURST software was used for cluster analysis of W-Beijing strains (http://eburst.mlst.net/).

6.2.2. Statistical Analysis

For analysis, cases with both pulmonary and extra-pulmonary disease were classified as having extra-pulmonary disease. Cases presenting with pleural effusion were categorised as having pulmonary disease. Fisher's exact test of probability was used for contingency analyses. The Mann Whitney test was used for comparison of median ages.

6.3. Results

Strains from a total of 285 children were isolated over the 3-year period. In all 40 cases where more than one positive culture was obtained from a single child, the infecting strains were identical by typing and included only once in the dataset. Extra-pulmonary tuberculosis accounted for 47% of cases, isolated pulmonary disease for 46% and isolated extra-pulmonary lymph node involvement for 7%. The most common manifestations of extra-pulmonary tuberculosis were meningitis (n=58), bone and joint (n=18), pericardial (n=12), peritoneal (n=5) and miliary disease (with or without localized manifestations, n=39). The median age at diagnosis was 2 years, with 75% of children less than 5 years of age. The age and sex distribution of cases is shown in Table 1.

Table 1. Sex and age distribution of cases.

Site of disease		Sex (% female)	Median age (months)
Pulmonary		41.6	22
Extra-pulmonary	Meningitis	37.0	31
	Lymph node	33.3	49
	Bone and joint	57.9	43
	Miliary	57.5	25
All cases		**43.7**	**28**

Children with isolated lymph node or bone and joint tuberculosis were significantly older than those with pulmonary disease (p=0.0128 and 0.0187 respectively). HIV testing (ELISA and/or PCR in children <18 months of age) was performed when clinically suspected (130 children). Amongst those tested, HIV infection was documented in 13% of children with isolated involvement of lymph nodes; 20% of those with pulmonary tuberculosis; and 19% of those with extra-pulmonary tuberculosis.

6.3.1. Comparison with the updated SpolDB4 database and global population structure

Of a total of 285 clinical isolates genotyped by both spoligotyping and MIRU-VNTR, 122 different MIRU alleles and 69 different spoligotypes were identified. A representative spoligotype blot is shown in Figure 6.2 and a representative MIRU-VNTR analysis in Figure 6.3.

Figure 6.2 A representative spoligotype blot. Each strain is represented by a horizontal series of up to 43 spacers. The presence of each spacer (1 to 43 running from left to right) is demonstrated by the presence of a spot on the film. The top row represents the positive control (H37Rv) and the second row the no template control. An example of a W-Beijing strain (spacers 1-34 absent) is indicated by a filled arrow and a LAM3/F11 strain (spacers 9-11, 21-24 and 33-36 absent) by an open arrow.

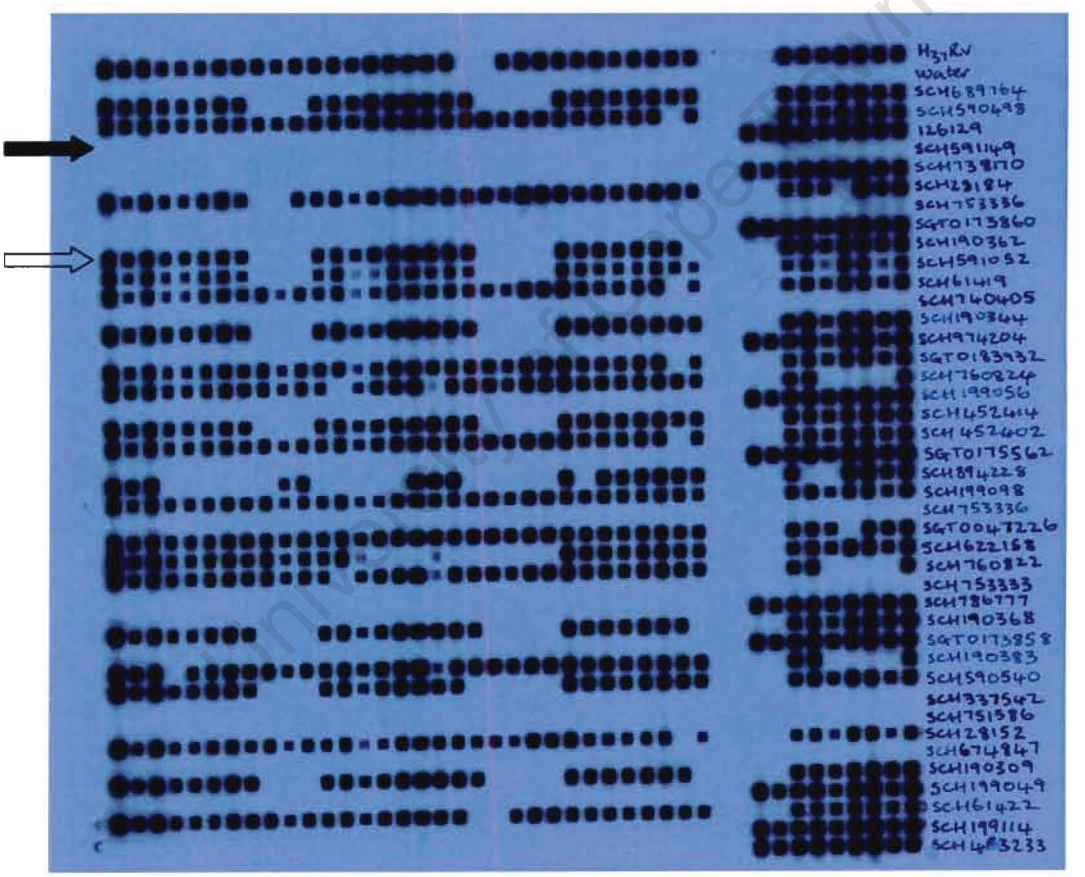
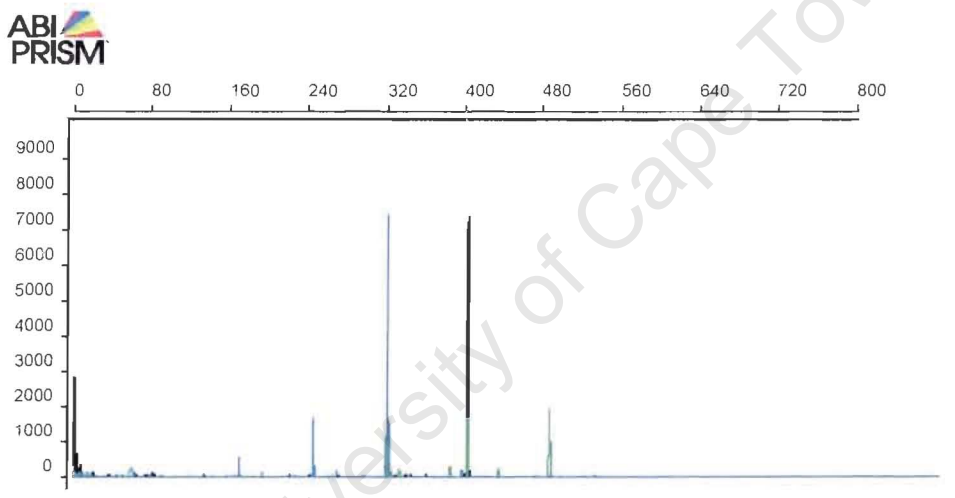


Figure 6.3 Analysis of MIRU fragments using GeneScan software. The blue, black and green peaks indicate the amplified fragments for loci 4, 26 and 40. The MapMarker size standard is not shown for clarity. The size of the amplified fragments is denoted on the top axis. Small "stutter' peaks leading up to the larger blue and green peaks are shown (these are generated by slippage of the polymerase). The black peak has a green component due to spectral overlap of the green and yellow (represented as black) dyes.



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
7B, 1	10.34	246.16	3164	36107	3876
7B, 2	12.55	321.17	7614	137398	4708
7G, 1	12.55	320.88	1578	4193	4705
7G, 2	12.57	321.55	1867	7824	4712
7G, 3	12.88	332.75	1847	18761	4829
7G, 4	12.90	333.71	875	8421	4839
7G, 5	14.33	384.71	1620	17586	5373
7G, 6	14.35	385.66	988	9901	5383
7G, 7	14.86	403.52	6390	50478	5571
7G, 8	15.73	435.42	1123	13086	5900
7G, 9	15.76	436.30	832	8096	5909
7G, 10	17.11	487.99	6694	115687	6416
7Y, 1	12.55	321.17	3768	23044	4708
7Y, 2	14.84	402.94	7083	58128	5565
7Y, 3	14.87	404.00	7143	60947	5576

Spoligotyping identified 11 genotype families (nomenclature from the 4th International Spoligotype Database)(167), namely LAM3/F11 (n=86), W-Beijing (n=70), X (IS*6110*-RFLP European low-copy family) (n=30), S/F28 (n=21), Zimbabwean (n=7), other LAM (n=8), Tuscany and Russian (n=12), Haarlem (n=11), T clade (n=20), CAS (Central Asian, n=5) and "unknown" clades (n=15)(170;171). Except for the "unknown" clades, a parsimonious genetic network linking all alleles within their respective family was created using BioLayout software (Figure 6.4).

6.3.2. Clustering analysis

If relevant clusters (historically or recently transmitted) are defined on the basis of perfectly identical MIRU- and spoligotyping alleles, a total of 27 clusters totaling 148 clinical isolates were found (52% of all the studied isolates). These clusters are defined by a combination of a SIT and a VIT allele number (Table 2).

Eight clusters belonging to the W-Beijing (including one prevalent cluster of 24 isolates) and five to the LAM3/F11 family (including two prevalent clusters of 32 and of 13 isolates) were identified. Four clusters belonging to the previously defined S/F28 family and three to the previously defined IS*6110*-RFLP low-copy clade, also called the X family were found. Finally, five clusters belonging to more or less well phylo-geographically-defined families (Central Europe 1, Russia 1, T3, Tuscany and Z) were also found.

Figure 6.4 A maximum parsimony genetic network built for the five most frequent family genotypes (W-Beijing, LAM3/F11, S/F28, X-low copy, and Zimbabwe). Reconstruction was performed manually, using spoligotype to identify major families and then by linking SIT-VIT genotypes and allowing for creation of intermediate missing links when required. The size of the nodes reflects the number of strains with an identical SIT-VIT genotype, for clusters with more than 10 representatives.

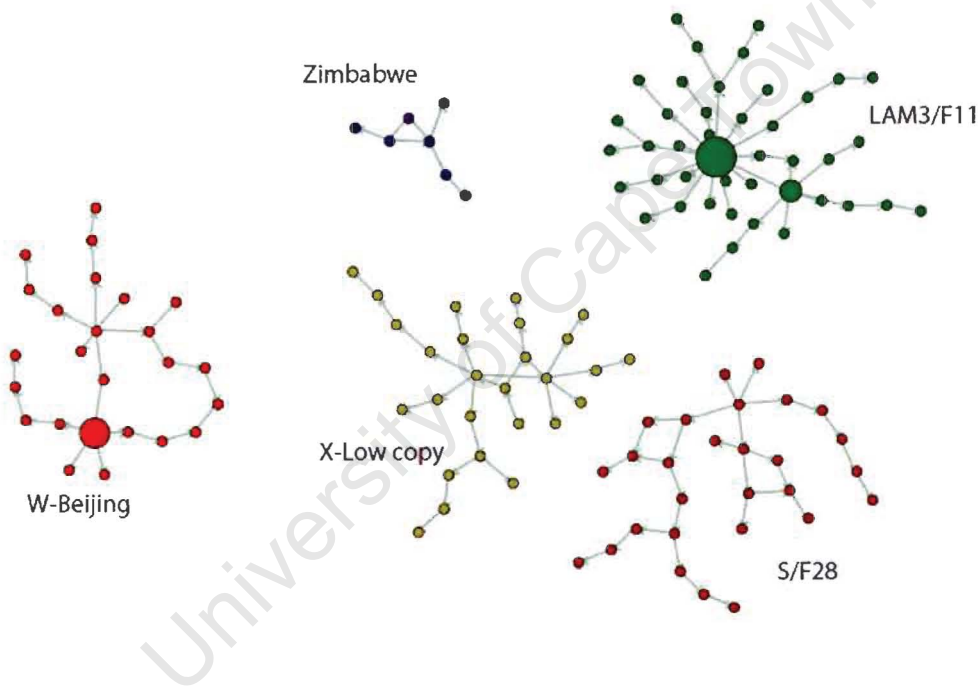


Table 2. Clusters of isolates based on identical MIRU- and spoligotyping alleles

Family designation*	cluster number	VIT number	SIT number	number of isolates	% of clustered isolates
Central Europe1	1	220	39	3	2.0
LAM3	2	236	33	6	4.1
LAM3	3	213	33	32	21.6
LAM4	4	249	130	2	1.4
LAM3	5	213	719	13	8.8
LAM3	6	213	1294	2	1.4
LAM3	7	213	2014	2	1.4
Russia 1	8	140	254	3	2.0
S	9	212	34	3	2.0
S	10	250	71	2	1.4
S	11	252	71	2	1.4
S	12	262	71	2	1.4
T3	13	257	73	2	1.4
Tuscany†	14	140	1737	2	1.4
W-Beijing	15	104	1	24	16.2
W-Beijing	16	223	1	5	3.0
W-Beijing	17	254	1	2	1.4
W-Beijing	18	83	1	3	2.0
W-Beijing	19	238	1	5	3.0
W-Beijing	20	17	1	9	6.1
W-Beijing	21	99	1	7	4.7
W-Beijing	22	245	1	2	1.4
X1	23	112	119	5	3.0
X1	24	117	119	2	1.4
X1	25	258	2019	2	1.4
X3	25	34	92	4	3.0
Z	26	237	811	2	1.4

* Family and VIT-SIT designations from SpolDB4(167)
†(171)

6.3.3. Correlation with clinical presentation

Complete clinical records were available for review in 281 of the 285 cases. Of these, 52 were known to have HIV co-infection and were analysed separately, since HIV infection is independently associated with extra-pulmonary tuberculosis. Strain families as groups did not appear to vary in their ability to cause extra-pulmonary disease in general or meningitis specifically. The proportion of cases of extra-pulmonary disease varied from 50 to 70% between families, with children infected by the two largest families LAM3 and W-Beijing having extra-pulmonary disease in 46 and 50% of cases respectively. The clinical presentations associated with infection by the major strain families of Mtb are detailed in Table 3.

Table 3. Site of disease amongst HIV-uninfected children classified according to strain family

Strain family	Number of strains (% of total strains)	Pulmonary only (% of family)	Extra-pulmonary (% of family)	Odds of developing extra-pulmonary disease (p-value†)	Major extra-pulmonary disease sites (% of total strains)			
					Meningitis	Lymph node	Bone and joint	Miliary
All HIV neg	229 (100)	101 (44)	128 (56)		51 (22)	18 (8)	18 (8)	23
LAM3/F11	72 (31)	33 (46)	39 (54)	0.9 (0.78)	16 (22)	4 (6)	6	4
W-Beijing	52 (23)	26 (50)	26 (50)	0.74 (0.34)	12 (23)	4 (8)	6	5
X	24 (10)	9 (38)	15 (62)	1.36 (0.52)	4 (16)	3 (13)	0	4
S	19 (8)	7 (37)	12 (63)	1.39 (0.63)	6 (32)	3 (16)	0	4
T	15 (7)	5 (33)	10 (67)	1.63 (0.43)	6 (40)	0 (0)	1	2
Other	47 (21)	21 (45)	26 (55)	0.97 (1)	8 (17)	4 (9)	5	4

* miliary disease frequently occurred together with localized disease at an extra-pulmonary site

† Fisher's exact test of probability

The proportion of children with W-Beijing or X family strains was greater amongst HIV-infected (W-Beijing 31%, X family 29%) vs. uninfected (W-Beijing 23%, X family 10%) children whilst the proportion with LAM3/F11 strains was lower amongst HIV infected children (19% vs. 31%). These differences were not statistically significant (p=0.1 for W-Beijing strains).

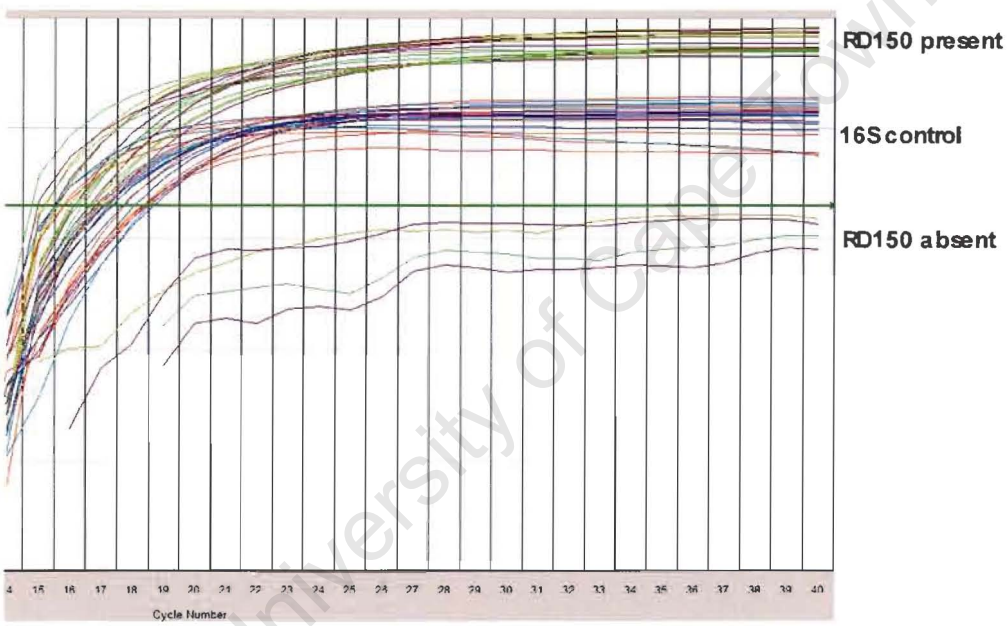
6.3.4. LSP analysis of LAM3/F11 and W-Beijing strains

We performed PCR amplification using primers flanking the RD761 region (Appendix F, Figure 6.5) to confirm that RD761 was deleted in all 86 LAM3/F11 strains studied. In addition, we examined all non-LAM3 strains and confirmed that this deletion was specific to LAM3 strains alone.

In addition, we determined the presence of RD181, RD150 and RD142 in all W-Beijing strains by real time PCR (RD150 and RD142, Figure 6.6) or conventional PCR (RD181). These regions have previously been used by Tsolaki et all to define the major sub-lineages within W-Beijing.

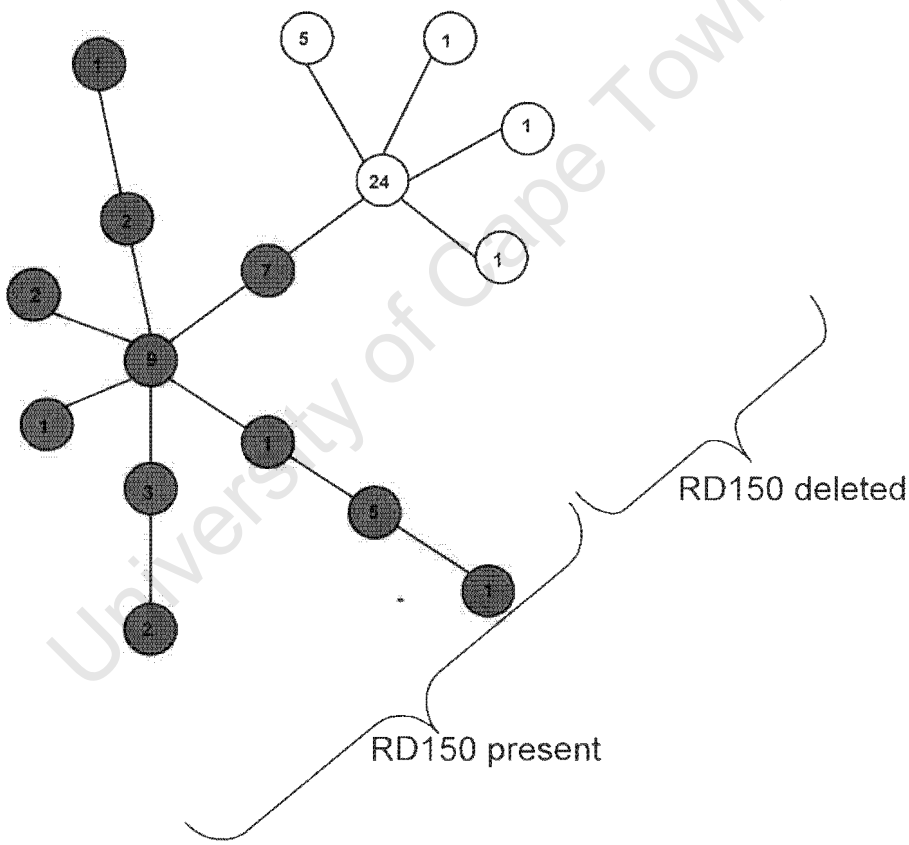
Figure 6.5 Agarose gel electophoresis of PCR products to determine the presence of the RD761 deletion. A fragment size of 1371bp (lanes 5 and 9) indicates the absence of the deletion (non-LAM3) whilst a fragment size of 277bp (lanes 1-4, 6-8) indicates the presence of the deletion (LAM3). Lane 10: no template control; lane 11: λpst size marker.

Figure 6.6 Amplification plot of real time PCR to detect presence of RD150 in 20 strains. The top green and brown lines indicate amplified product for the RD150 region (in 16 strains), the blue and red lines indicate amplification of the 16S control (in all 20 strains) whilst the bottom 4 lines indicate no product for RD150 (i.e. these four strains had a deletion of the RD150 region)



All but 2 of the W-Beijing strains studied had RD181 deleted, whilst RD150 divided Cape Town W-Beijing strains into 2 major sub-lineages. These sublineages coincided precisely with the eBURST cluster analysis of W-Beijing strains according to MIRU-VNTR genotype (Figure 6.7). RD142 was not deleted in any strains.

Figure 6.7 eBURST and RD cluster analysis of W-Beijing strains. Each circle represents a particular MIRU-VNTR genotype. Genotypes differing at a single locus are linked. The number in each circle represents the number of strains identified with the same genotype. Grey circles indicate strains with RD150 not deleted whilst clear circles represent strains with RD150 deleted. The MIRU-VNTR cluster analysis and RD analysis coincide exactly.



6.3.5. The relationship between sub-lineages of W-Beijing and clinical outcome

Whilst W-Beijing strains were no more likely than other strain families to be associated with extra-pulmonary infection in our study, sub-lineages within the W-Beijing family were associated with distinct clinical outcomes. HIV-uninfected children infected with W-Beijing strains characterized by the deletion RD150 were more likely to have tuberculous meningitis (13/32) than those infected with W-Beijing strains in which RD150 was not deleted (3/27, OR 5.47, 95% CI 1.36-22.03). There was no difference between children infected with RD150 deleted or non-deleted strains with regard to median age (30 vs. 25 months respectively, p=0.74), drug-resistance of infecting strain (1/32 vs. 3/27 respectively, OR 0.26, 95% CI 0.025-2.64) or place of residence (data not shown).

6.4. Discussion

Strains of *Mtb* from children in Cape Town are of interest for a number of reasons. Firstly, the Western Cape region represents an area of particularly intense tuberculosis transmission, with incidence rates approaching 1% per annum(172). The reasons for this are as yet unclear and may relate to host or bacterial determinants. The area has lower prevalence rates of HIV infection than much of the country and yet a higher burden of tuberculosis. Since tuberculosis in children is classically primary disease, following soon after exposure to an infectious adult source case, strains causing tuberculosis in young children likely reflect those transmitted by adults in the same community. Secondly, since

extra-pulmonary disease is frequent amongst children hospitalised with tuberculosis in Cape Town, this represents an important opportunity to study potential associations between strains and their ability to cause extra-pulmonary disease.

The strain families circulating in a small precisely defined area of Cape Town have previously been well documented(173-175), but there is little data regarding the broader Cape Town area. Since the referral area for Red Cross Children's Hospital encompasses a large part of Cape Town, our results may more generally represent this area. It is however possible that there is selective transmission of certain strains to children. In addition, if strains differ in their ability to cause severe or extra-pulmonary disease, then these may be over-represented in a collection from hospitalized children. We did not attempt to perform detailed evaluation of transmission patterns within the community. Warren and colleagues have previously used IS*6110* RFLP to extensively address issues relating to strain transmission in a well-defined area of Cape Town(175-178).

Our results concord with previous work of others(174) suggesting that two strain families, the LAM3/F11 family of strains (32% of all clustered cases) and W-Beijing (17% of all clustered strains) predominate in the Western Cape. Taken together, these two lineages represent half of all the clustered isolates. Among the 27 VIT-SIT clusters, three (VIT104SIT1, VIT213SIT33 and VIT213SIT719) are the most prevalent strains within this setting. The first of these belongs to the W-Beijing lineage and the other 2 to the LAM3/F11 lineage. This dominance may be due to ecological or historical factors or, alternatively, because they are specifically virulent in these populations, an issue further

addressed in the following chapter. These comparisons are helpful to better delineate the specificities of the local population structure of *Mtb*. Diversity within these families may be representative of *Mtb* microevolution in a high prevalence setting. In addition, the congruence between spoligotype and MIRU data provides further supportive evidence of a clonal population structure for *Mtb*.

There is accumulating evidence relating differences in virulence beween strains in animal models to the interaction with the host innate immune system. It has been suggested that the W-Beijing lineage of clinical isolates is more virulent in animal models(16;122). In our study we did not find an association between broad strain lineages such as W-Beijing strains and a propensity to cause extra-pulmonary disease or meningitis. We chose extra-pulmonary disease and meningitis as our outcome measures, since these were common, are easily defined and represent a clear spectrum of disease. It is possible that the use of alternative measures of disease severity might reflect more subtle differences between strains. In addition, these result should not be extrapolated to adult tuberculosis, where the strain distribution of extra-pulmonary cases may be different. Indeed, Kong and colleagues(114) found an association between extrapulmonary disease and W-Beijing strains in adults.

There is considerable diversity within strain families based on MIRU-VNTR and, more recently, deletion analysis. Tsolaki et al(179) have defined large deletions resulting in considerable genotypic diversity in 100 clinical strains of *Mtb* as well as deletions which subdivide the W-Beijing family into distinct sub-lineages(109). In our study these

deletions defined two major sub-lineages within W-Beijing strains in Cape Town. These two sub-lineages appeared to be differentially associated with the ability to cause tuberculous meningitis in children. The biological explanation for this phenomenon is not clear as yet, is discussed briefly in the following chapter and forms a focus of ongoing work by our group.

6.5. Conclusion

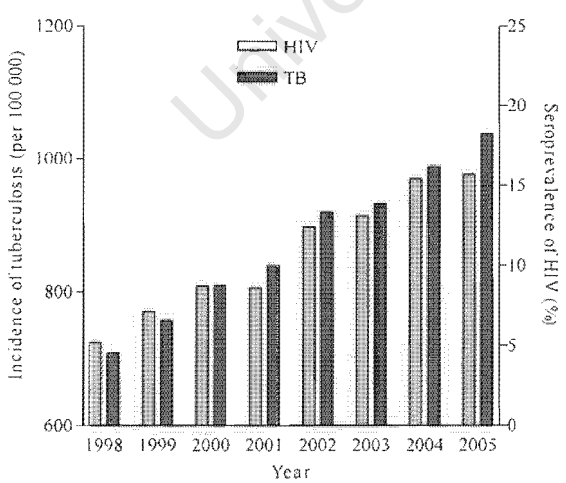
W-Beijing and LAM3/F11 strain families predominate amongst children presenting to hospital with tuberculosis in our setting. These strains are likely to reflect those currently transmitted in Cape Town. We found no significant association between broad strain family and site of disease, however we identified an association between sub-lineages of W-Beijing strains and a propensity to cause tuberculous meningitis in children.

Chapter 7. The recent and rapid emergence of W-Beijing strains of *Mycobacterium tuberculosis* in Cape Town

7.1. Introduction

The burden of tuberculosis in the Western Cape region of South Africa is amongst the highest in the world. In 2005, incident cases exceeded 1% of the population (1037 cases per 100 000 individuals)(3). There were more cases of tuberculosis in the city of Cape Town in 2005 than in the entire United States. Whilst rates of tuberculosis in Cape Town have historically been high, in the past 5 years there has been a rapid further increase in incidence associated with a concurrent HIV epidemic (Figure 7.1). The reasons for these high rates are unclear – the Western Cape region is socio-economically one of the richest in Africa and has a relatively lower HIV seroprevalence than many other parts of sub-Saharan Africa(3).

Figure 7.1 The incidence of active tuberculosis (per 100 000 population) and seroprevalence of HIV infection (percentage positive by ELISA) amongst antenatal clinic attendees in the Western Cape from 1998-2005



The outcome of infection with *Mtb* results from a complex interplay of host, environmental and bacillary factors. In the previous chapter we have shown that the major *Mtb* strain families in the region belong to two broad lineages, the W-Beijing lineage and the Euro-American lineage (the major representative of which is the Latin-American-Mediterranean-3/F11 family [LAM3]), confirming the findings of others in the field(140;155;180).

The W-Beijing family of strains is globally distributed(127) and well documented as a cause of tuberculosis outbreaks, including multi-drug-resistant cases, particularly in HIV-infected individuals(131). W-Beijing strains have recently been associated with extra-thoracic disease(114) and with HIV infection(142). BCG vaccine has been associated with relatively poor protection against subsequent challenge with a W-Beijing strain in experimental models(152). In addition, W-Beijing strains have been demonstrated to be virulent in animal models(19;181). The increased virulence of these strains has been attributed to the production of a phenolic glycolipid which is able to inhibit the innate immune response(16). More recently, W-Beijing strains have been shown to have constitutive upregulation of the DosR dormancy regulon(138) which may confer an survival advantage in anaerobic conditions. Despite this clear evidence from *in vitro* and animal studies, there are no unambiguous data from human studies demonstrating increased virulence.

One marker suggesting increased virulence might be the emergence or expansion of these strains within human populations. We therefore wished therefore to determine whether

W-Beijing strains were emerging in Cape Town. We identified the proportion of W-Beijing strains amongst children presenting to Red Cross Hospital annually over the study period to determine short term trends and to determine the proportion of W-Beijing strains amongst recently transmitted cases. Further, we typed strains present in archived histological specimens in order to determine a point in time emergence of W-Beijing strains in Cape Town. In order to determine whether the ability to subvert innate immune responses was a conserved phenotype of these strains, we evaluated cytokine induction by current clinical isolates of W-Beijing in a primary human macrophage model.

7.2. Methods

7.2.1. Sample selection:

7.2.1.1. Culture Isolates from Red Cross Children's Hospital:

Over the period 2000-2003 we collected all cultures positive for *Mtb* from children presenting with tuberculosis at this hospital, as described in the previous chapter.

7.2.1.2. Archived histological specimens:

We obtained a stratified selection of post-mortem tissue samples from each decade over a 76-year time period (1930 to 2005) from patients who had died with active tuberculosis from the archived collection in the Division of Anatomical Pathology, Groote Schuur Hospital, Cape Town, South Africa. Groote Schuur Hospital is one of two major tertiary referral hospitals serving the greater Cape Town region. After 1995 all samples were

from tissues tested HIV-negative at the time of autopsy. Prior to 1995 HIV testing was not performed. The first 100 patients who had died with tuberculosis were selected for each decade, where numbers permitted. Demographic details including age, gender, and ethnicity were recorded for each patient. Tissue samples were obtained from lung primarily, with extra-pulmonary sites selected if affected lung tissue was not available. Following selection, companion haematoxylin and eosin stained slides were reviewed to confirm evidence of tuberculosis infection. Samples with evidence of 'old tuberculosis' or in which we were unable to confirm the diagnosis of tuberculosis were excluded.

7.2.2. DNA extraction

Following selection of tissues, twelve 7μm specimen tissue sections were cut and two sections placed into each of six separate 2.0ml screw-cap tubes. Extraction of mycobacterial DNA from the paraffin-embedded tissues was performed using a method based on the use of Chelex100 (Bio-Rad, Marnes la Coquette, France) as documented in the spoligotype manual, Isogen LifeSciences (Appendix C). 150μl of a 5% Chelex100 suspension was added to a micro tube containing the tissue sections. The mixture was heated to 100^0C in a water-bath for 30mins and subsequently centrifuged for 10mins at 13,000g. The solution beneath the paraffin wax layer containing the extracted DNA was carefully transferred to a clean 1.5ml Eppendorf tube.

7.2.3. Spoligotyping

This was performed as previously described(99), details in Appendix C. W-Beijing strains are identified by an easily recognizable spoligotype pattern, with spacers 1-34 absent and 35-43 present. We classified our spoligotyping results according to the following criteria; 'Possible W-Beijing': absence of spacers 1-34 and presence of at least 1 spacer in the region 35-43; 'W-Beijing': absence of spacers 1-34 and presence of spacers 35-43 'Euro-American'(155): absence of spacers 33-36 and presence of flanking spacers around this region; 'Non-Beijing Other': Clear presence of multiple spacers between 1-34 (i.e. non-W-Beijing), where the deletion of spacers 33-36 was not clearly defined; and 'Unsuccessful': insufficient or no spacers present to identify a *Mtb* strain(94;103;155). We chose to use the broad "Euro-American" designation rather than assign these strains a specific classification according to the spoligotype database, since in many cases the spoligotype patterns were incomplete and would not allow specific sub-classification.

7.2.4. Quality control measures

A number of steps were taken to prevent cross contamination. Firstly, a tuberculosis-negative control tissue section was cut between samples to obtain a 'PCR-clean' surface. The microtome blade was replaced and gloves discarded after each sample and the work area wiped down with 10% bleach in preparation for the next sample. The following tissues were used for quality control (QC) in the extraction process: *positive control tissue:* a tissue section with evidence of tuberculosis infection by demonstration of acid

fast bacilli with Ziehl-Neelsen staining; *negative control tissue:* a tissue section without any evidence of tuberculosis infection or disease; *extraction batch control:* a suspension of Chelex100 in an tube processed in same way as the samples (but with no tissue) to ascertain that the extraction suspension was contamination free.

Additional precautions were taken to avoid and detect possible contamination during the PCR amplification. The DNA extraction and post-PCR product handling procedures were conducted in separate laboratories on separate days. Positive and negative tissue controls were included during DNA extraction and genotyping. All work areas were wiped down with 10% bleach and all procedures were performed wearing clean gloves.

A successful extraction was defined by an interpretable result on spoligotyping together with successful spoligotyping of the positive control tissue and a negative result for an extraction batch control and negative control tissue. In order to obtain an interpretable or complete result it was frequently necessary to repeat the DNA extraction and spoligotyping on independently extracted tissues.

7.2.5. Single Nucleotide Polymorphism (SNP) analysis

In order to confirm the spoligotype analysis, we amplified and sequenced a 91 base pair fragment of *Mtb* DNA to detect the presence of a discriminatory SNP(96) at position 4280708 (H37Rv) from all 13 samples in which W-Beijing strains and a random selection of 56 samples in which non-W-Beijing strains were identified by spoligotyping. W-Beijing strains contain an adenine and non-W-Beijing strains a guanine base at this

position. The following primer pair was used: forward CCTTGGTCGGGCACATTC (H37Rv 4280682-4280699), reverse TAGCGCAGAATCTCTAGGACC (4280772-4280752). The product was purified and cycle sequencing was performed (reverse primer only).

7.2.6. Macrophage infection model

We selected representative W-Beijing strains for evaluation of growth rates and cytokine induction in the MDM model on the basis of our previous work (Chapter 6) showing the existence of two major sub lineages of W-Beijing in Cape Town(140). The W-Beijing strains chosen were representatives of the largest clusters previously defined by 12-locus MIRU-VNTR analysis of the paediatric strains. Four strains were used for evaluation of growth rates and 6 strains for evaluation of cytokine induction (equal numbers with RD150 deleted or not deleted).

MDM were prepared from healthy blood donors (5 donors for intracellular growth assays and 7 donors for cytokine assays) and isolated, characterized and infected with W-Beijing strains or H37Rv exactly as previously described (Appendix D)(182). Cells were infected at a multiplicity of infection of 1:1 for 4 hours, followed by washing 3 times in warm RPMI to remove non-phagocytosed bacteria. Wilkinson et al have previously shown the washing procedure ensures that ~90% of bacilli subsequently recovered from cultures are cell associated rather than in the supernatant(183). To determine the rate of *Mtb* growth in cells, colony forming units (CFU) were determined at time 0, 3 and 7 d after infection; the supernatant was aspirated and the cell monolayer was lyzed for 12 min with sterile

water followed by serial dilution and plating on to 7H11 agar. For cytokine assays non-phagocytosed bacilli were not washed away from the cells in an attempt to eliminate differences in cytokine production between cell types that could be accounted for by differing initial uptake. After 72 h incubation at 37 °C, culture supernatants were harvested and sterile filtered for storage at –80 °C. Supernatants were analyzed for the presence of tumour necrosis factor (TNF) and interleukin-12p40 (IL12p40), by ELISA using antibody pairs from R & D Systems (Abingdon, UK). For assessment of growth rates and cytokine induction 5 and 7 donors were used respectively.

7.2.7. Statistical analysis

Statistical analysis was performed using Prism Software 4.0 (Graphpad Software) and STATA Version 9 (StataCorp LP). Non-parametric unpaired data was analyzed using the Mann-Whitney U test. The Fisher exact test was used to compare proportions of W-Beijing per periods. Chi square for trend was used to evaluate the changes in W-Beijing, ethnicity, age and strain distribution over time. The Kruskal-Wallis test was used for comparing median ages. We used logistic regression to control for the effects of age and ethnicity on the association between time (measured as a dichotomous variable, either by decade or per designated time period) and the number of W-Beijing strains identified. The Friedman test was used to compare cytokine induction by strains and growth in macrophages. Dunn's Multiple Comparison test was used for post-test comparisons. Significance was inferred by $p<0.05$. Approval for this study was obtained from the University of Cape Town Research Ethics Committee (Reference: 320/2005).

7.3. Results

7.3.1. Strains currently circulating in Cape Town

To assess recent transmission of *Mtb* strains in Cape Town, we genotyped all strains of *Mtb* cultured from children at the major regional paediatric referral hospital in Cape Town (Red Cross Children's Hospital) over the period 2000-2003. Isolates from 291 children were obtained (6 further isolates were identified in addition to the 285 described in Chapter 6), of which 64% were from pulmonary specimens (Table 1). The majority of children (267/291) gave home addresses in the greater Cape Town metropolitan region.

Overall, 23% of strains were identified as W-Beijing by spoligotyping. The proportion of W-Beijing strains as a percentage of total strains increased from 12.5% in 2000 to 32.8% in 2003 (p=0.026 comparing 2000/2001 to 2002/2003, Figure 7.2A). This increase was largely due to the expansion of W-Beijing strains with RD150 deleted.

Table 1. Strains from Red Cross Children's Hospital 2000-2003

Family designation*	n	HIV+† (%)	Culture specimen			Susceptibility		
			CSF‡	Pulmonary	Extra-pulmonary	INH mono-resistant	Rif § mono-resistant	MDR¶
X	30	6 (30)	4	17	9	2	1	1
W-Beijing	68	15 (22)	9	42	17	5	0	0
S	21	2 (10)	5	14	2	4	0	0
LAM3	86	13 (15)	8	59	19	3	0	0
Other	86	18 (21)	12	53	21	6	1	4
Total	**291**	**54 (19)**	**38**	**185**	**68**	**20**	**2**	**5**

* from SpolDB4(167)

† HIV ELISA (with confirmatory PCR if under the age of 1 year); testing was only performed if clinically indicated

‡ cerebrospinal fluid

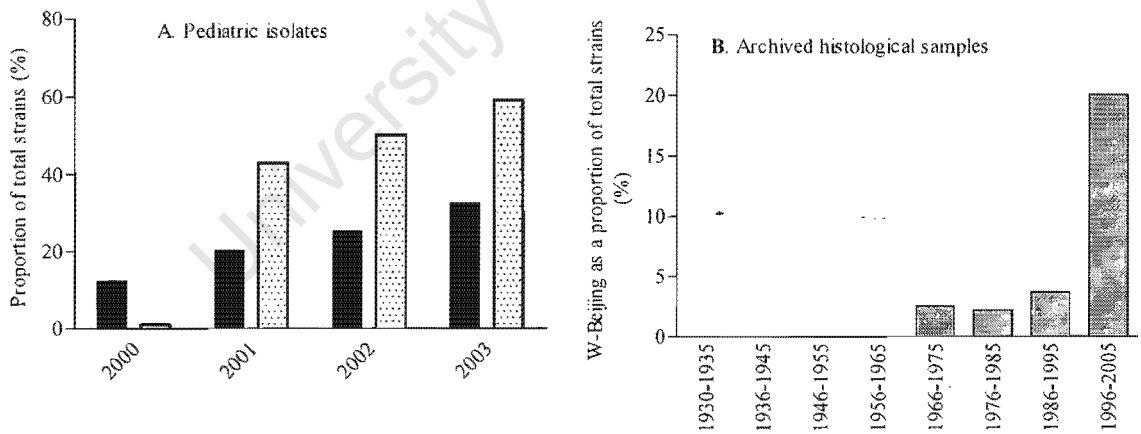
§ rifampicin

¶ multi-drug resistant (INH and rifampicin resistant)

Figure 7.2 Changes in the proportion of W-Beijing strains amongst total *Mtb* strains in Cape Town.

A. The proportion of W-Beijing isolates as a percentage of total isolates (solid bars) and the proportion of W-Beijing strains with RD150 deleted as a proportion of all W-Beijing isolates (shaded bars), amongst children with culture-confirmed tuberculosis at Red Cross Children's Hospital from 2000-2003 (Fisher exact test for 2000/2001 vs. 2002/2003 p=0.026);

B. The proportion of W-Beijing-positive histological samples (as a percentage of total *M.tb*-positive samples) from post-mortem tissues with histological evidence of tuberculosis 1930-2005 (Chi square test for trend p=0.0001)



7.3.2. Strains in archived histological samples

To further investigate whether the frequency of W-Beijing strains had recently emerged in the region, we genotyped strains of *Mtb* present in archived post-mortem tissue samples in which there was histological evidence of tuberculosis over the period 1930-2005. The following numbers of tissues per decade were chosen: 1930-1935 n=(45), 1936-1945 n=(78), 1946-1955 n=(65), 1956-1965 n=(55), 1966-1975 n=(52), 1976-1985 n=(56), 1986-1995 n=(71), and 1996-2005 n=(48). We were able to obtain an interpretable spoligotype pattern in 352 (74.7%) of the 471 individual tissue samples selected.

From 1930 to 1965, none of 169 *Mtb* patterns identified were W-Beijing (Table 2). Between 1966 and 1995, 4 of 138 (2.8%) patterns were identified as W-Beijing. However, in the decade 1996 to 2005 the W-Beijing pattern was identified in 9 of 45 (20%) tissue samples (Odds ratio 19.05 [95% CI 2.45-148.3], p=0.0001 when comparing the period 1930-75 with 1976-2005, Figure 7.2B). By comparison, the proportion of Euro-American strains identified declined throughout the study period (OR 0.79 [95% CI 0.48-1.3] comparing 1930-1975 with 1976-2005).

Table 2. Strain lineages identified by time period (n=351)

Time period	W-Beijing (%)	Euro-American (%)	Other Non-Beijing (%)
1930-1935	0 (0%)	28 (90.3%)	3 (9.7%)
1936-1945	0 (0%)	39 (83%)	8 (17%)
1946-1955	0 (0%)	39 (83%)	8 (17%)
1956-1965	0 (0%)	34 (77.3%)	10 (22.7%)
1966-1975	1 (2.5%)	30 (75%)	9(22.5%)
1976-1985	1 (2.2%)	33 (75%)	10(22.7%)
1986-1995	2 (3.7%)	38 (70.3%)	14(25.9%)
1996-2005	9* (20%)	33 (73.3%)	3 (6.7%)
Total	**13 (3.7%)**	**274 (77.8%)**	**65 (18.5%)**

*Chi square comparison of W-Beijing cases 1930-1975 vs. 1976-2005, OR= 19.05 (95% CI 2.45-148.3)

There were no strains classified as "Possible W-Beijing", i.e. all strains in which there was absence of spacers 1-34 had spacers 35-43 present. However, to investigate further and to confirm the spoligotype classification, we amplified and sequenced a discriminatory SNP in all W-Beijing containing and in 56 randomly selected non-W-Beijing containing tissue samples(96). We were able to obtain interpretable sequence from 11 of the 13 W-Beijing and 49 of the 56 non-W-Beijing tissue samples tested. In all cases the sequence data concurred with the spoligotype-assigned classification.

The demographic details of the patients are shown in Table 3. The median age was 33 years (range 0.3-93 years). The median age of patients was lower in the earlier decades ($p<0.0001$), primarily due to the large number of post-mortems performed during this period on children dying with tuberculous meningitis. The emergence of W-Beijing strains remained significant when excluding all cases of meningitis (Odds Ratio 14.04 [95% CI 1.79-110.5], $p=0.0016$ for the comparison of the periods 1930-1975 and 1976-2005).

Table 3. Demographic details of patients

Time period	n	Median age years (range)	Percent female	Ethnic background no. (%)			
				Black African	Cape Coloured*	European	Unknown
1930-1935	45	3.0 (0.3–55.0)	57.8	5 (11.1)	32 (71.1)	7 (15.6)	1 (2.2)
1936-1945	78	11.0 (0.3–69.0)	46.2	10 (13)	51 (65)	15 (19)	2 (2.5)
1946-1955	65	5.0 (0.3–69.0)	41.6	16 (24.6)	34 (52.3)	13 (20)	2 (3.1)
1956-1965	55	33.0 (0.3-93.0)	49.1	23 (41.8)	27 (49.1)	4 (7.3)	1 (1.8)
1966-1975	52	40.5 (0.4-75.0)	42.4	25 (48.1)	24 (46.2)	3 (5.7)	0 (0)
1976-1985	56	44.5 (0.8-77.0)	48.3	29 (51.8)	20 (35.7)	7 (12.5)	0 (0)
1986-1995	71	52.0 (2.5-84.0)	43.7	42 (59.2)	28†(39.4)	1 (1.4)	0 (0)
1996-2005	49	43.0 (18.0–82.0)	51.0	28 (57.2)	20 (42.8)	0 (0)	0 (0)

*See discussion for an explanation of this population group (184)

†Includes Asian n=1

We observed a change in ethnicity of subjects undergoing post-mortems with an increase in the number of Black Africans in the more recent time periods and a corresponding decrease in the number of European and Coloured patients (Chi-square test for trend for number of Black Africans $p<0.0001$). However we detected no significant association between ethnic group and strain type (Table 4).

After using logistic regression to control for the effects of age and ethnicity, time remained a significant predictor of the emergence of W-Beijing strains (OR = 16.54, 95% CI 1.90-144.0, p=0.011, for the comparison of the period 1930 to 1975 with the period 1976 to 2005 or, as expressed as an increase in odds per decade, OR = 3.08, 95% CI 1.58-5.98, p=0.001). Age and ethnicity were not predictors of W-Beijing genotype.

Table 4. Proportion of W-Beijing strains by ethnic group

Decade	**Ethnicity**	**W-Beijing**	**Other strains**	**Odds ratio (95% CI)***
1930-1995	Black African (n=103)	1 (1.0%)	102(99.0%)	0.51 (0.05-5)
	Cape Coloured† (n=160)	3 (1.8%)	157 (98.2%)	
1996-2005	Black African (n=27)	6 (22.2%)	21 (77.8%)	1.43 (0.31-6.64)
	Cape Coloured (n=17)	3 (16.7%)	15 (83.3%)	
Total	**Black African (n=130)**	**7 (5.4%)**	**123 (94.6%)**	**1.63 (0.54-4.98)**
	Cape Coloured (n=177)	**6 (3.4%)**	**172 (96.6%)**	

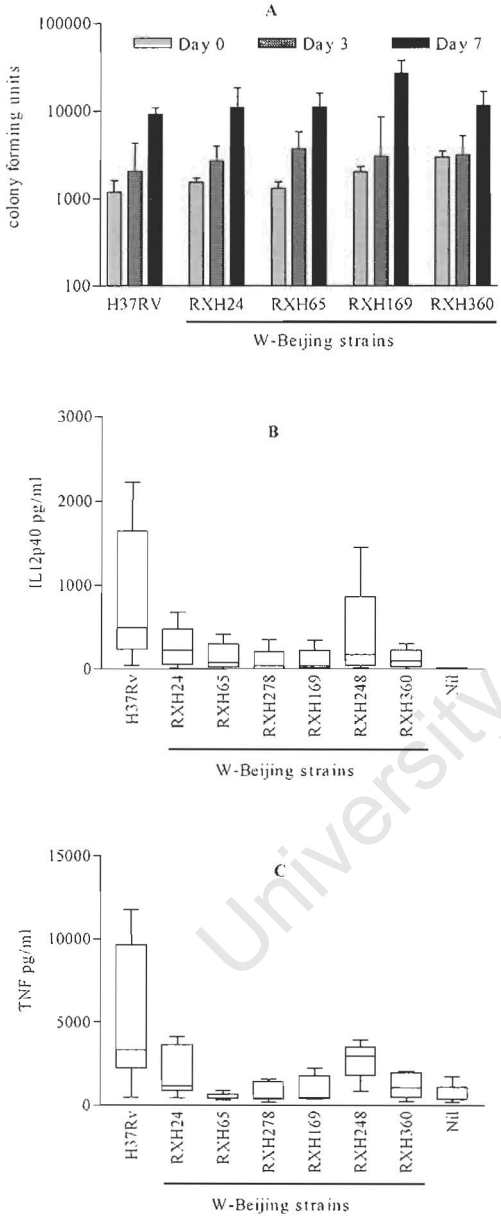
* Odds in favor of Black African patient having a W-Beijing strain (95% confidence interval)

† See discussion for an explanation of this population group (184)

7.3.3. Macrophage infection model

We infected primary human MDM in order to determine the ability of a representative range of W-Beijing strains from Cape Town to multiply within macrophages (4 strains in 5 donors) and to induce the protective cytokines IL12p40 and tumour necrosis factor TNF (6 strains in 7 donors). Growth of W-Beijing strains within macrophages did not differ significantly from that of the laboratory reference strain, H37Rv (Figure 7.3A) except for a single W-Beijing strain (RXH169) which showed faster growth than H37Rv at day 7 ($p<0.05$). Despite similar rates of intracellular growth, W-Beijing strains induced less IL12p40 ($p<0.0001$) and TNF ($p<0.0007$) when compared to H37Rv (Figure 7.3B, C). There was no difference in growth rate or cytokine induction between W-Beijing strains with RD150 deleted or not deleted.

Figure 7.3 Growth of strains within monocyte-derived macrophages and cytokine induction.



A. Intracellular growth of strains was similar, with only strain RXH169 showing a significant increase in growth compared with H37Rv on day 7 ($p<0.05$). Error bars show median and maximum;

B. IL12p40 induction by strains. W-Beijing strains all induced less IL12p40 than H37Rv ($p<0.0001$, Dunn's post test showed $p<0.05$ for RXH65, RXH278, RXH169, RXH360). Median and interquartile ranges are shown with error bars showing minimum and maximum.

C. TNF induction by strains. W-Beijing strains all induced less TNF than H37Rv ($p=0.0007$, Dunn's post test showed $p<0.05$ for RXH65, RXH278).

(RD150 not deleted in strains RXH 24, 65 and 278; deleted in RXH 169, 248 and 360)

7.4. Discussion

Since *Mtb* is slow-growing, rarely undergoes horizontal genetic exchange(92) and most frequently gives rise to unapparent infections, rapid changes in the population structure of *Mtb* within human communities are counterintuitive. However, here we present two independent lines of evidence suggesting that W-Beijing strains of *Mtb* have recently emerged in Cape Town and are rapidly expanding. W-Beijing strains were rare or absent in post-mortem specimens from Cape Town in the early and middle part of the 20th century, their first appearance being in the mid 1960s. In the last decade however, these strains have emerged as a major cause of death from tuberculosis.

Further, we observed that the proportion of W-Beijing strains amongst children diagnosed with tuberculosis increased from 12.5% to 32.8% over the period 2000-2003 at the major paediatric referral hospital in Cape Town. The figure for 2003 coincides precisely with that observed independently at the second paediatric referral centre in Cape Town(180) and is therefore likely to be representative of the region. Since tuberculosis in children is almost always due to progressive primary disease, this figure is likely to reflect the current extent of transmission of W-Beijing strains.

By contrast, our evaluation of strains present in archived histological material did not identify a single W-Beijing strain amongst the 116 children (younger than 10 years of age) in the period 1930-1965. Interestingly, a regression line drawn from the recent paediatric data has an intercept at 1998 which corresponds well with the period of rapid emergence (1996-2005) obtained from the post-mortem samples.

The younger age of patients undergoing post-mortem in the earlier years of this study is attributed to the large number of children dying with tuberculous meningitis during this period. It may be argued that this could influence our analysis, as certain strains may have a predilection for meningeal disease. However, when we excluded all samples from patients with meningitis, W-Beijing strains were still strongly over-represented in the later decades. This is in keeping with our previous work showing a lack of association between W-Beijing strain lineage and the propensity to cause meningitis in children(140). Anh and colleagues have shown that W-Beijing genotype was associated with younger age in Vietnam(135), suggesting that W-Beijing strains may be emerging in this region. In our post-mortem samples, age was not a predictor of W-Beijing genotype, but this may reflect the small number of samples from children in the more recent decades. Given the high proportion of W-Beijing strains in our paediatric collection, it is possible that our post-mortem data may underestimate the magnitude of the emergence of these strains.

We elected to use spoligotyping as the primary method for identifying W-Beijing strains, since this is generally regarded as highly accurate for defining this lineage(185) and is suitable for use on degraded DNA. DNA in paraffin-embedded tissues is variably degraded(186) therefore it is not possible to perform multiple interspersed repetitive unit (MIRU) typing, large sequence polymorphism analysis or IS6110 restriction length fragment polymorphism analysis on such specimens. The aim of this study was to determine whether W-Beijing strains as a whole were expanding in the region rather than to specifically ascertain routes of transmission.

There is recent evidence of a group of 'ancestral' W-Beijing strains which do not show the characteristic deletion of spacers 1-34, but share a common ancestry with 'modern' W-Beijing strains(155). However, since the W-Beijing strains which have recently emerged in Cape Town all have the characteristic spacer 1-34 deletion (we did not find a single strain with a spoligotype pattern compatible with 'ancestral W-Beijing' in our paediatric collection), it is this genotype that we sought to identify in the archived histological material.

Prior to European settlement of the Western Cape region 400-500 years ago the population density of indigenous Khoi-San peoples was very low and tuberculosis was rare or unknown. European settlement is believed to have introduced (or re-introduced) the disease, indeed, for some years, the Cape was promoted as a health resort for European consumptives(187). This coincides with our finding that the majority of strains found in the early years of the 20th century belong to the Euro-American lineage which predominates in Europe. Admixture between East Asian, Khoi-San, European and Black African populations gave rise to the Cape Coloured population of the province(184). W-Beijing strains are common in East Asia. It was therefore surprising that W-Beijing strains were uncommon (or absent) in the early decades of this study, since the majority of the tissues from this period were from patients of Cape Coloured origin. It is possible that W-Beijing strains have existed in isolated areas or at a low level since this time. W-Beijing strains are now common amongst Cape Coloured patients with tuberculosis(140;174;188).

This is the first study to document the emergence and expansion of W-Beijing strains within a tuberculosis-endemic region over an extended period of time. Such a rapid change in the population structure of *Mtb* within a short period of time is unexpected and suggests that these strains may possess a significant selective advantage.

Possible alternative explanations for the observed expansion of W-Beijing strains include migration, clonal spread and drug pressure. There has been no documented significant migration from Eastern Asia, Russia or Eastern Europe (where W-Beijing is prevalent) to the Cape Town region in recent years. Currently, the Asian population in the city constitutes 1.4% of the total population (Census 2001, Statistics South Africa). Prior to the 20th Century there was little history of settlement by black African people in the Western Cape region. However urbanization and rural displacement saw increasing migration (which peaked in the 1940s) such that the African population now predominates in many areas(189). This is reflected in the shift in ethnicity of post-mortem subjects over the period of study. This change long preceded the emergence of W-Beijing strains and so internal migration is unlikely to account for our observation. Moreover, there was no association between ethnicity and strain lineage on multivariate analysis.

Previous reports have largely focused on clonal outbreaks of W-Beijing strains in communities with a low incidence of tuberculosis(149). We have previously shown that the population structure of W-Beijing in the Western Cape is heterogeneous, including at least two major lineages, with considerable diversity within each lineage(140).The

current increase in W-Beijing tuberculosis is therefore not due to a single large clonal outbreak.

There is a well-documented association between W-Beijing strains and drug resistance(109;190-192). Marais and colleagues described an association between drug resistance and W-Beijing strains of *Mtb* in Cape Town, however most W-Beijing strains (83%) were drug-susceptible(180). Only 9% of the W-Beijing strains in our paediatric collection were resistant to either isoniazid or rifampicin (data not shown). Since the majority of W-Beijing strains in the region remain fully susceptible, this is unlikely to be the most important reason for the dramatic emergence of these strains, however drug-resistance may increasingly play a role in their expansion.

There is emerging evidence of an association between HIV infection and W-Beijing strains of *Mtb*. Caws and colleagues (2006) documented a strong association between the W-Beijing lineage and HIV infection amongst patients with tuberculous meningitis in Vietnam(142). The original description of the W-strain outbreak in New York City was primarily (86%) amongst HIV-infected persons(193). It is therefore interesting that the emergence of W-Beijing coincides with the arrival of the HIV epidemic in the Western Cape. HIV infection was uncommon until 1994, when seroprevalence amongst antenatal clinic attendees first exceeded 1%. Since then there has been a linear increase, with a prevalence of 15.7% measured in 2005(3). This mirrors the expansion of W-Beijing strains.

The tissues used for this study originated from HIV-uninfected persons (since full post-mortems are not performed on HIV-infected patients). It is therefore not possible to determine from our data whether HIV has contributed to this emergence. However it is likely that an increased burden of W-Beijing strains amongst HIV-infected persons, who presently account for 47% of cases of tuberculosis in Cape Town (Western Cape provincial data, unpublished), would 'spill over' into the HIV-uninfected population. Since HIV co-infection is associated with a high rate of progression to active disease, more rapid onset and severe illness(194), rapid changes in strain distribution may be more likely in a population with a high prevalence of HIV. There is no reliable data on the proportion of W-Beijing strains in other parts of South Africa. Since rates of HIV infection are higher in all of the other provinces, it will be important to document the presence of these strains beyond the Western Cape province.

Previous reports have suggested that isolated clinical W-Beijing strains induce low levels of pro-inflammatory cytokines both *in vitro* and *in vivo*(18;195). We extended this work by confirming this immune-subverting phenotype in 6 clinical strains representing the major sub-lineages of W-Beijing in Cape Town. There was no difference between the two major sub-lineages of W-Beijing prevalent in this region (RD150 deleted and not deleted), despite the apparent differences in the propensity of these strains to cause meningitis (Chapter 6). Interleukin-12p40 and TNF were chosen as representative pro-inflammatory cytokines since these have previously been shown to be key cytokines in the protective response to *Mtb* (Chapter 5). Recent work has suggested that the ability of W-Beijing strains to down-regulate Th1 responses in infection is related to the induction

of interleukin-10 producing regulatory T cells. Since W-Beijing strains are able to subvert the protective immune response, which plays a key role in maintaining latency(196), it is feasible that a high proportion of infections progress to active disease rather than becoming latent. This situation would likely be exacerbated by HIV co-infection.

Immune subversion may also be related to an increased bacillary load, as demonstrated in some animal models(19). HIV co-infection, which has been associated with increased bacillary burden(197), may have a synergistic effect. An increased bacterial load in the lung may increase transmissibility (and may also increase the likelihood of dissemination to extrapulmonary sites, reflected in the association between W-Beijing and extrapulmonary tuberculosis(114)).

Some authors have suggested that BCG vaccination might feasibly select for the spread of W-Beijing strains(152-154). Routine BCG vaccination in South Africa commenced in 1973 and is now almost universal(3), overlapping closely with the period over which W-Beijing strains have expanded. We do not, however, have any data specifically linking W-Beijing strains with prior BCG vaccination.

7.5. Conclusion

We have documented the emergence and rapid expansion of W-Beijing strains within a tuberculosis-endemic region. This finding suggests that these strains possess an important advantage in their ability to disseminate within a community. This emergence

has occurred in the context of a concurrent HIV epidemic. The cumulative impact of a highly susceptible host population and the rapid spread of W-Beijing strains is likely to place significant pressure on the tuberculosis control program. We are entering a new era in tuberculosis epidemiology, where HIV co-infection and drug resistance will play important roles in driving the expansion of specific lineages of *Mtb*. Our findings highlight the need to better understand the relationship between HIV infection, strain genotype and drug resistance and the implications for the spread of tuberculosis amongst HIV-infected and uninfected individuals.

Chapter 8. Conclusions and further work

This thesis focused on two aspects of childhood tuberculosis which pose a particular challenge to clinicians. Firstly, we evaluated the use of the ELISpot assay for the diagnosis of paediatric tuberculosis. We showed that ELISpot may be a useful adjunct to clinical diagnosis for the evaluation of children presenting to hospital with suspected tuberculosis. ELISpot responses frequently increased during early treatment and therefore a decline in response should not be used to monitor the efficacy of therapy. Furthermore, we demonstrated that the sensitivity of the assay is lower in very young children with milder disease in a community setting, where ELISpot did not appear to offer any advantage over TST. ELISpot responses, but not TST responses were strongly age-dependent, suggesting that ELISpot responses may be age-related in early life. A negative ELISpot response should not be used to exclude tuberculosis in young children.

Further work is required to confirm the age-dependent nature of ELISpot responses and to adequately determine the performance of the assay in HIV-infected children. Our data suggest, however, that the utility of this assay may be limited for the diagnosis of tuberculosis in very young HIV-uninfected children, where the sensitivity of TST may be better. Given the considerable costs of this assay and the relatively poor performance of ELISpot in such children, its utility in resource-poor settings is questionable.

In the second part of this thesis, we addressed the question of whether the outcome of tuberculosis in children, in particular the propensity to develop extra-pulmonary disease, is influenced by the genetic background of the infecting strain. We used a range of genotyping methods to characterize the strains of *Mtb* causing disease in children in Cape

Town, and showed that the majority of infections were caused by two *Mtb* strain lineages, namely W-Beijing and LAM3/F11. These two lineages did not appear to differ overall in their propensity to cause extra-pulmonary disease. W-Beijing strains in the region were, however, shown to belong to two major sub-lineages, defined by the large sequence polymorphism RD150. There was an association between sub-lineage and tuberculous meningitis, with strains characterized by the deletion RD150 more likely to cause meningitis than those without the deletion. All W-Beijing strains tested were shown to possess a common immune-subverting phenotype which has previously been described amongst isolated W-Beijing strains. There was no distinction between strains with and without the deletion RD150, with respect to their pattern of cytokine induction or growth within human monocyte-derived macrophages. Further work is required to determine the biological basis for the difference in clinical outcome.

In order to further investigate the preliminary observation that the proportion of W-Beijing strains amongst children presenting to hospital had increased in frequency over the period 2000-2003, we genotyped strains of *Mtb* present in archived histological samples from patients who died with tuberculosis over a 76 year period. We showed that W-Beijing strains have only recently emerged in Cape Town, and are rapidly expanding. This expansion suggests that these strains have a significant selective advantage, which may relate to their immune-subverting phenotype, their association with HIV infection, drug-resistance or the effect of BCG vaccination. Future work will focus on the interaction between HIV-infection and W-Beijing strains of tuberculosis.

Appendices

Appendix A. 'In-house' enzyme-linked immunospot (ELISpot) assay

A.1 Purpose

To detect interferon-γ (IFNγ)–secreting T cells in response to stimulation with phytohaemagglutinin (PHA), PPD and *Mtb*-specific antigens ESAT-6 and CFP10.

A.2 Principle

The method used is based on that described by Lalvani et al(48). A microtitre plate with PVDF membrane is coated with a monoclonal antibody to IFNγ. PBMCs from patients with suspected tuberculosis are added to the coated wells with stimulating antigens (PHA, PPD, ESAT-6 or CFP10) and incubated overnight. The secretion of IFNγ by individual T cells is detected by the addition of monoclonal detection antibody. Streptavidin alkaline phosphatase is added, followed by substrate. Colour development occurs representing the 'footprint' of individual IFNγ-secreting cells.

A.3 Test sample

2-5ml venous blood is collected into a Falcon tube with preservative free sodium heparin (10µl/ml blood) or into a commercial sodium heparin tube (green top). Samples are kept at room temperature and brought to the laboratory in a plastic sample bag to contain any leakage.

A.4 Reagents

Mabtech antibody kit for IFNγ (Mabtech, Nacka, Sweden)

Biorad Alkaline Phosphatase Conjugate Substrate Kit (Biorad, Hercules, CA, USA)

Coating buffer: 0.1M Carbonate-Bicarbonate buffer pH 9.6

Sterile phosphate buffered saline (PBS)

RPMI plus Penicillin/Streptomycin

R10 (RPMI plus 10% Foetal Calf Serum (FCS))

Sterile water for red cell lysis

Ficoll-Histopaque

Foetal Calf Serum (FCS)

A.5 Procedure

A.5.1. Day 1: Coating of plates

Coating procedure is performed in the Class II Biological Safety Cabinet to maintain sterility of plate and cells.

a. Add 100µl of capture antibody (1-DIK; required concentration: 15ug/ml in coating buffer)
b. Cover and seal in cling film and incubate overnight at 4°C or at 37°C for 1½ hours

A.5.2. Day 2: Blocking, preparing cell suspensions, washing and loading of plate

Blocking, preparation of cell suspensions, washing and loading of the plate are performed in Class II biosafety cabinet

a. Discard Coating buffer and wash once with 200µl blocking solution (10% FCS in RPMI).
b. Blot dry on paper towel. Add 200µl blocking solution to each well and incubate for 2 hours at room temperature or 1 hour at 37°C.

A.5.2.1. Separate and wash PBMCs:

a. Heparinised blood is centrifuged at 1500rpm for 10 minutes at 20°C with brake set in the 'low' position.
b. Plasma is then aspirated using a plastic pasteur pipette, taking care not to interrupt the buffy coat layer. Plasma is stored at -80°C.

c. The remaining red and white cells are diluted 1:1 in RPMI and layered onto 5 ml histopaque.

d. The suspension is centrifuged at 2400rpm for 15 minutes with the brake set in the 'Off' position, at 20°C.

e. The mononuclear cell layer is then carefully removed with a plastic pasteur pipette and suspended in 10ml sterile PBS in a universal tube. Remaining supernatant is aspirated and add to sterile PBS/cell suspension.

f. The volume is made up to 20ml with sterile PBS and tube is centrifuged at 1500rpm for 10 minutes at 4°C. (1st wash).

g. After centrifugation, the supernatant is decanted into biocide and the cells resuspended by tapping the tube. 10ml sterile PBS is added to the cell suspension which is mixed and centrifuged as before. (2nd wash).

h. Supernatant is carefully removed and discarded with a plastic Pasteur pipette to just above the cell button.

i. 1ml R10 is added and the suspension mixed by aspirating and expelling 5 times.

A.5.2.2. Red Cell Lysis:

The mononuclear layer (after centrifugation on Ficoll) can be contaminated with red cells particularly in patients with acute disease. If this occurs, the following procedure is performed after the first wash:

a. Resuspend the cells by tapping the tube.

b. Prepare 45ml of R10 in a 50ml centrifuge tube, i.e. 4.5ml FCS + 40.5ml RPMI

c. To the cell suspension add 5ml of sterile water and invert gently 10 - 12 times.

d. Immediately pour cells plus water into 45ml R10 and mix.

e. Centrifuge as before (this takes the place of the second wash).

A.5.2.3. Cell counts:

Using a Nunc round-bottomed cell culture plate, prepare the following wells per sample:

a. To one well for viability testing add 10µl 1.8% NaCl plus 10µl 1% Trypan Blue, add 20µl of cell suspension, mix well.

b. To one well for cell count, add 40µl 2% Glacial Acetic Acid with Methylene Blue add 10µl of cell suspension to make a 1 in 5 dilution.

c. Fill 2 Neubauer counting chambers per patient (1 with viability suspension, 1 with cell count suspension).

d. Use 10x objective to calculate the percentage viability (non-viable cells take up Trypan Blue dye)

e. Use 40x objective to count cells (all mononuclear cells, i.e., lymphocytes and monocytes are counted.

A.5.2.4. Preparation of antigens

Prepare antigen dilutions (dilutions all made in RPMI):

a. PHA (positive control; Sigma Cat. No: L-9134) Require final concentration of 5 µg/ml.

b. PPD (Evans PL00039/0439) Require final concentration of 10µg/ml.

c. ESAT-6 (Lionex Diagnostics and Therapeutics MT ESAT6). Require final concentration of 5µg/ml.

d. CFP10 (Lionex Diagnostics and Therapeutics MT-CFP10). Require final concentration of 2.5µg/ml

e. Add cell to antigen in 2ml round-bottomed NUNC tubes (Cat No.341173). Volumes sufficient for duplicate wells (Table 1).

f. Wash plate 6 times with sterile filtered PBS (in autoclaved squeeze bottle), Dry on paper towel

g. Add cell suspensions as per plate plan (Table 2).

Table 1. Concentration of antigens and cells used for ELISpot assay

	PHA	PPD $3x10^6$/ml	PPD $1x10^6$/ml	ESAT-6	CFP-10	Control
Concentration of cells	$1.0x10^6$/ml	$3x10^6$/ml	$1x10^6$/ml	$3x10^6$/ml	$3x10^6$/ml	$3x10^6$/ml
Dilution of $3x10^6$/ml	100µl + 200µl R10		100µl + 200µl R10*			
Volume of cells	250µl	250µl	250µl	240µl	240µl	200µl
Stimulant	50µl PHA	50µl PPD	50µl PPD	10µl ESAT6	2.4µl CFP-10	
Volume per well	120µl	120µl	120µl	105µl	105µl	100µl
Final concentration - of stimulant	5µg/ml	10µg/ml	10µg/ml	5µg/ml	2.5µg/ml	
Final number of cells/well	100 000	300 000	100 000	300 000	300 000	300 000

*RPMI plus 10% heat-inactivated foetal calf serum

Table 2. Plate Plan

	Control	**Patient 1**	**Patient 2**	**Patient 3**
Columns	1+2	3+4	5+6	7+8
PHA (positive control)	120µl	120µl	120µl	120µl
PPD $3x10^6$/ml	120µl	120µl	120µl	120µl
PPD $1x\ 10^6$/ml	120µl	120µl	120µl	120µl
ESAT-6	105µl	105µl	105µl	105µl
CFP-10	105µl	105µl	105µl	105µl
No antigen control	100µl	100µl	100µl	100µl

The following controls are included:

a. Positive control: Cell suspension + PHA

b. No antigen control: Cell suspension only

c. Background control: Sterile culture medium, i.e., R10

d. Detection antibody control: R10 instead of detection antibody

(Note: Controls c. and d. are not necessary once assay is established.)

Incubate overnight in incubator at 37°C containing 5% CO_2

A.5.3. Day 3 (or 2 if same-day coating protocol was followed)

a. The rest of the assay can now be carried out on the bench. Wash plate 2 times with sterile filtered PBS using multichannel pipette. Wash 6 times with filtered PBS containing 0.05% Tween20. Dry on clean towel

b. Detection antibody: 7-B6-1 (Stock at 1mg/ml). Diluent: 0.5% FCS in filtered PBS (100µl in 20ml PBS). Dilute 1 in 1000. Add 100µl per well.

c. Incubate in room temperature incubator (24°C) for 2½ hours

d. Wash plate 6 times with sterile filtered PBS containing 0.05% Tween20. Dry on paper towel

e. Conjugate: Streptavidin Alkaline Phosphatase. Dilute 1 in 1000 in 0.5% FCS in filtered PBS. Add 100µl per well.

f. Incubate in room temperature incubator (24°C) for 1½ hours

g. Wash plate 6 times with sterile filtered PBS. Dry on paper towel.

h. Substrate: BIO-RAD Alkaline Phosphatase Conjugate Substrate kit Cat No. 170-6432. Prepare sufficient volume of diluted development buffer for number of patients, 1 hour before use and keep at room temperature. Make 1 in 25 dilution of concentrated buffer, e.g., 4.8ml distilled water + 200µl concentrated development buffer, add 50µl each of Solution A and Solution B to 5ml development buffer immediately before use, mix well. Add 100µl/well

i. Incubate 10 - 15 minutes in the dark

j. Stop reaction by washing 4 times in running tap water to stop development of spots. Allow to air dry completely

A.6 Interpretation of results

The number of spots in each well is determined by two observers using a stereomicroscope, the average of the counts of both observers is calculated. The number of spot-forming cells is expressed per million PBMCs.

Expected responses:

PHA: graded from +/- to 5+. If no response = failed assay

PPD: varies from no spots to innumerable depending on exposure or BCG vaccination

Negative control: <10 spots per well

A positive result is indicated by a minimum of 10 spots per well and at least twice the number of spots counted in the negative control. An average of 10 spots per well translates to 33 spot forming cells (SFCs) per million PBMCs. The cut-off for a positive result is 33 SFC's/10^6 PBMCs. An ELISpot assay is reported as positive if either the ESAT-6 or CFP10 wells are positive by these criteria.

A.7 Limitations of procedure

If an individual's response to an antigen is very strong, the spots are innumerable.

A blue haze around the rim of the well can obscure some spots leading to inaccurate counts.

Spots are sometimes not well defined (e.g. blurred or hazy) which can affect the accuracy of the count.

A.8 Optimization of procedure

The assay was relatively simple to set up and optimise. The only significant problem was the need to introduce the red cell lysis step in order to deal with PBMC samples significantly contaminated with red cells. These resulted in hazy, indistinct spots if not lysed (presumably related to red cells preventing close approximation between the membrane and the IFNγ-secreting T cell).

Appendix B. T-SPOT.*TB* ELISpot Assay

The principle of this assay is exactly the same as that of the 'in-house' assay described above. The major differences between the in-house and commercial assays are as follows.

a. Peripheral blood mononuclear cell (PBMC) preparation tubes (CPT tubes, Becton Dickinson) are used (according to the manufacturer's specifications) in place of histopaque centrifugation for the separation of PBMC.
b. T-SPOT.*TB* uses a commercially developed set of two panels of overlapping peptides derived from ESAT-6 and CFP10, rather than the recombinant antigens used in the 'in house' assay.
c. T-SPOT.*TB* includes ELISpot plates pre-coated with the IFNγ capture antibody so that a coating step is not required.
d. T-SPOT.*TB* incorporates use of a detection antibody linked to an enzyme conjugate, obviating the need for a separate step in which avidin-peroxidase enzyme is added.
e. The cut-off value for a positive test as specified by the manufacturer is somewhat less stringent than that applied using the 'in house' assay: a panel is considered positive if the number of spots per well minus the number of spots in the background well is 6 or more (where the background well had 0-5 spots) or if the number of spots per well was greater than or equal to twice the number of spots in the background well (where the background well has 6 or

more spots). A T-SPOT.TB assay is reported positive if either panel of peptides is positive.

The method used is exactly as specified in the manufacturers instructions and is therefore not included here.

Appendix C. Spoligotyping assay

This method is that of Kamerbeek and colleagues(99) which has been adapted and described in a spoligotyping manual (Isogen Biosciences, B.V.). The details of the method as described in the manual are summarised below.

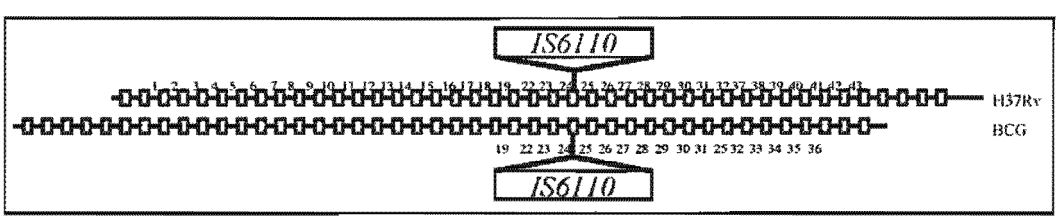
C.1 Purpose

To genotype clinical strains of *Mtb* in order to define the major strain lineages.

C.2 Principle

Spoligotyping is based on DNA polymorphism present at the "Direct Repeat" (DR) locus, present in *Mtb* complex bacteria (Figure C.1). The locus consists of repeated sequences of 36 base pairs (DRs), interspersed by non-repetitive DNA spacers, (each 35 to 41 base pairs in length).

Figure C.1 The DR locus in the genome of *M. tuberculosis* H37Rv and *M. bovis* BCG. The green rectangles depict the 36 base pair DR (from spoligotyping manual, Isogen Biosciences, B.V.)



Polymorphism in the DR region results in the variable presence or absence of each of 43 unique spacers. PCR is used to amplify the 43 spacer regions using primers directed against the DR elements. The presence or absence of the spacers is detected by hybridization of amplified spacer DNA (Figure C.2) to a set of immobilized oligonucleotides, representing each unique spacer sequence. One is able to detect the presence or absence of each of these spacers (Figure C.3). The reverse primer is biotin labeled so that the presence of each spacer can be detected by chemiluminescence following incubation with streptavidin-peroxidase.

Figure C.2 Amplification of spacers within the DR region of *M. tuberculosis* complex bacteria using primers directed against DRs (from spoligotyping manual, Isogen Biosciences, B.V.))

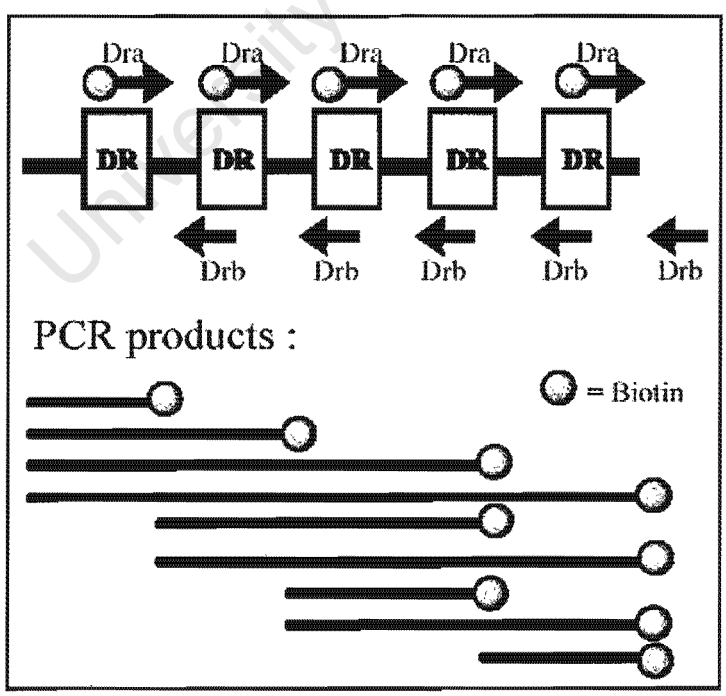
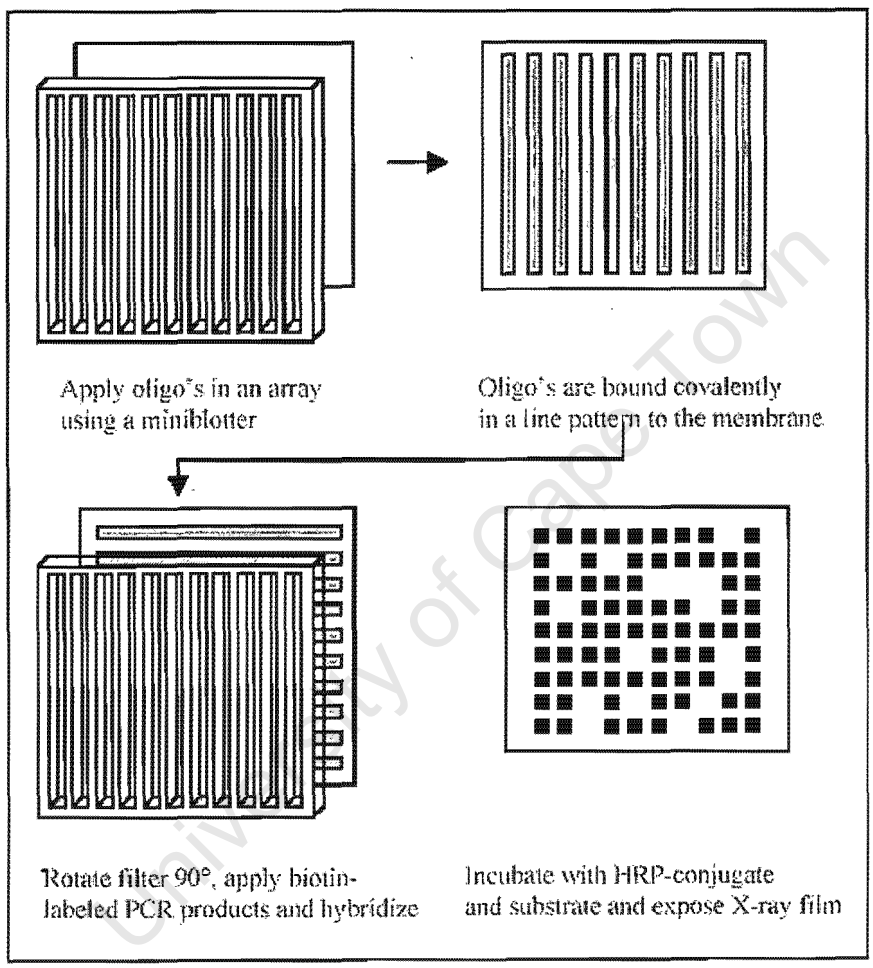


Figure C.3 Detection of amplified products (from spoligotyping manual, Isogen Biosciences, B.V.))



C.3 Reagents

- Spoligtyping kit (Isogen Biosciences) containing two positive controls (*M. tuberculosis* strain H37Rv and *M. bovis* BCG), primer Dra (biotinylated) and primer DRb and one spoligo-membrane.

- HotstarTaq kit (Qiagen 203205 containing hot start Taq polymerase, PCR buffer, $MgCl_2$, solution Q)
- dNTP mix (Fermentas R0192)
- SDS ultrapure (Sigma L6026-250G)
- SSPE 20x concentrate (Sigma S2015-1L)
- Streptavidin peroxidase (Boehringer 1089153)
- ECL Detection reagent (Amersham RPN2105)

C.4 Procedure

(adapted from spoligotyping manual, Isogen)

DNA extraction is performed either by formal extraction of chromosomal DNA or by preparing a crude lysate directly from cultures using heat to disrupt the mycobacterial cell wall.

C.4.1. Chromosomal extraction

a. Use a sterile swab to swab the surface of a Lowenstein-Jensen slope and agitate within a Sarstedt tube containing sterile water
b. Heat 1 hour at 80°C to kill the cells, and cool to room temperature
c. Add 70 µl 10 % SDS and 50µl proteinase K (10 mg/ml) and mix on thermomixer for 1 hour at 60°C, low mode shaking
d. Add 100 ul 5M NaCl, mix thoroughly, followed by 100ul 10% CTAB [N-cetyl-N,N,N,-trimethylammoniumbromide], mix thoroughly
e. Incubate 15 min at 60°C on thermomixer

f. Freeze (15 min at -70°C), thaw and reincubate 60°C 15 mins shaking

g. Cool, add 700 µl of chloroform/isoamyl alcohol (24:1), invert 20 times and centrifuge at room temperature for 10 min, 12,000 *g*

h. Transfer the aqueous supernatant to a fresh tube containing 700 µl cold isopropanol, mix, set at -20°C for at least 30 min

i. Centrifuge 15 min at room temperature

j. Discard the supernatant and wash the pellet with 1 ml of 80% ethanol and centrifuge 5 min at room temperature

k. Discard the supernatant and dry the pellet

l. Redissolve the pellet in 50 µl of 1xTE buffer and store at 4°C until further use

C.4.2. Preparation of lysates from colonies or liquid media

a. Resuspend cells in distilled water in a Sarstedt tube

b. Kill the cells by incubation at 80°C for 1 hour

c. Centrifuge the tube at 13000 rpm for 2 min, discard the supernatant and resuspend the pellet in 500 µl of sterile water

d. Microwave on high for 1 minute

C.4.3. Amplification of spacer DNA by PCR

a. PCR is performed on 10 ng purified chromosomal Mycobacterial DNA or using neat lysates from heat-killed cultures

b. Dilute the DNA samples to the required concentration. Include chromosomal DNA of H37Rv and BCG as positive controls. Use water as a negative control

c. Prepare the master mix: per reaction use -

i. 1µl primer DRa (20 pmol) and 1µl primer DRb

ii. 0.5µl dNTP-mixture (final conc. 0.2 mM each dNTP)

iii. 0.1µl HotSarTaq (0.5 units/reaction)

iv. 2.5µl 10x PCR buffer

v. 5µl Solution Q

vi. 1µl of 25mM MgCl2 (final Mg concentration 2.5mM)

vii. 11.9µl water

d. Add 23µl per PCR tube, add 2 µl DNA template

e. Cycling conditions: 15 min 96°C followed by 25 cycles of 1 min 96°C; 1 min 55°C; 30 sec 72°C; followed by 5 min 72°C (if using crude lysates use 30 cycles)

C.4.4. Hybridization and detection

a. All buffers should be prewarmed. Prepare the following buffers

i. 500ml 2xSSPE/0.1% SDS, 60°C,

ii. 1000ml 2xSSPE/0.5% SDS, 60°C,

iii. 500ml 2xSSPE/0.5% SDS, 42°C,

iv. 500ml 2xSSPE, room temperature.

b. Add 150 µl 2xSSPE/0.1% SDS to PCR products

c. Heat-denature the diluted PCR product for 10 min at 99°C and cool on ice immediately.

d. Wash the membrane for 5 min at 60°C in 250 ml 2xSSPE/0.1% SDS

e. Place the membrane and a supportcushion into the miniblotter, so that the slots are perpendicular to the applied oligonucleotides.

f. Remove residual fluid from the miniblotter by aspiration.

g. Fill the slots with the diluted PCR product (take care not to cause air bubbles) and hybridize for 60 min at 60°C on a horizontal surface

h. Remove the samples from the miniblotter by aspiration and take the membrane from the miniblotter using forceps.

i. Wash the membrane twice in 250 ml 2xSSPE/0.5% SDS for 10 min at 60°C .

j. Place the membrane in a rolling bottle and allow it to cool down to prevent inactivation of the peroxidase.

k. Add 2.5 µl streptavidin-peroxidase conjugate (500U/ml) to 10 ml of 2xSSPE/0.5% SDS, add to rolling bottle with membrane for 45 to 60 min at 42°C.

l. Wash the membrane twice in 250 ml of 2xSSPE/0.5% SDS for 10 min at 42°C.

m. Rinse the membrane twice with 250 ml of 2xSSPE for 5 min at room temperature.

n. For chemiluminiscent detection of hybridizing DNA, incubate the membrane for 1 min in 20 ml ECL detection liquid.

o. Cover the membrane with cling film and expose a light sensitive film to the membrane for 10-40 min.

p. If the signal is too weak or too strong the membrane can be used again directly to expose another film for a shorter or longer period.

C.4.5. Regeneration of the membrane

a. The membrane can be used 10-20 times

b. Wash the membrane twice by incubation in 1% SDS at 80°C for 30 min (the temperature of the buffer is critical!)
c. Wash the membrane in 20 mM EDTA pH 8, for 15 min at room temperature.
d. Store the membrane at 4°C until use (sealed in cling wrap)

C.5 Interpretation of results

The positive controls (H37Rv and BCG) are assessed: H37Rv should have spacers 20-21 and 33-36 absent, whilst BCG should have spacers 3, 9, 16 and 39-43 absent. The 'no template control' should not show the presence of any spacers. If the controls fail the run should be repeated.

The presence or absence of each spacer (1 to 43) for each strain is coded and entered into an excel spreadsheet. This is then submitted to the International Spoligotype Database for interrogation and lineage assignment.

C.6 Limitations of procedure

Spoligotyping is less discriminatory than other methods used for the genotyping of *Mtb* strains (such as IS*6110* RFLP and MIRU-VNTR)(104). It is therefore less useful for epidemiological investigations where it is important to differentiate between closely related but different strains. Deletions of multiple spacers may occur due to a single recombination event, so that closely related strains may have very different spoligotype patterns. It is however highly reproducible, rapid and technically undemanding(198). Since the patterns are easily coded they may be shared and combined to form international databases(167). Moreover, there is a high degree of consistency in the

identification of the major lineages of *Mtb* between spoligotyping, large sequence polymorphism and single nucleotide polymorphism analysis(96).

C.7 Optimization of procedure

There were no major problems encountered in the optimization of the spoligotyping procedure, however the following problems were encountered during the course of the study:

a. A batch of membranes produced by Isogen did not have spacer 32 hybridised onto the membrane. We identified this problem and Isogen produced a new batch of membranes.

b. Initially, problems were encountered in adequately regenerating the membrane. We identified the temperature of the stripping buffer as the critical issue. Subsequently both the buffer and the plastic container in which the stripping takes place were adequately pre-warmed prior to stripping.

Appendix D. Mycobacterial interspersed repetitive unit-variable number of tandem repeat (MIRU-VNTR) genotyping

D.1 Purpose

Second line genotyping of strains of *Mtb* following spoligotyping

D.2 Principle

The genome of *Mtb* contains repetitive 40-100 base pair sequence elements known as 'mycobacterial interspersed repetitive units" (MIRU) which are found as tandem repeats at multiple intergenic loci throughout the chromosome(100). These loci demonstrate hypervariability in the number of repeats, leading to the use of the term "variable number of tandem repeats" (VNTR).

The typing method MIRU-VNTR is based on the detection of the number of tandem repeats present at several different loci. The most commonly used method examines 12 of these loci. MIRU-VNTR is performed by amplifying each of these loci, determining the size of the amplicon and hence the number of repeats at each locus(100). This method is adapted by performing the amplification in a series of multiplex PCR reactions (each

amplifying 3 loci), using fluorescently-labeled primers(101). The size of the fragments is then detected using an automated capillary sequencer. The method used for this study was based on that of Supply et al(101), which we modified for use on an Applied Biosystems ABI3100 Bioanalyser.

D.3 Reagents

- HotstarTaq kit (Qiagen 203205 containing hot start Taq polymerase, PCR buffer, $MgCl_2$, solution Q)
- dNTP mix (Fermentas R0192)
- HiDi formamide (Applied Biosystems)
- Genescan-2500 Rox size standard (Applied Biosystems), or
- MapMarker1000 (Bioventures, Murfreesboro, TN, USA)
- Fluorescent primers for 12 loci (Applied Biosystems) (Table 1)

Table 1. Details of primers used for 12-locus MIRU-VNTR analysis

Mix	Locus	MIRU length	Primers	Label
A	4 (a)	77	GCGCGAGAGCCCGAACTGC	FAM
			GCGCAGCAGAAACGC*CAGC	
	26 (b)	51	TAGGTCTACCGTCGAAATCTGTGAC	
			CATAGGCGACCAGGCGAATAG	HEX
	40 (c)	54	GGGTTGCTGGATGACAACGTGT	NED
			GGGTGATCTCGGCGAAATCAGATA	
B	10 (a)	53	GTTCTTGACCAACTGCAGTCGTCC	
			GCCACCTTGGTGATCAGCTACCT (FAM)	FAM
	16 (b)	53	TCGGTGATCGGGTCCAGTCCAAGTA	
			CCCGTCGTGCAGCCCTGGTAC	HEX
	31 (c)	53	ACTGATTGGCTTCATACGGCTTTA	
			GTGCCGACGTGGTCTTGAT	NED
C	2 (a)	53	TGGACTTGCAGCAATGGACCAACT	
-			TACTCGGACGCCGGCTCAAAAT	FAM
	23 (b)	53	CTGTCGATGGCCGCAACAAAACG	HEX
			AGCTCAACGGGTTCGCCCTTTTGTC	
	39 (c)	53	CGCATCGACAAACTGGAGCCAAAC	
			CGGAAACGTCTACGCCCCACACAT	NED
D	20 (a)	77	TCGGAGAGATGCCCTTCGAGTTAG	FAM
			GGAGACCGCGACCAGGTACTTGTA	
	24 (b)	54	CGACCAAGATGTGCAGGAATACAT	

			GGGCGAGTTGAGCTCACAGAA	HEX
	27 (c)	53	TCGAAAGCCTCTGCGTGCCAGTAA	
			GCGATGTGAGCGTGCCACTCAA	NED

* The MIRU 4 reverse primer was changed to 5_GCG CAG CAG AAA CGC CAG C, as this resulted in more specific bands(199)

D.4 Procedure

D.4.1. DNA extraction

(as per spoligotyping protocol)

D.4.2. Amplification of MIRU loci

a. PCR is performed on 10 ng purified chromosomal Mycobacterial DNA or DNA extracts from lysed bacteria
b. Dilute the DNA samples to the required concentration. Include chromosomal DNA of H37Rv and BCG as positive controls. Use water as a negative control
c. Prepare the master mix (Table 2)
d. To 24µl master mix per PCR tube, add 1 µl DNA template
e. Cycling conditions: Initial denaturing 95°C 15min followed by 30 cycles of denaturing 94°C 1:30min; annealing 59°C 1:30min; extension 72°C 2:00min and final termination 72°C 10min

Table 2. PCR master mixes for MIRU-VNTR

	Mix A (µl)	Mix B (µl)	Mix C (µl)	Mix D (µl)
Taq (5 U/µl)	0.1	0.1	0.1	0.1
Solution Q	5	5	5	5
dNTP (final concentration 0.2mM)	0.5	0.5	0.5	0.5
10x PCR buffer	2.5	2.5	2.5	2.5
Primer (a) F&R	0.2	0.5	0.5	0.5
Primer (b) F&R	0.5	0.5	0.5	0.2
Primer (c) F&R	0.5	0.5	0.5	0.5
$MgCl_2$ (25mM)	1.5	0.5	1	0
Water	11.9	12.4	11.9	12.9

D.4.3. Analysis of fragments

a. Dilute reaction products 1:70 in distilled water

b. For each multiplex reaction, 2µl of diluted product is mixed with 0.2µl Genescan-2500 Rox size standard or 0.2µl MapMarker1000 and 8µl HiDi formamide

c. Denature for 2 minutes at 95°C

d. Run on ABI3100 analyzer for 40 minutes using dye set D

e. Calculate fragment size using GeneScan software (Applied Biosystems)

f. Determine number of MIRU repeats at each locus using table 3:

Table 3. Fragment sizes for corresponding number of MIRU repeats at each locus

No. of repeats	MIRU 02	MIRU 04	MIRU 10	MIRU 16	MIRU 20	MIRU 23	MIRU 24	MIRU 26	MIRU 27	MIRU 31	MIRU 39	MIRU 40
0	402	175	482	565	437	150	395	285	498	492	540	354
1	455	252	537	618	514	200	447	336	551	545	593	408
2	508	329	590	671	591	253	501	387	604	598	646	462
3	561	406	643	724	668	306	555	438	657	651	699	516
4	614	483	696	777	745	359	609	489	710	704	752	570
5	667	560	749	830	822	412	663	540	763	757	805	624
6	720	637	802	883	899	465	717	591	816	810	858	678
7	773	714	855	936	976	518	771	642	869	863	911	732
8	826	791	908	989	1053	571	825	693	922	916	964	786
9	879	868	961	1042	1130	624	879	744	975	969	1017	840
10	932	945	1014	1095	1207	677	933	795	1028	1022	1070	894
11	985	1022	1067	1148	1284	730	987	846	1081	1075	1123	948
12	1038	1099	1120	1201	1361	783	1041	897	1134	1128	1176	1002
13	1091	1176	1173	1254	1438	836	1095	948	1187	1181	1229	1056
14	1144	1253	1226	1307	1515	889	1149	999	1240	1234	1282	1110
15	1197	1330	1279	1360	1592	942	1203	1050	1293	1287	1335	1164

D.5 Interpretation of results

The number of MIRU tandem repeats (allele number) at each locus is expressed as a number from 0-9. The MIRU-VNTR genotype of the strain is then denoted by a 12-digit number representing the allele number at each locus (in numerical order, starting with

locus 4). These are submitted to the International Spoligotype and MIRU-VNTR database.

D.6 Limitations of procedure

MIRU-VNTR is considerably more discriminatory than spoligotyping, for example, we documented 15 different MIRU-VNTR types amongst the W-Beijing strains identified in our collection (all of which have the same spoligotype). This method is therefore suitable for epidemiological investigations as well as to discriminate the major sub-lineages within each spoligotype-defined cluster. However, MIRU-VNTR has recently been shown to correlate relatively poorly with phylogenetic analysis conducted using single nucleotide polymorphism analysis(96), and is therefore probably not sufficiently robust for defining the broader global phylogeny of Mtb. This may relate to the potential for gain or loss of repeats at each locus, leading to the possibility of convergent evolution (broadly different strains acquiring similar numbers of repeats at most loci).

D.7 Optimization of procedure

The method used was essentially that defined by Supply et al(101). Several modifications were made to the published method:

D.7.1. PCR Conditions

a. The MIRU 4 reverse primer was changed to 5_GCG CAG CAG AAA CGC CAG C, as this resulted in more specific bands(199)

b. Final reaction volume was reduced to 25µl (for economy)

c. PCR conditions were changed to improve amplification of longer fragments (annealing and extension times were increased)

d. Primer concentrations were altered in Mix A and Mix D, where there was preferential amplification of shorter fragments (from locus 4 and locus 24) leading to poor amplification of other loci. In order to improve amplification of longer products, the following changes in concentration were made:

   i. In primer mix A, primers for locus 4 were used at 0.16µM (=0.2µl per reaction) vs. 0.4µM (0.5 µl per reaction) for other primers

   ii. In primer mix D. primers for locus 24 were used at 0.16µM (=0.2µl per reaction) vs. 0.4µM (0.5 µl per reaction) for other primers

D.7.2. Genotyping on the ABI 3100

The method of Supply(101) was adapted for use on the ABI3100. This involved initial dilution of products 1 in 70 in water (since this system is more sensitive). A change was also made from Rox2500 size standard to MapMarker1000 size standard, which had more peaks in the relevant range and gave more accurate sizing than Rox2500.

in order to best approximate physiological infection. The rates of growth of *Mtb* strains within macrophages are determined by CFU analysis and the induction of cytokine production measured by ELISA.

E.3 Reagents

E.3.1. *Mtb* culture

- Middlebrook 7H9 broth and 7H11 agar (Difco)
- ADC (albumin, dextrose, catalase) and OADC (oleic acid, albumin, dextrose, catalase) supplement (Becton Dickinson)
- 25ml Univeral screw cap bottles
- 2ml Sarstedt tubes
- Glycerol
- Tween-80

E.3.2. Macrophage isolation and culture

- 50ml conical centrifuge tubes
- 10, 25 ml plastic pipettes
- plastic Pasteur pipettes
- Corning Costar 24 and 48-well tissue culture plates
- Corning long handled cell scrapers
- Corning large tissue culture flasks
- Ficoll paque (GE Healthsciences)

E.5 Procedure

E.5.1. Establishing frozen mycobacterial stocks

a. Grow the strain to late-log phase (O.D.600 of 0.7-1) in Middlebrook 7H9 broth supplemented with ADC, glycerol and Tween-80 at 37°C with gentle shaking (120rpm)
b. Freeze in 1 ml aliquots in final concentration of 15% glycerol (add 500 µl of culture to 500 µl of 30% glycerol in water) in 2ml Sarstedt screw cap tubes
c. Freeze at -80°C
d. After at least 24 hours, thaw to perform CFU analysis

E.5.2. CFU analysis

a. This is performed on frozen strains to establish CFU content as well as on macrophage lysates to determine growth rate of strains)
b. Prepare square CFU agar plates by adding 25-30ml 7H11 agar (with OADC and glycerol) to each square petri dish (pour in laminar flow cabinet). Allow to solidify and cool before refrigerating.
c. Estimate the likely CFU content of sample and prepare plate for dilutions:
    i. place square CFU agar plates (4 strains can be plated on each plate) in 37°C incubator to warm
    ii. use 96-well round bottom, sterile tissue culture plate to perform dilutions

iii. add 45µl of sterile water to appropriate wells (e.g. to determine CFU content of frozen stock, add water to the first two wells of rows A, B, C, D, E, F of a 96-well plate)

iv. vortex thawed *Mtb* stock (making sure that lid of Sarstedt is tightly closed and vortex is placed within biosafety cabinet)

v. add 5µl of *Mtb* stock to each duplicate well in row A, mix thoroughly (at least 5 times with 40µl volume)

vi. transfer 5µl to row B for each duplicate, mix thoroughly

vii. change tips, transfer 5µl to row C, mix as before

viii. repeat for required dilution factor (e.g. for frozen stocks need to dilute to 10^{-6}), changing tips between each dilution step

ix. carefully transfer 10µl from relevant dilutions (e.g. if plating out frozen stock use 10^{-4}, 10^{-5} and 10^{-6} dilutions) in duplicate to appropriate squares on 7H11 plate

x. incubate at 37°C (CO_2 not needed) for 2-4 weeks. Check daily after 1 week

xi. count colonies under 10x magnification (using stereomicroscope) when colonies easily visible

E.5.3. Monocyte-derived macrophage isolation and maturation

a. Obtain buffy coats from SA Blood Transfusion Services. These need to be requested the previous day.

b. Dilute 12-15ml buffy coat to 35ml with RPMI in as many tubes as required

c. Layer each tube on to 10ml Ficoll

d. Spin at 2400rpm for 20 minutes (brake off)

e. Aspirate PBMC layer with plastic Pasteur pipette and collect into 2 tubes of 50 ml RPMI per Ficoll tube

f. Wash (spin at 1500rpm for 10 minutes, decant supernatant, resuspend in 50ml RPMI) and pool into 1 tube

g. Wash again and resuspend in 50ml RPMI

h. Count cells (expect 600-800 x 106 PBMC per buffy coat)

i. Adhere in large tissue culture flasks ($175cm_2$) at 300 x 10^6 PBMC per 25 ml RPMI per flask for 2 hours at 37°C in CO_2

j. Pipette out non-adherent cells with 10ml pipette

k. Carefully add 10ml warm RPMI to the flask (squirt on upper wall, not on cells). Lay flask flat and gently tilt. Stand it up again and remove the RPMI.

l. Repeat wash 2 more times

m. Add 10ml cold PBS and refrigerate flask for 20 minutes

n. Using long-handled scraper, scrape the monocytes and pool in 50ml tube in PBS

o. Spin, resuspend in 10ml R10

p. Count, resuspend to concentration of 1 x 10^6/ml in R10

q. Plate out (1 x 10^6 per well in 24-well flat bottom tissue culture plate)

r. Incubate for 6 days at 37°C in CO_2 incubator, after which stage macrophages may be infected with *Mtb*. Prior to infection, ensure that macrophages look healthy by visualizing under inverted microscope (look for plump cells, some of which will have dendritic processes).

E.5.4. Macrophages infection and lysis

E.5.4.1. Infection of macrophages

a. Thaw frozen stocks
b. Prepare dilution of frozen stock in warm R10 (based on prior CFU analysis) so that final CFU will be 1 x 10^6/ml. If infection is performed for growth assays, non-inactivated serum should be used when making up R10, to enhance opsonization and phagocytosis.
c. Carefully aspirate of media from relevant wells of tissue culture plate and add 1ml of diluted strains to each well
d. Always include H37Rv as positive control and R10 alone as a negative control
e. Incubate (37°C in CO_2) as required
f. If CFU analysis is being performed, wash after 4 hours
    i. Aspirate supernatant
    ii. Gently wash 5 times with warm R10
    iii. Re-incubate for relevant time period

E.5.4.2. Lysis of macrophages

(this is performed in order to release *Mtb* to determine growth rate in macrophages)

i. At relevant time points aspirate supernatant gently
ii. Add 1ml of 0.1% SDS to lyse macrophages
iii. Mix thoroughly with pipette until homogeneous
iv. Leave for 10 minutes to allow complete lysis to occur
v. Immediately perform dilutions and plate out for CFU as above

E.5.5. Cytokine analysis

a. At the relevant time points, aspirate supernatant from cultures

b. Filter through 0.22µm filter and aliquot into Sarstedt tube

c. Freeze at -80°C until use

d. ELISA (IL-12p40, TNF, IL-1β, IL-10)

e. R&D DuoSet ELISA systems used exactly as per manufacturer's protocol

f. or IL-12p40, IL-10 use neat supernatants, for TNF use 1:10 and for IL-1 β use 1:20 dilutions

g. For ELISA reagents and dilutions see Table 1.

Table 1. Summary of ELISA reagents and relevant dilutions

	capture Ab dilution	wash buffer	block buffer	reagent diluent	detection Ab dilution	streptavidin-HRP dilution	substrate solution	stop solution	top standard
IL12p40	61.1ul in 11ml PBS per plate	0.05% Tween20 in PBS*	1% BSA in PBS with 0.05%NaN_3‡ (33ml per plate)	0.1%BSA, 0.05% Tween20 in TBS	61.1ul in 11ml RD	55ul in 11ml RD	1:1 Colour Reagents A&B	2N H_2SO_4	(at 40ng/ml) for 2000pg/ml add 25ul to 475ul RD
TNF	61.1ul in 11ml PBS per plate	0.05% Tween20 in PBS	33ml RD per plate	1% BSA in PBS	61.1ul in 11ml RD	55ul in 11ml RD	1:1 Colour Reagents A&B	2N H_2SO_4	(at 310ng/ml) for 1000pg/ml add 3.3ul to 997ul RD

* 500ul Tween20 per litre PBS

† 9g NaCl, 500ul Tween20 per litre water

‡ 165ul 10% NaN3 per 33ml 1% BSA in PBS

E.6 Interpretation of results

CFU analysis: the growth of strains in human MDM is calculated, expecting roughly one doubling every 1 to 2 days.

ELISA: negative control should have low to undetectable levels (<100pg/ml) of IL-12p40 and IL-1β, with low levels (<500pg/ml) of TNF and IL-10. H37Rv and CDC1551 should induce similar levels of each cytokine. The levels of cytokines induced by clinical strains vary.

E.7 Limitations of procedure

Preparation of stock cultures: Growing clinical strains of *Mtb* is not uncomplicated. Clinical strains grow at very different rates, some growing very slowly so that the 7H9 media is exhausted prior to achieving OD of 0.7-1. For these strains, subculture during early log phase to new media should be performed to enhance growth. Clumping is also a problem. The addition of Tween-80 to the media is helpful. In cases where visible clumping occurs, tubes should be briefly centrifuged and the clear media at the top of the tube used to inoculate a new tube of media. Media should always be prewarmed prior to subculturing.

Monocyte isolation and maturation: There is variation from donor to donor in the quantity and quality of monocytes obtained. Where small numbers of monocytes are obtained, or where there is clumping or fibrinous clots formation, the monocytes should

be discarded. If more or less confluent monolayers of MDM are not obtained after 6 days maturation, these should not be used for infection with *Mtb*.

This is an *in vitro* model which, in many ways does not represent physiological infection, e.g., cells are infected in isolation, with none of the other components of the immune system (e.g. soluble mediators) present.

E.8 Optimization of procedure

The major optimization related to the growth of clinical strains of *Mtb* as noted above (see Limitations). The monocyte maturation and infection was an established assay which had previously been by described by Wilkinson et al(182).

Appendix F. Large sequence polymorphism analysis

F.1 Purpose

To assign strains of *Mtb* to distinct phylogenetic lineages (in particular, to define the major sub-lineages of W-Beijing strains circulating in Cape Town and to confirm that the LAM3/F11 family of strains is monophyletic).

F.2 Principle

The method used is based on that described by Tsolaki et al(109) and Gagneux et al(155). Since modern strains of *Mtb* rarely undergo horizontal exchange of genetic material, the genome evolves primarily by deletion and duplication(93). The implication is that if a region of DNA is deleted from the genome of a strain of *Mtb*, this region can not be re-acquired and all descendants of this strain (forming a strain lineage) will have the identical deletion. There are therefore characteristic deletions which define the major lineages and sub-lineages of *Mtb*(94). These regions are known as "regions of difference" (RD). The deletions can be detected by large sequence polymorphism (LSP) analysis.

The method used in this study relies on the use of real-time PCR to simultaneously detect amplification of a conserved region of DNA (encoding the 16S ribosomal subunit) and a region within the deleted region of interest. The amplification of the 16S fragment acts as an internal positive amplification control, whilst the failure to amplify the deleted region implies that this particular strain has the relevant deletion. The presence of product is detected using the presence of probes specific for the amplified products on the Applied Biosystems 7000 sequence detection system (TaqMan), as described in Chapter 6.

F.3 Reagents

- Primers and probes (for RD181, RD150 and RD142 as described by Tsolaki(109); for RD761 these were designed using Primer Express Version 2.0 [Applied Biosystems], Table 1)
- Taqman Universal PCR Master Mix (Applied Biosystems)
- Water for molecular biology (Sigma)
- HotstarTaq kit (Qiagen 203205 containing hot start Taq polymerase, PCR buffer, $MgCl_2$, solution Q)
- dNTP mix (Fermentas R0192)

Table 1. Details of LSP regions and relevant primers and probes

LSP or control	Start of RD*	End of RD	Size	Genes deleted	L primer	R primer	Probe or product sizes
RD142	1332182	1335033	2,851	Rv1189-Rv1192	CGG CAA GAT CAC AAA AAT CCA	CCC GTA ACG GGT CCA ATG T	FAM -TCT TAG TGC AGC CTT C
RD150	1896862	1899349	2,487	Rv1671-Rv1674c	TGC TCG GTG GGC AAC TG	CGG CAC AGC GAG ACG AA	FAM-AAC GAC ATC CAC GCA TT
RD181	2535429	2536140	711	Rv2262c-Rv2263	CGCAACGGCCGCGGTGAACTCT	CGGGCGGCTGCGGGAACCTT	Expected fragment sizes: H37Rv 1712bp, if deleted 100bp
RD761	1502787	1503881	1,094	Rv1334-Rv1336	GCCGGCGTGCTCAATGCTCAG	CCTAGGCCGGCGACGAAGTGC	Expected fragment sizes: H37Rv 1371bp, if deleted 277bp
16S rRNA	N/A	N/A	N/A	N/a	CGT TCC CGG GCC TTG TAC	CGG GTG TTA CCG ACT TTC ATG	VIC-CAC CGC CCG TCA CG

*Region of Difference

F.4 Test samples

Extracted chromosomal DNA (see Appendix B) from strains of *Mtb* from children presenting to Red Cross Children's Hospital (Chapter 6)

F.5 Procedure

F.5.1. TaqMan real-time PCR (for RD150 and RD142)

F.5.1.1. Master mix (per reaction)

- 1µl genomic DNA
- 0.25µl primer RD-F
- 0.25µl primer RD-R
- 0.5µl probe RD
- 0.25µl primer 16S-F
- 0.25µl primer 16S-R
- 0.5µl probe 16S
- 12.5µl Taqman 2x PCR Master Mix
- 9.5µl water

F.5.1.2. Reaction conditions (Universal cycling conditions for Taqman):

- 50°C 2 minutes then 95°C 10 minutes
- followed by 40 cycles of: 95°C 15 seconds and 60°C 1 minute

F.5.2. PCR for RD181 and RD761

F.5.2.1. Master mix (per reaction)

- 0.1µl HotStarTaq
- 5µl Solution Q
- 0.5µl dNTP mix
- 2.5µl buffer
- 1µl primer RD-F
- 1µl primer RD-R
- 1µl genomic DNA
- 1.5µl $MgCl_2$ (25mM)
- 12.4µl water

F.5.2.2. Reaction conditions:

- Initial denaturing 95°C 15 min followed by 35 cycles of denaturing 95°C 30sec; annealing 72°C 1:30 min; extension 72°C (for RD181) or 60°C (for RD761) 1:30min and final termination 72°C 10 min

F.5.2.3. Agarose gel electrophoresis

- 1% agarose, λpst size marker (see Figure 2, Chapter 6)

F.6 Interpretation of results

Real time PCR detection of LSP: For each strain, product should be clearly detected for the 16S positive control. If there is no 16S product this constitutes a failed reaction. The water control should show no product except in cycles 38-40 where some amplification may be detected. The absence of product for the relevant RD in the presence of product for 16S of the same strain is interpreted as showing that the relevant deletion has occurred.

Agarose gel electrophoresis: Water control – no product, H37Rv positive control 1712bp product (for RD181 PCR) or 1371bp product (for RD761 PCR). Strains with RD181 deleted – 100bp product with RD181 PCR; strains with RD761 deleted – 277bp product with RD761 PCR.

F.7 Limitations of procedure

The presence of overlapping but distinct deletions will show an identical pattern using the real-time PCR detection system. However this is likely to be a relatively rare occurrence and is unlikely to have significantly influenced results.

F.8 Optimization of procedure

The Taqman system is remarkably robust and required no particular optimization relying on universal cycling conditions. Both PCR reactions (for RD181 and RD761) were uncomplicated and worked using the initial set of reaction conditions as described.

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