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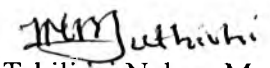
PHENOTYPIC AND GENOTYPIC CHARACTERISATION OF *MYCOBACTERIUM TUBERCULOSIS* STRAINS IN RELATION TO THE TRANSMISSION OF TUBERCULOSIS IN SOUTH AFRICAN MINES

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A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy.

DECLARATION

I declare that this thesis is my own, unaided work. It is being submitted for the degree of Doctor of Philosophy in the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.



Tshilidzi Nelcus Muthivhi

ABSTRACT

Background

The prevalence of tuberculosis in South African miners is substantially higher than that of in the general population. Through exposure to dust which leads to different degrees of silicosis, and by working in enclosed spaces where coughed out bacilli can survive in droplet nuclei and be inhaled by other workers, miners are especially prone to to become infected with *M. tuberculosis* and develop the disease. It is not only the working conditions which promote transmission of *M. tuberculosis*, but the living conditions as well. Most miners live and sleep in rooms shared by up to eight other men, which increases the opportunity for transmission, leading to both primary and reinfection tuberculosis. A serious complication of tuberculosis in the mines has been the emergence of multidrug-resistant (MDR) strains which fortunately, at this stage, is still at a relatively low level. In addition to increased risk of becoming infected with tubercle bacilli, miners, because of mine-related stress factors and the common occurrence of intercurrent viral infection which depress cell-mediated immunity may also experience reactivation of dormant tuberculosis more commonly in the mining environment. This process will be further enhanced by the rapidly increasing incidence of HIV disease.

In order to understand and devise strategies for the control of tuberculosis in the miners and other high- incidence populations, it would be useful to determine the relative frequencies of primary infection or recently-acquired reinfection and reactivation tuberculosis and to identify risk factors associated with transmission and drug resistant. Such, knowledge could

lead to the optimal use of scarce health care resources and prioritisation of interventions for tuberculosis control.

Objectives

The objectives of this study were: 1) to determine by IS6110-based DNA fingerprinting, the extent of homogeneity/ heterogeneity i.e RFLP polymorphism which existed amongst approximately 50% of *M. tuberculosis* isolates which were available for the study from the Freegold Health Region mines during a six-month period in 1994. 2) to similarly determine the population structure of drug-resistant *M. tuberculosis* isolates in the Freegold Health Region mines during two study periods in 1994 and 1995-1977; 3) to identify possible risk factors which may be associated with transmission of *M. tuberculosis* ; 4) to determine in mouse model, possible differences in virulence between three MDR strains within the highly prevalent Family 1 isolates and differences between those isolates and an H37Rv international strain of *M. tuberculosis* ; 5) to identify a protein or proteins in culture filtrates of *M. tuberculosis* of varying degree of virulence in the mouse model obtained from Colorado State University, USA that may be linked to virulence.

Methods

The insertion sequence IS6110- based RFLP analysis of *M. tuberculosis* was used to characterise all the isolates in these studies. Possible risk factors relating to geographic location of patients, working place and treatment history in clustered and non-clustered patients for isolates with fingerprints of more than five bands were assessed statistically by

univariate and multiple logistic regression analysis.

Virulence was assessed in a pilot study according to growth rates in the lungs of intravenously challenged BALB/c mice and survival times in these experimental animals

Profiles of culture filtrate proteins prepared by two dimensional gel electrophoresis were analysed to establish possible associations between specific proteins and degrees of virulence of strains previously determined in animals, and between these two parameters and the RFLP patterns of the strains.

Results

One hundred and thirty-nine of the patients (72%) in 1994 study period had isolates with unique RFLP patterns, while 78 (40.4%) had isolates that belonged to 24 clusters of identical IS6110-based RFLP patterns designated identical band pattern (IBP) strains. Based on clones, defined as clusters comprising isolates with identical RFLP band patterns but exhibiting more than five bands per isolate, there were 65 isolates in 20 clones (33.7%). The 20 clones consisted of 8 clusters of two isolates, 7 clusters of three isolates, 2 clusters of five isolates and 1 cluster each of four, six and eight isolates. Based on strict criteria of IS6110-based clones-based clustering, an estimated minimum of 26.2% of tuberculosis cases were due to recent transmission within the mining community.

Miners from the Free State were associated with a significantly higher risk of being in a clone-based cluster (P=0.01), while the Eastern Cape as a source of miners was associated with a significantly lower risk of being in clusters (P=0.02) compared to other provinces. Patients who had failed treatment at entry to the study were more likely to be in cluster (adjusted odds ratio 2.38, P=0.04, 95% confidence interval (1.04-5.40).

In several instances, epidemiological links between patients whose isolates were found to be in clone based clusters were apparent. Approximately 18% of all evaluable clone-based clustered cases could be linked to a mine shaft and 7.8% cases originated from the same village, strongly suggesting a common source of recent infection. The corresponding figure for sharing a mine or a geographic region was 53.2% and 62.5% respectively.

Many strains with different RFLP patterns showed common band patterns. These strains were therefore grouped into RFLP families, such that all the members of each family were identical or differed by one to three bands and showed ≥ 80% homology based on RFLP band patterns. This yielded 23 families which varied in size from 2 to 50 per family. The percentage transmission over time, based on RFLP family clusters was calculated to be 69.9% while 81.2% of isolates could be grouped in families. Twenty percent come from the same village while approximately 40% shared a mine shaft and 80% a mine or a geographical region respectively.

Two major families (41.2% of total isolates) of closely related isolates were identified in

1994 (Family 1 and Family 2). The strains in each of the families shared the majority of their IS6110 DNA-containing restriction fragments. These two RFLP families contained 7 and 3 clones respectively, and 8, 6 and 5 isolates were found in the largest clone-based clusters, providing evidence of micro-epidemics. It is proposed that members of families which did not fall within a clone as defined in this study, are strains that have become endemic in the mines and over a period, during which minor genetic changes in strains could have occurred . These strains represent either recent transmission or transmission in the past followed by recent reactivation while in the mine.

There was an equal risk of infection with a defined *M. tuberculosis* clone for HIV-seropositive and HIV-seronegative individuals. There was no evidence of an RFLP-identifiable clone infecting HIV-positive rather than HIV-negative patients which could have been associated with decreased virulence.

The finding that retreatment patients were significantly associated with clustering has important implications. These patients are likely to expectorate large numbers of tubercle bacilli for prolonged periods of time and constitute a high priority target group for future preventive planning.

Southern blot analysis of drug- resistant *M. tuberculosis* strains showed considerable diversity in banding patterns. The 19 drug-resistant isolates from the 1994 period revealed eighteen patterns while the 26 MDR strains isolated during 1995-1997 study period exhibited

sixteen IS6110 banding patterns. Three and 12 isolates from 19 drug-resistant and 26 MDR strains respectively, showed identical or near identical patterns. At least four MDR clones and another INH mono-resistant clone were identified in the mines and , based on identical band patterns, 12 possible transmission linkages amongst drug-resistant isolates could be demonstrated.

M. tuberculosis clinical isolates with defined *in vivo* growth characteristics in mouse lungs were examined for their IS6110-based RFLP patterns and protein profiles. Of the 31 clinical isolates subjected to RFLP analysis, 23, 5, and 3 were previously grouped (in terms of virulence) as rapid, moderate and slow growing strains in mouse lungs, respectively. In all, the number of the IS6110 copies per isolate ranged from 1 to 20. Among the 23 clinical isolates displaying the rapid *in vivo* growth phenotype, 14 different IS6110 RFLP patterns were observed, while all strains categorised as having slow or moderate *in vivo* growth phenotypes showed different RFLP patterns.

Protein profiles of culture filtrates demonstrated only minor differences between individual strains. However, one basic protein at approximately 12 kDa was notable in that it was abundant in eight highly virulent strains and weakly expressed in further six strains. Among the latter group, one strain (CSU 24), categorised as of low virulence while the other five were classified as highly virulent). This protein was however, absent from number of highly virulent strains, including H37Rv. Other proteins of 55, 35, and 16 kDa appeared to be differentially expressed by the clinical isolates. All moderately virulent strains (five) and two

out of three weakly virulent strains (CSU 24 was the only exception) failed to express the 12 kDa protein.

Conclusions

In the six mines studied, evidence is presented that recently transmitted tuberculosis may account for approximately 37% of the cases of active tuberculosis and approximately 42% of drug- resistant cases. Recent transmission of tuberculosis, and not only reactivation of dormant disease, therefore contributes substantially to the high incidence of tuberculosis in the mines. The findings that only 19% and 6.5% of IBP clusters could be linked by proximity in the work place and village respectively, suggest that most instances of transmission may occur on a casual basis without evidence of prolonged close contact. However, clusters could also have been formed following by repeated cycles of transmission and therefore may not necessarily indicate recent transmission.

The study revealed that more than 1/3 of tuberculosis patients in the six mines were infected by two RFLP families of *M. tuberculosis*. These genotypes became prevalent in the mines through the expansion of clones generated either cyclically over a period of time or as a result of recent spread. There as no evidence that this expansion was related to the recent escalation of HIV/AIDS.

Although many strains in the mines are closely related, there is sufficient diversity to make RFLP analysis, using IS6110, useful for the detection of clustering of human isolates of

M. tuberculosis. It is therefore a technique that holds great promise for the monitoring of the transmission of tuberculosis in the mines and thereby contributing substantially to improved disease control.

There were several limitations applicable to the studies presented in this thesis. The most important of these are the fact that only about 50% of isolates during 1994 period were available for study and that the duration of that study was only six months. These shortcomings resulted in an underestimate of recent transmission rates in the mining communities studied.

It was also unfortunate that of isolates from clone 3 in RFLP family 1 and other clones in this family and RFLP family 2 were not available to the author while he was in USA for including in his culture filtrate studies which found the 12kDa protein to be prominent in several highly transmissible international strains.

ACKNOWLEDGMENTS

It is with sincere appreciation that the following persons are acknowledged:

Professor K.P. Klugman, my early supervisor for having recommended me for this project and his excellent guidance which made my work most pleasant.

Professor H.J. Koornhof, my co-supervisor and more recently sole supervisor for devoting so much of his precious time guiding and advising me in this investigation. This study would not have been completed without his encouragement and understanding. It was a valuable experience to have a supervisor with such a broad understanding and experience in the field of tuberculosis.

Dr P.B. Fourie the Director of the National Tuberculosis Research Program, Pretoria for his support and for encouragement and the use of laboratory facilities required for this study.

Drs P. Brennan and J. Belisle for the six months I spent in the Mycobacteriology Research Laboratories, Department of Microbiology, Colorado State University, Fort Collins laboratory. Experiments relating to culture filtrate proteins of virulent strains with high transmissibility potential were performed when I visited this laboratory.

The Freegold Health Region(FHR) management and in particular, Dr G. Churchyard who

is in charge of a detailed epidemiological study on the transmission of tuberculosis in FHR for the supply of research materials and access to information on the location of patients with tuberculosis in the FHR mines.

The South African Institute for Medical Research (SAIMR) for permission to perform this study and access to their laboratory facilities.

Mr C. Constaninou for his assistance in the use of Gelcompare software

Dr J. Levin and Ms S. Moti for their help with statistical analysis

The SAIMR, Medical Research Council and the University of the Witwatersrand for funding the research project.

My wife Linda, my daughter Rendani and my son Khuliso for their patience and endless support.

My mother, my brothers and sister for their confidence in my ability to complete this study.

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1: GENERAL INTRODUCTION

1.1. History of tuberculosis

Tuberculosis probably occurred as an endemic disease among animals long before it infected human (Steele and Ranney, 1958). *Mycobacterium bovis* was the most likely species involved, and the first human infections may have been with *M. bovis*. Since *M. tuberculosis* infects all primates species it is also possible that this species existed in subhuman primates before it became established in human populations.

Tuberculosis probably occurred as a sporadic and unimportant disease of humans in its early history. Epidemic spread began slowly with the increasing population density. This spread and the selection pressure it exerted have occurred at different times around the globe. The epidemic slowly expanded worldwide because of infected Europeans travelling to and colonising distant regions (Diamond 1992). In the 1700s and the 1800s tuberculosis prevalence peaked in Western Europe and the United States and was the largest cause of death (Bloom and Murray 1992). One to two centuries later, tuberculosis had spread in full force to Eastern Europe, Asia, Africa, and South America (Daniel *et al.* 1994).

Donald, (University of Stellenbosch Medical School, Cape Town) related some intriguing concepts on the natural history and epidemiology of tuberculosis in South Africa (Donald 1997). From a pan-Africa perspective, tuberculosis is well documented to have been present in Egypt from as far as back as 3000 BC. However, in spite of the long established shipping links between central and eastern Africa and Asia, there is scant evidence that tuberculosis ever became established along the east coast of Africa. Reports by many reliable observers show that

it is unlikely that tuberculosis occurred to any great extent among indigenous peoples of South Africa but was rather established following the colonisation of Africa by the European settlers and then the rapid urbanisation that followed (Livingstone 1857). Thus colonisation introduced sources of infection and caused rapid urbanisation for purposes of commence and trade. Besides the explosion of tuberculosis in indigenous peoples, the turn of the century saw a large influx of tuberculosis sufferers from Europe to South Africa. By the 1920s, tuberculosis was showing endemic features in indigenous rural population of South Africa and beyond. About that time, tuberculosis was recognised as a highly common health problem between the black and coloured peoples of South Africa.

National notification commenced in 1921 in South Africa, and an incidence of 43/100 000 cases was observed to rise to 365/100 000 in 1958, which declined to 162/100 000 in 1986, mirroring global trends at that time, before rising again to 221/100 000 in 1993.These figures do not include the independent homelands. An exceptionally high incidence has been recorded among the coloured population of the Western Cape Province in 1993. Various rates up to as high as 713/1000 000 were proposed for this group compared to the national incidence of 225/100 000 (Donald 1997).

Although the infectious nature of tuberculosis was established by Villemin around 1865, the protean nature of its clinical manifestations delayed understanding of the disease until Koch's discovery of the causative agent in 1882. Koch regularly found the organism associated with the clinical disease, isolated it in pure culture, reproduced the disease in animals, and recovered the bacillus in pure culture from the experimental animals (Koch 1932).

1.2. The burden of tuberculosis in Africa

1.2.1. General aspects

In their 1997 reports on the tuberculosis epidemic and on anti-tuberculosis drug resistance in the world, WHO paints a bleak picture of the global failure of health service providers to deal with the burden of tuberculosis (WHO, 1997). In the 216 reporting member countries of the WHO, representing a total population of 5.72 billion, there were an estimated 7.4 million new cases of tuberculosis in 1995. This represents a rate of 130 cases among every 100 000 persons.

In Africa the case rate was reported 216 per 100 000 in 1997. The 11 countries of the southern Africa subregion contribute approximately 275 000 cases every year to the total case load in Africa. Almost half of these come from South Africa. In an analysis of tuberculosis trends and the impact of HIV infection on the situation in the subregion, the National Tuberculosis Research Programme of the MRC in South Africa estimates that by 2001 the smear positive case rate would have increased from an estimated 198 per 100 000 for the region as a whole in 1997, to 681 per 100 000 if tuberculosis control efforts are not optimised (WHO 1997). To aggravate the situation, 69% of these cases would be directly attributable to HIV-infection.

A serious complication of the tuberculosis problem in the world, including southern Africa has been the emergence of multidrug-resistant (MDR) strains of the organism causing the disease. Patients infected with MDR require prolonged chemotherapy with very expensive medication that will at best cures only half of them. Such treatments cost at least ten times as much as the cost of curing an ordinary tuberculosis patient infected with drug-sensitive bacteria. Very few countries can afford this additional burden.

In order to determine the magnitude of the MDR problem in southern Africa, and the implications for National Tuberculosis Programmes (NTPs), surveys are being conducted in various countries as part of the activities of the WHO/IUATLD Global Working Group on tuberculosis Drug Resistance Surveillance (WHO, 1997). To date, information is available for four countries in Southern Africa: Botswana, Lesotho, South Africa, and Swaziland.

Results confirmed that *initial* resistance to first line drugs is relatively low in southern Africa compared to some other regions in Africa and Asia where the problem is up to five times more common. Resistance rates range between 4% and 12% for isoniazid, and between 4% and 7% for streptomycin. For rifampicin it is 1% and for ethambutol 1%; MDR is fortunately still low at 1%, suggesting that resistance strains are uncommonly transmitted from person to person. On the other hand, rates for *acquired* resistance, that is resistance which has arisen in patients previously inadequately treated for tuberculosis, are at least three times higher than in patients not previously exposed to anti-tuberculosis medications. The high rates of an acquired resistance point to a failure of control programmes to effectively manage case-holding and treatment adherence.

1.2.2. The South African situation

1.2.2.1. General aspects

South Africa is burdened by one of the worst tuberculosis epidemics in the world, with disease rates more than double those observed in other developing countries and up to 60 times higher than those currently seen in the USA or Western Europe (WHO 1997). In 1998, the MRC National Tuberculosis Programme estimated that the country had 195 776 cases (less than 55% reported), or 446 per 100 000 of the total population. Of these, 35.8% (73 679 cases) were also infected with HIV. The Western Cape Province is the worst affected at the moment but the epidemic is now growing fastest in Kwazulu-Natal and in the central Provinces (Table1.1). Although South Africa has lagged behind other African countries in terms of time and initially incidence (probably because of geographical, social and political barriers), the HIV epidemic has increased rapidly and exponentially during the last six years in South Africa which currently has one of the highest incidence figures of HIV/AIDS in the world.

Table 1.1. Estimated incidences of tuberculosis according to HIV infection status in South Africa

Provinces	Smear -positive cases/100 000 (Reported cases)	Smear- negative cases/100 000 (Unreported cases)	Proportion of HIV-positive Tuberculosis cases
Eastern Cape	270	329	30.1%
Free State	150	183	41.8%
Gauteng	201	245	34.9%
KwaZulu-Natal	214	261	54.7%
Mpumalanga	152	185	49.2%
Northern Cape	183	223	23.3%
Northern Province	140	171	26.4%
North West	144	176	35.6%
Western Cape	302	369	21.7%
South Africa	201	245	37.7%

Source: National Tuberculosis Research Program, Medical Research Council, Pretoria, 1998

Number based on 1998 estimates

The tuberculosis problem in South Africa should be viewed in the context of historical neglect and poor management systems, compounded by the legacy of fragmented health services. Prior to the introduction of the tuberculosis register in 1995 cure rates were unknown, and consequently control efforts could not challenge poor performance. The implication of this failure is evident from the fact that in 1997 a cure rate of only 54% could be recorded, with the consequence of continued high rates of transmission and drug resistance in the country.

Tuberculosis was declared a top health priority by the Department of Health in November 1996 and National Health Minister Zuma committed her Department to implementing a new control programme based on the directly observed therapy (DOTS) strategy of the World Health Organisation (Department of Health 1996). The pace and extent of implementation of the programme have, however, proved to be less than optimal. Estimates by the MRC National Tuberculosis Programme indicate that current trends in the epidemic will continue unless effective control is achieved, resulting in 3.5 million new cases of tuberculosis over the next decade and at least 90 000 patients dying (WHO 1997). The financial implications are staggering: Given that more than US$100 million is spent annually on tuberculosis in South Africa, in excess of US$3 billion would be required over the next ten years if current increases in tuberculosis rates are allowed to continue unabated. On the other hand, significant reductions in transmission of HIV infection together with effective tuberculosis control would mean a turnaround in the tuberculosis epidemic by the year 2003. At least 1.7 million tuberculosis cases will be prevented and more than US$400 million would be saved.

1.2.2.2. Situation in the South African gold mining industry

Tuberculosis has long been recognised as an important health hazard in the South African gold mining industry and has been notified since 1911(Watkins-Pitchford 1927). The incidence of tuberculosis in this industry had more than doubled since the advent of the HIV epidemic, and is currently estimated to be in the order of 2 000 per 100 000 population per year (Churchyard *et al.* 1999; Murray *et al.* 1999). Among the South African gold miners with tuberculosis, the prevalence of HIV infection has increased rapidly, to approximately 50% of all cases (Churchyard *et al.* 1999).

Gold miners are exposed to silica-containing dust, and silicosis has been shown to be a strong risk factor for overt tuberculosis (Cowie 1994; Snider 1978). It is likely that the high tuberculosis incidence in miners preceding the HIV epidemic was largely due to the effect of silica exposure which poses a risk, even in miners who do not show evidence of silicosis (Murray *et al.* 1996). Silicosis prevalence in South African miners has been shown to be high: in an autopsy-based study of miners dying of trauma, Murray *et al.* showed that the prevalence of silicosis increased from 9.3% to 12.8% between 1975 and 1991(Murray *et al.* 1996). Silicosis prevalence increased with age and duration of service. The authors concluded that in view of current labour stabilisation in the South African gold mining industry, miners are exposed to silica for longer periods of time, and thus we can expect further increases in the prevalence of silicosis and therefore tuberculosis.

In addition to these risk factors, significant ongoing transmission of tuberculosis, despite active screening programmes and DOT, appears to be contributing to the high incidence of tuberculosis in the South African gold-mining community. Recent molecular epidemiological studies of tuberculosis in the miners have shown clustering of identical strains of between 50-67%, suggesting that at least 50% of tuberculosis cases were due to transmission within mine population (Godfrey-Faussett *et al.* 2000). The congregate, social and working conditions of miners are likely to potentiate the transmission of tuberculosis.

1.2.3. Comparative rates elsewhere in the world

Although case rates of tuberculosis in sub-Saharan Africa are very high and the burden of disease large, the largest number of tuberculosis cases is to be found in Asia and Western Pacific and HIV is beginning to spread into these regions. The seroprevalence of HIV in tuberculosis patients in Bombay, India, has risen from 2% in 1988 to 9% in 1992-93, and in Northern Thailand from 5%

in 1989 to 26% in early 1992 (Raviglione *et al.* 1995).

If there is no improvement in the current tuberculosis control programmes, it has been estimated that tuberculosis cases will rise from 7.5 million in 1990 to 10.2 millions in 2000 (Raviglione *et al.* 1993). Deaths will increase from 2.5 million to 3.5 million. This represents an extra 90 million new cases and 30 million deaths in the decade. The proportion of cases attributed to HIV is estimated to increase from 4%in 1990 to 13% in 2 000. Therefore, although HIV is having a much greater impact in some areas compared with others, at global level the lack of investment in effective control programmes in developing countries in the last 20-30 years, combined with continued population growth in these regions, is largely responsible for the deteriorating situation.

In the developed world, notably in the USA but also in a number of European countries, including The Netherlands and England, tuberculosis cases have been increasing since the mid-1980s. This increase in developed countries is probably mainly due to immigration from developing countries (Harries, 1994). HIV is also playing a part in the increase of cases in the developed world, particularly in the USA, where the co-infection of the tubercle bacillus and HIV is increasing in some inner city areas, particularly New York (Weltman and Rose, 1994). This is being compounded by multi-drug resistance. An increase in poverty, unemployment, the numbers of people living below critical income level and homelessness are factors contributing to this situation.

1.3. Epidemiology of tuberculosis

Tuberculosis is primarily acquired by inhalation of dried residues of droplets containing tubercle bacilli expelled in an aerosol created by coughing, sneezing, or talking (Bloom and Murray,

1992). These droplet nuclei remain suspended in the air for prolonged periods, and particles 1 to 10μm in diameter are sufficiently small to reach the alveoli and initiate infection. The most important source is an undiagnosed infectious person with cavitary tuberculosis.

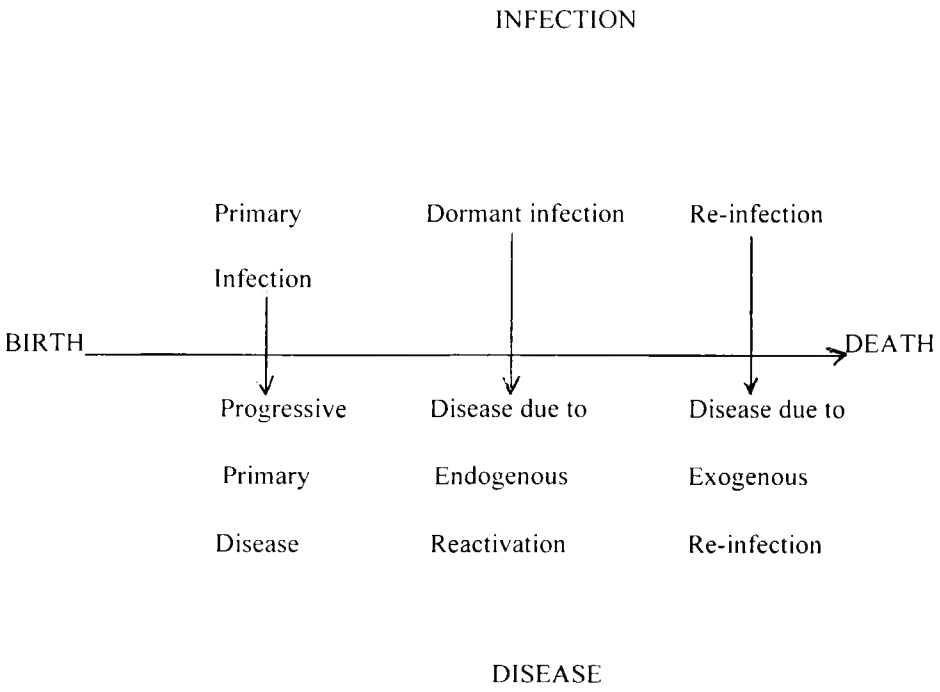


Figure 1.1. Diagrammatic representation of natural history of tuberculosis in individuals as they age (Modified from Smith and Moss, 1994)

As shown in Figure 1.1 tuberculosis develops in an individual as a consequence of one of three processes: Progression of primary infection, endogenous reactivation or exogenous reinfection (Smith and Moss, 1994).

The occurrence of primary infection depends on the number of active infectious tuberculosis cases in the community. Following primary infection, a relatively small proportion of individuals

develop progressive primary disease mostly within two to five years (Medical Research Council 1972). Estimates of the size of the risk of progressive primary disease following primary infection vary from five to 10 percent (Smith and Moss 1994). Most people do not develop disease following infection and mount an effective immune response to the initial infection that limits proliferation of the bacilli, and leads to long-lasting immunity both to further infection and reactivation of dormant bacilli remaining from the original infection.

At later stages, the immunity of some persons who have been previously infected may wane, and they are then at risk of developing active tuberculosis because of either reactivation of dormant bacilli or following exogenous reinfection.

Recent advances in molecular typing techniques have been used to elucidate the occurrence of recent or reactivation tuberculosis as well as to define transmission patterns in outbreaks of tuberculosis See section 1.6. 6)

1.4. Host factors for the development of tuberculosis

Resistance to tuberculosis is expressed in two ways: resistance to the acquisition of infection and resistance to the development of disease after infection is established. Although the mechanisms by which uninfected persons resist infection with
M. tuberculosis are uncertain, macrophages are believed to play a central role in this process, initially through ingestion of bacilli, and ultimately through production of cytokines that enhance the immune defences.

Racial differences in susceptibility to tuberculosis infection have been reported. Blacks have been shown to be approximately twice as likely as whites to develop tuberculosis infection after equivalent exposure to a source case, possibly because macrophages of blacks are more permissive of growth of *M. tuberculosis* (Rich 1944; Stead *et al.* 1990). This racial difference in susceptibility has been attributed to the prolonged selection pressure brought about on whites by the tuberculosis epidemic throughout Europe for many generations prior to the first introduction of tuberculosis into sub-Saharan Africa. Recently, Bellamy *et al.* (1998) described a significant association between alleles of the human *NRMP1* gene and the susceptibility of an African population to tuberculosis.

Many studies based on clinical observations (Dooley *et al.* 1992; Fischl *et al.* 1992; Pape *et al.* 1983; Piot *et al.* 1984; and Pitchenikn *et al.* 1993), and on epidemiological surveys of tuberculosis and HIV seroprevalence (de Cock *et al.* 1992; Onorato *et al.* 1992 and Selwyn *et al.* 1989) have provided evidence that HIV infection is a risk factor for the development of active and often lethal tuberculosis. This might be explained by increased reactivation of previously acquired dormant mycobacteria and enhanced susceptibility to both reinfection and primary infection. It was previously suggested that reactivation is responsible for the majority of HIV associated tuberculosis (Selwyn *et al.* 1989). However, the study of DNA fingerprints of *M. tuberculosis* isolates obtained from AIDS patients using the technique of restriction fragment length polymorphism (RFLP) analysis showed that reinfection and new infection also occur in AIDS patients (Daley *et al.* 1992; Godfrey-Faussett and Stoker 1992; and Small *et al.* 1993).

Other factors such as malnutrition, alcoholism, diabetes mellitus and heavy tobacco smoking have

been stated to increase the risk of reactivation of tuberculosis twofold to fivefold (Barnes *et al.* 1996; Drobniewski. 1997; Haney *et al.* 1996; Torrea *et al.* 1996 and Vogetseder *et al.* 1994).

1.5. Mycobacterial virulence factors

In developing countries, tuberculosis is most prevalent in young adults and this is partly related to the increased risk of tuberculosis in those who are HIV seropositive. However, even among HIV-negative young adults, the incidence of active disease is proportionately higher in young adult than in other age groups. In these presumably healthy people, the manifestations of disease may depend on the properties of the organism rather than on host factors as this group has no known underlying immunological defect that would predispose to active disease. In fact, the incidence of active disease in a population may be influenced greatly by the distribution of clones with certain virulence properties (Valway *et al.* 1998; Zhang *et al.* 1999).

With the advent of whole genome sequencing of *M. tuberculosis* (Cole *et al.* 1998) and considerable information on the nucleotide sequences of less virulent *Mycobacterium bovis*, *Mycobacterium africanum* and *Mycobacterium microti* several genes and their products have been identified as potential candidates for virulence determinants which still need to be confirmed (Gordon *et al.* 1999).

Several putative mycobacterial virulence determinants have however been studied in some detail and are summarised below :

1.5.1. Resistance to oxidizing agents and virulence

Resistance to hydrogen peroxide as a virulence determinant emerged from comparison of strains of high and low virulence in the guinea pig. Extensive studies of these strains of

M. tuberculosis showed a very high correlation between virulence in the guinea pig and susceptibility to killing by hydrogen peroxide (Mitchison *et al.* 1963; Nair *et al.* 1964). A similar conclusion came from a study of strains mutated to be isoniazid-resistant, which are attenuated in the guinea pig and have increased sensitivity to killing by peroxide (Morse *et al.* 1954). Further evidence that peroxidase resistance was a virulence attribute came when it was shown that peroxide sensitive mutants of *M. tuberculosis* survive less well than peroxide-resistant parents in the organs of guinea pigs (Jackett *et al.* 1981).

1.5.1.1. *katG* as a tuberculosis virulence gene

For many years, there has been evidence that strains of the *M. tuberculosis* complex with defective catalase activity have reduced virulence in experimental animals, presumably because of their increased sensitivity to oxidative stress. Recently evidence to confirm that *katG* is a virulence gene has been obtained. Transformation of INH-resistant and avirulent strains with a *katG* gene resulted in recombinants able to produce catalase / peroxidase and virulence to guinea pigs, verifying that *katG* is a virulence determinant for at least some strains of *M.bovis* (Wilson *et al.* 1995). However, there are many clinical isolates of the *M. tuberculosis* complex that have lost KatG activity but remain virulent. In these cases, it is likely that the dual selection pressures of surviving in an infected host and developing isoniazid resistance have produced strains that have lost KatG activity but have some compensating mutations that enable them to remain virulent. Recently, in some isoniazid-resistant strains mutations in the promoter region of a gene that appears to encode an alkylhydroperoxidase activity were identified (Wilson and Collins, 1996). These mutations caused upregulation of gene expression, and it is possible that this is the mechanism by which KatG-inactive strains maintain virulence.

1.5.1.2. Nitric oxide and reactive nitrogen intermediates

There are many reports that nitric oxide is lethal to microorganisms, including mycobacteria. Recent studies have shown that virulence of *M. avium* in the mouse (Doi *et al.* 1993) and virulence of *M. tubeculosis* in the guinea pig (O'Brien *et al.* 1994) correlates with resistance to killing by nitric oxide. The importance of nitric oxide killing of *M. tuberculosis* in human macrophages has been firmly established (Rich *et al.* 1997): "Thus *M. tuberculosis*-infected human alveolar macrophages are capable of producing NO and NO production correlates with intracellular growth inhibition of *M. tuberculosis* in alveolar macrophages suggesting that NO may serve either directly or indirectly as a mycobactericidal mediator in human tissue macrophages". Similar findings were reported by Rhodes and Orme (1997) using strains of *M. tuberculosis* of different virulence exposed to murine bone marrow-derived macrophages. Their results indicate that reactive nitrogen intermediates (RNIs) can kill *M. tuberculosis* under stringent conditions but under less harsh physiological conditions the effects of RNIs range from partial to negligible inhibition.

1.5.2. Mycobacterial cell envelope components

The cell envelope of mycobacteria is rich in lipids. For many years lipids have been suspected to play a role in the virulence of mycobacteria. An association between the fatty acid composition of mycobacterial lipid and virulence has been suggested, virulence being associated with low unsaturated to saturated fatty acids (Nandedkar 1982).

The ability to stimulate release of a tumour necrosis factor (TNF-α) is noteworthy because it has been proposed that much of the pathology seen in tuberculosis is due to the unregulated action of

TNF-α (Rook 1990). Thus the ability of mycobacterial cell envelope components to stimulate excessively release of this cytokine may represent an important virulence mechanism involving the type of tuberculosis in which the Th1 response predominates. Virulence of *M. tuberculosis* has been associated with lipoarabinomannans (LAM) (Brennan and Nikaido 1995). LAMs from the more virulent strains of *M. tuberculosis* do not induce as much TNF-α, when compared with less virulent strains (Adams *et al.* 1994; Chattterjee *et al.* 1992 ; Roach *et al.*1994). TNF-α together with INF-α and other Th1 immunoregulatory cytokines play an important role in host resistance to *M. tuberculosis* and other pathogens. Conversely a switch to Th2 responses involving IL-4, IL 10 and other mediators results in tuberculosis lesions seen in patients with AIDS which are diffuse and associated with systemic spread. Two structural forms of LAM have been described on the basis of the arrangement of terminal sugars (Chatterjee *et al.* 1991; Chatterjee *et al.* 1992). One form (AraLAM) has predominately arabinose termini, while in the other form (ManLAM), the arabinose residues are extensively capped with mannose residues. Several recent studies have reported that mannose capping alters the biological activity of LAM. Thus, ManLAM from *M. tuberculosis* Erdman and *M.leprae* is 100-fold less effective in inducing TNF-α production (Adams *et al.* 1993; Chatterjee *et al.* 1992). The ability of LAM to act as a second signal for macrophage activation and to induce early response genes in macrophages also is abrogated by the presence of a mannose cap (Adams *et al.*1993; Anthony *et al.* 1994; Roach *et al.* 1994).

In addition to LAM, the mycobacterial cell envelope contains other factors with biological activities suggesting that they may be virulent factors. Mycobacteria produce a number of glycolipids of unusual structure (Besra and Brennan 1994). Two classes of acylated toxins have attracted attention as virulence factors. Trehalose 6, 6'-dimycolate or cord factor has a range of biological activities (Goren 1982), including lethal toxicity for mice, but its role and that of other

glycolipids in virulence has tended to be discounted (Grange 1985) because they are found in both virulent and saprophytic species of mycobacteria (Goren 1972).

Mycobacterial sulpholipid also has been considered as a virulence determinant. Sulpholipid is toxic to phagocytes and it may be suggested as contribute to inflammation by stimulating oxidative activity of phagosomes at sub-lethal concentrations (Zhang *et al.* 1988). It is also responsible for the inhibition of phagosome-lysosome fusion by *M. tuberculosis* by virtue of its polyanion nature (Goren *et al.* 1982). Initially a strong association was found between virulent strains of *M. tuberculosis* and high levels of sulpholipids (Goren *et al.* 1974). Furthermore, attenuation of H37Ra compared with H37Rv was accompanied by a substantial decrease in sulpholipids (Goren *et al.* 1982). Subsequent studies however, showed that Western strains were fully virulent in the guinea pig yet had low amounts of sulpholipid (Grange *et al.* 1978; Goren *et al.* 1982). Thus, sulpholipids as a determinant of virulence were discounted (Goren *et al.* 1982).

1.5.3. Interaction with phagosomes and lysosomes

A key characteristic of *M. tuberculosis* that contributes to its virulence is its ability to grow inside monocytes and macrophages. The question of how *M. tuberculosis* enters macrophages is still unanswered. The balance of evidence indicates that *M. tuberculosis* is probably phagocytosed by the normal phagocytic processes mediated by C3b or iC3b opsonization, but the possibility that the bacteria stimulate their own uptake by phagocytes using invasins cannot be ruled out. Uptake via complement factors has been suggested as a virulent attribute by providing a cell- entry mechanism avoiding triggering a potentially lethal respiratory burst (Schlesinger *et al.* 1990). However, in some circumstances, *M. tuberculosis* can trigger a special respiratory burst but here there was no correlation between extent of triggering and virulence (O'Brien *et al.* 1991). Also,

binding to mannose receptors occurs with some strains of *M. tuberculosis*.

Following attachment and subsequent phagocytosis *M. tuberculosis*, sustained intracellular bacterial growth depends on the ability to avoid destruction by lysosomal enymes, ROI, and reactive nitrogen intermediates (RNI). A capacity to block the fusion of mycobacterium-containing phagosomes with lysosomes could be critical for the survival. A careful study of the fate of the bacteria in the phagocytes has shown that there may be two stages to the invasion process. In the first stage, the bacteria are found in phagolysosomes (McDough *et al.* 1993). Some of them are killed and degraded by the phagocytes, but others survive and multiply. In the second stage, some of the bacteria that have survived in the phagolysosome are seen budding off from the phagolysosome in vesicles whose membranes appear to be impaired.

Importantly, *M. tuberculosis* appears to have the ability to disrupt the normal functioning of the phagosome, preventing them from developing into hydrolase-rich compartments. Several laboratories have reported a failure of mycobacterium-containing vesicles to fuse with endosomal vesicles containing other ingested material, such as electron dense colloids(Armstrong and Hart 1971; de Chastellier *et al.* 1993; Clemens and Horwitz 1995). This restricted capacity of mycobacterial phagosomes to fuse with other vesicles suggested that their biochemical composition is altered, blocking association of phagosomes with host molecules that are harmful to the bacteria. This possibility was confirmed by studies that revealed that vacuolar membrane surrounding the bacilli lacked a proton-ATPase, which may be responsible for phagosomal acidification. Moreover, containment of viable within these specialised vesicles may reduce the capacity of mycobacterial antigens to be processed, associated with major histocompatibility complex (MHC) class 11

proteins, and/or transported to the cell surface. While one study also reported the presence of free within the macrophage cytosol, other investigators have been unable to replicate this findings.

1.6. Molecular Epidemiology

1.6.1. Introduction

An important consideration for tuberculosis control programmes should be to analyse the transmission of the disease in epidemiological studies in which individual strains can accurately be traced. Four to eight weeks are required for the growth of *M. tuberculosis* strains, thus the utilization of phenotypic markers requires a similar lapse in time. Such studies have however, until recently, been limited due to the lack of highly polymorphic strain markers able to distinguish the various bacilli infecting unrelated individuals. Previously, the only method available to type *M. tuberculosis* strains was phage typing (Snider *et al.* 1984). However this method has been used by few laboratories due to considerable technical problems and also the fact that only a limited number of phage types can be distinguished.

1.6.2. Ribotyping

Ribosomal RNA genes have been shown to be present in multiple copies in many bacterial species. Since part of their sequence are conserved among very distant species, they have been used as probes for generating RFLP fingerprinting. This method, which has been named ribotyping, is not useful for the differentiation of *M. tuberculosis* strains and provides very limited polymorphism since this species harbours only a single RNA gene copy.

1.6.3. Chromosomal DNA-based typing methods

Several molecular methods can be used to demonstrate relatedness or differentiate between bacterial strains. Genotypic fingerprinting utilizes slight differences in the total chromosome that are generally not related to phenotypic differences. The cleavage of *M. tuberculosis* total DNA by restriction enzymes (which cut the DNA at specific sites), and the separation of the fragments by electrophoresis on a gel, produces a banding pattern following ethidium bromide straining of the gel. However the mobility of the vast majority of the fragments overlaps, and thus at least four different restriction enzymes are needed to obtain reasonable strain differentiation.

1.6.4. Pulse-field gel electrophoresis

Use of restriction enzymes with long AT-rich recognition sequences (Mazurek *et al.* 1991) generates fewer fragments, but separation of such large fragments can be accomplished only by using the cumbersome technique of pulsed-field gel electrophoresis.

1.6.5. Repetitive sequences in *M. tuberculosis*

Various repetitive DNA elements that contribute to strain variation have been discovered in *M. tuberculosis* (Table 1.2). Four of these are insertion sequences, and the remainder are short repetitive DNA sequences with no known function or phenotype.

Table 1.2. Repetitive DNA sequences in *M. tuberculosis* complex

Element	Size(bp)	Source	Copy no.	Related to
IS6110	1,355	*M. tuberculosis*	0-25	IS family of Enterobacteriaceae
IS1081[a]	1,324	*M.bovis*	5-7	IS256 of *S.aureus*
DR	36	*M.bovis* BCG	10-50	
MPTR[b]	10	*M. tuberculosis*	>100	Chi and REP[c] from *E.coli*
PGRS	30	*M. tuberculosis*	>100	

[a]Also being found in *M.xenopi*

[b]Also being found in *M.gordonae, M. kansasii, M.asiaticum, M. gastri*, and *M. szulgai*

[c]Chi, *E. coli* recombination signal; RFLP, repetitive extragenic palindrome

1.6.5.1. Insertion sequence IS6110

IS6110 is a 1,355-bp IS that was initially identified in *M. tuberculosis* (Thierry *et al.* 1990) and subsequently found to be distributed throughout the *M. tuberculosis* complex (Cave *et al.* 1991; Hermans *et al.* 1990; van Soolingen *et al.* 1991; Zainuddin and Dale 1989). Two well known and virtually identical insertion elements to the IS6110 are IS986 isolated from another strain of *M. tuberculosis* (Zainuddin and Dale 1989) and IS987 demonstrated in *M.bovis* BCG (Hermans *et al.* 1991). These three sequences differ in only a few base pairs and therefore are considered to be essentially the same elements. Sequence comparison shows that IS6110 is related to the IS3 family of insertion elements, an IS family initially discovered in Gram-negative bacteria (McAdam *et al.* 1990; Thierry *et al.* 1990).

The IS6110 is usually present in multiple copies in various positions in the genomes of the clinical isolates of *M. tuberculosis* but only a single or few copies in *M.bovis* (Collins *et al.* 1993; Cousins *et al.* 1993; Zainuddin and Dale 1989). BCG strains may be divided into two groups with either one or two copies of IS6110 (Fomukong *et al.* 1992). IS6110 sequences were previously thought to be randomly inserted in the *M. tuberculosis* genome but it is known that there are preferred sites for insertion(McHugh and Gillespie 1998)

IS6110 shows highly polymorphic banding patterns in *M. tuberculosis* isolates, due to insertion at a very wide range of chromosomal sites. The degree of polymorphism is well suited to epidemiological analysis and the fingerprint patterns are readily analysed using a computer programme such as GelCompar (Applied Maths, Kortijk, Belgium). The polymorphism of IS6110 is presumed to be due to its ability to transpose within the genome with little target sequence specificity (Mendiola *et al.* 1994), and indeed transposition of clone IS986 has been experimentally verified in both *M. smegmatis* and BCG (Fomukong and Dale 1995). Therefore it might be expected that the profiles would change over time. However, the patterns appear to be stable during prolonged growth *in vitro* or *in vivo* (Hermans *et al.* 1990; van Soolingen *et al.*1991) and development of drug resistance does not alter the fingerprint pattern (Cave *et al.* 1994). On the other hand, minor variations in fingerprint patterns have sometimes been observed in epidemiologically related isolates (van Soolingen *et al.* 1991).

Although most *M. tuberculosis* strains carry multiple copies of IS6110, a significant minority of strains carry only one or two copies (Fomukong *et al.* 1994; Gillespie *et al.* 1995; van Soolingen *et al.* 1993; Yuen *et al.* 1993). These strains show much less polymorphism; in this case the use of

other probes (especially PGRS or DR) enables differentiation of epidemiological unrelated strains. Furthermore, a small number of *M. tuberculosis* isolates lack IS6110 altogether, which is especially significant in relation to the use of IS6110 as target for PCR.

1.6.5.2. Insertion sequence IS1081

IS1081 is the only other known transposable element in *M. tuberculosis*. It was discovered by Collins while attempting to clone *M.bovis*-specific DNA sequences (Collins and Stephens 1991). This element is 1.324 bp long with 15 bp inverted repeat ends and contains a large open reading frame (ORF). The IS1081 is present in all *M. tuberculosis* complex strains and *M. xenopi* (Small and van Embden 1994). The insertion sequence is repeated 5 to 7 times in *M. tuberculosis* complex. However, very little polymorphism is observed in the locations of this sequence.

1.6.5.3. IS1547 and IS-like elements

IS1547 and the IS-like element are present in one or two copies per genome(Cole *et al.* 1998; Fang *et al.* 1998, Mariani *et al.*1993). The discrimination of IS1547-associated restriction fragment lengthy polymorphism was found to be low in comparison to IS6110-associated RFLP (Fang *et al.* 1998).

1.6.5.4. New insertion sequences

The establishment of the complete genomic DNA sequence of H37Rv disclosed the presence of 25 unknown ISs which are present in one to three copies, two prophages, and a novel type repetitive sequence, the REPI3E12 family (Cole *et al.* 1998). The REPI3E12 elements ranged in size from

1352 to 1436 bp and are present in seven copies throughout the genome of (Cole *et al.* 1998; Gordon *et al.* 1999).

1.6.5. 6. Repetitive DNA

Five types of short repetitive DNA associated with some degree of genetic diversity have been identified in complex.

1.6.5.6.1. The direct repeat (DR) cluster and spoligotyping

The insertion site of IS6110 in BCG is found in an unusual chromosomal region containing a variable number of directly repeated sequences (DR), 36 bp long, interspersed with unique spacer sequences of 36-41bp (Herman *et al.* 1992). The majority of *M. tuberculosis* complex strains, including those of *M. tuberculosis* and *M.bovis* with a single copy, contain a copy of IS6110 within one of the DR sequence in this region. It has been suggested that this is a preferred site for integration of IS6110, or alternatively it may represent the original site of insertion when IS6110 was first acquired by the ancestor of the *M. tuberculosis* complex. This region is polymorphic, both in the number of the DR copies, and in the structure of the spacer region; this polymorphism may arise from interaction between adjacent or distant DRs as well as by IS-mediated rearrangements (Groenen *et al.* 1993).

A novel method to simultaneously detect and type complex, designated "spoligotyping" was described by Kamerbeek *et al.* (1997). In this method the DRs are used as a target for *in vitro* DNA amplification and the variation in the spacer is exploited to obtain different hybridisation patterns of the amplified DNA with multiple synthetic oligonucleotide, which are convalently bound to the

membrane. DR-based probes are valuable for differentiation of strains of *M. tuberculosis* for which IS6110 does not provide sufficient discrimination (van Soolingen *et al.* 1993, 1994).

1.6.5.6.2. The major polymorphic tandem repeats (MPTR)

MPTR were first located downstream of the gro EL-1 gene (Doran *et al.* 1992; Hermans *et al.* 1990; Shinnick 1987) and are structurally similar to the DR clusters; consisting of 10bp direct repeats separated by a 5 bp unique DNA spacer. The MPTR copy number has been estimated at about 80 per genome (Hermans *et al.* 1992), although mapping studies with ordered cosmid suggest that they are confined to nine loci (Poulet and Cole 1994). Unlike, the previously described elements, MPTR have been detected in species other than those of *M. tuberculosis* complex such as *M. gordonae* and *M. kansasii.*

1.6.5.6.3. The polymorphic GC-rich repetitive sequence (PGRS)

The PGRS elements appear to be the most abundant repetitive sequences in the *M..tuberculosis* complex, occurring at 26 to 30 loci. They display significant polymorphism (Ross *et al.* 1992; Poulet and Cole 1994; van Soolingen *et al.* 1993), which makes them potentially useful in epidemiological studies. Several copies have been cloned and sequenced (De Wit *et al.* 1990; Doran *et al.* 1993; Poulet and Cole 1994; Ross *et al.* 1992), and these vary both in size (0.7 to 1.3 kb) and nucleotide sequence, exhibiting about 70% sequence identity. A characteristic feature of PGRS is its GC-richness (about 80%). The host range of PGRS extends from the *M. tuberculosis* complex to other mycobacteria species, such as *M. kansnsii*, *M. gordonae*, *M. marinum*, *M. microti*, *M. gastri*, and *M. szulgai* (Poulet and Cole 1994; Ross *et al.* 1992). MPTR and PGRS elements share some similarities in their nucleotide consensus sequence, host range, high copy number,

polymorphism, and stability.

1.6.5.6.4. Exact tandem repeats (ETR)

Six ETR loci has been identified (Frothingham and Meeker-O'Connell 1998; Gordon *et al.* 1999). Unlike MPTR and PGRS, these ETR loci contain tandem repeat of identical DNA sequences. Each locus has a unique repeated sequence, ranging in size from 53 to 79 bp

1.6.5.6.5. GTG repeat.

A repeat of triplet GTG is present at multiple chromosomal loci and could form the basis for typing of *M. tuberculosis* strains (Wiid *et al.*1994).

1.6.5.7. PCR-based methods

Numerous amplification-based methods are being developed for typing mycobacteria, as they do not require the extraction of large amounts of DNA and potentially enable typing to done from clinical specimens. PCR with arbitrary primers (Random Amplified Polymorphic DNA, RAPD) has been shown to produce polymorphic banding patterns with *M. tuberculosis*, confirming the epidemiological relationships in several clusters of *M. tuberculosis* infection (Palittapongarnpim *et al.* 1993).

Ross and Dwyer (1993) described a *M. tuberculosis* typing method using primers derived from IS6110; polymorphic amplified products were observed, resulting from priming between IS6110 and nonspecific sites on the genome. Another approach using primers based on MPTR and nested primers from IS6110 is described (Plikaytis *et al.* 1993) Termed Isamplipriting , this technique

yields reproducible banding patterns directly from clinical samples. Mixed-linked PCR (Haas *et al.* 1993) uses one primer specific for IS6110 and a second that is complementary to a linker ligated to the restriction digested DNA; one strand of the linker contain uracil and is removed by uracil N-glycosylase to ensure specific amplification. A similar technique termed ligation-mediated PCR (Palittapongarnpim *et al.* 1993) employs a non-phosphorylated linker.

1.6.6. Use of molecular fingerprinting in epidemiological studies.

Discovery of repetitive elements in *M. tuberculosis* genome has enabled strains differentiation by restriction fragment length polymorphism (RFLP). RFLP typing using the insertion sequence IS6110 has been used successfully to examine nosocomial infections (Dooley *et al.* 1992; Hewlett *et al.* 1991; Edlin *et al.* 1992) institutional outbreaks (Campbell *et al.* 1993; Greifinger *et al.* 1992; Valway *et al.* 1994) and transmission of multidrug-resistant (MDR) tuberculosis (Beck-Sague *et al.* 1992; Brian *et al.* 1992; Coronado *et al.* 1993)

Several population-based epidemiological studies that combine conventional and molecular biology methods have been published and suggest that patients in developed countries whose *M. tuberculosis* strains have identical RFLP patterns could be classified into epidemiologically linked clusters, although few cases of transmission has been identified by conventional contact tracing(Small *et al.* 1994; Genewein *et al.*1993; Alland *et al.* 1994; Yang *et al.* 1992). Clustering in this settings reflects recent transmission or a common source of infection. These combined studies have shown that recent transmission contributes substantially to the increase in the incidence of tuberculosis in some areas.

Analysis of microbiological, clinical, social, and demographic data associated with these epidemiologically-related cases can be used to great advantage in defining risk factors contributing towards transmission of strains shown to be identical on molecular fingerprinting techniques.

2 RESTRICTION FRAGMENT LENGTH POLYMORPHISM STUDY OF *MYCOBACTERIUM TUBERCULOSIS* IN THE FREEGOLD HEALTH REGION USING IS6110 AS A PROBE

2.1. Introduction

Gold mining is an important industry in South Africa, employing about 300 0000 men. The South African gold mining industry has reported a TB incidence in excess of 2000 per 100 000 population (Churchyard *et al.* 1999; Murray *et al.* 1999). After trauma, tuberculosis is the single largest cause of mortality among mine workers (Leon *et al.* 1995). Among South African gold miners with tuberculosis, the prevalence of HIV has increased rapidly to approximately 50% of all cases (Churchyard *et al.* 1999). Gold deposits are found in quartz seams and miners are exposed to silica-containing dust. Silicosis has been shown to be a strong risk factor for developing clinical overt tuberculosis (Cowie 1994; Snider 1978) to the extent that even without clinical evidence of silicosis, silica exposure was shown to predispose to tuberculosis (Murray *et al.* 1996). The prevalence of silicosis in South Africa is high, and increasing, probably as a result of changes in labour recruitment policies, resulting in a more stable and therefore older workforce than was a the case in few decade ago (Corbett *et al.* 1999; Murray *et al.* 1996).

It is not only the working conditions which make miners more prone to active tuberculosis, but the living conditions as well. Most miners live and sleep in rooms shared with up to eight other men, which increases the opportunity for transmission and reinfection tuberculosis. Evidence of extensive transmission of tuberculosis in a gold-mining population in South Africa has recently been documented (Godfrey-Faussett *et al.* 2000). These miners fall victim to tuberculosis, and they carry

the organism back to their families and communities, further adding fuel to the current tuberculosis epidemic in the country.

Transmission of tuberculosis from person- to- person can be documented by restriction fragment length polymorphism (RFLP) analysis of the distribution of the conserved insertion sequence IS6110 (described in the general introduction), which varies in copy number and location in the *M. tuberculosis* genome. There is broad variability of RFLP patterns of strains from epidemiologically unrelated patients, whereas identical RLFP patterns are found in isolates from patients who were clearly infected from a common source involving either several patients or reflecting recent person-to-person transmission with the same strain. This molecular technique therefore provides a valid measure of recent infection which has progressed to active tuberculosis (Alland *et al.* 1994; Daley *et al.* 1992; Mazurek *et al.* 1991; Small *et al.* 1994). Matching fingerprints may however also demonstrate transmission in the past between patients who acquired primary tuberculosis, followed by dormancy and subsequent reactivation, often years after the primary infection. This situation has been shown to occur in populations where tuberculosis is highly prevalent e.g. in Ethiopia and Tunisia (Hermans *et al.* 1995) and French Polynesia (Torrea *et al.* 1995). In this context of poor countries where tuberculosis is common, Godfrey- Fausset in a commentary in The Lancet in 1999, emphasized that " clusters will be formed by repeated generations of transmission limited by emigration and stability of the DNA fingerprint itself. Unless there is much mobility of population, clusters may have little to do with recent infection"(Godfrey-Fausset 1999). In a recent review article on the use of RFLP-based DNA fingerprinting in developing countries, Cohn and O'Brien (1998) discussed differences in the transmission of tuberculosis between such poor regions and industrialized counties. Based on a comparison between African countries and the Netherlands (van Soolingen *et al.* 1991), they concluded that more recent transmission occurs in areas of high

incidence while more reactivation disease and importation of cases are reflected in DNA fingerprinting-based studies in areas of low incidence. Unlike Godfrey-Faussett (1999), Cohn and O'Brien (1998) did not discuss the process of cycles of transmission over generations which could lead to occurrence of dominant clones in high incidence regions.

A recent study involving 28522 working men in four gold mines in South Africa concluded that at least 50% of tuberculosis cases were due to transmission withing mining community. The main risk factor for clustering in this study was found to be treatment failure. Patients with multiple-drug-resistant disease were more likely to have failed treatment but were less likely to be clustered than those with a sensitive strain. HIV was common but not associated with clustering.(Godfrey-Faussett *et al.* 2000)

The main objectives of the study recorded in this chapter were:

- To determine by IS6110-based DNA fingerprinting, the extent of homogeneity/ heterogeneity i.e RFLP polymorphism which existed amongst approximately 50% of M. tuberculosis isolates available from the Freegold Health Region mines during a six-month period in 1994 and
- To identify possible risk factors which may be associated with the transmission of *M. tuberculosis* clones (i.e isolates with identical IS6110-based RFLP band patterns comprising ≥ 6 bands/isolates) in these mines.

2.2. Materials and methods

2.2.1. Study population, patients and cultures

Patients investigated during the study period were predominantly black migrant miners working for

the Freegold Health Region (FGHR) mines situated in the Free State Province of South Africa. The incidence of tuberculosis in miners from mines in that region in 1994 was approximately 1200/100 000 per annum for the population (Churchyard *et al.* 1999). The FGHR has an estimated working force of 53000 persons (G.J.Churchyard, Aurum Research, Welkom, personal communication). Ninety percent of miners, all males work underground in six different mines. Miners live in a single sex hostels located at each mine shaft and return home once a year from leave.

2.2.1.1. Management of tuberculosis in FGHR mines

The Ernest Oppenheimer Hospital administration provides the sole source of tertiary care for mine employees, and designated members of the hospital administration manage the TB control programme.

Directly observed therapy, short-course (DOTS) policy was introduced in 1993 and this strategy is relatively easy to accommodate and administer in the well-regimented mining industry.

Some cases of TB are detected by active case finding through the mass miniature radiograph (MMR) screening programme (done once a year). Patients with new chest radiographic changes are investigated further with standardise-size chest radiograph, sputum microscopy and culture. Most cases in the present study were however, identified by passive case detection i.e patients who presented with symptoms to the medical service rather than being identified through a screening programme.

2.2.1.2. Patients included in study

A group of 193 patients admitted to the EOH of the Freegold Health Region mines from whom

M. tuberculosis was isolated cultured during the period between 1 January and 31 June 1994 and whose isolates were available to the author was included in this study. These isolates constitute about 50 percent of all patients hospitalised at EOH with culture-proven tuberculosis during that period. They represent available cultures which remained viable and uncontaminated after either having been isolated directly and stored at the SAIMR laboratories in Johannesburg or having survived transportation from the EOH laboratories in Welkom to the SAIMR in Johannesburg. Some cultures were listed as having been stored but could not be retrieved and were lost to the study.

The period was limited to six months as the initial design of the project was confined to the description of the population structure of *M. tuberculosis* isolates based on IS6110-derived RFLP polymorphism of *M. tuberculosis* strains in miners, linking clusters mainly to work place and place of origin. It did not initially include, nor anticipate, a detailed epidemiological component such as assessment of risk factors contributing towards transmission as those aspects were to form part of a separate study under the direction of another author. It is realised that in order to assess rates of recent transmission a study period in excess of 24 months would have been more appropriate, especially in light of the recent decision to extend the scope of the study when the previously planned separate study involving risk factor was reassigned to the present author (see discussion, section 2.4)

2.2.1.3. Data collection

Patient data concerning sex, age, findings of microscopy for acid-fast bacilli, status of patients' treatment (new treatment or retreatment cases), seropositivity for infection with the human immunodeficiency virus (HIV) in consenting patients, work place relating to mine and mine shaft,

and place of origin, were compiled by the hospital administration and were extracted from a central computerised database. Using a biostatistical approach, these data were analysed to establish possible epidemiological linkages, involving the transmission of organisms in mine-related settings and risk factors related to transmission.

2.2.1.4. Treatment information

Computerised information on treatment was limited to patients who received treatment at the mine hospital for the first time (new treatment cases) and those that were treated for tuberculosis before their present admission (retreatment cases). This group includes treatment failures and patients with recurrent episodes of tuberculosis.

2.2.1.5. *M. tuberculosis* strains

Sputum and other samples from patients were cultured using a culture system based a radiometric detection signal (BACTEC)(Johnston Laboratories, Cockeysville, Maryland, USA) either at the Ernest Oppenheimer Hospital (EOH) or at the South African Institute of Medical Research (SAIMR) in Johannesburg. Positive cultures were subcultured on Lowenstein-Jensen (LJ) slants. The drug susceptibility of the isolates to ethambutol streptomycin, isoniazid, rifampicin, ethionomide, rifabutin, kanamycin and pyrazinamide were tested using the BACTEC system (Siddiqi 1988). Cultures were stored on L-J slopes at 4 °C.

2.2.2. DNA fingerprinting

DNA fingerprinting of the investigated isolates was performed as described previously (van Embden *et al.* 1993; van Soolingen *et al.* 1994) except that the markers used for estimating the sizes of separated fragments were digoxigenin-labelled marker III and internal markers were not used. The

technique of fingerprinting entails the growth of *M. tuberculosis*, extraction of DNA, restriction endonuclease digestion, Southern blotting of the digested fragments and probing with the IS element (van Embden *et al.* 1993; van Soolingen *et al.* 1994).

2.2.2.1. DNA extraction using SDS

Colonies were scraped from L-J slopes and harvested into 0.5 ml of lysis buffer (15% sucrose, 0.05M Tris pH8.5, 0.05M EDTA, 1mg/ml D-cycloserine). After SDS was added to a final concentration of 2%, the solution was incubated up to 24 hours at 37 °C and 30 minutes at 80 °C. The lysate was purified by two extractions with phenol:chloroform solution (1:1 v/v) and once with chloroform alone. The DNA was dialysed overnight against distilled water at 4 °C

2.2.2.2 Restriction endonuclease digestion

Approximately 1 *ug* of DNA was digested overnight at 37 °C with 10 units of *Pvu* II in a buffer supplied by the manufacturer (Boehringer Mannheim).

2.2.2.3 Separation of fragments by electrophoresis

The digested fragments were separated by electrophoresis through 1% agarose gel (20cm gel) in TAE buffer (0.04M Tris-acetate, 0.001M EDTA, pH 8). The gel was run at 1.5 V/cm for 16 - 24 hours at 4 °C. The markers used for estimating the sizes of separated fragments were digoxigenin-labelled marker III (a mixture of fragments from Lambda DNA digested with Eco RI and Hind III) which consists of 13 fragments. These were 21.226, 5.148, 4.973, 4.268, 3.530, 2.027, 1.904, 1.584, 1.375, 0.947, 0.831, 0.564 and 0.125 kilobase pairs each in size (Boehringer Mannheim).

2.2.2.4. Synthesis of the probe

A 245bp probe was prepared by PCR from IS6110 using primer (5'-CGTGAGGGCATCGAGGTGGC) that corresponds to base pair 631 through 650 and primer 2 (5'GCGTAGGCGTCGGTGACAAA) that corresponds to base pair 856 through 875 of the IS6110 insertion (van Soolingen *et al.* 1994). The PCR mixture consisted of 50nM NaCl; 5mM MgCl2; 10mM Tris-HCl; 0.01% (w/v) gelatin pH 9.6; dNTP mix, (2.5mM each), primers (50ng/l each) and 1.25U Taq polymerase.

The cycling parameters of denaturing-annealing-synthesizing cycles were as follows:

3 min. 94 °C		Once
1 min. 94 °C	|	
1 min. 65 °C	|	25 cycles
2 min. 72 °C	|	
4 min. 72 °C		Once

The PCR product was run on 2% gel and the fragment with the correct size (245-bp) was cut from the gel and purified by Gene-clean II kit as recommended by the manufacturer (Bio 101 Inc.). The probe was non-radioactively labelled by the use of the digoxigenin labelling system as described by the manufacturer (Boehringer Mannheim).

2.2.2.5. Southern blotting

Prior to transferring the DNA to the Hybond-N, nylon membrane (Amersham life science), the gel was treated in depurination solution (0.25M HCl), for 15 minutes, denaturation solution (0.5M NaOH, 1M NaCl) for 30 minutes and finally in neutralising solution (0.5M Tris-HCl, 1.5M NaCl,

pH 7.4) for 30 minutes.

The Hybond-N membranes for Southern blotting were prepared according to the manufacturer's recommendation (Amersham Life Science) and the DNA was transferred by capillary action using 20X SSC (3M NaCl, 0.3M sodium citrate) a transfer buffer overnight. After blotting, the nucleic acids were fixed to the membrane by baking at 120 °C for 30 minutes.

2.2.2.6. Prehybridisation and hybridisation

The membranes were prehybridised for at least 2 hours at 68 °C in a hybridisation solution containing 5XSSC, 1%w/v blocking reagent, 0,1% N-lauroysarcosine, and 0.2% sodium dodecyl sulfate (SDS). After prehybridisation heat-denatured DNA probe was added and the membrane was incubated overnight at 68 °C. Following hybridisation the membrane was washed twice for 5 minutes at room temperature with 2XSSC, 0.1% SDS and twice for 15 minutes at 68 °C with 0.1x SSC, 0.1% SDS.

2.2.2.7. Detection procedures

The presence of digoxigenin-labelled DNA probe was determined with alkaline phosphatase conjugate antibody as described by the manufacturer (Boehringer Mannheim).

2.2.2.8. Computer-assisted analysis of DNA fingerprints

The IS6110 fingerprint pattern were digitised with HP11c scanner(Hewlet-Packard, Hopkin, Min). Conversion of digitised fingerprint pattern, normalisation and cluster analysis were carried out with Gelcompare software (Window 95 , Version 4.0, Applied Maths, Kotrijk, Belgium). The fingerprint patterns were analysed for similarity by using the Dice coefficient, and a dendrogram was calculated

with the unweighted-pair group method using average linkage(UPGMA) according to the supplier's instructions. Band positions were determined by using the peak finder function of the Gelcompare software and were controlled manually by comparison with the original IS6110 fingerprint blots.

2.2.2.9. Statistical analysis

Statistical analyses were performed by Epi-Info, version 6.02 (Centers for Disease Control and Prevention, Atlanta, GA, USA). Chi-square determinations were performed to test the univariate risk factor for belonging to a cluster or family. When cell sizes were expected to be smaller than five, Fisher' exact test was used. Adjusted odds ratios with 95% confidence intervals were calculated in comparison of categorical variables.

Multiple logistic regression models were fitted to identify the most important risk factors for clustering. Potential explanatory variables considered were place of origin, mine shaft, HIV-status, drug-resistant profile, treatment episode, duration of employment and age.

2.2.2.10. Failure to perform secondary DNA fingerprinting.

When the initial IS6110-based DNA fingerprinting was performed n 1994, secondary typing of isolates exhibiting ≤ 5 RFLP bands was not envisaged. By the time it was considered to be relevant, stored DNA from isolates had been degraded to such an extent that attempts at secondary typing proved to be unsuccessful. Unfortunately, in the process of moving from SAIMR laboratories in Johannesburg to the MRC laboratories in Pretoria the original cultures were lost.

2.2.2.11. Definition of terms

Isolate. A pure culture of bacteria in monoculture from a primary clinical material, identified to the species level

Strain. An isolate or a group of isolates showing traits distinctive from those of other isolates of the same species

Clone, general definition. A set of microbial isolates that have been recovered independently from different sources, in different locations and perhaps at different times, but showing so many identical phenotypic and genetic traits that the most likely explanation for this identity is a common origin(Leger 1992).

Clone, specific definition. A clone in the context of the present study is confined to a set of *M. tuberculosis* isolates which exhibits an identical IS6110-based RFLP pattern, provided more than five bands are present in each of the isolates.

Identical band pattern (IBP) isolates. Strains which are indistinguishable, i.e. 100% identical, on IS6110-based fingerprinting.

Low copy number isolates. Isolates exhibiting the same number of copies of IS6110 fragments of identical molecular weight but which contained less than six copies per isolate

RFLP family strains. Strains which share at least 80% of IS6110- based RFLP band patterns including the position and grouping of bands.

Clustering of isolates was based on three different genotypic criteria forming three different types of clusters which will be clearly specified in the text of the thesis:

IBP clusters. Groups of two or more isolates exhibiting the same number of copies of IS6110 fragments of identical molecular weight i.e. identical RFLP patterns.

Clone-based clusters or clone clusters. Groups of two or more IBP isolates with more than

5 copies (RFLP bands) in each of the isolates.

RFLP-family clusters. Groups of two or more isolates exhibiting near-identical RFLP band patterns defined as ≥80% RFLP band pattern homology, based on number, position and grouping of bands

Recent infection. Infection acquired recently without necessarily exhibiting overt disease.

Recent transmission. Evidence of recent spread of a strain from one patient to another, based on the demonstration of clone-based clusters of pulmonary-derived isolates, i.e. strains with identical band patterns and ≥6 IS6110 copies.

Recently acquired tuberculosis. Clinically overt tuberculosis disease which was recently acquired, with the implication that the period between infection as a result of transmission and disease manifestation is short.

2.3. Results

2.3.1. Patient characteristics

All 193 patients were males. The age distribution of the patients is shown in Figure 2.1. The average age of the mine workers was 38 years. All patients presented with pulmonary tuberculosis.

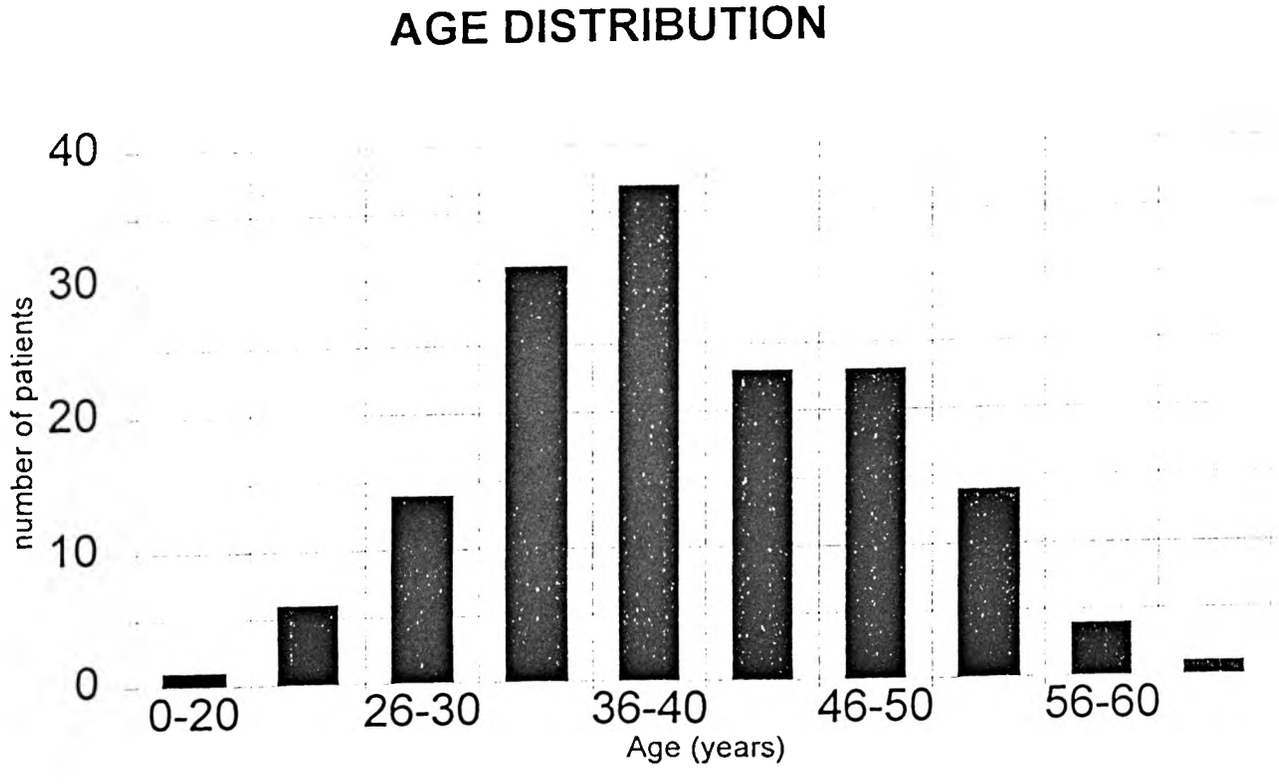


Figure 2.1. Age distribution of tuberculosis patients from Freegold Health Region mines

Geographical origin was determined for 186 patients(96%): 70 were from Eastern Cape Province, 66 from Lesotho, 38 from Free State Province, 3 from Mozambique, 3 from Kwazulu-Natal, 4 from Swaziland and 1 from Gauteng Province.

All patients in this study were working underground in six different mines. Of the 181 patients on which information relating to mine or mine shaft was available, 23 worked in FSG, 21 in FSS , 28 in Freddies, 36 in Pres Brand , 39 in Pres Steyn and 34 in W Holdings. Table 2.1 show the distribution of miners in each mine shaft. A total of 34 mine shafts was recorded in this study.

Table 2.1. Distribution of miners with tuberculosis in different mine shafts

	01	**02**	**03**	**04**	**05**	**06**	**07**	**08**	**09**	**10**
FSG	8	5	3	4	1	NE	1	1	NE	NE
FSS	1	6	11	3	NE	NE	NE	NE	NE	NE
Freddies	2	NE	NE	NE	15	NE	3	NE	6	2
Pres Brand	5	9	5	8	9	NE	NE	NE	NE	NE
Pres Steyn	7	10	2	18	1	1	NE	NE	NE	NE
W Holdings	9	6	1	7	6	3	2	NE	NE	NE

NE = Mine shaft does not exist /function or no miner was recorded from that mine shaft

Due to the mobility of the work force in the mines, the number of miners in each mine or shaft could only be approximated and as these figures were not required for analysis of risk factors associated with transmission of *M. tuberculosis*, no attempt was made to access these data. The miners lived in hostels and each of hostel accommodated miners from a particular shaft. A small number of miners may however have been transferred from one shaft to another, yet remained in the original hostel, while others may have been reassigned to a different hostel on closure of either a shaft or a hostel. A

hostel comprised rooms of varying size, each of which being occupied by 2 to 8 men.

HIV serological status was available for 180 (93%) patients, and was negative for 153 (85%), positive for 27 (15 %) and unknown for 13 patients.

2.3.2 IS6110- associated DNA polymorphism of *M. tuberculosis* isolates collected from Freegold Health Region miners.

The genetic polymorphism of 193 *M. tuberculosis* isolates obtained from patients was investigated by Southern blot analysis of the genomic DNA with the IS6110 probe. Hybridisation of *Pvu-II*-digested DNA with right arm of IS6110 showed that the copy number of IS6110, as determined from the number of bands hybridising the probe, ranged from 1 to 19 according to the strain, with a median of 13 copies per isolate (Figure 2.2). Most strains (172 total) had more than 5 copies of IS6110 per genome and only one isolate carried a single copy of the insertion sequence. The heterogeneity of isolates based on the ratio of band patterns to clusters was lower for isolates with 1 to 5 IS6110 copies (Table 2.2), suggesting that secondary typing of low copy number strains may yield additional unique patterns.

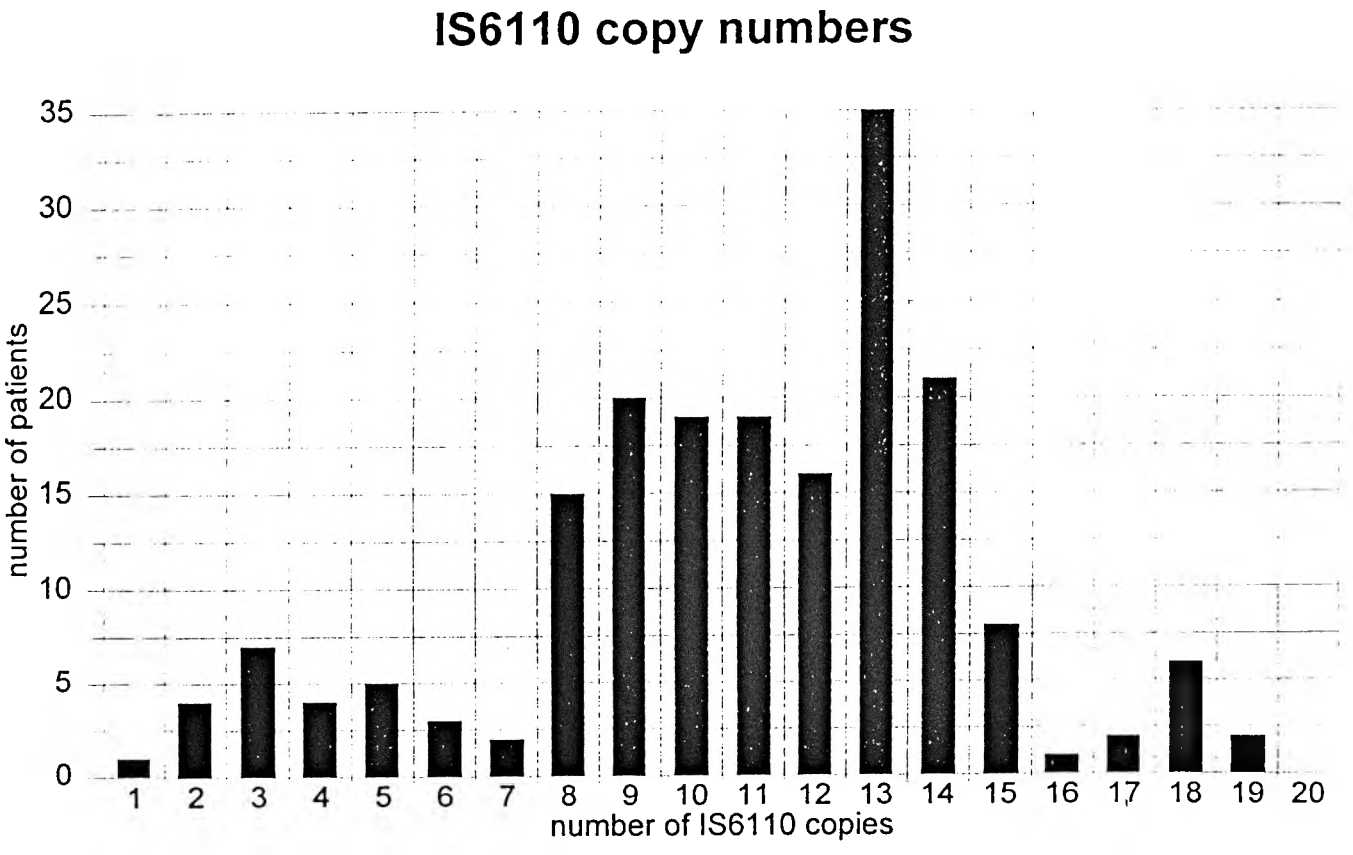


Figure 2.2. Number of IS6110 copies in *M. tuberculosis* from Freegold Health Region miners

Table 2.2. Distribution of isolates, IBP clusters and IS6110-based band patterns.

No.of IS6110 copies/pattern	No of isolates	Total isolates in clusters	No of IBP clusters	Total patterns	Heterogeneity[a] patterns: clusters
1-5 bands	21	13	4	10	2.5
6-10 bands	57	20	5	44	8.8
11-15 bands	104	42	14	76	5.4
16-19 bands	11	3	1	13	13.0

a) Degree of heterogeneity based on the ratio of number of patterns to number of clusters

On the basis of computer-assisted comparison of DNA fingerprints for *M. tuberculosis* isolates, a dendrogram showing the similarity between any two isolates or groups of isolates was constructed (Figure 2.3). One hundred and thirty- nine distinct IS6110 fingerprint patterns were distinguished in the 193 isolates analysed; 24 banding patterns were shared by ≥ 2 isolates (i.e. clusters with identical band patterns (IBPs). These 24 IBP clusters involved 78 isolates (Table 2.4 and Table 2.5). IBP clusters varied in size from 2 to 8 isolates. The majority of clusters were small: 10 clusters consisted of 3 isolates and 8 clusters of 2 isolates (Table 2.3).

Table 2. 3. Band frequency and size of 24 IBP clusters found in the study population

No. of isolates per cluster	No. of IS6110 bands	No. of clusters
2	6-15	8
3	2-19	10
4	3-14	2
5	8-13	2
6	13	1
8	14	1

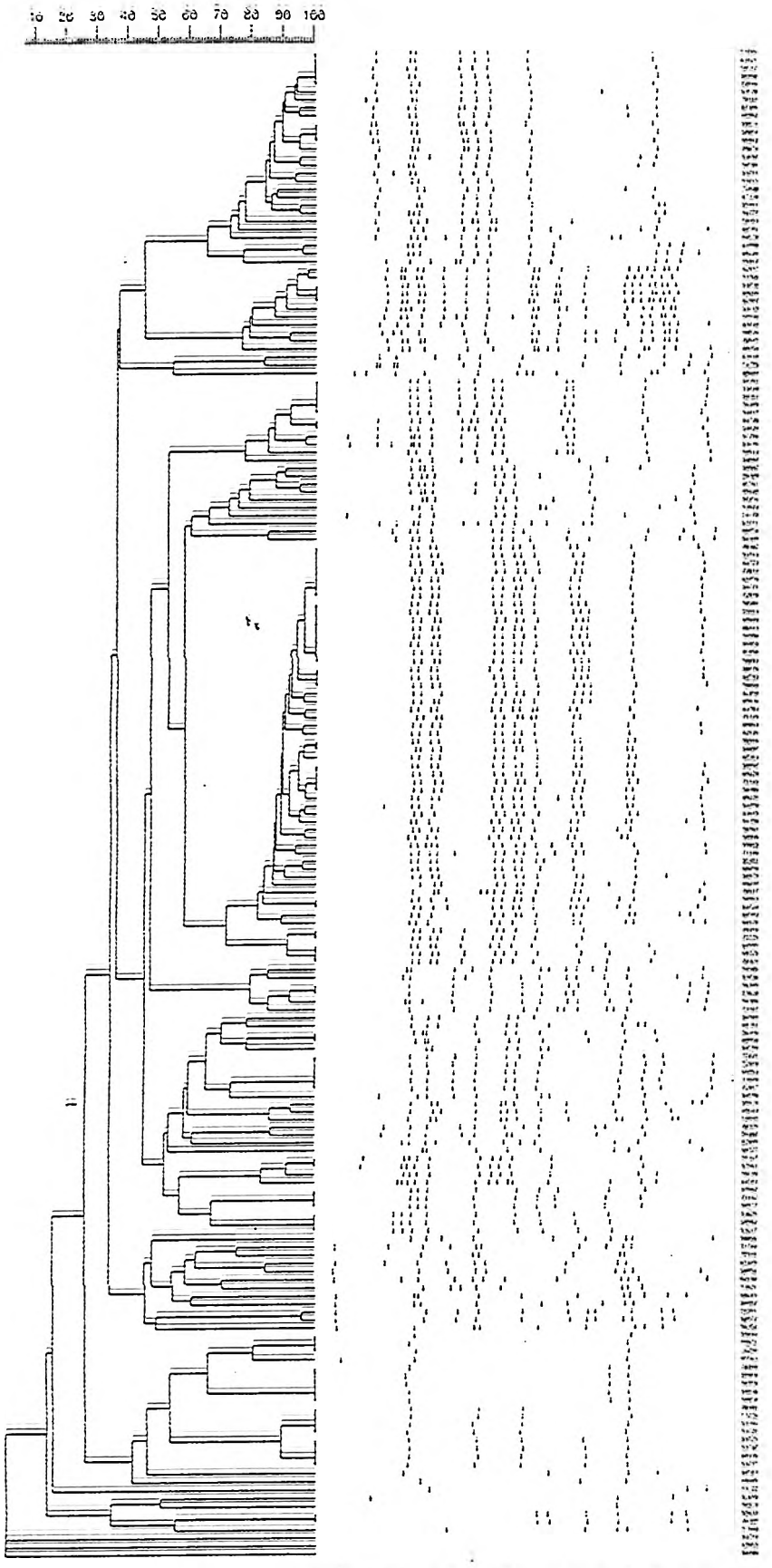


Figure 2.3. Dendrogram based on computer-assisted comparison of IS6110 DNA fingerprints of 193 isolates of *M. tuberculosis*

Table 2.4. Distribution of total and evaluable numbers of *M. tuberculosis* isolates in RFLP families and IBP and clone clusters from mining environment and place of origin.

Isolates in genotype groups	Total	Percentage
Isolates with IS6110-based fingerprint	193[c]	100
Evaluable isolates[d] in mines/ shafts	181	94
Evaluable isolates[d] according to region	186	96
Evaluable isolates[d] according to village	151	78
Isolates in 23 RFLP families	158	82
Evaluable isolates[d] in RFLP families in mines /shafts	148	94
Evaluable isolates[d] in RFLP families in regions	154	97
Isolates in 24 IBP clusters	78	40
Evaluable isolates[d] in IBP clusters in mines/shafts	75	96
Evaluable isolates[d] in IBP clusters in regions/villages	77	99
Isolates in 20 clones	65	34
Evaluable isolates[d] in clones in mines/ shaft	62	95
Evaluable isolates[d] in clones in regions/villages	64	99
Isolates in RFLP families shared in mines	116	78
Isolates in RFLP families shared in shafts	60	41
Isolates in RFLP families shared in regions	115	75
Isolates in RFLP families shared in village	30	20
Isolates in IBP clusters shared in mines	36	48
Isolates in IBP clusters shared in shafts	14	19
Isolates in IBP clusters shared in regions	48	62
Isolates in IBP clusters shared in villages	5	6.5
Isolates in clone clusters shared in mines	33	53
Isolates in clone clusters shared in shafts	11	18
Isolates in clone clusters shared in regions	40	63
Isolates in clone clusters shared in villages	5	7.8

a) There were 6 mines involved

b) A total of 34 shafts were recorded where patients with TB worked

c) There were 139 distinct IS6110 patterns amongst the 193 isolates

d) Evaluable isolates denote isolates from patients on which information relating to mines shafts or villages and geographic region was available.

Table 2.5 Distribution of identical band pattern (IBP) clusters amongst mines and mine shafts

NUMBER OF IBP STRAINS IN MINE/SHAFT																	
Cluster compositin				Shared in mine number						Not shared	Shared in shafts in mine number						NE
IBP Cluster	IS6110 copies[a]	RFLP family	Total isolates	1	2	3	4	5	6		1	2	3	4	5	6	
CL 1	14	FM 1	8	0	0	3	0	3	0	2	0	0	2	0	3	0	0
CL 2	13	FM 1	3	0	0	0	0	0	0	3	0	0	0	0	0	0	0
CL 3	13	FM 1	6	0	2	0	0	0	0	3	0	0	0	0	0	0	1
CL 4	14	FM 1	2	0	0	0	0	0	0	2	0	0	0	0	0	0	0
CL 5	14	FM 1	4	0	0	0	0	0	0	4	0	0	0	0	0	0	0
CL 6	16	FM 1	2	0	0	0	0	0	0	2	0	0	0	0	0	0	0
CL 7	9	FM 2	3	0	0	0	0	0	2	1	0	0	0	0	0	0	0
CL 8	8	FM 2	5	0	0	2	0	0	2	1	0	0	2	0	0	0	0
CL 9	10	FM 2	2	0	0	0	0	0	0	2	0	0	0	0	0	0	0
CL 10	12	FM 3	2	0	0	0	0	0	0	2	0	0	0	0	0	0	0
CL 11	11	FM 3	3	2	0	0	0	0	0	0	0	0	0	0	0	0	1
CL 12	19	FM 4	3	0	0	0	0	0	0	3	0	0	0	0	0	0	0
CL 13	4	FM 5	3	0	0	0	0	0	0	3	0	0	0	0	0	0	0
CL 14	5	FM 5	3	2	0	0	0	0	0	1	0	0	0	0	0	0	0
CL 15	13	FM 6	2	0	0	0	0	0	0	1	0	0	0	0	0	0	1
CL 16	11	FM 7	5	0	0	0	3	0	2	0	0	0	0	0	0	0	0
CL 17	11	FM 9	2	0	0	0	0	0	0	1	0	0	0	0	0	0	0
CL 18	12	FM 10	3	0	2	0	0	0	0	1	0	2	0	0	0	0	0
CL 19	10	FM 10	2	0	0	0	0	2	0	0	0	0	0	0	0	0	0
CL 20	2	FM 11	3	0	0	0	0	0	0	3	0	0	0	0	0	0	0
CL 21	3	FM 12	4	3	0	0	0	0	0	1	3	0	0	0	0	0	0
CL 22	9	FM 13	3	0	0	2	0	0	0	1	0	0	2	0	0	0	0
CL 23	9	FM 20	3	0	2	0	0	0	0	1	0	0	0	0	0	0	0
CL 24	6	FM 23	2	0	2	0	0	0	0	0	0	0	0	0	0	0	0
TOTAL			78	7	6	7	3	5	8	39	3	2	6	0	3	0	3
Total shared in mines/ shafts				36							14						

NE: Not evaluable; FM: family; Mine 1 FSG; Mine 2 ISS; Mine 3 Deddies; Mine 4 President Brand; Mine 5 President Steyn; Mine 6 Western Holdings
a) Total number of isolates in IBP clusters shared in mines and shafts respectively

Table 2 6 Distribution of RFLP Family-based clusters in mines and mine shafts

RFLP Family	Isolates per RFLP family	NUMBER OF RFLP FAMILY STRAINS SHARED IN MINES/SHAFTS													NE[2]
		IN MINE NO[1]						Total Strains shared in mines	IN SHAFT IN MINE NO[1]						Total strains shared in shafts	
		1	2	3	4	5	6		1	2	3	4	5	6		
1	50	4	5	7	8	13	9	46	0	4	6	7	12	5	34	4
2	24	2	2	6	3	3	7	23	0	0	4	0	3	4	11	1
3	9	2	2	0	0	0	0	4	0	0	0	0	0	0	0	0
4	9	0	2	0	2	3	0	7	0	0	2	2	0	0	4	0
5	7	3	0	2	0	0	0	5	2	0	0	0	0	0	2	0
6	6	2	0	0	0	0	0	2	0	0	0	0	0	0	0	1
7	5	0	0	0	3	0	2	5	0	0	0	0	0	0	0	0
8	4	0	0	0	0	2	0	2	0	0	0	0	0	0	0	0
9	5	2	0	0	2	0	0	4	0	0	0	0	0	0	0	0
10	5	2	0	0	0	3	0	5	2	0	0	0	0	0	2	0
11	4	0	0	0	0	2	0	2	0	0	0	0	2	0	2	0
12	4	3	0	0	0	0	0	3	3	0	0	0	0	0	3	0
13	3	0	0	2	0	0	0	2	0	0	2	0	0	0	2	0
14	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
16	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
17	2	0	0	0	0	0	0	0	0	0		0	0	0	0	0
18	2	0	0	0	2	0	0	2	0	0	0	0	0	0	0	0
19	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
20	3	0	2	0	0	0	0	2	0	0	0	0	0	0	0	0
21	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
22	2	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0
23	2	0	2	0	0	0	0	2	0	0	0	0	0	0	0	0
TOTAL	158	20	18	17	20	26	18	116	7	4	14	9	17	9	60	7

NE - Not evaluable FM Family Mine 1 ESG Mine 2 ESS Mine 3 Freddies Mine 4 President Brand Mine 5 President Steyn Mine 6 Western Holdings

2.3.3. Evidence of recently-acquired tuberculosis

2.3.3.1. Quantification of transmission

To calculate a percentage estimate of recently-acquired tuberculosis, it is assumed that the patient who recently transmitted its strain to other members represented by a cluster (source case) acquired his/her tuberculosis infection elsewhere in the past, with subsequent development reactivation tuberculosis (Small *et al.* 1994; Godfrey-Faussett *et al.* 2000). This assumption should be qualified by the possibility that more than one patient who acquired infection with the same clone some time in the past would develop reactivation tuberculosis during the relatively brief study period. This could happen when strains have become endemic in the mining community. Based on the premise the latter scenario is unlikely, the minimum percentage of tuberculosis cases due to recently acquired tuberculosis in the miners (calculated according to the formula proposed by Small *et al.* 1994, as [Total No. in clusters]-[No. of clusters]/Total No. of strains) was 26.2% based on clones and 28.0% based on IBP clusters. If one assumes that all patients in different clones or IBP clusters developed recently acquired tuberculosis and that none of the members of a IBP cluster was an index case with reactivation tuberculosis i.e. that the source case was not identified in the present study, the percentage of tuberculosis cases due to recently acquired tuberculosis in the Freegold Health Region miners could have been as high as 37.8% in the case of clones and 40.4% for IBP clusters. This would be possible if the source cases were not captured in the study by being undiagnosed and/or left the mine before the study commenced or not on the mine while visiting a local community or in the eventuality of miners visiting a migrant area when on leave.

2.3.3.2. Evidence of geographically-linked transmission

The epidemiological relationship among patients in IBP clusters was obtained through the examination of information relating to patients originating from the same geographic region or village and working in the same mine or mine shaft (the latter group would likely to have lived in the same hostel).

In several instances, epidemiological links between patients and IBP clusters were apparent. Of all evaluable IBP clustered cases 14 out of 75 (18.7%) could be linked to a mine shaft and 5 out of 77

(6.5%) IBP clustered cases originated from the same village, strongly suggesting a common source of infection (Table 2.4 and Table 2.5). The corresponding figures for sharing a mine or a geographical region was 48.% and 61.5% respectively.

Based on clustering of clones as defined in this study, 11 out of 62 (17.7%) could be linked to a mine shaft and 5 out of 64 (7.8%) originated from the same village. The figure for sharing a mine or a region was 53.2% and 62.5% respectively.

2.3.3.3. Description of two large clone-based clusters

As cluster 1 and cluster 3 had 14 and 13 IS6110 copies respectively, both clusters meet the definition of a clone-based cluster (≥6 copies). The isolates that formed the largest cluster (Cluster 1, Family 1) came from 8 miners. Six members of this cluster originated from different parts of the southern region, while two came from the same village suggesting in the latter instance a common source of infection. Overall six patients could be epidemiologically linked, giving direct evidence of recent transmission: five sharing a mine shaft (Table 2.5) and another patient with a CL 1 fingerprint who came from the same village as one of the five miners who shared a mine shaft.

The second largest cluster (Cluster 3, Family 1) involved six patients. All members of this cluster come from different regions and none could be linked to a mine shaft. Two worked at the FSS mine (Table 2.5).

2.3.4. Risk factors associated with clustering

2.3.4.1. Univariant analysis of risk factors

Sixty- five clustered stains defined as clones for the purpose of this study, were compared with 105 non-clustered strains from mine patients to identify factors which may pose a risk of transmission.

Table 2.7 presents the results of univariant analysis to identify potential risk factors for transmission. Miners from the Free State were associated with a significantly high risk of being in a cluster (P = 0.01). In contrast to this finding, the Eastern Cape as a source of miners was associated with a

significantly lower risk than other regions of being in clusters (P=0.02). No significant association between clustering and age, drug resistance profile, period of employment in the mine, previous history of tuberculosis and or HIV seropositive status of patients could be demonstrated.

Table 2.7. Univariate analysis of risk factors for a patient to belong to a cluster/clone

Characteristic	No. of patients		Odds Ratio	95% Convidence Interval	P value
	Clustered	Nonclustered			
MINE					
FSG	7	9	1.24	0.39-3.92	0.68
FSS	10	10	1.67	0.59-4.71	0.28
Freddies	11	10	1.88	0.68-5.19	0.18
Pres Brand	10	24	0.58	0.24-1.42	0.20
Pres Steyn	13	24	0.81	0.35-1.85	0.58
W Holdings	11	20	1.91	0.80-4.53	0.11
PLACE OF ORIGIN					
Eastern Cape	16	45	0.43	0.20-0.90	0.02
Free State	20	14	2.73	1.18-6.36	0.01
Lesotho	24	37	0.99	0.49-1.99	0.97
Natal	0	2	Undefined	0.00-8.16	0.52
Swaziland	1	0	Undefined	Undefined	0.40
Mozambique	2	2	1.55	Undefined	0.65
HIV Seropositive	14	11	2.16	0.84-5.58	0.08
Retreatment	13	15	1.56	0.63-3.85	0.29
Drug resistant	8	10	1.24	0.41-3.65	0.67
INH only	5	6	1.38	0.35-5.38	0.61
INH + RMP	0	3	Undefined	Undefined	Undefined
INH + Others	3	1	Undefined	Undefined	Undefined
Period in the mine					
1-5 years	5	4	2.18	0.44-11.39	0.30
6-10	20	34	0.97	0.46-2.1	0.92
11-15	12	26	0.68	0.29-1.57	0.33
16-20	13	17	1.35	0.56-3.24	0.47
21-25	5	8	0.96	0.62-1.46	1.00
25	4	9	0.72	0.15-2.74	0.77

2.3.4.1. Multiple logistic regression analysis of variables.

Treatment episode (retreatment versus new untreated cases) was significantly associated with transmission [OD] 2.38, P=0.04, 95% confidence interval =1.04-5.40) while HIV status (seropositive versus seronegative) approached statistical significance when variables which could be linked to clustering by multiple logistic regression models were examined (OD 2.24, P=0.07, 95% confidence interval =0.95-5.3).

2.3.5. RFLP- based Families

2.3.5.1. Closely related isolates and concept of RFLP families

In order generate a visual impression of strain relatedness between the banding patterns of *M. tuberculosis* strains, a diagonal similarity matrix was generated (Figure 2.4). This matrix shows the degree of relatedness of each IS6110 fingerprint banding pattern with any other in the collection. Similar strains generate a triangle below the diagonal, which vary in shade, darker shades representing closer relatedness. Two major clusters and six clusters of moderate size are clearly visible while several small clusters of closely related isolates are also evident. These near -identical clusters were designated families based on the following observations and criteria: Many strains with non-identical RFLP patterns showed common band patterns, including the position and grouping of bands. Characterisation of families required that all the members of a family were identical or differed by only one to three fingerprint bands, while still maintaining the general band structure of the fingerprint. Further stipulations were the sharing of at least three bands in identical positions when there were four or less bands, or, when strains had more than four bands, the requirement of at least 80% homology between the strains. This exercise yielded 23 families which varied in size from 2 to 50 (Table 2.6).

2.3.5.2. RFLP family clustering as evidence of transmission over time

Based on the assumption that an isolate belonging to RFLP family is likely to constitute an endemic strain, an RFLP- family cluster would serve as evidence of transmission in the recent or more distant past (Figure 2.6 and Discussion 2.4.4). The percentage transmission of endemic strains based on the Small *et al.* (1994) formula for the 158 isolates in 23 RFLP families out of a total of 193 isolates.

would be 69.9%. Furthermore, 158 of 193 isolates (81.9%) were found in RFLP family clusters, giving an indication of the high proportion of endemic isolates being responsible for transmission in miners, either in the mining environment or from sources outside, including regions from where miners were recruited. The proportion of recent transmission based on IBP or clone clusters (78 isolates in 24 IBP or 65 isolates in 20 clone clusters out of 158 isolates in 23 RFLP families) is 34. 2% or 28.5% respectively.

Forty -one percent evaluable miners whose isolates were in RFLP- families shared a mine shaft and 20% (30 out of 151) come from the same village, presenting indications of the existence of common sources of infection and/ or endemicity of strains (Table 2.4 and Table 2.6). The corresponding figures for sharing a mine or a geographic region were 116 out of 151 (77%) and 115 out of 154 (78%) respectively.

2.3.6. Analysis of two dominant *M. tuberculosis* RFLP families from the mines.

The largest family (Family 1) included 50 isolates (25.9% of total) and the IS6110 copy number in this family ranged from 13 -16 per isolate (Figure 2.5). Twenty-five (50%) isolates with Family 1 type patterns occurred in seven clone-based clusters comprising two or more isolates, suggesting recent transmission involving these strains. The two largest IBP clusters consisted of 8 and 6 strains respectively, and are very likely to have caused microepidemics (Section 2.3.3.3).

Patients within Family 1 originated from adjoining central and eastern regions in southern Africa, comprising Lesotho (14), Free State (11), and Eastern Cape (20) a well as from Mozambique (1) and Swaziland (1). Family 1 isolates are also widely distributed in the six different mines. However, 73.9.0% (34 out of 46) of all evaluable cases with the Family 1 fingerprint pattern occurred in 2 or more miners working in the same mine shaft, suggesting possible linkage between the cases over time (Table 2.6). Eighteen (39.1%) recruited from the same village) providing additional evidence in favour of either transmission, recent or in the past. Taken together, a total of 42 (91.3%) of patients in Family 1 could be linked to either mine shaft or village of origin. None of the miners in families shared both mine shaft and village of origin.

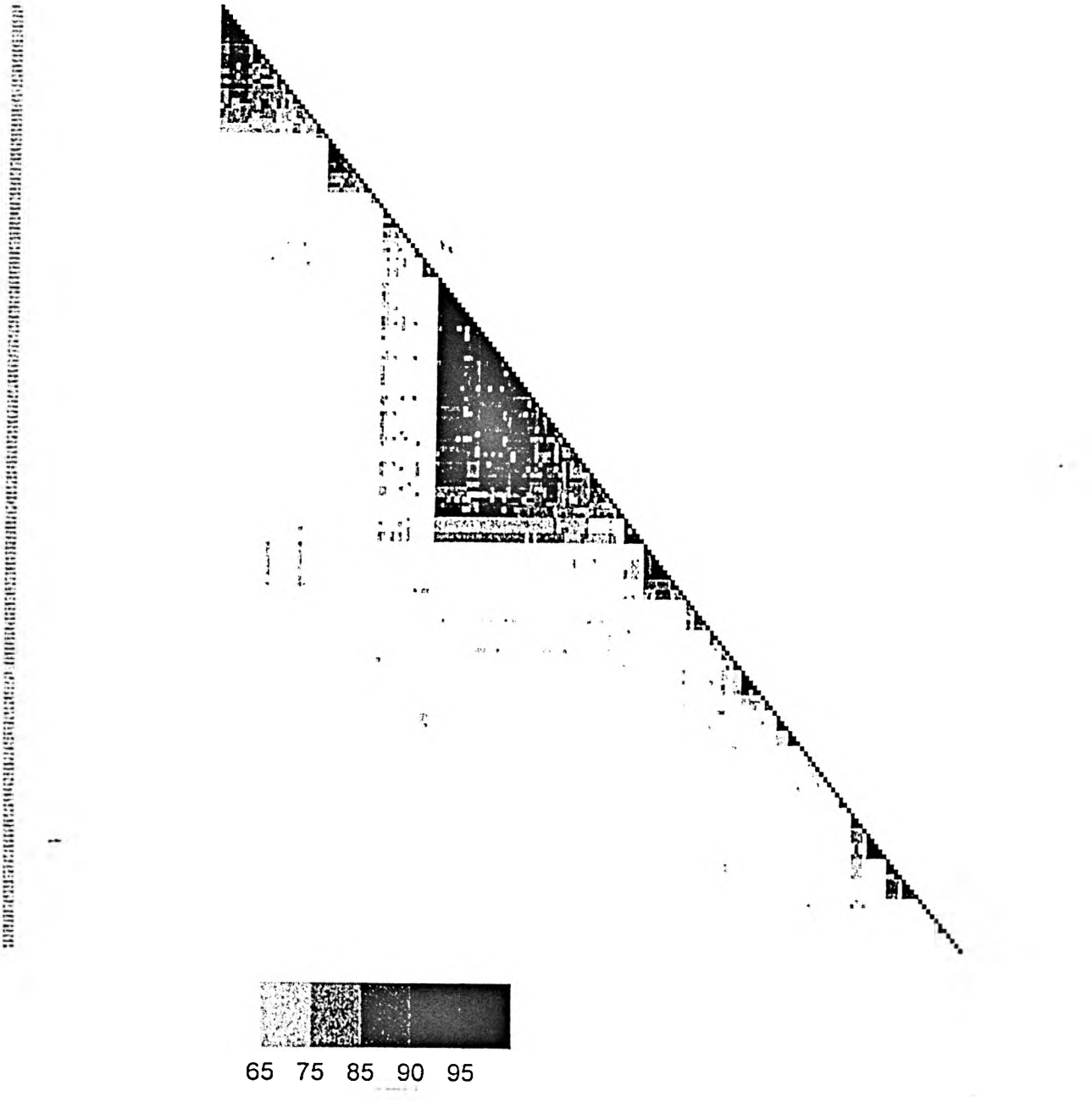


Figure 2.4. Similarity matrices of 193 isolates from Freegold Health Region miners. Similarity coeffients from 65% to 100% are shown by 5 different gray tones in matrices. Diagonal is formed by 100% similarity coeffient values of corresponding strains.

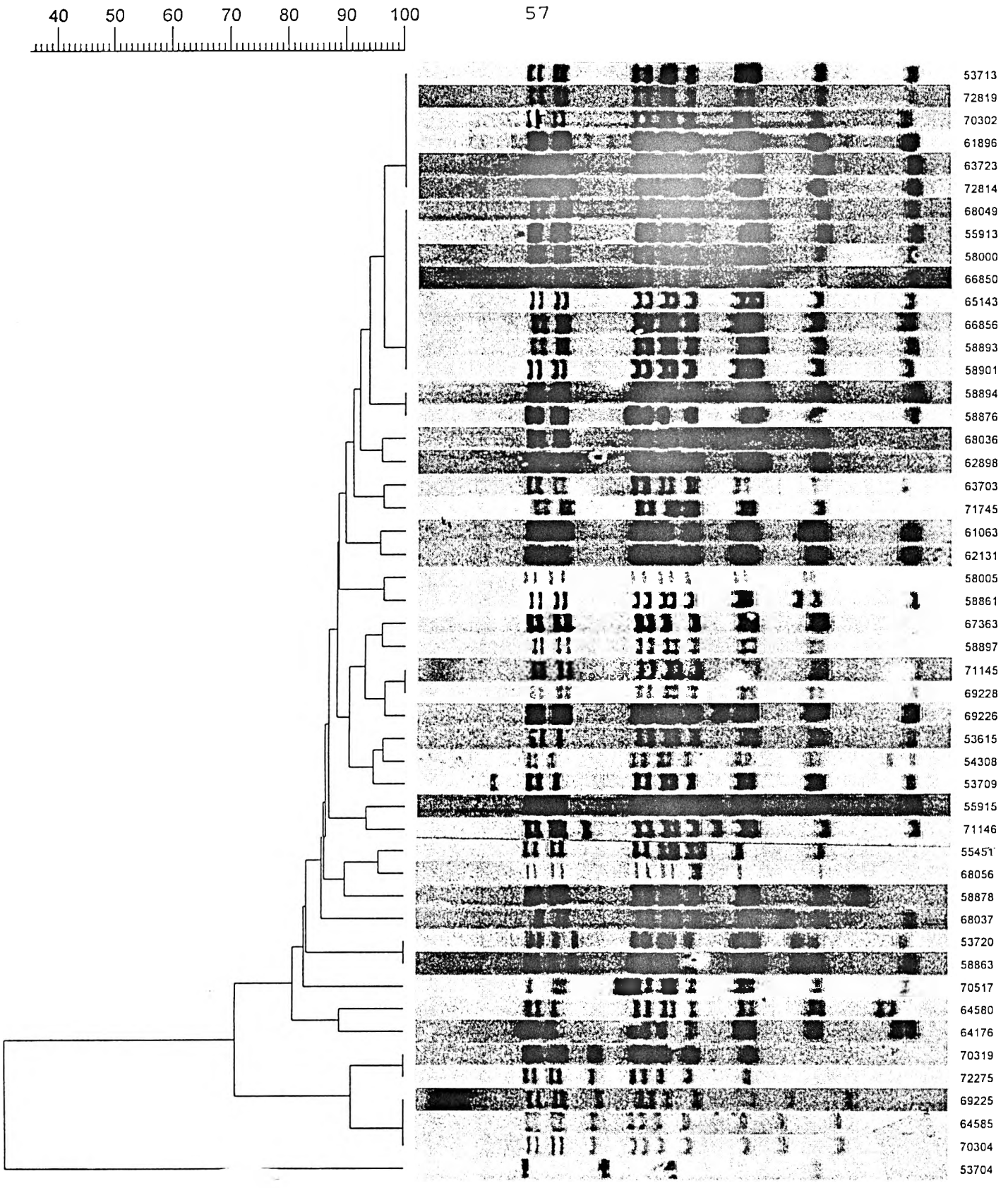


Figure 2.5. Dendrogram illustrating relationship between *M. tuberculosis* strains belonging to Family 1 type pattern. Strains from 53713 to 64176 included in Family 1(≥ 80% homology)

The second dominant family, Family 2 (Figure 2.6), consisted of 24 isolates with IS6110 copies, ranging from 7 -10 per bacterial cell. Ten strains within Family 2 formed three clone-based clusters consisting of 5, 3 and 2 isolates respectively. Patients in these were distributed unevenly in the six mines with more than half of all cases located in two mines (Table 2.5). Of 11 cases that shared a mine shaft, 2 were identical and 7 differed by one or two bands (Figure 2.6). Patients with the Family 2 pattern originated from Lesotho (10), and the Eastern Cape (10) and Free State provinces (4) and 9 could be linked to a village or town.

Comparison of patients whose isolates were in dominant Family 1 and Family 2(74) with patients who were in the heterogenous group (98 total) reveal that working in Freddies mine (7 miners have isolates belonging to Family 1 and 6 to Family 2) was associated with a significant risk of belonging to dominant families [OD] 3.03; 95% confidence interval 8.89-1.07; $P = 0.02$).

No significant difference in age, prior tuberculosis treatment, HIV- *M tuberculosis* co- infection status, drug resistance profiles, or period of employment in the mine was observed between patient in dominant families and patients in the heterogenous group (Table 2.8).

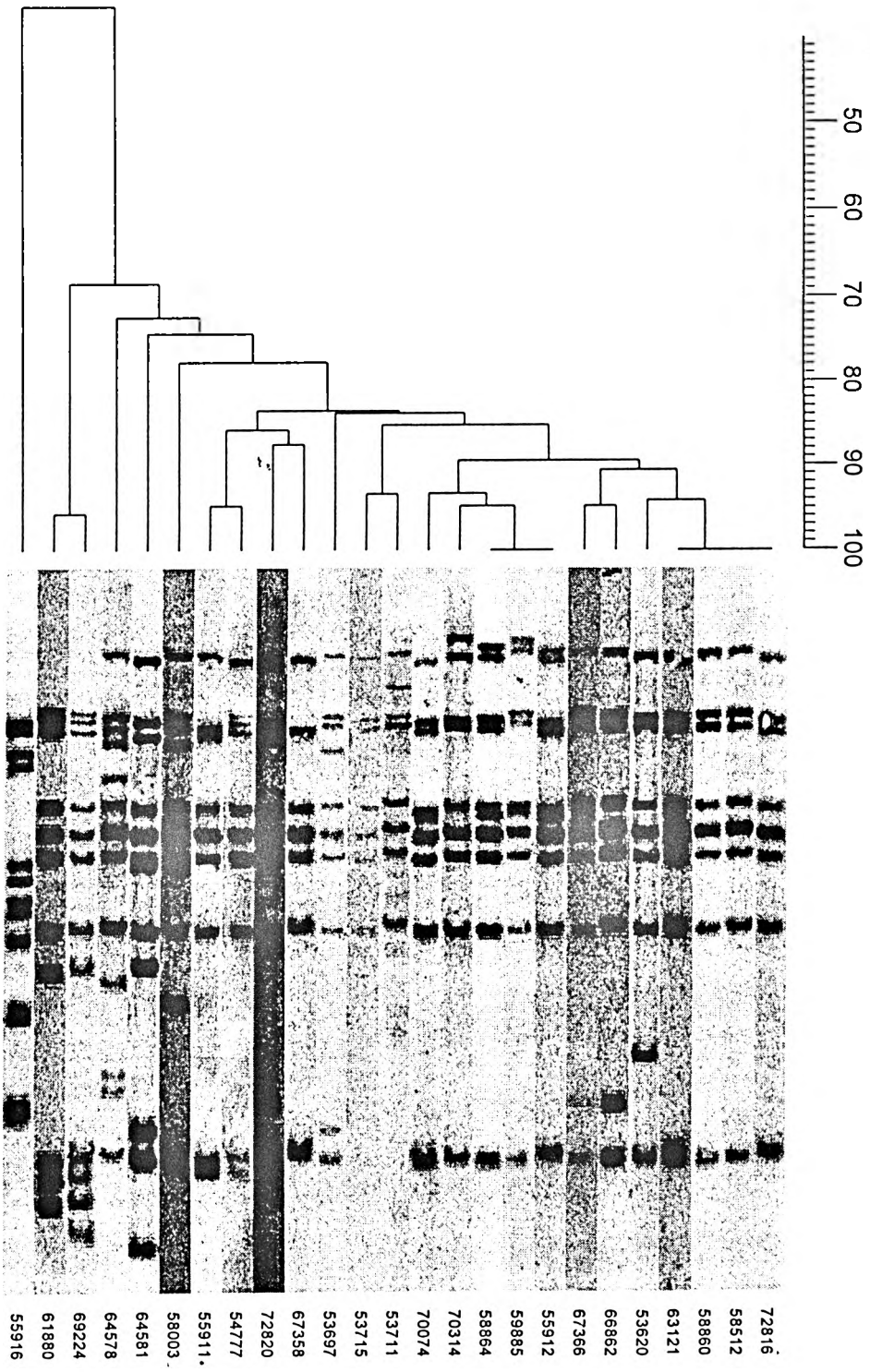


Figure 2.6. Dendrogram illustrating relationship between *M. tuberculosis* isolates belonging to Family 2 type pattern. Strains from 72816 to 55911 included in Family 2 (≥ 80% homology)

Table 2.8. Univariate analysis of risk factors associated with dominant RFLP families

Characteristic	No. of patients		Odds Ratio	95% Convidence Interval	P value
	Family 1+2(N=74)	Heterogenous (N=98)			
MINE					
FSG	7	9	1.03	0.33-3.23	0.95
FSS	7	13	0.68	0.23-1.97	0.44
Freddies	13	7	2.77	0.96-8.89	0.03
Pres Brand	11	23	0.57	0.24-1.34	0.16
Pres Steyn	17	20	1.16	0.53-2.57	0.69
W Holdings	16	15	1.53	0.67-3.57	0.28
PLACE OF ORIGIN					
Eastern Cape	30	31	1.55	0.78-3.06	0.18
Free State	15	19	1.06	0.46-2.4	0.89
Lesotho	24	37	0.79	0.40-1.57	0.47
Natal	1	2	undefined	undefined	undefined
Swaziland	1	3	undefined	undefined	undefined
Mozambique	1	2	undefined	undefined	undefined
HIV Seropositive	13	12	1.53	0.60-3.87	0.33
History of tuberculosis	14	14	1.40	0.58-3.39	0.42
Drug resistant	8	10	1.07	0.36-3.13	0.90
INH only	6	5	1.64	0.42-6.52	0.42
INH + RMP	1	2	undefined	undefined	undefined
INH + Others	1	2	undefined	undefined	undefined
Period in the mine					
1-5 years	4	5	1.24	1.01-1.51	1.00
6-10	25	29	1.21	0.60-2.44	0.56
11-15	13	25	0.62	0.27-1.40	0.21
16-20	15	15	1.06	0.46-2.40	0.89
21-25	7	6	1.36	0.40-4.57	0.58
25	2	11	0.22	0.02-1.06	0.04

Undefined= Figures too low for defined statistical analysis.

2.3.7. RFLP patterns of *M. tuberculosis* strains isolated from HIV -seropositive patients

2.3.7.1. Spectrum and cluster patterns

The RFLP patterns of the isolates from 26 HIV-seropositive patients with tuberculosis are shown in Figure 2.7. A total of 21 different IS6110 RFLP patterns was found. The number of IS6110 copies per isolate of *M. tuberculosis* varied from 3 to 19; 25 of the 26 isolates exhibited 6 or more copies. Nine isolates within the HIV-seropositive group shared identical RFLP patterns with one or two isolates and were grouped into four clusters: CL 1 (3 isolates), CL 3 (2 isolates), CL 8 (2 isolates) and CL 16 (2 isolates). An additional 7 clusters (19 isolates) observed in the study included isolates from both HIV-seropositive and HIV-seronegative patients (Table 2.9). These were CL 21 (4 isolates); CL 11, CL 14 and CL 18 (3 isolates each) and clusters CL 15, CL 19, CL 24 (2 isolates each).

Twenty-four fingerprints of isolates from HIV-seropositive patients were scattered within RFLP families (Family 1 to 24) and 12 out of 26 isolates from HIV-seropositive patients belonged to the two largest families, Family 1 and Family 2 (Table 2.9).

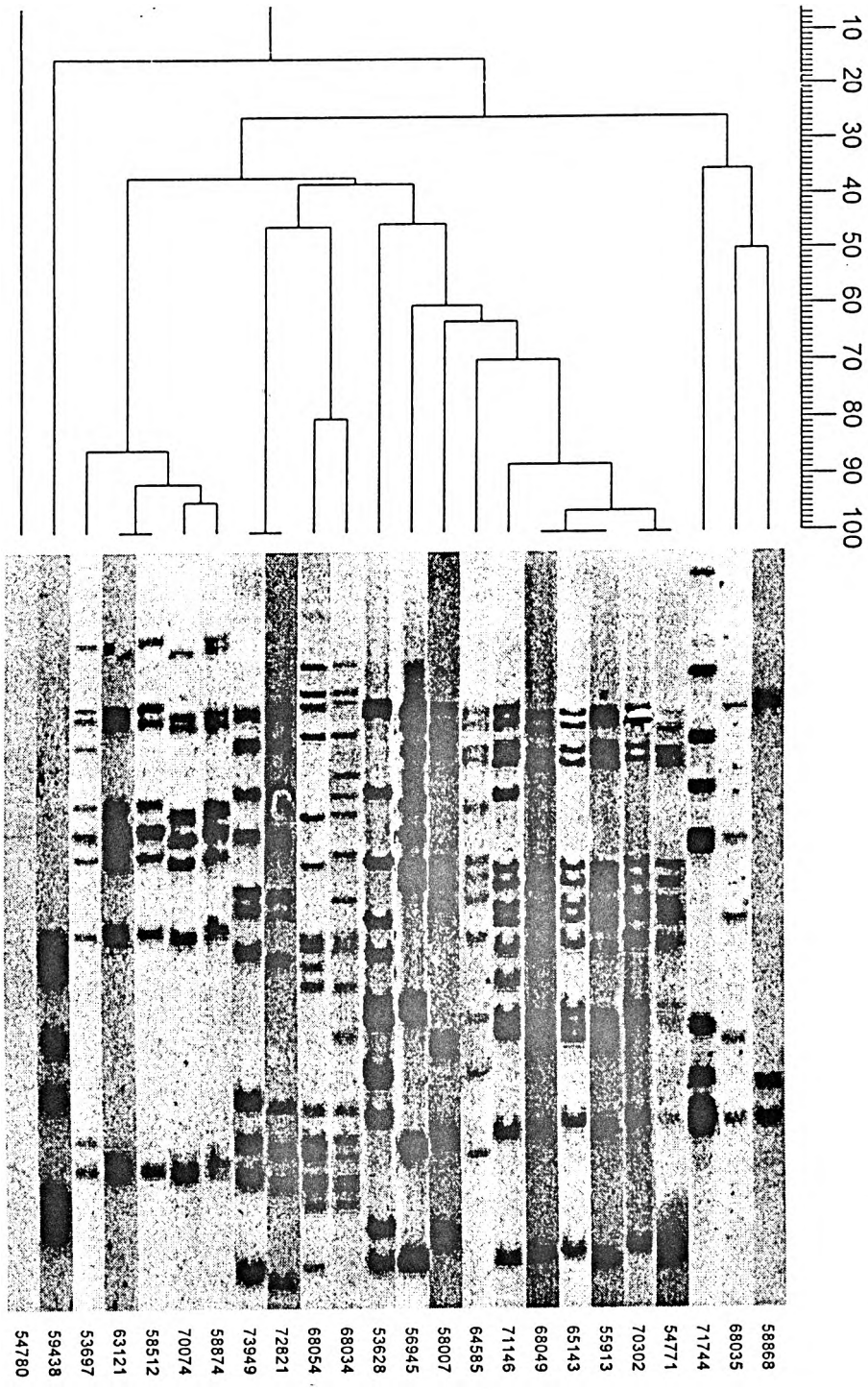


Figure 2.7. Dendrogram showing 23 isolates obtained from HIV-sero-positive patients.

Table 2.9. Drug resistance profiles, IS6110 clusters status and location in mines of 26 HIV-positive tuberculosis patients

LAB NO	Susceptibility	Cluster	Family	Copy number	Mine/shaft
54780	Sensitive	Unique	Unique	11	W Holdings 06
71744	Sensitive	Unique	Unique	10	Pres Brand 03
53709	Sensitive	Unique	FM 1	15	FSS 03
71146	INH-resistant	Unique	FM 1	15	Pres Steyn 04
55913	Sensitive	CL 1	FM 1	14	Pres Steyn 02
65143	Sensitive	CL 1	FM 1	14	Freddies 05
68049	Sensitive	CL 1	FM 1	14	Freddies 09
54771	Sensitive	CL 3	FM 1	13	FSS 02
70302	Sensitive	CL 3	FM 1	13	Freddies 05
53697	Sensitive	Unique	FM 2	10	Freddies 05
58874	Sensitive	Unique	FM 2	9	Freddies 05
70074	Sensitive	Unique	FM 2	9	Freddies 05
58512	Sensitive	CL 8	FM 2	8	FSS 03
63121	Sensitive	CL 8	FM 2	8	Freddies 05
56945	Sensitive	CL 11	FM 3	11	FSG 01
68034	Sensitive	Unique	FM 4	19	FSS 03
68054	Sensitive	Unique	FM 4	16	Pres Steyn 02
68035	Sensitive	CL 14	FM 5	5	FSG 04
53628	Sensitive	CL 15	FM 6	13	FSG 08
72821	INH-resistant	CL 16	FM 7	11	Pres Brand 02
73949	Sensitive	CL 16	FM 7	11	Pres Brand 05
58007	Sensitive	Unique	FM 8	10	W Holdings 05
64585	Sensitive	CL 18	FM 10	12	FSS 02
72275	Sensitive	CL 19	FM 10	10	Pres Steyn 01
58868	Sensitive	CL 21	FM 12	3	FSG 01
59438	Sensitive	CL 24	FM 23	6	FSS 01

Abbreviations: INH: Isoniazid; CL: IBP (identical band pattern) cluster; FM : RFLP (restriction fragment length polymorphism) family.

2.3.7.2. Comparison of RFLP patterns between HIV-seropositive and HIV-seronegative tuberculosis patients

The level of diversity of the DNA fingerprints observed in the HIV sero-negative tuberculosis group (106 patterns among the 134 isolates) was comparable to the level of diversity in the HIV-seropositive tuberculosis group (20 patterns among 25 isolates)($P = 0.92$).

The risk of belonging to a group of patients within a clone-based cluster was 56% (14 out 25) for the HIV-seropositive group and 37.3%(50 out 134) for the HIV- seronegative group ($P=0.07$), suggesting a possible trend of increased transmissibility in the HIV seropositive group which did not reach statistical significance. There were only two antimicrobial-resistant isolates in the HIV-seropositive group, both resistant to INH. One of these belonged to CL 16 and is linked to a drug-susceptible isolate in this cluster (Table 2.5 and Table 2.9).

2.4. Discussion

2.4.1. Heterogeneity of isolates

The present study revealed a total of 139 distinct RLFP patterns among 193 isolates analysed, indicating high strain diversity. The degree of strain diversity found in the present study is probably inflated because the short study period may have failed to reveal additional clusters likely to have occurred in subsequent months due to delayed reactivation of dormant/incubating tuberculosis. It was however, found that the isolates of 78 of the 193 patients (40.4%) belong to 1 of 24 clustered RFLP patterns (IBP clusters) and thus may have been epidemiologically linked. An estimated 28.0- 40.4% of the 193 cases was due to recent infection that had progressed to active disease during the six months study period.

High clonal diversity of *M. tuberculosis* has previously been reported in high incidence communities of Ravensmead and Uitsig, Cape Town, South Africa (Warren *et al.* 1996) as well as in countries with low prevalence of tuberculosis and contrasts with a lower genetic heterogeneity reported in so-called high prevalence countries in Africa (Tunisia, Ethiopia) (Hermans *et al.* 1995) and in high-risk populations such as urban homeless (Barnes *et al.* 1996), hospitals (Beck-Sague *et al.* 1992; Coronado *et al.* 1993) and shelter dwellers (Nardell *et al.* 1986; Nolan *et al.* 1991) among whom interhuman transmission is frequent. The "unexpected" high clonal diversity encountered in communities in Cape Town as well as in miners found in the present study may well reflect the heterogeneous origins of these populations. These comprise immigrants from different European and Asian countries and sailors and visitors/tourist to the Cape and migrant workers from different regions in southern Africa and elsewhere in the case of the mining industry. Reports by many reliable observers show that it is unlikely that tuberculosis occurred to any great extent among indigenous peoples of South Africa but was rather established following the colonisation of Africa by the European settlers and then

accelerated due to the rapid urbanisation that followed (Livingstone 1857). Thus colonisation and rapid urbanisation for purposes of commence and trade introduced multiple sources of infection into southern Africa. In South Africa, black miners have traditionally been a migrant work force and are recruited from in areas with a high incidence of tuberculosis. It is likely that many of these miners acquired their tuberculosis infection from diverse sources with European origins.

2.4.2. Low copy number isolates

Seventy-five percent of the 193 investigated isolates of *M. tuberculosis* in the present study were found to carry 8 to 14 copies of the IS6110 element, while the proportion of isolates with a low (≤ 5) copy number was 10.8%. This low figure is comparable with those of other studies of strains from a South African gold-mining group (11.5%)(Godfrey-Faussett, *et al.* 2000), Denmark (8.6%)(Yang *et al.* 1992) Tunisia (5.5%)(Hermans *et al.* 1995), from Cape Town, South Africa(5%)(Warren *et al.* 1996 and from Nambia,(13%) (Haas *et al.* 1999). The low patterns to clusters ratio showed that, compared with the higher copy number isolates, relatively more clusters were present in the low copy number group. This low heterogeneity index emphasises the need for secondary typing of this group of isolates in order to identify unique patterns within the clusters. This was unfortunately not done in the present study (See section 2.2.2.10). Polymorphic GC- rich sequence (PGRS) and spoligotyping are favoured methods for such subtyping (Ross, *et al.* 1992; Kamerbeek *et al.* 1997).

In the present study, a single copy of IS6110 insertion sequence was observed for one isolate recovered from a 48-years old man who originated from Mount Frere, Eastern Cape in South Africa. Such strains are relatively rare and have previously been reported in India (Van Embden *et al.* 1993).

2.4.3. Evidence of recent infection

The true magnitude of an increased burden of tuberculosis due to recent infection in the Freegold Health Region is probably greater than our estimate (28.0%-40.4%) provided in the present study. The incidence of tuberculosis in South African miners (approximately 1200/100 000 in 1994) is extremely high and substantially higher (approximately four fold) than that in the general population. The working and sleeping conditions in the mines are conducive to interhuman transmission of *M. tuberculosis* and one might expect a large number of tuberculosis cases caused by relatively few strains circulating in a population with a high rate of recent transmission (high percentage of clustering). This situation would be equivalent to countries with a high endemicity of tuberculosis (Hermans, *et al.* 1995)

Although evidence of direct transmission could not be demonstrated conclusively in majority of cases in the present study, geographical links relating to patients whose isolates were in IBP or clone clusters make transmission plausible. Approximately 19% (14 out of 75) of all evaluable IBP-clustered cases, involving 14 miners with two clusters of three miners and four clusters of two each, occurred in two or more patients in the same mine shaft while 5 out of 77(6.5%) IBP clusters cases originated from the same village, strongly suggesting recent transmission. The figures for sharing a mine or a geographic region were 48% and 62% respectively.

Transmission could have occurred in many different settings other than mine shafts, such as in hostels, dining halls, bars, etc. A limitation of the present study is that interviews with relevant miners in clusters were not performed. Only limited information regarding possible linkage between cases was obtained from affected miners by the mine authorities and an attempt to establish such linkages through an appropriate questionnaire did not form part of this study. In a recent study in rural Kwazulu-Natal,

43% of isolates from 246 patients were found to be in clusters, a figure which is very close to the 40.2% of the present study (Wilkinson *et al.* 1997; Wilkinson 1999).). The authors also could not demonstrate direct epidemiological links between most of these clustered cases, although patients with close or intimate contact, such as a relative, were more likely to be in clusters. They interpreted the presence of clusters as evidence of recent infection and the lack of epidemiological linkage as transmission through casual and not prolonged or intimate contact (Wilkinson *et al.* 1997; Wilkinson 1999). An alternative possibility is that *M. tuberculosis* strains with matching RFLP patterns may be epidemiologically related as a result of transmission that occurred some time in the past, either directly or indirectly, and that the affected cases under the stressful conditions in the mines then coincidentally reactivated during the period of the investigation.

More relevant to the present findings is a study in another group of mines in South Africa which has been published recently (Godfrey-Faussett *et al.* 2000). These researchers found that 67% of isolates from 371 miners could be assigned to 62 clusters with identical fingerprint patterns. An estimated 50% of isolates were therefore putatively involved in recent transmission (62 source cases subtracted from total number of isolates). The transmission rate amongst this group was higher than that found in the present study (50% vs 28%) and included all isolates for the duration of the study (one year, 1995). Plausible explanations why transmission appeared to be less frequent in Freegold Health Region mines relate to the longer duration of the Godfrey-Faussett *et al.* study (one year as opposed to 6 months) and the incomplete complement of isolates (± 50%) available in the present study. A six-month extension of the present study would have provided extra time for appreciably more cases to have preceded to overt disease following infection, allowing for more clusters to be formed, while the exclusion of the 21 out of 193 isolates (10.8%) low copy number isolates would also have reduced the estimated percentage of recent transmission.

2.4.4. Endemicity of *M. tuberculosis* strains, as evidenced by dominant RFLP families.

2.4.4.1. Two dominant RFLP families in the mines

The criterion selected for the definition of a cluster in relation to recent transmission was the requirement of 100% RFLP pattern matching. However, the results demonstrate a relatively high level of relatedness among a large number of different patterns. If banding patterns differing by one to three bands, as defined earlier, were considered to be phylogenetically linked, 82% of Freegold Health Region isolates fall into 23 families of identical or nearly identical patterns.

Two dominate families of genetically related strains, Family 1 and Family 2, were identified and harboured 50 and 24 isolates respectively. Isolates in these two families account for 38.3% of the total number of isolates from the Freegold Health Region. Strains in each family share more than two-thirds of their IS6110-containing restriction fragments, suggesting that *M. tuberculosis* strains in these two RFLP family groupings might have descended from expanded clones generated either cyclically in the past and as a result of recent spread. This interpretation assumes that horizontal transfer of IS6110 or genes in *M. tuberculosis* does not play a dominant role in the evolution of clones over time. Unfortunately, it is not yet possible to translate the RFLP IS6110 patterns in terms of time that has passed since the branching of a common ancestral clone. To do that, it would be necessary to know the average pace of the molecular clock of IS6110, that is, its average transposition frequency (Small and van Embden 1994).

The nature of the force (s) that contributed to the selection and dissemination of strains of these two families is unknown. Patients from these two dominant RFLP families did not differ significantly in their demographic characteristics from patients whose isolates were in other families or patients who harboured individual strains. Cases within Family 1, members of Family 2 and isolates in the

heterogenous group were also equally distributed between HIV-seropositive and HIV-seronegative miners. This is in keeping with previous studies in Tanzania and Thailand (Yang *et al.* 1995) which could not demonstrate different patterns of IS6110- associated RFLPs between strains from HIV-positive patients and isolates infecting patients who did not contract HIV infection.

The recent spread of HIV in the Freegold Health Region mines at the time of the present study was therefore unlikely to be the main driving force behind the expansion of the clones that became Family 1 and Family 2. Although retreatment of patients was shown to be associated with cluster formation in the present study and by Godfrey-Faussett *et al.* (2000) erratic treatment of tuberculosis is also not likely to be an important factor in the spread of these families in the mines, as DOTS is applied consistently to all miners with tuberculosis. Duration of employment in miners was also not shown to be significantly associated with these clusters.

A possible clue to the dominance of these two RFLP families is their statistically significant association with the Freddies mine. Three miners with CL 1 isolates in FM 1 and two with CL 8 isolates in FM 2 provide direct evidence of recent transmission involving miners from this mine. These two clones were also the largest encountered in the mines and comprised eight and five isolates respectively. It is possible that both RFLP families have become dominant in the mines because of recent and possibly past outbreaks in the mines (as evidenced by FM 1 and FM 2 -related clusters in the Freddies mine and the largest clusters in the study belonging to these two families) rather than acquisition of the strains elsewhere and the patients subsequently presenting with reactivation tuberculosis in the mines.

Possible properties of these two families that may account for the propensity to spread, such as the

ability to cause disease in BCG-vaccinated patients or tendency to be more infectious in causing pulmonary disease remain to be studied. Based on the findings reported by Valway *et al.* (1998) repudiated to some extent by Manca *et al.* (1999) and North *et al.* (1999), rates of growth of representatives of these families in the lungs of an aerosol animal model or lung, spleen and liver counts in an intravenous model as well as those of Zhang *et al.* (1999) in human macrophages, may help to elucidate aspects of the transmission and pathogenicity of *M. tuberculosis* strains (see Chapter 4). Studies to identify the genes and gene products could lead to the identification of virulence factors of the tubercle bacillus that are important for aggressive *in vivo* growth or the propensity for easy transmission of disease. This in turn, could lead to the development of a subunit vaccine that targets a critical virulence factor. Furthermore, if specific host factors which interact with virulence factors are identified, tests to identify persons at high risk for active disease could be developed, as was suggested by Bellamy *et al.* (1998) who linked variations in the NRAMP1 gene encoding the natural-resistance-associated macrophage protein 1 with susceptibility to tuberculosis in West Africans.

2.4.4.2. Generation of endemic strains

Figure 2.8 gives a diagrammatic illustration of how over an extended period of time a series of transmission episodes would result in clusters comprising strains from multiple sources from the past. Following primary infection of contacts, mainly children, by the original clonal strain, reactivation of dormant bacilli may occur in a small proportion of infected persons following different periods after primary infection. These cases could again infect a number of persons following which similar cycles could occur as a result of reactivation of dormant cases. As can be seen from the diagram, cycled strains which belong to the same clone can be isolated at the same time, without any obvious or direct epidemiological link between them. When such a clone is introduced into a population, it may spread over a number of generations and become established in a community. The many cycles described

above, during which extensive multiplication of strains would have occurred, would have provided ample opportunity for the introduction of minor insertional changes of IS6110 into the chromosome of strains, resulting in RFLP families of near identical fingerprints.

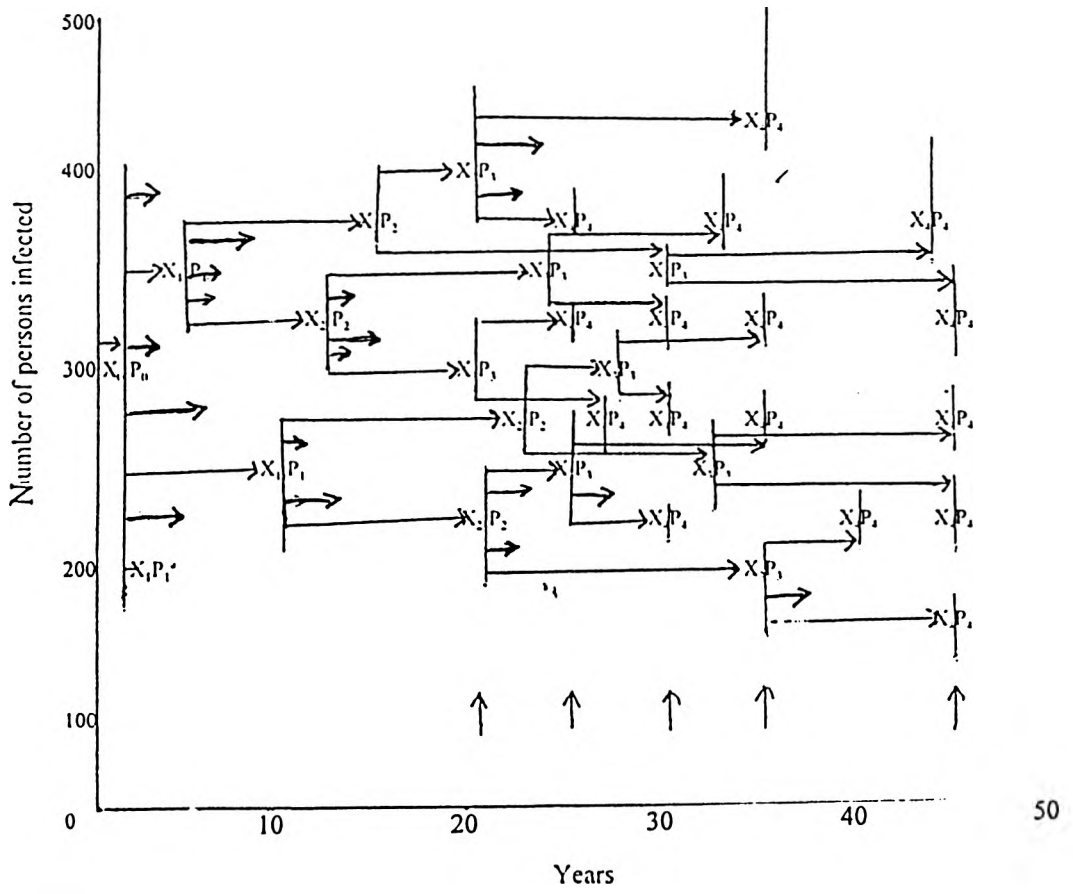


Figure 2.8: Patterns of clonal spread of *M. tuberculosis*, in patients with reactivation tuberculosis following dormancy of longer than two years.

X= Reactivation with disease; X_0, X_1, X_3, etc. denote numbered cycles of reactivation
P = Primary infection followed by dormancy which may end in reactivation
→ = Progression of dormancy to reactivation
| = A bar = number of persons infected
↑ = Clusters observed at a specific period in time
a =About 80 percent of infected individuals who develop clinical TB within two years after primary infection

2.4.4.3. Reactivation tuberculosis and endemicity of tuberculosis

The frequency of reactivation of dormant tuberculosis would affect the extent of endemicity of strains in the mine population. Conditions in the mining environment are likely to facilitate reactivation of dormant tuberculosis. Stress, both physical and emotional as well as viral infections which may suppress cell-mediated immunity could induce reactivation while the recognised effect of silica dust and the common practice of migratory miners to return to the same mine could further augment the establishment of the clones in the mines.

Stress, both physical and psychological has been shown to affect immunity to infection adversely. In a review article, Peterson *et al.* (1991) pointed out that considerable research had been carried out identifying a variety of psychological stressors e.g. bereavement, academic pressure and loss of self-esteem and their immunosuppressive effects through several immune-regulatory mediators and neuroendocrine hormones (Bonneau *et al.* 1990; Khansari *et al.* 1990). Several aetiologies have been shown to induce immune suppression, including respiratory and herpes viruses while several bacterial and parasitic infections have also been incriminated (Peterson, *et al* 1991).

Specific examples of viral disease which include influenza A virus and respiratory syncytial viral infections have been shown to suppress cell-mediated immunity (Roberts *et al.* 1986; Preston *et al.* 1992) and the basis for such effects has been reviewed by McChesney and Oldstone (1987). Another example is the well documented immuno-suppressive effect of measles virus in patients with tuberculosis (Kempe and Fulginiti 1995; Smithwick and Berkovich 1966).

Animal experiments in mice and rats have shown that both physical (forced exercise) and psychological stress (crowding) suppress immunity to tuberculosis, leading to increased mortality due

to this organism (Tobach *et al.* 1956; Kreis *et al.* 1965)

2.4.4.4. Endemicity of dominant strains and interpretation of DNA fingerprinting

Evidence in keeping with the concept of the establishment of dominant RFLP families/clones was provided by studies involving Ethiopia and Tunisia where tuberculosis is highly prevalent and relatively few RFLP-families were found, compared with a much larger percentage of unique strains in the Netherlands where the incidence of tuberculosis is low (Hermans *et al.* 1995).

RFLP analysis to identify patterns of tuberculosis transmission within a community is based on the premise that epidemiologically unrelated cases will have occurred as a result of reactivation of dormant infection and thus have unique RFLP patterns, whereas cases that are linked as a consequence of recent infection will have the same patterns (i.e. appear in defined clusters). As mentioned in the introduction of this chapter, in poor countries where tuberculosis is highly endemic, clusters may be formed by repeated cycles of transmission and may not necessarily indicate recent infection (Godfrey-Fausset, 1999; Hermans *et al.* 1995). Godfrey-Faussett implied in his commentary in the Lancet (1999) that the validity of linking clustering to recent infection in highly endemic regions would be strengthened by "much mobility of population". The fact that miners are recruited from many diverse regions is therefore likely to improve the predictive value of linking IBP clusters to recent infection. Utilisation of migratory labour at the EOH is decreasing but still applies to a large number of miners employed by the mines. There are, however, factors inherent to the mining environment and to the labour management of mines which could promote the establishment of "endemic strains" reflected by RFLP-based families, including IBP strains within families, and thus reduce strain diversity. Such factors include the practice of migratory miners from defined rural communities returning to the same mines where they worked before, often for periods from 18 months or longer (G. J. Churchyard, personal

communication). Furthermore, mining conditions which promote reactivation of dormant cases would lead to an increased "infectious pool" and thus favour further transmission. Such cycling of miners with dormant or overt tuberculosis to create a common infectious pool would facilitate the establishment of endemic strains in mines.

The degree to which this process is operating in mines is uncertain but on balance it appears likely that many and probably the majority of cases in an IBP /clone cluster would represent recent infection, while near identical strains defined as RFLP families would more likely give an indication of the extent to which endemic strains have become established. It is however, possible that some near identical isolates within a RFLP-family could be linked to recent infection as minor changes as a result of transposition may be encountered in outbreaks (Cave *et al.* 1994 Chevrel-Dellagi *et al.* 1993; Daley *et al.* 1993; Das *et al.* 1993; Hermans *et al.* 1990; Mazurek *et al.* 1991; Small *et al.* 1993; and Takahashi *et al.* 1993). The high percentage of isolates in Family 1 (73.9%) and Family 2 (70%) of miners sharing a shaft (Table 2.5) provides epidemiological evidence that this could have been the case in the present study. Furthermore, the finding that 35 out of 74 isolates (47.3%) of RFLP- family 1 and RFLP -family 2 incorporated ten clone clusters (2 to 8 isolates per cluster), provides convincing evidence of recent transmission involving endemic strains (clone-based clusters within predominant RFLP-families).

2.4.5. Risk factors of recent transmission

2.4.5.1. Recent transmission in the Free State and Eastern Cape mining recruits.

Univariant analysis of the findings of the present study suggests that being a patient from Free State region was associated with clustering involving clones. The reason for this is not clear. Analysis of isolates from Free State reveals that 13 isolates among Free State miners showed RFLP patterns

identical to one or more isolates and were grouped into 5 clusters (4,3,2,2,2 per cluster respectively)

Miners from the Free State whose isolates were in clusters were distributed evenly in the mines (5 from Freddies, 2 from W Holding, 3 from Pres Brand, 3 from Pres Steyn, 2 from FSG and 1 from FSS mine) and none could be linked to a mine shaft. Furthermore patients in clusters originated from different villages or towns and only two miners in one cluster could be linked to a specific village or town. The lack of evidence linking clustering to work place and or geographical region is consistent with reactivation of endemic strains, but may also be due to casual contact between patients.

A possible explanation for the relatively high percentage of clusters in mine recruits from the Free State, may relate to the proximity of those recruits to the mines which have a much higher prevalence of tuberculosis than the rural recruiting areas. The Free State recruits are therefore more likely to be exposed to infectious (sputum-positive) persons in the immediate mining environment than their counterparts from distant regions.

The same reasoning may explain the relative fewer clusters in mine recruits from the Eastern Cape who are less exposed to the high prevalence mining environment. They may recently or over time, have acquired a greater variety of strains in their region where tuberculosis is also very common. Reactivation of their dormant tuberculosis of diverse origin, while in the mines, will result in greater heterogeneity of strains with less evidence of cluster formation.

2.4.5.2. Retreatment of patients as a risk of recent transmission

The most important risk factor found to be important for clustering in multiple logistic regression model in this study was retreatment episode. Retreatment patients in the present study include those

who failed treatment as well as reinfection after a successful treatment episode. Patients on retreatment schedules remain culture positive for prolonged periods, even when their isolates remain susceptible to antituberculosis drugs, and therefore pose a considerable risk to contacts. Control measures should be introduced to reduce transmission originating from this group which has been identified as a high risk in this and other studies (Godfrey-Fausset *et al.* 2000).

2.4.5.3. HIV infection and recent transmission

According to the literature, increased clustering of isolates from patients who rapidly progress from infection to active disease as a consequence of HIV infection can be expected (Beck-Sague *et al.* 1992; Daley *et al.* 1992; Small *et al.* 1994). Based on multiple logistic regression analysis, isolates from HIV- infected patients in the present study are estimated to be about twice more likely to be in clusters than HIV-seronegative patients, although it is likely that the limited data from the present study did not allow statistical significance at the 5% level. At 16.6%, the prevalence of HIV infection in miners with tuberculosis was found to be relatively low in the Freegold Health Region mines during 1994 study period. Since then, the rate of HIV infection in miners at these mines increased dramatically to 45% in patients with tuberculosis in 1996, while the incidence of tuberculosis in the mines increased to 2476/100 000 in 1996 (Churchyard *et al.* 1999). Based on experience elsewhere, it is likely that HIV infection and AIDS would, in time have a major impact on the transmission of tuberculosis in Freegold Health Region miners.

2.4.6. Usefulness of DNA fingerprinting for monitoring of control programme

Molecular typing methods have proved to be powerful tools in the investigation of important aspects of the activity of *M. tuberculosis* in a population. Linked to understanding aspects the pathogenesis of the disease, such as the differentiation between reactivation from dormant infection and recent

transmission (Daley *et al.* 1992), molecular techniques have been used in specific situations to determine the relative frequency of recent infection in a community (Small *et al.* 1994). Studies utilising such methods also complemented our understanding of environmental and social factors that influence tuberculosis transmission which could have a bearing on the design of improved tuberculosis control strategies, i.e., the identification and modification of settings that foster spread of the disease. Once different strategies are undertaken to control transmission, the monitoring of the efficacy of tuberculosis control programme can be accomplished with ongoing RFLP-based typing of isolates. This can be achieved by the early identification of micro-epidemics and by noting increasing or declining trends in cluster formation, especially in relation to larger clusters such as cluster 1 and cluster 3 of the present study. An increasing trend would denote the likelihood of a deficient control programme while a decrease in cluster formation over time would reflect favourably on the programme. In a broader context, prospective fingerprinting of isolates in longitudinal studies could give a more accurate picture of transmission patterns involving endemic strains (IBP clusters within larger RFLP -families) as opposed to transmission of strains from diverse sources (IBP-clusters outside RFLP families).

Ongoing DNA fingerprinting of isolates may also be useful in individual miners with tuberculosis, where consecutive isolates with the same fingerprint showing a change to resistance to a single antituberculosis agent would confirm the occurrence of acquired drug resistance. This may be seen when treatment failures occur or in cases with relapse. Primary drug resistance could be assumed if the initial isolate from a patient is already resistant to one or more drugs. If, however, such a strain forms part of an existing IBP cluster in the mine, recent transmission most likely occurred. Such findings could also provide evidence of an impending outbreak involving an MDR strain as was seen in MDR outbreaks in the USA (Hewlett *et al.* 1993; Campbell *et al.* 1993; Valway *et al.* 1994). These cases

would constitute reinfection with primary drug-resistant strains.

2.4.7. Dominant RFLP families in the mines

The criterion selected for the definition of a cluster in relation to recent transmission was the requirement of 100% RFLP pattern matching. However, the results demonstrate a relatively high level of relatedness among a large number of different patterns. If banding patterns differing by one to three bands, as defined earlier, were considered to be phylogenetically linked, 82% of Freegold Health Region isolates fall into 23 families of identical or nearly identical patterns.

Two dominate families of genetically related strains, Family 1 and Family 2, were identified and harboured 50 and 24 isolates respectively. Isolates in these two families account for 38.3% of the total number of isolates from the Freegold Health Region. Strains in each family share more than two-thirds of their IS6110-containing restriction fragments, suggesting that *M. tuberculosis* strains in these two RFLP family groupings might have descended from expanded clones generated either cyclically in the past or as a result of recent spread. This interpretation assumes that IS6110 or genes are not horizontally transferred in *M. tuberculosis*. Unfortunately, it is not yet possible to translate the RFLP IS6110 patterns in terms of time that has passed since the branching of a common ancestral clone. To do that, it would be necessary to know the average pace of the molecular clock of IS6110, that is, its average transposition frequency (Small and van Embden 1994).

The finding that a high percentage of isolates in Family 1 (78%) and Family 2 (70%) of miners sharing a mine shaft provides good epidemiological evidence that some near identical isolates within a RFLP-family could be linked to recent infection. Although, IS6110 fingerprint patterns were stable after multiple passages of *M. tuberculosis* strains in guinea pigs for two months, and in macrophage and

liquid culture for six months, this element is potentially capable of transposition, duplication and excision within the genome. Sequential isolates can yield one or more additional bands in chronic excretors (Cave *et al.* 1994; Das *et al.* 1993) and during outbreaks of tuberculosis (Chevrel-Dellagi *et al.* 1993; Daley *et al.* 1993; Mazurek *et al.* 1991; Small *et al.* 1993; and Takahashi *et al.* 1993).

In the present study, clustered isolates comprising identical band patterns were more common among members of the dominant Family 1. The largest cluster of 8 isolates (Table 2.2) was found among this group of strains. This phenomenon of clusters within family may be interpreted as evidence that the members within each cluster in a family had some selective advantage. However, this might also be an artefact created during the expansion of a clone into RFLP family which may, as a matter of course, also contain identical strains (i.e., IBP clusters).

The nature of the force (s) that contributed to the selection and dissemination of strains of these two families is unknown. Patients from these two dominant RFLP families did not differ significantly in their demographic characteristics from patients whose isolates were in other families or patients who harboured individual strains. Cases within Family 1, members of Family 2 and isolates in the heterogenous group were equally distributed between HIV-seropositive and HIV-seronegative miners. This is in keeping with previous studies in Tanzania and Thailand (Yang *et al.* 1995) which could not demonstrate different patterns of IS6110- associated RFLPs between strains from HIV-positive patients and isolates infecting patients who did not contract HIV infection.

The recent spread of HIV in the Freegold Health Region mines at the time of the present study was therefore unlikely to be the main driving force behind the expansion of the clones that became Family 1 and Family 2. Although retreatment of patients was shown to be associated with cluster formation

in the present study and by Godfrey-Faussett *et al.* (2000) erractic treatment of tuberculosis is also not likely to be an important factor in the spread of these families in the mines, as DOTS is applied consistently to all miners with tuberculosis. Duration of employment in miners was also not shown to be significantly associated with these clusters.

A possible clue to the dominance of these two RFLP families is their statistically significant association with the Freddies mine. Three miners with CL 1 isolates within FM 1 and 2 with CL 8 isolates in FM 2 provide direct evidence of recent transmission involving miners from this mine. These two clones were also the largest encountered in the mines and comprised 8 and 5 isolates respectively. It is possible that both RFLP families have become dominant in the mines because of recent and possibly past outbreaks in the mines (as evidenced by FM 1 and FM 2 -related clusters in the Freddies mine and the largest clusters in the study belonging to these two families) rather than acquisition of the strains elsewhere and the patients subsequently presenting with reactivation tuberculosis in the mines.

Possible properties of these two families that may account for the propensity to spread, such as the ability to cause disease in BCG-vaccinated patients or tendency to be more infectious in causing pulmonary disease remain to be studied. Based on the findings reported by Valway *et al.* (1998) repudiated to some extent by Manca *et al.* (1999) and North *et al.* (1999), rates of growth of representatives of these families in the lungs of an aerosol animal model or lung, spleen and liver counts in an intravenous model as well as those of Zhang *et al.* (1999) in human macrophages, may help to elucidate aspects of the transmission and pathogenicity of *M. tuberculosis* strains (see Chapter 4). Studies to identify the genes and gene products could lead to the identification of virulence factors of the tubercle bacillus that are important for aggressive *in vivo* growth or the propensity for easy transmission of disease. This in turn, could lead to the development of a subunit vaccine that targets

a critical virulence factor. Furthermore, if specific host factors which interact with virulence factors are identified, tests to identify persons at high risk for active disease could be developed, as was suggested by Bellamy *et al.* (1998) who linked variations in the NRAMP1 gene encoding the natural-resistance-associated macrophage protein 1 with susceptibility to tuberculosis in West Africans.

2.4.7. Strains isolated from HIV-seropositive patients

The pathogenesis of HIV-related tuberculosis involves primary or secondary exogenous infection and reactivation of previously acquired dormant bacteria in highly susceptible HIV-infected individuals. Patients that are being infected simultaneously by HIV and *M. tuberculosis* are likely to experience an accelerated progression to overt tuberculosis. A large proportion (61.9%) of isolates from HIV-seropositive patients in this study belonged to RFLP clusters based on full identity. The results are consistent with recent data from New York City, showing that 57% of new tuberculosis cases in HIV-seropositive patients were due to recent transmission (Alland *et al.* 1994). Although, recent transmission was high among HIV-seropositive individuals in the present study, the prevalence of HIV (13.4%) at the time of the study was found to be relatively low in the Freegold Health Region mines. In the absence of evidence that HIV co-infection played a prominent role in accelerating the escalation of tuberculosis in miners in 1994, it may be postulated that factors promoting transmission in the mine environment (silica dust, overcrowding and the type of ventilation) interacting with sources of the organism (mainly cases with cavitary tuberculosis), facilitated the generation of a pool of infective aerosols, and, that once miners became infected, stress-related factors (Petersen *et al.* 1991) and/or frequent viral and other infections (McChesney and Goldstone, 1987) including infection with HIV, could have suppressed cell-mediated immunity, leading to a shorter period between infection and development of active tuberculosis. Since 1994, however, the rate of HIV infections in miners at Freegold Health Region mines increased dramatically to 45% in patients with tuberculosis in 1996,

while the incidence of tuberculosis in this mines increased to 2476/100 000 in 1996 (Churchyard *et al.* 1999). The contribution of HIV infection and AIDS to this recent escalating in the transmission of tuberculosis in Freegold Health Region miners has not been clearly documented but it may have been considerable.

By comparing the RFLP patterns of HIV-related *M. tuberculosis* with the RFLP patterns of non-HIV-related isolates, it was found that the DNA fingerprints exhibited similar polymorphism in the two groups. Fourteen IBP clusters were shared by two or more isolates in the HIV-positive patients and eleven in the HIV-seronegative groups. This difference was not statistically significantly (P= 0.08). Yang *et al.* (1995), reported an similar risk of infection with a defined *M. tuberculosis* clone for HIV-seropositive and HIV-seronegative individuals.

It might be expected that HIV-related tuberculosis could also be associated with low-virulent clones of mycobacteria which would be able to infect immunocompromised hosts. However, from the present study no indication was found that particular clones of *M. tuberculosis* were predominant in cases of HIV-related tuberculosis.

2.4.9. Limitations of the study

The current study has several limitations which have been discussed earlier in various sections of this chapter. Two major failings however, require emphasis. First, only approximately 50% of all patients hospitalised at EOH with culture-proven tuberculosis during 1994 were included in the present study. Apart from the fact that the lower number of cases available for analysis could have affected the number of clusters in the mining community at the time, the low number also reduced the power of statistical analyses. Second, a six months' survey will underestimate recent transmission. Because

RFLP analysis can only be used to analyse microbiologically-confirmed cases, persons who became infected but whose infection remained dormant during the six months of the study cannot be identified. As the high-risk period for developing tuberculosis is considered to be approximately 2 years after acquisition of infection, a period of at least 2 years would be required for optimal evaluation of the rate of transmission. In populations with active transmission, the proportion of clustered isolates increases when the period is longer than six months, as demonstrated previously in a French Polynesia population (Torrea *et al.* 1995).

Another important limitation was the failure to perform secondary DNA typing on low copy number isolates, in order to identify subclusters or eliminate 'false clusters' of apparently identical isolates. Furthermore, the lack of detailed information based on patient interviews using an appropriate questionnaire which could have identified additional risk factors.

3. THE POPULATION STRUCTURE OF DRUG-RESISTANT *MYCOBACTERIUM TUBERCULOSIS* ISOLATES IN RELATION TO RECENT TRANSMISSION AND PRIMARY AND ACQUIRED DRUG RESISTANCE

3.1. Introduction

The introduction of effective chemotherapy in the 1960's and subsequent advances in the use of drug combinations made tuberculosis a curable disease, while intermittent therapy on an outpatient basis and more recently the success of directly observed therapy short course (DOTS) constitute progress in the successful management of tuberculosis. Despite this potentially favourable situation, the incidence of tuberculosis in South Africa is increasing, and a most disturbing aspect of the problem is the emergence of multidrug-resistant (MDR) tuberculosis caused by strains resistant to both isoniazid and rifampicin, which poses a threat to individual patients(prolonged, toxic and expensive treatment with poor cure rates) to communities and to control programme efforts (World Health Organisation 1997). With the incipient HIV/AIDS explosion in South Africa, extensive spread of drug resistance as encountered in outbreaks in the USA (Hewlett *et al.* 1993; Campbell *et al.* 1993; Valway *et al.* 1994) could occurred in the South African mines and could make tuberculosis extremely difficult to control. It is therefore both timely and appropriate to reappraise the problem of drug resistance in order to learn more about the source of infection and delineate outbreaks due to these resistant strains.

It is currently accepted, on the basis of concepts formulated during the pre-HIV era, that drug resistance in patients with tuberculosis can take on two forms. Firstly, acquired drug resistance occurs as a result of treatment which is poorly conceived or poorly adhered to, allowing the emergence of naturally occurring drug-resistant mutants causing clinical disease (Lipsitch and Levin 1998; Mitchison 1998)

The second situation arises when a resistant strain from a patient with pulmonary tuberculosis subsequently infect and cause disease in another individual who may or may not have been previously infected with *M. tuberculosis*. This results in the occurrence of primary drug resistance, i.e. the strains infecting patients are already drug-resistant at the time of infection. Small *et al.* (1993) demonstrated through the RFLP analysis of *M. tuberculosis* isolates that primary drug- resistant tuberculosis can result from exogenous reinfection with a new multidrug- resistant strain of *M. tuberculosis* during or after therapy for an episode of drug-resistant tuberculosis due to another drug-resistant strain. If infection with an MDR strain involves a patient under treatment for disease caused by another strain, the process has been referred as superinfection.

The relationship between clustering, strain diversity and drug susceptibility patterns may be used to examine recently transmitted drug-resistant strains between miners or drug resistance acquired during treatment. Clonal dissemination of recently transmitted drug-resistant isolates i.e. primary drug-resistant strains, will exhibit identical or near identical RFLP profiles and limited RFLP diversity among drug-resistant strains in a population. Resistant strains that have become endemic in mines over a prolonged period may also contribute to such limited clonal diversity. Acquired drug resistance, arising from inadequate treatment of individual patients, on the other hand, will tend to be associated with a high proportion of strains with unique RFLP patterns. Recent findings by Van Rie *et al.*(2000) however, have suggested that acquired drug resistance may include in addition to patients infected with strains whose resistance was truly acquired during treatment, also patients who were initially infected with or reinfected with a drug-resistant strain.

The two main objectives of the investigations reported in this chapter are:

- To determine by molecular typing, using IS6110-based RFLP, the population structure of drug-resistant *M. tuberculosis* isolates in the Freegold Health Region mines during two periods of

1994 and 1995-1977

To determine the by molecular typing the distribution and patterns of recent transmission of such strains in the Freegold Health Region mines and to relate these to the drug resistance status (acquired or primary drug resistance) of patients' isolates.

An assessment of risk factors involved in the transmission of MDR isolates was also attempted but the small number of MDR cases available for analysis, especially during the 1994 investigation militated against reliable findings. A risk factor analysis involving the 1995-1997 study period is however, included in this chapter.

3.2. Materials and methods

3.2.1. Strains

a) A total of 19 isolates from patients admitted to EOH from 1 January to 30 June 1994 which showed resistance to one or more antimycobacterial drugs were included for study. The isolates formed part of a group of 193 isolates from patients who constituted about 50% of all confirmed tuberculosis patients hospitalised during the study period.

b) An additional 26 MDR cultures were isolated from the same hospital during 1995-1997 and submitted to the National Tuberculosis Research Program (NTBRP) bank of MDR strains. All viable cultures of *M. tuberculosis* isolated at EOH and submitted to the SAIMR laboratories as well as isolates from this hospital submitted directly to the SAIMR during this period were tested for susceptibility to antituberculosis drugs at the SAIMR and all isolates found to be MDR were sent to NTBRP bank for long-term storage and for references purposes. These 26 isolates were used in the present study.

The incidence of and factors associated with drug-resistant tuberculosis in the mines involved in the present study featured in recent publication by Churchyard *et al.* 2000. In this publication the authors indicated that 83.1% of cultures obtained from patients during the period 1994 to 1997 were submitted for drug susceptibility testing to at least INH and RMP (Churchyard *et al.* 2000).

3.2.2. Methodology and definitions

The 19 isolates from the 1994 were charcaterised by DNA fingerprinting as described in Chapter 2 while the additional 26 MDR isolates from the NTBRP bank were similarly typed following the same approaches used on the 1994 isolates. All fingerprint profiles were analysed using Gelcompare software for the presence of clusters of identical and closely related strains (see Chapter 2.2.).

In patients who had not previously received antituberculous drugs (listed as new cases), bacterial resistance was assumed to be probably primary. In patients with a record of previous treatment(listed as retreatment cases), the bacterial resistance was classified as probably acquired.

Multidrug-resistant tuberculosis was defined as resistance to at least isoniazid and rifampicin while single-drug resistance or mono resistance denoted resistance to only one antituberculous agent.

3.3. Results

3.3.1. Population structure of drug-resistant *M. tuberculosis* isolates during 1994

Drug-resistant *M. tuberculosis* cases were analysed by the standard DNA fingerprinting method with IS6110 as genetic probe. The generated IS6110 fingerprint patterns were highly variable among these isolates. The number of IS6110 copies per isolate varied from 4 to 17. The majority (94.7%) contained 6 or more copies. Eighteen distinct patterns among 19 drug-resistant isolates were identified (Figure 3.1.)

3.3.1.1. Drug resistance profiles of 1994 isolates

All 19 isolates were resistant to INH and of these 12 were mono-resistant. Of all the isolates resistant to more than one drug, three complied with the definition of MDR(resistant to both INH and RMP). One of these MDR isolates was in addition resistant to ethambutol (EMB) and another to streptomycin (SM). The other four isolates, apart from being resistant to INH, were also resistant to streptomycin and ethambutol (one isolate), or in addition resistant to streptomycin (two isolates) or apart from INH resistance also resistant to EMB (one isolate) (Table 3.1.)

3.3.1.2. Acquired and primary drug resistance in 1994 isolates.

Listing of patients on admission regarding their treatment history as "New" or "Retreatment" is given in Table 3.1 and Table 3.2. It was assumed that in the case of retreatment patients, resistance would most likely be acquired, while new (previously untreated) cases were categorised as primary drug resistance.

3.3.1.3. Geographical distribution of drug-resistant isolates of 1994

Geographical data of the patients with drug-resistant tuberculosis isolated during 1994 study period are shown in Table 3.1. Seven were from Eastern Cape , 5 from Lesotho, 3 from the Free State, 1 from Gauteng and 2 from Mozambique. Information on the mines where the patient worked was available for 18 patients. One worked in FSG, 2 in FSS, 2 in Freddies, 4 in Pres Brand, 4 in Pres Steyn and 3 in the W Holdings mine (Table 3.1 and Table 3.2)

Table 3.1. Characteristics of 19 drug -resistant *M. tuberculosis* strains isolated during 1994 study period

Strain	Episode type	INH	RMP	SM	EMB	Cluster	Family	HIV status	Mine/shaft	Place of origin
58901	New	+		+		CL 1	FM 1	Negative	W.Holding 05	Free State
58863	Retreatment	+				CL 6	FM 1	Negative	Pres Steyn 04	Eastern Cape
71146	New	+					FM 1	Positive	Pres Steyn 04	Eastern Cape
64580	NA	+					FM 1	Negative	Pres Brand 04	Lesotho
55451	New	+					FM 1	Negative	FSG 01	Eastern Cape
53715	New	+				CL 8	FM 2	Negative	W Holdings 05	Eastern Cape
53620	Retreatment	+					FM 2	Negative	Pres Steyn 04	Lesotho
58003 (MDR)	Retreatment	+	+	+			FM 2	Negative	Pres Brand 03	Eastern Cape
53718	NA	+				CL 11	FM 3	Negative	NA	NA
57216	New	+					FM 3	Negative	Pres Stein 04	Gauteng
53716 (MDR)	New	+	+				FM 3	Negative	Freddies 07	Free State
60526	NA	+			+		FM 4	Negative	FSS 02	Eastern Cape
53276	New	+		+	+	CL 13	FM 5	Negative	Freddies 09	Mozambique
72821	New	+				CL 16	FM 7	Positive	Pres Brand 02	Free State
66853	Retreatment	+				CL 16	FM 7	Negative	W Holdings 05	Mozambique
65138	New	+					FM 8	Negative	Pres Steyn 01	Lesotho
64582 (MDR)	Retreatment	+	+		+		FM 9	Negative	Pres Brand 05	Eastern Cape
69225	NA	+		+		CL 18	FM 10	Negative	FSS 02	Lesotho
70309	New	+				CL 24	FM 23	Negative	FSS 03	Lesotho

Abbreviations: INH: Isoniazid; RMP: Rifampicin; SM: Streptomycin; EMB: Ethambutol; ETH: Ethionomide; CL: Clusters based on 100% identical; FM : Families; NA : Information not available
The shaded areas comprise isolates belonging to four RFLP families including one cluster. CL 16; MDR multidrug-resistant

Table 3.2. Distribution of 19 drug-resistant *M. tuberculosis* isolates during 1994 study period according to treatment history

Characteristic	Treatment history Not available(NA) (n=4)	Retreatment cases Acquired drug resistance (n=5)	New cases Primary drug resistance (n=10)
Unique RFLP	2	1	3
Miners in 8 clusters(9)	2	2	5
RFLP family size≥3(11)	2	3	6
HIV-seropositive(2)	0	0	2
Lesotho(5)	2	1	2
Eastern Cape(7)	1	3	3
Free State(3)	0	0	3
FSS(3)	2	0	1
FSG(1)	0	0	1
Freddies (2)	0	0	2
Pres Steyn (5)	0	2	3
President brand (4)	1	2	1
W.Holdings(3)	0	1	2

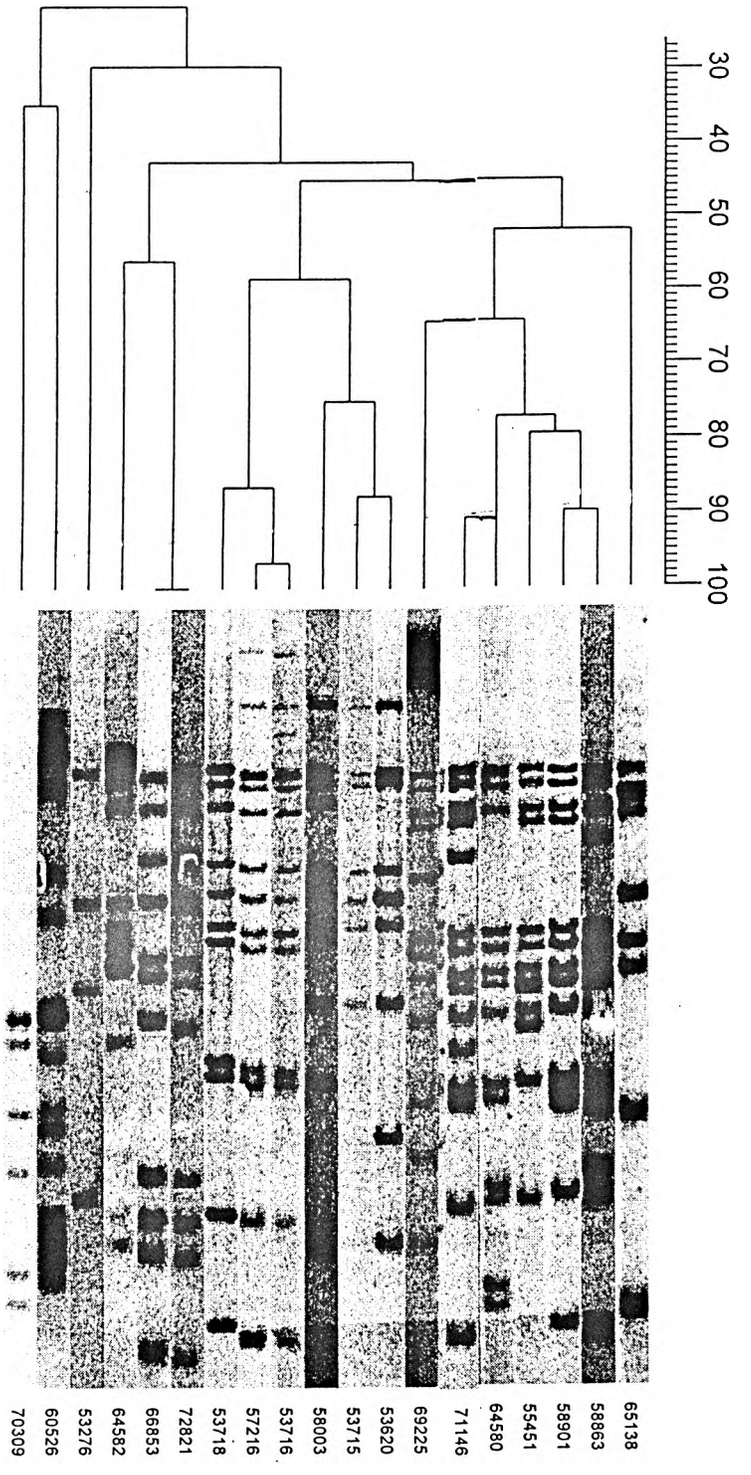


Figure 3.1. RFLP patterns of 19 drug- resistant *M. tuberculosis* isolated during 1994 study period

3.3.1.4. RFLP-based evidence compatible with recent transmission involving primary and acquired drug-resistant isolates

Computer-assisted analysis of the fingerprints revealed a single clone (two isolates) based on identical RFLP band patterns among drug-resistant isolates. The interesting sequence of transmission events involving clone 16 where the most likely scenario suggests transmission from a patient with acquired drug resistance(patient 66853) to an HIV-seropositive patient with no previous treatment history (patient 72821) is presented in detail in section 3.3.1.5 of this chapter.

A total of four RFLP families comprising identical or near identical strains corresponding to the RFLP-based families of drug-susceptible isolates were identified (Families1, 2, 3 and 7) (Table 3.1. and Table 3.3.). Amongst the four RFLP families there were eight clones which shared drug-susceptibility isolates with resistant isolates. One of these clones, (CL 16), contained two drug-resistant isolate, in addition to three drug-susceptible isolates (Table 2.5 and Table 3.1).

Ten possible transmission linkages based on genetic relatedness and, in addition, on identical resistance profiles, historic time of isolation of strains and treatment history could be established (Table 3.3). Of the seven involved possible transmission to patients categorised clinically as primary drug-resistant. Only two patients in Family 1 were working in the same mine shaft (Pres Steyn 04) (Table 3.1.). These miners both came from the Eastern Cape but from different towns.

If one assumes, based on the findings of Van Rie *et al.* (2000) that recent transmission may evolve strains that on clinical grounds, are categorised as acquired drug resistance (retreatment cases), then an additional 12 transmission linkages between isolates with identical resistance profiles are possible, 9 from primary drug-resistant strains to acquired drug-resistant strains and 3 between acquired drug-

resistant isolates (see data in Table 3.1)

Three MDR tuberculosis isolates, available for RFLP typing from the Freegold Health Region in the 1994 study period, exhibited different IS6110 DNA fingerprint patterns, and therefore failed to show direct evidence of spread of MDR tuberculosis during 1994. Two patients with MDR-tuberculosis reported previous treatment of tuberculosis i.e. probably acquired resistance while the third was a new case (presumably primary resistance).

Eight RFLP patterns among drug-resistant strains were in clones shared by drug susceptible strains (Table 3.1.). As indicated earlier, only in clone CL 16 were more than one drug-resistant isolate represented. The two drug-resistant isolates in CL 16 were from patients working in the President Brand and Western Holdings mines respectively. Five CL 16 isolates were found in these two mines, three in President Brand, two of which being drug susceptible and two in the Western Holdings mine (one susceptible)(Table 2.5 and Table 3.1).

3.3.1.5. HIV status, resistant category and recent transmission

Two patients, both with isolates mono-resistant to INH, were HIV seropositive. Their isolates were both in the primary drug-resistant category. From Table 3.1 and Table 3.3 it can be seen that one of these, patient No 71146, shared the President Steyn mine 04 shaft with patient No 58863 whose resistance was listed as being acquired. Both isolates belonged to RFLP family 1, the latter belonged to clone 6. These two isolates were closely related; isolate 58863 has an additional low molecular band fragment, while isolate 71146 has one additional band that had a somewhat larger molecular weight. The band positions of all the other fragments between the two isolates were identical (Figure 3.1).Chronologically patient 58863's isolate preceded that of patient 71146. Although both patients

came from the Eastern Cape, their towns of domicile (Butterworth and Bizana) are about 230 km distant from each other. The evidence as presented above suggests that the HIV-seropositive patient 71146 (primary drug-resistant, strain isolated later than isolate 58863) may have acquired his infection from patient 58863, who, because of his retreatment/acquired drug resistance status, was probably sputum culture positive for a prolonged period (Retreatment of patients in this study was shown to be a statistically significant risk factor for recent transmission). The possibility is that patient 71146 acquired his infection from another unidentified source cannot however be excluded.

More convincing evidence of having been involved in recent transmission is presented by the second HIV-seropositive patient, patient No 72821(Table 3.1). This patient's isolate was in the same clone (CL 16) as that of patient 66853 who suffered from acquired INH resistance. The latter culture was isolated before that from patient 72821. The likely sequence of events involving these two patients was that patient 66853 acquired INH resistance during therapy and transmitted his resistant clone to patient 72821, who because of his HIV-seropositive and compromised immune status presented clinically early, within the six-month period of the study.

Table 3.3. Possible linkages between drug-resistant isolates from 1994, based on treatment history

Linkage candidates	Clone	RFLP family	Resistance profile (HRES)[a]	Treatment history New(P)[b] Retreated (A)[c]	Type of linkages[d]
66853	16	7	H	A	A-P
72821	16	7	H	P	
55451	-[e]	1	H	P_1	
58863	6	1	H	A	P_1-P_2; (P_1-P_3)[f];
64580	-	1	H	NA[g]	A-NA[g]; A-P_2,
71146	-	1	H	P_2	NA[g]-P_2; (P_3-NA)[f]
58901	1	1	HS	P_3	P_1-NA[g]
53620	-	2	H	A_1	A_1-P
53715	8	2	H	P	
58003	-	2	HRS	A_2	
53716	-	3	HR	P_1	NA[g]-P_2
53718	11	3	H	NA[g]	
57216	-	3	H	P_2	

a HRE or S resistant to isoniazid, rifampicin, ethambutol or streptomycin respectively
b New case, not previously treated, presumed primary drug resistance, designated P, P_1 or P_2
c Retreatment case: history of previous treatment episode/s, presumed acquired drug resistance, designated A, A_1 or A_2
d Possible linkages based on genetic relatedness and in addition, identical resistance profile, historic time of isolation of strains and treatment history
e Dash indicates that the linkage candidate strain was not a member of a clone
f Linkages in brackets indicates unlikely transmission as resistance profile are not identical
g NA=Information on treatment history not available.

3.3.2. Population structure of MDR-tuberculosis isolates during the 1995-1997 study period

3.3.2.1. Drug resistance profiles of MDR isolates

All 26 isolates fitted the definition of MDR (resistant to both INH and RMP).One of these MDR isolates was in addition resistant to EMB and another to SM.

3.3.2.2. Geographical distribution of MDR isolates.

Forty-eight percent of 23 patients with MDR tuberculosis come from Lesotho and 40% from Eastern Cape. The patients worked in four different mines (Table 3.4).

3.3.2.3. Primary and acquired drug resistance in MDR isolates

The majority of patients (20/25) received antituberculosis treatment prior to the diagnosis of MDR-TB and could therefore have been examples of acquired drug resistance. Five did not receive prior treatment and were therefore classified as having primary drug resistance (Table 3.4). Information related to MDR isolates available from National Tuberculosis Research Programme indicated that in sixteen patients with acquired MDR-TB, the strain responsible for the first episode was found to be sensitive to all drugs while in four patients the strain was initially resistant to INH only. As susceptible isolates from patients who subsequently expectorated MDR strains, were not DNA fingerprinted, it is possible that some of the MDR patients acquired MDR strains through suppression of their susceptible strains followed by reinfection or supeinfection with MDR strains as happened in several outbreaks involving MDR tuberculosis (Hewlett *et al.* 1993; Campbell *et al.* 1993; Valway *et al.* 1994). Based on available DNA fingerprinting evidence , eight isolates in four clusters provide definitive evidence of recent transmission. Some of the twenty isolates belonging to seven families could also possibly have been involved in recent transmission. Two presumed primary drug-resistant cases (387/97 and472/96) are likely candidates while seventeen of the nineteen isolates in RFLP families on which treatment history

was available were categorised as presumably acquired drug resistance and some of these may have been involved in reinfection or superinfection (Table 3.4). Table 3.5 presents examples of possible transmission episodes among presumed acquired drug-resistant cases.

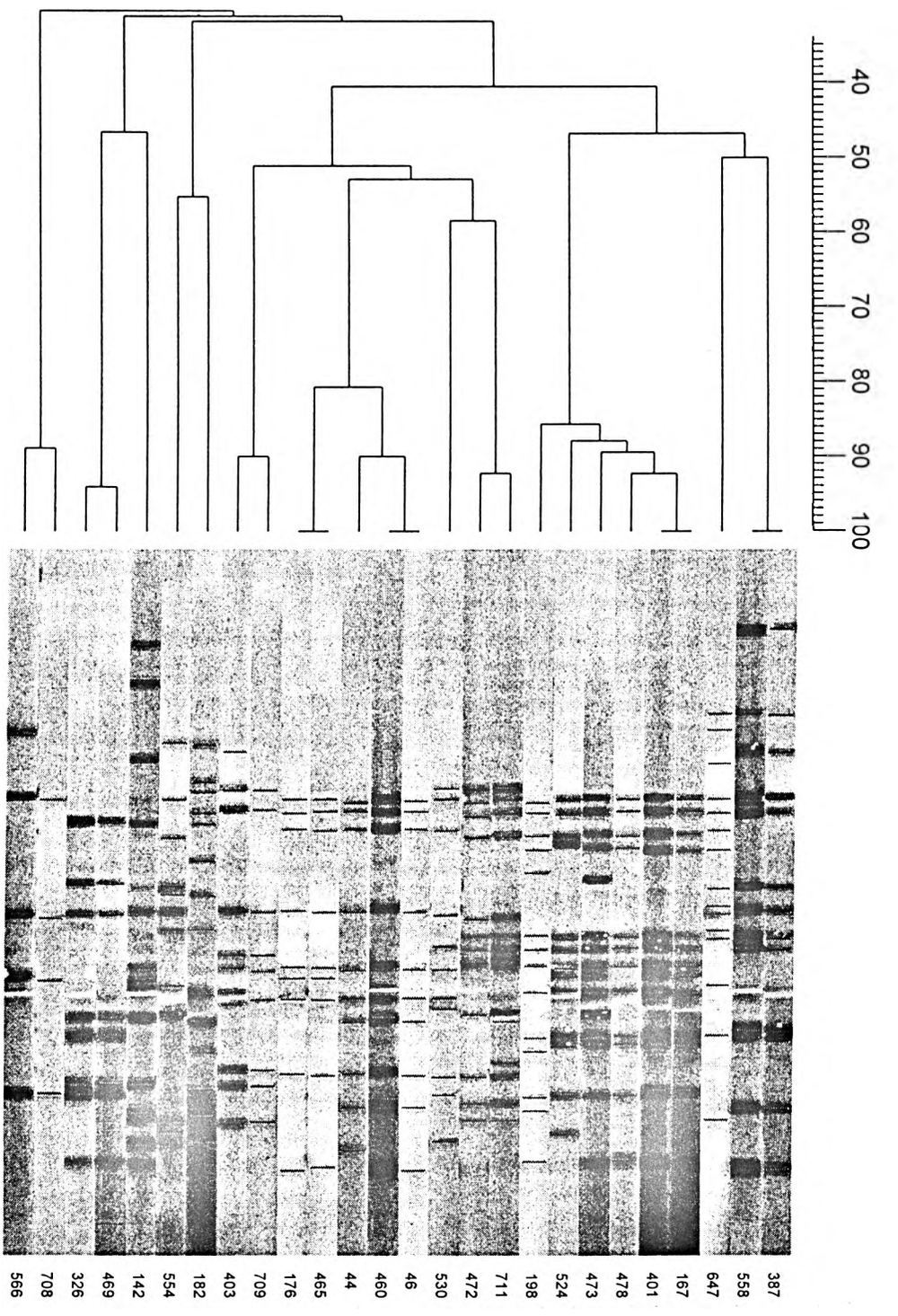


Figure 3.2. Dendrogram showing relationship between MDR-tuberculosis isolated during 1995-1997 study period

Table 3.4. Characteristics of 26 MDR *M. tuberculosis* strains isolated during 1995-1997 study period

Strain	Episode type	INH	RMP	SM	EMB	Cluster	HIV status	Family	Mine/shaft	Place of origin	Period in mine in years
167/96	Retreatment	+	+			CL 3[a]	Negative	FM 1[b]	Freddies 05	Lesotho	16
401/97	Retreatment	+	+			CL 3	Negative	FM 1	Pres Steyn 05	Eastern Cape	26
473/96	Retreatment	+	+				Negative	FM 1	W Holdings 02	Eastern Cape	30
478/96	Retreatment	+	+				Positive	FM 1	Pres Steyn 01	Eastern Cape	7
524/96	Retreatment	+	+		+		Positive	FM 1	Freddies 07	Eastern Cape	NA
566/95	Retreatment	+	+				Negative	FM 5*	NA	NA	NA
198/97	New	+	+				Negative	FM 1	Pres Steyn 03	NA	NA
708/96	Retreatment	+	+				Negative	FM 5	NA	NA	NA
460/96	Retreatment	+	+			CL 25	Negative	FM 20*	W holding 06	Lesotho	NA
44/97	Retreatment	+	+			CL 25	Negative	FM 20	FSS 03	Lesotho	16
176/96	Retreatment	+	+			CL 23[a]	Negative	FM 20	W Holdings 03	Eastern Cape	31
465/96	Retreatment	+	+			CL 23	Negative	FM 20	FSS 03	Lesotho	10
558/95	Retreatment	+	+			CL 26	Positive	FM 25	FSS 03	Natal	8
387/97	New	+	+			CL 26	Negative	FM 25	Pres Steyn 04	Lesotho	11
472/96	New	+	+				Negative	FM 26	W Holdings 03	Lesotho	27
711/96	Retreatment	+	+				Negative	FM 26	W Holdings 03	Lesotho	17
709/95	Retreatment	+	+	+			Negative	FM 27	Pres Stein 04	Lesotho	NA
403/97	Retreatment	+	+				Positive	FM 27	W Holdings	Free State	8
326/96	Retreatment	+	+				Negative	FM 28	FSS 03	Lesotho	20
469/96	Retreatment	+	+				Negative	FM 28	W Holdings	Free State	8
142/96	Retreatment	+	+				Negative	Unique	Pres Steyn 04	Eastern Cape	15
182/96	Retreatment	+	+				Negative	Unique	Pres Steyn 05	Lesotho	22
647/97	Retreatment	+	+				Positive	Unique	Pres Steyn 04	Eastern Cape	25
46/97	New	+	+				Negative	Unique	Pres Steyn 07	Eastern Cape	28
530/96	Retreatment	+	+				Positive	Unique	W Holdings	Lesotho	15
554/95	New	+	+				Negative	Unique	Pres Stein 03	Eastern Cape	NA

Abbreviations INH Isoniazid, RMP rifampicin, SM streptomycin, EMB Ethambutol, ETH Ethionomide, CL Clusters based on 100% identical, FM Families, NA Information not available

a) Cluster 3 and cluster 23 were also represented among the 1994 isolates

b) Family 1, Family 5 and Family 20 were also represented among the 1994 isolates and FM 1 &FM 5 also included drug-resistant strains

3.3.2.4. RFLP-based evidence compatible with recent transmission amongst primary and acquired drug-resistant isolates .

Sixteen IS6110 banding patterns were identified in 26 strains suggesting that the emergence of MDR-tuberculosis in the mines have occurred in multiple clones.

Computer analysis of IS6110 DNA fingerprint patterns showed that eight out of 26 patients (30.8%) patients were infected with *M. tuberculosis* strains belonging to one of four clones (two isolates each in CL 3, CL 23, CL 25 and CL 26) and 18 (69.2%) were not. Clone 3 and clone 23 were also represented among the 1994 isolates. Taking into account the time of isolation of an isolate and its treatment history, eleven examples of likely transmission amongst the eight patients represented in the four clones can be envisaged (Table 3.5)

Several strains showed common bands and near identical RFLP patterns and were assigned to seven families, such that all members of each family were identical or differed by one to three bands. Twelve isolates were found in three of the seven families (RFLP families 1, 5 and 20 respectively), and these families were already encountered in 1994 study. Drug-resistant isolates from the latter study were also represented in RFLP family 1 and RFLP family 5. The four families not observed in 1994 study (RFLP families 25-28; Table 3.3), were considered to be unique and were presumably introduced more recently in the mine. Families of strains with identical or nearly identical patterns varied in size from two to five, but most groups consisted of pairs of isolates.

Possible linkages between MDR isolates from 1995-1997 study, based on genetic relatedness and in addition, identical resistance profile, historic time of isolation of strains and treatment history are shown in Table 3.5

The two miners in CL 25 were from Mafateng in Lesotho but were working in different mines. No other geographical links between transmission candidates could be established.

When RFLP families together with clones were considered to provide evidence of genetic homology which could form the basis for transmission 5 likely transmission episodes within clones and 22 possible episodes within families transmission could be identified (Table 3.5)

3.3.2.5. HIV status, resistance category and recent transmission

Twenty- four percent (six isolates) of MDR tuberculosis patients were known to be infected with HIV. One HIV- seropositive patient (Patient 558/95) shared a clone with an HIV-seronegative patient (387/97). Chronologically the isolate of patient 558/95 preceded that of patient 387/97, suggesting that the HIV-positive patient 558/95 (retreatment, acquired drug-resistance) had infected patient 387/979 (primary drug resistant). However, the two patients come from different regions and work in different mines. Further evidence of possible recent transmission is presented by the second HIV-positive patient, patient No 403/97. The isolate of this patient and that of patient 709/95 were both in the same RFLP family (FM 27). The isolate from the latter patient was in addition to INH and RMP also resistant to SM. Transmission was therefore only possible if this isolate lost its SM resistance (unlikely) or if the discrepancy in resistance profile between the two isolates was due to a laboratory error.

Table 3.5. Possible linkages between MDR isolates from 1995-1997

Linkages candidates	Clone	RFLP family	Resistance profile (HRES)	Treatment history New (P)[b] Retreated (A)c	Type of linkages[d]
167/96 401/97	3 3	1 1	HR HR	A_1 A_2	$\underline{A_1 \rightarrow A_2}$[e]
176/96 465/96	23 23	20 20	HR HR	A_1 A_2	$\underline{A_1 \rightarrow A_2}$
460/96 44/97	25 25	20 20	HR HR	A_1 A_2	$\underline{A_1 \rightarrow A_2}$
558/95 387/97	26 26	25 25	HR HR	A P	$\underline{A \rightarrow P}$
167/96 401/97 473/96 478/96 524/96	3 3 - - -	1 1 1 1 1	HR HR HR HR HRE	A_1 A_2 A_3 A_4 A_5	$\underline{A_1 \rightarrow A_2}$[c]; $A_1 \rightarrow A_3$; $A_1 \rightarrow A_4$; $A_1 \rightarrow A_5$; $A_2 \rightarrow A_3$; $A_2 \rightarrow A_4$; $A_2 \rightarrow A_3$; $A_3 \rightarrow A_4$; $A_3 \rightarrow A_5$; $A_4 \rightarrow A_5$
566/95 708/96	- -	5 5	HR HR	NA_1 NA_2	$NA_1 \rightarrow NA_2$
176/96 460/90 465/96 44/97	23 25 23 25	20 20 20 20	HR HR HR HR	A_1 A_2 A_3 A_4	$\underline{A_1 \rightarrow A_2}$[c]; $\underline{A_1 \rightarrow A_3}$[c]; $\underline{A_1 \rightarrow A_4}$[c] ; $\underline{A_2 \rightarrow A_3}$[c]; $\underline{A_2 \rightarrow A_4}$[c]; $\underline{A_3 \rightarrow A_4}$[c]
709/95 403/97	- -	27 27	HRS HR	A_1 A_2	
326/96 469/96	- -	28 28	HR HR	A_1 A_2	$A_1 \rightarrow A_2$

a HRE or S resistant to isoniazid, rifampicin, ethambutol or streptomycin respectively
b New case, not previously treated, presumed primary drug resistance, designated P
c Retreatment case, history of previous treatment episode/s, presumed acquired drug resistance, designated A_1, A_2, A_3 or A_4
d Possible linkages based on genetic relatedness and in addition, identical resistance profile, historic time of isolation of strains and treatment history
e Underlined linkages involve clones with identical RFLP band patterns
f Dash indicates that the linkage candidate strain was not a member of a clone

3.3.3. Assessment of risk factors associated with MDR tuberculosis

No difference in age, history of prior treatment for tuberculosis, period in the mine and HIV- *M. tuberculosis* coinfection was observed between clustered patients and patients infected with individual strains. These data are shown in Table 3.6.

Table 3.6. Univariate analysis of risk factor s for a patient with multi-drug resistant tuberculosis to belong to a tuberculosis transmission group based on clustering involving clones

Characteristic	No. of patients		Odds Ratio	95% Convidence Interval	P value
	Clustered	Nonclustered			
MINE					
FSG					
FSS	3	2	4.80	0.44-61.15	0.15
Freddies	1	2	1.07	0.0-23.96	1
Pres Brand					
Pres Steyn	2	7	0.61	0.05-5.17	1
W Holdings	2	4	1.56	0.100-17.38	1
PLACE OF ORIGIN					
Eastern Cape	2	8	0.61	0.05-5.17	0.6
Free State	0	2	0	0.00-11.55	1
Lesotho	5	7	3.06	0.40-25.93	0.39
Natal	1	0	0	Undefined	0.32
Swaziland					
Mozambique					
HIV Seropositive	1	5	0.34	0.01-4.24	0.62
Retreatment	6	14	0.92	0.10-12.95	1
New	1	4	1.56	0.01-10.4	1
Period in the mine					
1-9 years	1	1	2.29	0.03-190.42	1
10-19	4	7	1.43	0.19-10.62	1
20-29	1	4	0.49	0.01-6.21	1
30-39	1	2	1.07	0.02-23.93	1

3.4. Discussion

3.4.1. Relationship between drug resistance and RFLP types

In accordance with previous reports in the literature, drug-resistant isolates were shown to share cluster/s with isolate/s that were susceptible to a particular drug. These data are consistent with observations of van Soolingen *et al.* (1991), Godfrey-Faussett *et al.* (1993), and Chevrel-Dellagie *et al.* (1993) who showed that after *in vivo* selection of drug resistance by *M. tuberculosis*, the DNA fingerprint patterns did not change.

Conversion of antibiotic susceptibility to antibiotic resistance in *M. tuberculosis* strains is due to selection of spontaneous mutants during noncompliance with chemotherapy. Resistance to INH has been associated mainly with mutations in two genetic markers: the *kat*G gene, encoding a catalase-peroxidase, or the *inh*A gene which encodes a mycolic acid biosynthesis enzyme (Banerjee *et al.* 1994; Rouse and Morris, 1995; Zhang *et al.* 1992). Mutations in genes encoding the ribosomal S12 protein (*rpsl*) and 16S rRNA (*rrs)* have been detected in most streptomycin-resistant strains of the tubercle bacillus (Finken *et al.* 1993; Nair *et al.* 1993; Honore and Cole 1994). Reduced sensitivity to rifampicin has been correlated with perturbations in the *B* subunit of RNA polymerase (Telenti *et al.* 1993). Missence mutations in *pcn*A, a gene encoding pyrazinamidase/nicotinamidase, cause resistance to the anti-tuberculosis drug pyrazinamide. Such mutations would not be expected to change the fingerprint pattern, especially if they were point mutations, unless they accomplish this by introducing, depending on the chromosomal positions, a new *Pvu*11 restriction site close to an IS6110 sequence. A deletion or insertion may be associated with a change in band position.

3.4.2. Incidence of drug resistance

Resistance to anti-tuberculosis drugs can develop during the treatment of initial or subsequent episodes

of tuberculosis providing an example of acquired drug resistance. Resistant strains may also be transmitted to patients not previously infected with *M. tuberculosis*, resulting in primary drug resistance or to previously infected individuals, constituting reinfection with drug-resistant strains of *M. tuberculosis*. Exogenous reinfection with multidrug-resistant *M. tuberculosis* may occur either during therapy of the original infection or after therapy has been completed (Small *et al.* 1993). In the 1994 study, resistance to one or more anti-tuberculosis drugs was found in 9.9% of the tested isolates. Isolates resistant to both INH and RMP accounted for 1.6% of the total number of isolates involved in the study. The prevalence of drug-resistant *M. tuberculosis* in the present study is relatively low and is similar to earlier reports on drug-resistance in the South African gold mines (Cowie 1990) and in patients in the Western Cape (Weyer *et al.* 1995). More detailed information on drug resistance in the EOH mining community during the period July 1993 to June 1997 has been provided in a recent publication by Churchyard *et al.* (2000). During this period 2241 miners had culture-positive tuberculosis and 7.3% and 14.3% of the isolates exhibited primary and acquired resistance to INH respectively. Patients with retreatment pulmonary tuberculosis were significantly more likely to have tuberculosis with organism resistant to any drug or MDR(odds ratio 9.82, 95% CI 2.97-33.5 and 18.74, 95% CI 1.76-475 respectively). In this study, HIV infection was not significantly associated with primary or acquired drug resistance and there was no trend of increasing resistance over time. The overall 8.8%(85 out of 2095) incidence of INH resistance was, as explained, similar to the present figure of 9.8% (19 out of 193) from the much smaller sample size study of 1994. The incidence of MDR isolates in the Churchyard *et al.* (2000) study was 1.1% (23 out of 2095) of which 0.67% were primary and 0.42% acquired drug resistance. The MDR incidence in the present study was 1.55% (3 out of 193).

Analysis of MDR cases shows that during the July 1993 to June 1997 period of the Churchyard *et al.*

(2000) study, 23 MDR cases were identified while during the slightly shorter 1995-1997 study period of the present investigation, 26 MDR cases featured. This apparent discrepancy was explained by Dr Churchyard (personal communication) who indicated that those patients with MDR tuberculosis who received repeated treatment courses over a prolonged period of time and who were identified as MDR cases prior to the study period were excluded from their study. Several MDR cases in the present study received multiple courses of treatment, some of which pre-dating 1995.

3.4.3. Clonal diversity of isolates

Both isoniazid and multidrug-resistant strains showed great diversity in banding patterns. The 19 drug-resistant isolates analysed in 1994 reveal eighteen patterns while the 26 MDR strains isolated in a 1995-1997 study period exhibited sixteen IS6110 banding patterns.

Clonal diversity of drug-resistant *M. tuberculosis* has also been reported in San Francisco where resistance was relatively uncommon and occurred primarily in elderly immigrants (Small *et al.* 1994). This was in contrast with low genetic heterogeneity of *M. tuberculosis* strains reported in New York, where drug resistance was shown to be most common in relatively young HIV-infected persons whose immune status and social behaviour facilitated transmission of such clones (Moss *et al.* 1997).

The results of the present study show that there were at least five (CL 16, CL 3, CL 23, CL 25 and CL 26) drug-resistant *M. tuberculosis* clones present in the mines. In two of these (CL 3 and CL 23) isolates of four and six patients respectively constituted these clones. Altogether twelve likely transmission episodes (one in 1994 and eleven during 1995-1997 involving these clones could be identified. Acquired drug resistance, arising from inadequate treatment of individual patients, will potentially give rise to a high proportion of unique RFLP patterns among a pool of drug-resistant

strains and could reflect the degree of strain diversity in the study population. It is likely that a significant number of drug-resistant strains could have arisen from inadequate treatment in the mine setting. Eighty percent of all MDR-TB patients received antituberculosis treatment prior to the diagnosis of MDR-TB. This high proportion of presumed acquired drug resistant occurred in the context of directly observed therapy (DOT) that is practised meticulously at EOH (Dr Churchyard, personal communication). Smear-positive patients are initially hospitalised, after which they report every weekday to the primary health-care facilities, where DOT is administered and recorded. Treatment consists of two months of isoniazid, rifampicin, pyrazinamide, and ethambutol, followed by 4 months of isoniazid and rifampicin. All patients, regardless of a history of previous tuberculosis treatment, received the same regimen except for those infected with MDR strains, who were treated according to their drug sensitivity results, which were available within 3 to 6 weeks.

Despite evidence of recent transmission (see 3. 4. 4.), it is possible that some drug-resistant, including MDR strains, may have acquired part or their full complement of resistance in the past, outside the mining fraternity and then appeared in the mines following reactivation of their initial drug-resistant infection. Many workers in the gold mines are migrant labourers who are recruited from areas where high frequencies of initial drug resistance have been reported, including the Eastern Cape (Kleeberg and Olivier 1984; Fourie *et al.* 1980) and Lesotho (Fourie and Knoetze 1986). These men could be expected to develop reactivation tuberculosis with rurally-acquired drug-resistant strains thus contributing towards strain diversity.

The dominant Family 1 pattern of closely related strains described in the 1994 study period constituted 25% of all MDR-tuberculosis isolates in the 1995-1997 study. This family therefore could be typical of *M. tuberculosis* strains circulating in the mines after becoming endemic in this environment over

a relatively short period. On analysis of the band patterns of Family 1 strains, the isolates showed similar profiles, being either identical or differing only by one to three bands, suggesting that they may have shared a common ancestor that had undergone clonal expansion. Band differences in RFLP families may be due to the ability of IS6110 to transpose to other sites in the chromosome, giving rise to the changes noted in strains within families. Such changes may occur during transmission of bacilli between patients (Chevrel-Dellagi *et al.* 1993; Daley *et al.* 1993; Mazurek *et al.* 1991; Small *et al.* 1993; and Takahashi *et al.* 1993).

In a recent study in Cape Town, community-acquired transmission of an MDR strain labelled U was documented (Van Rie *et al.* 1999). This strain showed great RFLP similarity with the W (961-0874) strain responsible for an MDR outbreak in New York City (Frieden *et al.* 1996). The authors also studied mutations which led to resistance to anti-tuberculosis drugs and showed that the mutation conferring resistance to streptomycin in strain U(*rrs* 573) was different to that in strain W (rpsL43) and inferred from this that these two genomically-related strains arose independently in two different geographical locations in two very different patients groups (the Cape Town patients were HIV seronegative and acquired their strain in the community while the New York patients were predominately HIV seropositive and their strain was either nosocomically or institutionally acquired). These findings suggest that the W-like genomic family is prone to the development of mutations conferring drug resistance. Direct comparison of the RFLP fingerprints of drug-resistant isolates in the present study with those of W or U strains failed to show close similarity.

3.4.4. Evidence of transmission

Despite great diversity of the RFLP patterns, one clone of two isolates and an additional three clones in RFLP families (representing 5, 3, 3 isolates) were identified among drug-resistant isolates in the

1994 study. One likely casual transmission linkage in CL 16 and an additional possible five linkages amongst the RFLP family clusters were identified. Among the 26 MDR-tuberculosis patients whose *M. tuberculosis* strains were submitted for RFLP in 1995-1997 study period, 30.8% of isolates fitted into three clones, and 73% into six RFLP families of identical or nearly identical band patterns. Eleven clone-related and a possible 22 RFLP family-related transmission linkages were suggested by the available information (Table 3.4)

Convincing evidence of having been involved in recent transmission of an MDR strain two isolates in clone-based cluster is presented by the HIV-seropositive patient, patient No 72821 as set out in Section 3.2.1.5. Statistical analysis of the small number of HIV-seropositive patients, however, failed to show that HIV infection is significantly linked with clustering. In a larger study involving 448 South African miners with tuberculosis and a close to 50% HIV infection rate, Godfrey-Faussett *et al.* (2000) also found that HIV infection was not significantly associated with clustering. This is in contrast to the findings in outbreak situation in the USA were HIV infection was shown to be a risk factor (Daley *et al.* 1992; Beck-Saque *et al.* 1992). Interestingly, Mallory *et al.* (2000) found in the same mining population as the present study, covering the period 1 April 1993 to 31 July 1996 that HIV-seropositive patients had a significantly higher recurrence rates of tuberculosis episodes than HIV seronegative patients (8.2 vs 2.2 per 100 persons-years) with a multivariate-adjusted incidence rate ratio of 4.9 and a 95% confidence interval of 3.0 to 8.1. They also found that recurrence rate was significantly higher in patients receiving three antituberculosis drugs (INH, RMP and PZA) compared with the four-drug regimen which included EMB in addition to the other three drugs. Some of the "recurrences" encountered in the study may well have been as a result of reinfection or superinfection with drug-resistant strains.

The present study presents clear evidence of considerable transmission of drug-resistant strains (isolates within clones), especially amongst MDR isolates during 1995-1997 period. Churchyard *et al.* (2000) also in the same mining population, but comprising large study group, showed that patients with retreatment tuberculosis were significantly more likely to have drug-resistant tuberculosis. This is not surprising as retreatment patients harbour viable bacilli for prolonged periods, providing opportunities for not only the emergence of drug-resistant mutants, but also for transmission of *M. tuberculosis*, including drug-resistant strains.

Although information on the workplace with one possible exception, involving Family 1 isolates from 2 patients sharing a mine shaft (in the Pres Steyn mine shaft 04), failed to reveal a link between the members of a clone or family, it is most likely that more undetected transmissions occurred within and around the mine environment. It is general practice that all persons recruited to the mines are screened for tuberculosis and other diseases and are putatively free of tuberculosis. It is therefore likely that stress conditions in the mine may lead to reactivation of dormant drug-resistant bacilli in some infected persons who acquired tuberculosis outside the mining fraternity. Such cases could again infect a number of persons in a mine, resulting in clustering of drug-resistant strains. The link established by labour practices in South Africa between rural regions and specific mines to which miners may return several times in a life time, may have helped to establish clones in a mine and in this way contributed towards diminished clonal diversity. Similar situations have been reported in regions where tuberculosis is highly endemic, e.g., Ethiopia, Tunisia and French Polynesia (Hermans *et al.* 1995).

Although statistically significant evidence implicating drug resistance as a risk factor for transmission was lacking, transmission of drug-resistant strains may eventually contribute substantially to the

escalation of drug resistance problems. Selective pressures inherent to antimicrobial therapy could further augment the process and result in further increases in drug-resistant tuberculosis in the mines.

The evidence of microepidemics of MDR-tuberculosis reported in the present study underscores the need for more resources to be committed to a) cohort separation of new cases on treatment for the first two weeks, ideally until smear microscopy shows marked reduction in acid-fast bacilli or preferably when it is completely negative; b) suspected or known cases of MDR, as well as patients requiring retreatment including drug failures and recurrent cases should be isolated from smear-positive patients on treatment for drug-susceptible tuberculosis: c) use of rapid diagnostic tests for detection of drug resistance; d) revise treatment policies especially with regard to retreatment cases. At least two drugs to which isolates are susceptible should be administered in combination to the patient; e) ensure that drugs are taken correctly, especially during intensive treatment phase (Churchyard *et al.* 2000).

3.4.5. Primary and acquired drug resistance in the mines

In 1994 study, a higher proportion of cases of primary resistant tuberculosis (mainly mono-resistant strains) than of acquired resistant tuberculosis was recorded by clinical definition. The use of RFLP analysis of these isolates showed only one IBP/clone cluster comprising two isolates. The low rate of transmission of singly resistant strains may be related to the fact that patients infected with these strains, in contrast with to those infected with MDR organisms, are not infectious for long periods because the initial phase of a standard treatment regimen will provide at least two drugs to which the bacterium is sensitive.

Twenty out of 25 MDR (80%) in 1995-1997 study were retreatment (acquired drug resistance) cases. This finding accords with those of surveillance studies worldwide (Pabloz *et al.* 1997; Cohn *et al.*

1997).

Although the isolates associated with retreatment could be related to treatment failures and therefore be regarded as examples of acquired drug resistance, they may also be due to reinfection with drug-resistant strains(Van Rie *et al.*2000). Eight MDR cases were found to be in 4 clusters of 2 isolates each. The clusters seen in this group of patients strongly suggest recent transmission of drug -resistant strains in patients receiving treatment for drug-susceptible tuberculosis. Such treatment would afford a selective advantage to MDR strains and could cause outbreaks as was seen in the USA (Hewlett *et al.* 1993; Campbell *et al.* 1993; Valway *et al.* 1994). These findings contrast with the generally accepted idea that multidrug-resistant tuberculosis occurs only exceptionally in new cases but mainly in chronic cases, as a consequence of sequential selection of drug-resistant mutant of *M. tuberculosis* organism (Crofton *et al.* 1996). Recently Van Rie *et al.* (2000) recorded a similar experience where they found in a highly tuberculosis endemic population in Cape Town, clusters containing drug-resistant *M. tuberculosis* cases. Some of these would have been classified as acquired drug resistance based on their treatment history.

3.4.6. Risk factors

Because of small numbers, it was not possible to properly assess retreatment of patients as a risk factor for drug-resistant tuberculosis. However, in a study comprising a much larger group of patients, from the EOH mining population, patients who failed primary antituberculosis treatment, were found to be significantly associated with drug-resistant tuberculosis by Churchyard *et al.*(2000). This is not surprising, as retreatment cases are more likely to have harboured large numbers of tubercle bacilli for prolonged periods, providing opportunities to select for drug-resistant mutants during retreatment episodes. As shown in Chapter 2, the present study did show that retreatment of patients was

significantly associated with the risk of transmission of *M. tuberculosis* strains. Although this finding applied to predominantly drug-susceptible strains, it is likely that it would also hold for drug-resistant strains, which could, as was the case for as the susceptible strains, be expectorated for prolonged periods by retreatment patients. As mentioned earlier, Godfrey-Faussett *et al.* (2000) also found retreatment of tuberculosis patients to be a significant risk factor for transmission of tuberculosis.

A possible role for HIV infection as a risk factor for transmission in the present study could also not be established and the small numbers available for analysis again compromised statistical evaluation. With the escalation number of HIV/AIDS in South Africa and in the mines, it can be anticipated that more infectious tuberculosis cases will be hospitalised or attend HIV/AIDS clinics and also meet at social or sporting gatherings where opportunities for transmission are favourable, resulting in an increase in MDR tuberculosis will increase. As some cases will be on anti-tuberculosis treatment, selection of resistant clones will be facilitated and may spread to contacts who are being treated for tuberculosis, creating conditions favourable for the selective transmission of MDR strains. Furthermore, the shorter period between infection and active disease in immunocompromised HIV-positive patients could lead to a rapidly increasing the infectious pool of *M. tuberculosis* strains and in this way promote the spread of MDR strains.

A factor which was not studied in the present study nor assessed as a risk factor for acquisition of drug resistance in other reports in the literature, but which may be important in this country in future is possible interference with pharmacokinetics of anti-mycobacterial drugs by anti-retroviral agents used in the treatment of HIV-disease (Garcia *et al.*1997). Rifampicin is an essential component of the recommended regimen for tuberculosis. The protease inhibitors used for treatment of HIV-disease increase the serum levels of the rifabutins (rifampin and rifabutin) and thus increase the risk for toxicity.

Rifampicin, through the induction of the hepatic cytochrome p450, increases the metabolism of protease inhibitors and thus decrease serum concentrations-often to subtherapeutic levels.

3.4.7. Concluding remarks

In this chapter the author recorded a relatively low incidence of drug resistance in *M. tuberculosis* in the EOH mining population (9.9.%). The majority of resistant isolates were mono-resistant to INH, while 1.6% of isolates during 1994 were resistant to both INH and RMP (MDR strains). The majority of INH mono-resistant isolates were categorised as primary drug-resistant, while 80% of MDR isolates during 1995-1997 were retreatment cases and therefore presumed to be examples of acquired drug resistance.

The high proportion of drug-resistant isolates involving ten patients during 1994 suggest a high rate of recent transmission but only one small clone-based cluster was found. However, based on RFLP family clusters, seven possible transmission linkages could be demonstrated. Although there is strong evidence of predominantly acquired drug resistance amongst MDR strains of *M. tuberculosis* in miners, reflecting poor treatment outcome despite strictly administered DOTS, there is also convincing evidence of transmission of drug-resistant strains. Evidence of recent transmission was based on both clustering (four clones involving 13 isolates during 1994 and clones involving MDR isolates during 1995-1997) and the demonstration of six possible linkages in retreatment MDR cases, strongly suggest that some of the patients acquired their MDR strains through reinfection or superinfection.

The presence of dominant RFLP families of drug-resistant isolates in the mines (73% of MDR isolates could be linked to six RFLP families) suggest that, over time, many episodes of transmission involving these strains occurred , leading to their establishment as endemic strains in the mines.

Although a significant impact of HIV infection on the prevalent of drug resistance, including MDR, could not be demonstrated in the present study, Mallory *et al.* (2000) found that HIV infection amongst the same miners was significantly associated with recurrence of tuberculosis which in turn were dependent on whether a three-drug or four-drug regimen was used for treatment. In the light of the present findings, identifying several clone-based clusters amongst drug-resistant strains, it is suggested that some of the recurrences were due to recent transmission resulting in reinfection or superinfection.

4. VIRULENCE STUDIES ON STRAINS OF *MYCOBACTERIUM TUBERCULOSIS* FROM THE DOMINANT RFLP FAMILY

4.1. Introduction

Advances in the molecular biology of mycobacteria have provided new tools that facilitate more definitive studies of the virulence of *M. tuberculosis*. The molecular epidemiology approach of DNA fingerprinting based on restriction fragment length polymorphism (RFLP), has made it possible to identify specific isolates of *M. tuberculosis* involved in outbreaks of disease (Alland *et al.* 1995; Daley *et al.* 1992; Small *et al.* 1993, 1994). In some outbreaks, the transmission of *M. tuberculosis* was limited, whereas in others, high rates of transmission occurred. Variability in transmission has been attributed to the environment in which outbreaks occur, including the ability of strains to resist dessication, and the clinical characteristics of source patients. Some investigators reported that strains involved in such outbreaks may be especially virulent by showing increased growth rates in infected mouse lungs (Valway *et al.* 1998) or enhanced capacity to grow in macrophages (Zhang *et al.* 1999).

Despite extensive research, we still understand poorly the capacity of the human immune response to eliminate *M. tuberculosis* and the virulence mechanisms by which the organism evades host defences. These two aspects of the pathogenesis of infection converge at the level of the mononuclear phagocyte-*M. tuberculosis* interaction. Following activation by lymphocytes, mononuclear phagocytes serve as the final effectors in the killing of tubercle bacilli in the intracellular environment of human blood monocytes and tissue macrophages (Fenton and Vermeulen 1996). Nevertheless, the organisms can survive, and even thrive, in these host phagocytes.

Virulence may be defined as the capacity of a microorganism to overcome host defences. The mouse

and the guinea pig have become favoured animals for studying the virulence of mycobacteria. In mice, virulent strains of *M. tuberculosis* grow aggressively in the lungs of immunocompetent animals producing sustained pulmonary pathology, whereas avirulent strains show only limited growth (North and Izzo 1993; Pierce *et al.* 1953). These observations are independent of the route of infection used in the experimental model. In the guinea pig model, on the other hand, following challenge by the intramuscular route, virulence is associated with the capacity of the organism to disseminate from the site of infection (Dunn and North 1995). Virulence may also be defined in terms of the extent to which an *M. tuberculosis* strain is able to cause lung pathology in a given period of time (Manca *et al.* 1999; Mitchison *et al.* 1960, 1963) or be measured in terms of host survival in mouse experiments (Dunn and North 1995).

Numerous attempts have been made to investigate virulence by *in vitro* studies (Silver *et al.* 1998; Zhang *et al.* 1998, 1999). In such experiments, the virulence of strains has usually been correlated with their behaviour inside phagocytes. This is because *M. tuberculosis* is a facultative intracellular pathogen and its fate within mononuclear phagocytes is an important factor in determining the outcome of infection. It is therefore reasonable to suppose that any characteristic enabling the bacterium to survive within the phagocytes will contribute to virulence.

The research recorded in this chapter represents a pilot study to determine in an animal model, possible differences in virulence between three MDR strains within the highly prevalent Family 1 isolates, between themselves, as well as compared with an international control H37Rv strain which has previously been shown to be fully virulent in animal studies (Manca *et al.* 1999; Ordway *et al.* 1995).

As, for reasons of economy, only a limited number of strains could be tested for virulence, MDR

isolates from the present study which were incriminated as probably having caused micro-epidemics in miners (Chapter 3) were thought to be appropriate test strains. These strains form part of a collection of MDR cultures in a registry of the National Tuberculosis Research Programme (NTRP) of the South African Medical Research Council and are available to researchers in South Africa and elsewhere. Limited but important information on the treatment history of patients and laboratory data on isolates is available from the NTRP registry of MDR isolates. Three isolates on which such good information was available were chosen ffor virulence studies.

The demonstration of differences in virulence amongst the three Family 1 MDR strains, which are genetically closely related, could form the basis of molecular studies on virulence determinants in these strains in the future.

Excluded from the present pilot study, but a high priority for the future would be an extension of animal studies to include virulence testing of other appropriate candidate strains such as the expanded clone 3 within Family 1

4.2. Materials and Methods

4.2.1. *M. tuberculosis* cultures

Three clinical isolates belonging to the RFLP Family 1 designated MDR 473(15 IS6110 copies), MDR 478 (12 IS6110 copies), and MDR 524 (14 IS6110 copies) as well as H37Rv were studied. Although the three Family 1 strains harboured 12 to 15 IS6110 copy numbers, the band patterns were very similar and the three strains share eleven bands in the same positions. Twelve bands are shared by strains MDR 473 and MDR 524 and one of these is not present in MDR 478. In addition MDR 473 exhibits two prominent bands not present in MDR 478 and MDR 524 while MDR 524 shows unique

band not seen in MDR 473 or MDR478 (Figure 3.2). *M. tuberculosis* stock cultures for virulence testing were grown in broth (Middlebrook 7H9; Difco, Detroit) enriched with 5% albumin-dextrose catalase (ADC) additive and 0.05% Tween 80 for 8-10 days at 37°C and stored at -70°C in 1ml ampules until required. The viability of frozen suspensions was determined by slowly thawing randomly selected ampules, followed by sonication for 5s in water bath sonicator to break up any clumped bacilli. Serial 10- fold dilutions were then performed by plate counts of colony forming units (CFUs) on agar (Middlebrook 7H11; Difco). The plates were incubated at 37°C in sealed plastic bags for 3-4 weeks when the colonies could be counted.

4.2.2. Catalase testing

The presence of catalase was determined by observation of the ability of several loopfuls of an *M. tuberculosis* culture suspended in 0.5 ml phosphate buffer to to produce oxygen(bubbles) within 20 minutes after the addition of 0.5 ml of reagent consisting of an equal volume of 30% hydrogen peroxide and 10% Tween 80 (Witebsky and Kruczak-Filipov 1996). The test was performed without heating at 68 ^{O}C, a step which is used for the identification of *Mycobacterium* species.

4.2.3. Experimental animals

Inbred specific pathogen-free BALB/c female mice, 6-8 weeks old, were obtained from South African Vaccine Producers, Sandringham, Johannesburg. Twenty mice were used for the assessment of the virulence of the strains tested: 12 mice for the determination of growth rates/organism loads in lungs of intravenously challenged animals, while survival time after challenge was assessed in 8 mice.

4.2.4. Survival times

Mice were infected intravenously in the lateral tail vein at a dose of 10^5-10^6 colony forming units (CFU) of a bacterial suspension in 0.1 ml saline. The concentration of organisms in the infective dose was determined in terms of CFU/ml. Mass measurements were taken daily to assess morbidity. Mortality was monitored for up to fifteen weeks after which surviving mice were euthanased by means of cervical vertebra dislocation in order to remove organs for autopsy investigation including histology for evidence of tuberculosis.

4.2.5. Determination of growth rates of *M. tuberculosis* strains in mouse organs

BALB/c mice were infected intravenously with 10^5 -10^6 CFU of each selected strain. The lungs and spleen were removed aseptically from 3 randomly selected mice at each of 10 day, 20 day, 30 day and 40 day intervals and homogenised separately in saline. The organs were first cut into small slices aseptically and then sonicated for 5 seconds. A standardised volume of homogenate was diluted in 10-fold steps, plated on 7H11 agar, and incubated at 37°C in sealed plastic bags for 3-4 weeks, when colonies were counted. The colony counts were expressed as means ± standard errors of the means (SEM) of the CFUs/ml obtained from 3 mice used for each strain at day 10, day 20, day 30 and day 40 respectively.

4.3. Results

4.3.1. Growth of *M. tuberculosis* strains in the lungs and spleen of mice inoculated intravenously

The first experiment compared the four *M. tuberculosis* strains (H37Rv, MDR 473, MDR 478 and MDR 524) in terms of their ability to grow in the lungs and spleen of immunocompetent mice after the mice were inoculated intravenously with 10^5 -10^6 CFU. The course of infection was followed for 40 days. All strains multiplied essentially at the same rate in the lungs of the infected mice over the first

20 days after which MDR 473 and MDR 524 grew faster than the other strains, reaching $\log_{10}$ 7 30 days after infection (Table 4.1; Figure 4.1)

Table 4.1. Growth of *M. tuberculosis* strains in the lungs of mice inoculated intravenously

	Mean viable count in $\log_{10}$ CFU/ml ± SEM[a] at various times (Days)			
Strain	**Day 10**	**Day 20**	**Day 30**	**Day 40**
H37RV	2.32 ± 0.32	5.20 ± 0.24	6.42 ± 0.06	6.29 ± 0.19
MDR 478	2.10 ± 0.09	5.50 ± 0.15	6.14 ± 0.15	6.03 ± 0.04
MDR 473	2.70 ± 0.04	5.65 ± 0.01	6.94 ± 0.07	6.93 ± 0.19
MDR 524	2.73 ± 0.01	5.90 ± 0.01	7.53 ± 0.03	6.92 ± 0.19

a) SEM=Standard errors of the mean

For all four strains viable *M. tuberculosis* as evidence by CFU were detected in the spleen at 10 days. It was found that the elimination process involving MDR 473 and H37Rv in the spleen was slower compared to that of MDR 524 and MDR 478.

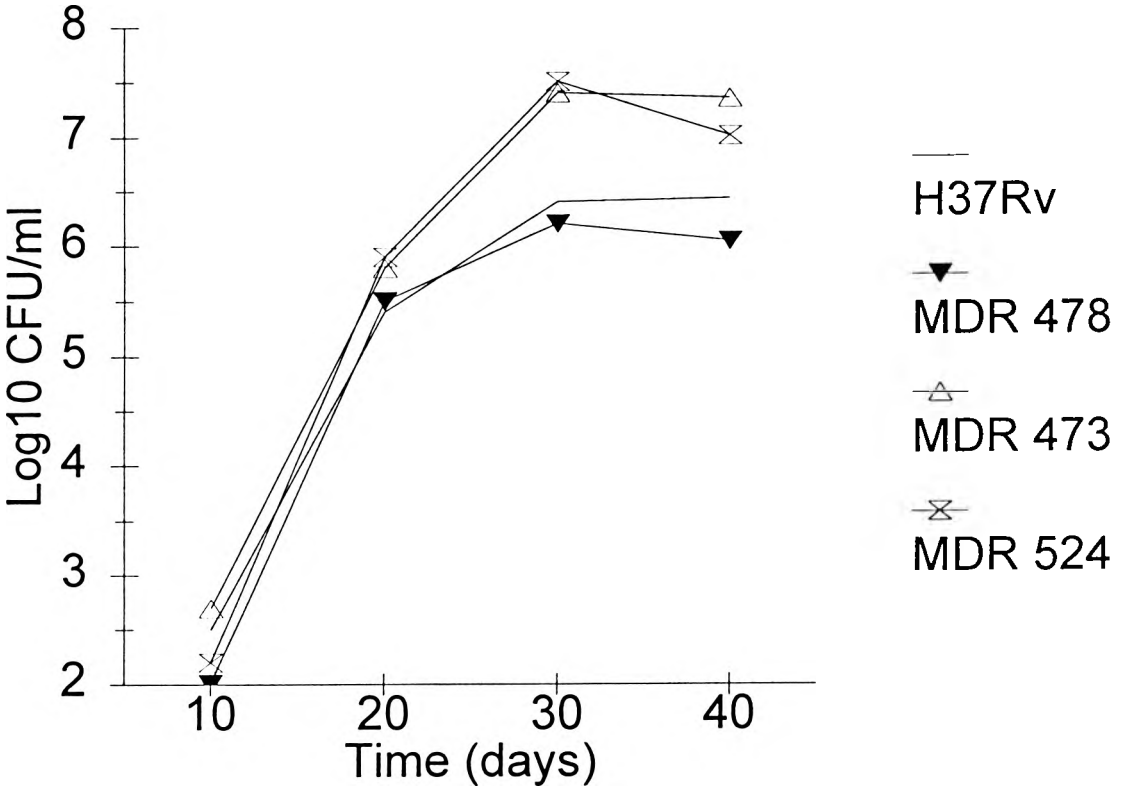


Figure 4.1. Comparison of growth of four strains of *M. tuberculosis* (H37Rv, MDR473, MDR478 and MDR 524) in lungs of immunocompetent mice infected intravenously with 10^{5}- 10^{6} CFU. Data shown are mean values for three mice. See Table 4.1 for the standard errors of the mean.

The population doubling times of the different strains during the first 20 days (calculated CFU/ml on day 10 and 20 days) were the similar (22-25h). From 20-30 days MDR 473 and MDR 524 grew much faster (doubling time 45 hours) compared to H37Rv (72h) and MDR 478 (104 h)(Table 4.2).

Table 4.2. Population doubling times of different strains of *M. tuberculosis* in lungs of infected mice.

Strain	DAYS		
	10-20	**20-30**	**30-40[a]**
H37Rv (14)[b]	24.7h	72h	Stationary
MDR 473 (15)[b]	22.5h	45h	Stationary
MDR 478 (12)[b]	21.2h	104h	Slight decline
MDR 524 (14)[b]	22.5h	45h	Slight decline

Mice were infected intravenously with 10^5 -10^6 CFU inoculum. Population doubling times were calculated from the data points (mean CFU) obtained for days 10, 20, 30 and 40

[a] Growth during this period was either stationary or showed slight decline in $\log_{10}$ CFU/ml (Figure 4.1)

[b] Figures in brackets denote IS6110 copy number per strain

4.3.2. Survival time

To determine whether there were differences in survival, mice were inoculated intravenously with the different strains and monitored for 15 weeks. Mice infected with MDR473 and MDR 524 began to die 45 days after infection whereas mice infected with MDR 478 did not begin to die until day 70 (Figure 4.2.). None of the mice challenged with H37Rv died during 105 days observation period. The results show that on the basis of survival time of immunocompetent mice, MDR 473 and MDR 524 were the most virulent (median survival times [MST], 70 and 64 respectively) followed by MDR 478 [MST, 91 days] and *M. tuberculosis* H37Rv[MST>105 days].

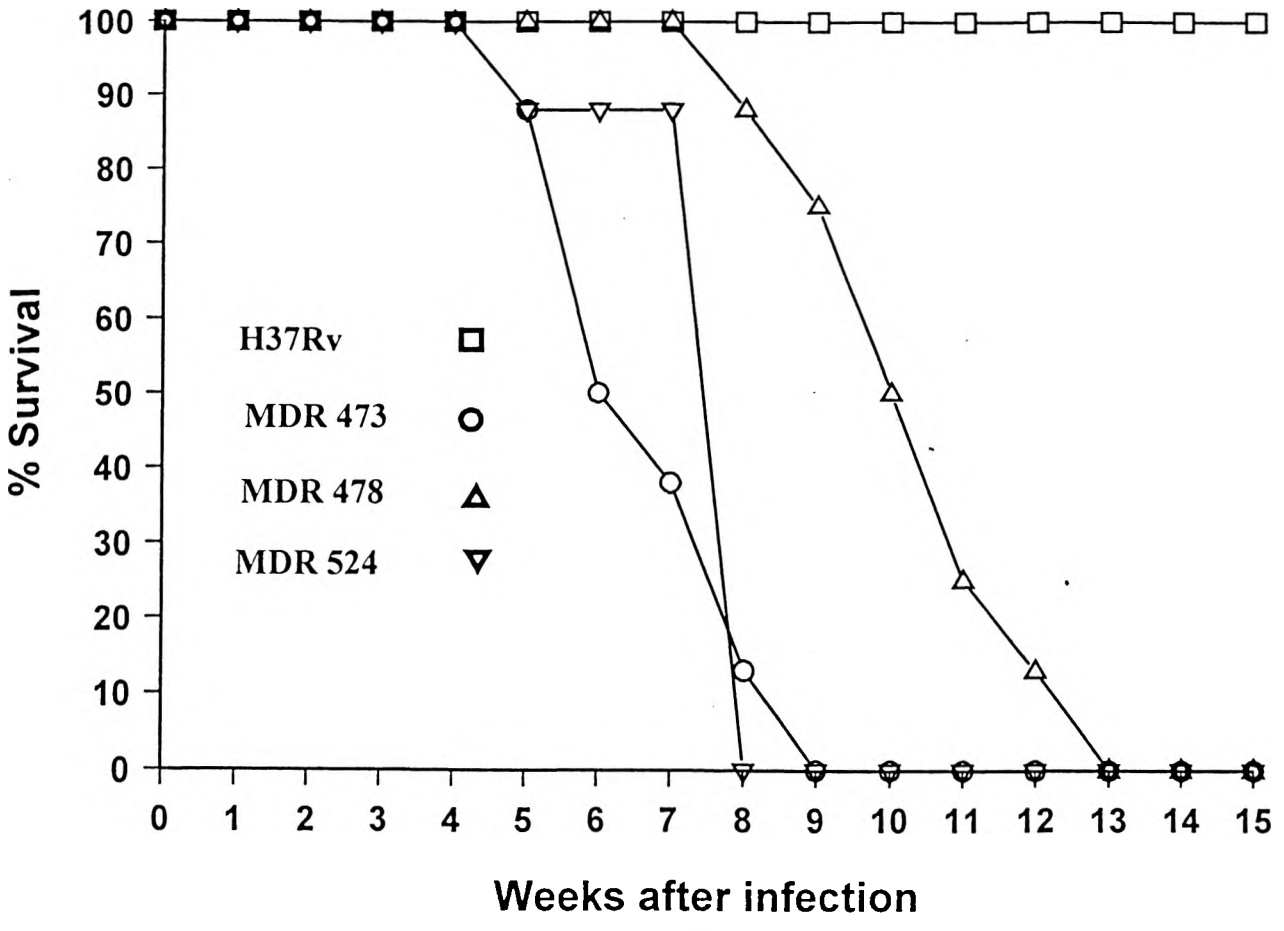


Figure 4.2. Survival times of immunocompetent mice infected intravenously with the strains used in the experiment in Figure 4.1. Median survival times were much shorter in mice infected with MDR 524, MDR 473 and MDR 478. H37Rv failed to kill mice during 105-day period of study. Results were obtained with 8 mice per group

4.4. Discussion

4.4.1. Virulence in animal models in relation to transmission of *M. tuberculosis* strains.

M. tuberculosis strains vary in the extent to which they spread in different population settings (Bafina *et al.* 1996; Valway *et al.* 1998; Zhang *et al.* 1999). This variability has been ascribed to differences in the generation of infectious aerosols, environmental conditions that affect organism survival and contact with the source patient, the likelihood of inhaling aerosols of critical size, and the susceptibility of exposed persons. Recent reports, however suggest that bacterial factors may play a role in variability of transmissibility of mycobacteria (Manca *et al.* 1999; Valway *et al.* 1998; Zhang *et al.* 1999)

4.4.1.1. Findings of the present study

In the present study three MDR clinical isolates belonging to Family 1(Chapter 3) and a control the H37Rv laboratory strain, were compared in terms of their growth rates in the lungs of infected mice, as well as the time taken to kill mice. The growth rates in lungs of mice challenged with the four strains were similar during the first 20 days of infection. However, when the organism loads of strain MDR 473 and strain MDR 524 were determined in lungs of mice at 30 days after infection, a 10- fold higher number of bacilli were found compared to the number of bacilli isolated from the lungs of mice infected with MDR 478 and H37Rv laboratory strain. On the basis of host survival time alone, mice infected by the three clinical isolates of the present study were more virulent and survived for much shorter periods than those infected with H37Rv.

Strain MDR 478 was less virulent than the other two MDR strains. This was demonstrated both in terms of generation time 20-30 days after initiation of infection (104h for MDR 478 versus 45 h for MDR 473 and MDR 524) the doubling time was also slower than that of the 72h for the H37Rv strain and with

respect to MST (91 days vs 70, 64 and 105 days respectively for MDR 473, MDR 524 and H37Rv). These findings suggest that the MDR 478 strain had undergone a genetic change compared with other two member of Family 1 examined in this study, and that, the MDR 478 may therefore prove to be valuable in identifying gene sequences that may be important in virulence and possibly also in terms of transmission. It was noted that the less virulent MDR 478 strain had fewer IS6110 copies (12) than the other two test strains (15 and 14 copies respectively). Whether this finding is related to the decreased virulence of MDR 478 is uncertain. Theoretically mutation affecting a *Pvu* 11 restriction site close to IS6110 sequence in the chromosome or deletion or insertions could change band patterns (see section 3.4.1.)

The recent explosion of nucleotide sequence information on *M. tuberculosis* (whole genome sequence) (Cole *et al.* 1998) and the less virulent *M. bovis, Mycobacterium africanum* and *Mycobacterium microti* provided evidence of 10 deletions possibly related to virulence. In *M. bovis* BCG all the deletions relative to *M. tuberculosis* (RD1-RD10) are present, while 7 corresponding deletions have been demonstrated in *M. bovis* (RD4-RD10) and fewer corresponding deletions in *M. africanum* and *M. microti*. Among the gene products related to these deletions are the mycobacterial invasin Mce, four phospholipases, including phospholipase D, epoxide hydrolase and several other proteins (Gordon *et al.* 1999). These exciting observations demonstrate how powerful studies relating to comparative genomics, including subtractive hybridisation (Mahairas *et al.* 1996), could be in the search for the genetic basis of physiological characteristics, host range and virulence in *M. tuberculosis*. The difference in virulence in the three MDR isolates of the present study could form the basis for comparative genomic studies involving the genes identified as candidates for virulence.

4.4.1.2 Transmissibility in relation to virulence: Findings of others

Strains of high transmissibility causing extensive outbreaks may exhibit virulence properties related to high growth rates in macrophages (Zhang *et al.* 1999) or in the lungs of non-immunocompromised mice (Valway *et al.* 1998; Manca *et al.* 1999; North *et al.* 1999). In a macrophage model, strain 210 which caused a large outbreak in Los Angeles, USA, multiplies significantly more rapidly in macrophages than isolates from small-cluster outbreaks in the same city. This finding however, was not consistent as the CDC1551 strain which caused a large outbreaks in Kentucky and Tennessee did not multiply significantly faster in macrophages than small cluster strains or unique strains that did not show a propensity to transmit tuberculosis to other patients, despite ample opportunity for transmission (unique strains were from patients from homeless shelters). Similarly, the findings in the mouse lung model were inconsistent as Manca *et al.* (1999) found that the CDC1551 strain did not multiply faster than H37Rv in mouse lungs and that experimental animals infected with this strain survived longer. It did, however cause more earlier granuloma formation than H37Rv in the lungs of mice. North *et al.* (1999) using the CDC1551, H37Rv and *M. bovis* strains in a mouse model also found that CDC1551 did not appear to be more virulent than the other strains. Based on these findings it cannot be assumed that the initial rate of growth in the lungs of mice infected by the two clinical isolates (MDR 473 and MDR 524) is necessarily related to their high transmissibility in the mining community. Unfortunately, the present pilot study was not designed to examine histological changes in mouse lungs during different phases of pulmonary invasion by the experimental strains.

4.4.2. Pathogenesis of early pulmonary disease in animal models

The basis of reduced survival times of mice infected with the three MDR strains compared to H37Rv in the present study remains speculative. It has been demonstrated previously (Dunn *et al.* 1995) that

the *M. bovis* has the ability to cause earlier death than H37Rv. In the latter study, the ability of an *M. bovis* strain to cause earlier death than H37Rv was related to its ability to induce more rapid development of lung pathology and thereby cause earlier loss of lung function. Although, histology was not performed during the various stages of the experiments, it is possible that a similar process was set in motion by the three MDR strains. On this basis, it could be speculated that less protective immunity was elicited by the experimental *M. tuberculosis* strains in BALB/c against the two more virulent MDR 473 and MDR 524 clinical isolates compared to H37Rv, (and MDR 478), allowing establishment of pathology in the lungs at an earlier stage in the presence of cytokine responses to *M. tuberculosis* cell components which differed in the respective experimental strains.

4.4.3 Cytokine production and Th clone response

The mechanisms underlying early establishment of lung pathology of experimental mice were not investigated in the present study but they may relate to an altered capacity of the strains to induce the production of cytokines and chemokines by the cells they invade, resulting in differently modulated Th1, Th2 or mixed Th1-Th2 responses (Barnes *et al.* 1993; Cher *et al.* 1987; Maggie *et al.* 1992; Mosmannan and Moore 1991; Kariyone *et al.* 1999; Cooper *et al.* 1993; Dalton *et al.* 1993; Flynn *et al.* 1993; Mc Donough *et al.* 1993; Nathan *et al.* 1983; Orme *et al* 1992). Protective immunity characterised by early granuloma formation is mediated through a Th1-type pro-inflammatory response which results in reduced spread/dissemination of the tubercle bacilli. Cytokines involved in Th1-type response are IL-12 from monocytes which stimulates INF-γ production in T-lymphocytes. INF-γ activates macrophages to produce TNF-α, IL-2 and IL-6. Activated macrophages will arrest rapid intracellular multiplication of *M. tuberculosis* and lead to increased protective immunity. Th1-type cytokines are also induced by LAM and other lipids through upregulation of CD14 receptors on macrophages. Th2-type response which is anti-inflammatory in nature operates through IL-10 which

inhibits the Th1-type response and IL-4 whose effect leads to reduced granuloma formation but increased ability to multiply inside macrophages, followed by dissemination and death. Published evidence in keeping with this idea of differential cytokine inducing capacity is seen in the demonstration that *M. tuberculosis* CDC1551 strain induced well-organised granulomas with high levels of TNF-α, IL 6, IL10, IL-12 and INF-γ mRNA sooner in lungs of mice than those infected with the other strains including H37Rv (Manca *et al.* 1999). It therefore seems reasonable, as mentioned in Section 4.4.2., to suggest that the two virulent MDR strains in the present study with their high multiplication rate in mouse lungs and shorter survival times of experimental mice, caused a more rapid progression in early lung pathology which led to early death compared with the H37Rv strain. The two virulent MDR strains were therefore likely to have induced a more prominent Th2-type oriented host response than the H37Rv and CDC1551 strains.

4.4.4. Virulence determinants involved in early evolution of lung lesions.

In order to offer explanations of our findings at the molecular level, present concepts on the early evolution of lung lesion will be discussed briefly. The production of cytokines and chemokines by the infected cells is initiated by bacterial cell constituents and metabolites that *M. tuberculosis* strains liberate in their host cells. One such metabolite is lipoarabinimannan (LAM), a cell wall component that is considered to be a key virulence factor (Chan *et al.* 1991). When ingested by macrophages, it induces the production of TNF- α (Chatterjee *et al.* 1992; Moreno *et al.* 1989 ; Roach *et al.* 1993) while it may also inhibits lysosome-phagosome fusion. It is likely that acquired immunity, as expressed through the activation of infected lung macrophages, would be more capable of inhibiting less virulent compared with more virulent strains of *M. tuberculosis*. Macrophage activities induced by such strains will presumably be modulated by the secretion and effects of mycobacterial products like LAM. There is also evidence (Chan *et al.* 1991) that LAM may affect the activation of the mycobactericidal activity

of macrophages by inhibiting the transcription of gamma interferon-inducible genes. Thus, in lung macrophages, this mycobacterial product, and perhaps others, might activate the transcription of inflammation-inducing cytokine genes on the one hand, yet suppress transcription of genes necessary for the expression of mycobactericidal function on the other.

Other mycobacterial factors may play a role in virulence by inducing inflammatory cytokines involved in host tissue damage. These include the heat shock protein(HSP) and other culture filtrate proteins. The mycobacterial HSPs, *M. tuberculosis* HSP70, *M. leprae* HSP65, and *M.bovis* HSP65, when added to cultures of murine macrophages, induce the expression of mRNA for IL-1α, IL-1β, IL-6, TNF-α and granulocyte/macrophage colony stimulating factors. On the other hand, culture filtrate proteins are also likely to be key antigens required for inducing protective immunity (Retzlaff *et al.* 1994). Kariyone *et al.* (1999) have shown recently that a 25 peptide epitope of antigen α (or 85B antigen) of *M. tuberculosis* react with $V\beta11^{11+}$ Th1 cells, resulting in the expansion of these cells and the production of IL-2 and INF-γ. Immunisation with amino acid residues of this antigen resulted in markedly decreased number of CFU/ml in the mouse lungs during the first 7 days after infection. Other culture filtrate proteins from *M. tuberculosis* may also play a role and are discussed in Chapter 5.

4.4.5. Drug resistance in relation to virulence

It is generally accepted that many isoniazid-resistant strains of *M. tuberculosis* possess reduced expression of catalase-peroxidase activity and that this loss of activity was associated with a lower level of virulence in guinea-pigs(Mitchison *et al.* 1963. Isoniazid resistance has been shown (Heym *et al.* 1995; Morris *et al.* 1995) to be associated with either a deletion or point mutations in the *katG* gene that encodes the heat-labile catalase-peroxidase enzyme of *M. tuberculosis*. As an intracellular pathogen capable of surviving and multiplying within host macrophages, tubercle bacilli have to survive

exposure to macrophages killing mechanisms, including the oxidative stress response. The catalase-peroxidase *katG* is the component of the bacterial OxyR response which is induced in oxidative stress and protects the bacteria against oxidative killing. Thus the loss of a functional *katG* in an INH - resistant strain of *M. tuberculosis* would be expected to lead to a reduced capacity of the pathogen to survive within the intracellular environment. This was however shown not to be necessarily the case as drug-resistant strains may be fully virulent (Ordway *et at.* 1995). In the context of this study, the findings also do not support the association of drug resistance with loss of virulence. Although the strains used in the present study were resistant to INH, the expression of catalase (as determined by the whole cell bubble assay) was positive in the three MDR strains. As is the case with INH-resistant strains, there is no evidence that resistance to other anti-tuberculosis drugs is associated with loss of virulence of *M. tuberculosis* in humans.

4.4.6. Concluding remarks

In this study it was shown that two MDR strains from the RFLP Family 1 associated with high transmissibility are more virulent than H37Rv in terms of host survival and increased multiplication in mouse lungs after twenty days of infection. It was however, not clear whether these findings may be related to increased transmissibility. Up to date, no reliable *in vitro* or *in vivo* model has been described to assess this phenotypic trait. Discovering markers associated with increased transmissibility in *M. tuberculosis* would represent an important step in advancing our understanding of mycobacterial virulence. Although the use of strain MDR 478 in future studies could assist in identifying virulence determinants related to mouse-lung model, it may not relate to transmissibility. As mentioned earlier (Section 4.4.1.2), recent study by Zhang *et al.* (1999) demonstrated the usefulness and limitations of a human-macrophage model for transmission-related virulence in *M. tuberculosis* strains. They interpreted their findings as evidence that the capacity to grow intracellularly in

macrophages is not the only factor that affects spread of *M. tuberculosis*.

In an interesting recently-described *in vitro* model utilising a fibroblast-*Mycobacterium* microcolony assay, Byrd *et al.* (1998) assessed the ability of *M. tuberculosis* to spread from cell to cell. The authors suggested that their model could prove to be useful in the study of mycobacterial pathogenesis, including possible correlation with clinical presentations (e.g.,miliary versus cavitary tuberculosis). It is also possible that the growth patterns in this model may relate to transmissibility. There is unfortunately as yet no reliable animal or *in vitro* model which consistently correlates with degrees of transmissibility. Further work in this field is warranted.

Future studies on the virulence and transmissibility of MDR strains to complement the investigation presented here should ideally include correlation of early pulmonary growth rates of strains and survival times with lung pathology and an assessment of cytokine production by strains with different transmissibility potential, similar to those conducted by Manca *et al.* (1999). The inclusion of macrophage studies on the strains investigated thus far is also envisaged, if appropriate resources become available. Direct comparison of our strains and the CDC 1551 strain and possibly other strains such as the Los Angeles 210 in terms of the survival time, lung pathology and cytokine production could be rewarding. Further attempts at identifying virulence factors directly linked to transmissibility exhibited by clones including our own Family 1 strain and others such as the W-strain from New York (Bafina *et al.* 1996 and the U strain from Cape Town (van Rie *et al.* 1999) should also receive a high priority.

5: MOLECULAR AND BIOCHEMICAL CHARACTERISATION OF ISOLATES WITH DIFFERENT VIRULENCE LEVELS IN MICE

5.1. Introduction

As demonstrated by Ordway *et al.* (1995), clinical isolates of *M. tuberculosis* exhibit a broad range of *in vivo* growth rates in target mouse organs, which followed no clear trend in terms of geographical source, degree of drug resistance or rate of growth *in vitro*. This led to the grouping of strains into three virulence categories based on the growth rates of *M. tuberculosis* strains in experimental animals (slow, moderate and rapid growth). Those strains classified with moderate growth rates had *in vivo* growth characteristics similar to the laboratory strain (Erdman) of *M. tuberculosis*, whereas rapidly growing virulent strains tended to grow much faster over the first 20 days of the infection (Ordway *et al.* 1995). It has been suggested that the latter group of strains could be responsible for the rapid progression of disease and early fatal outcomes sometimes seen in immunocompromised patients with tuberculosis.

Culture filtrate proteins (CFPs) including, early secretory proteins (ESP) of standard ATCC strains of *M. tuberculosis* have been studied extensively (Abou-Zeid *et al.* 1988; Andersen *et al.* 1991; Collins *et al.* 1988; Nagai *et al.* 1991; Rosenkrands *et al.* 1998). Some of these proteins are recognised by T cells and are thought to play an important role on the pathogenesis of tuberculosis (Andersen *et al.* 1991; Haslϕv *et al.* 1995; Rosenkrands *et al.* 1998; Sϕrensen *et al.* 1995).

Sonnenberg and Belisle of the Mycobacteriology Research Laboratories, Department of Microbiology, Colorado State University, Fort collins, investigated the culture filtrate proteins (CFPs) profile of

M. tuberculosis H37Rv. When the author of this thesis was studying at Fort Collins, he was assigned the task to characterise CFPs from strains exhibiting different degrees of virulence in the mouse lung model. It was anticipated that isolates from Family 1 which showed extensive spread in the Welkom mines, would be included with sstrains of varying degrees of virulence available at Fort Collins. Unfortunately because of recently imposed rigid restrictions on the importation of potentially dangerous microbial organisms to the USA, the strains could not be made available in time for inclusion in the studies. It was however, considered appropriate to include the findings of the Fort Collins studies conducted by the author of the present thesis as they relate to strains with an increased potential for transmission and could, at a later stage be applied to the Welkom strains.

The main aim of the study recorded in this chapter was to identify a protein or proteins in culture filtrates of *M. tuberculosis* strains of varying degrees of virulence in the mouse model that may be linked to virulence and therefore serve as a candidate virulence determinant/s for future study.

A secondary aim was to ascertain whether fingerprint patterns of these strains could be linked to distinct protein profiles or unique strain-associated proteins. At the time when this study was initiated at Fort Collins, no information was available on possible links between the presence of virulence determinants in strains and DNA IS6110-based fingerprinting of isolates. It had, however, already been established that strains expressing certain phenotypic characteristics with known genetic bases, such as resistance to antimicrobial agents, could occur within RFLP clusters with identical band patterns together with members which fail to express the phenotypic trait. Despite this knowledge, it was decided that attempts to link DNA fingerprint with virulence could still yield useful information, as it is theoretically possible that the position of virulence -related genes on the *M. tuberculosis* chromosome could affect RFLP band patterns.

5.2. Materials and methods

The initial virulence studies on the isolates were performed at the Colorado State University (CSU), Fort Collins, USA. For the *in vivo* experiments conducted by Ordway *et al.* (1995) at Fort Collins, bacterial isolates were grown in nutrient 7H9 broth containing 0.05% Tween 80. Aliquots were frozen at -70°C, thawed and diluted in sterile pyrogen-free saline to a concentration of 5x 10^4 viable bacilli per ml. A volume of 10ml was then added to the venturi nebulizer unit of a Middlebrook Aerosol Generation device (Glas-Col, Terre Haute, Ind.) and C57BL/6 mice were exposed to an aerosol for 30-min period. This routinely resulted in the implantation of 20 to 50 bacilli in the lungs of these animals. The number of bacteria in the lungs of test animals was monitored against time by harvesting of mice by CO_2 inhalation, plating of serial dilutions of individual whole-organ homogenate on nutrient 7H11 agar, and then counting of bacterial colony formation 2 to 3 weeks later after incubation at 37°C in humidified air. For comparison, the growth of the virulent laboratory strain *M. tuberculosis* Erdman was also determined. Growth patterns fell into three categories, low, moderate and highly virulent.

All subsequent experimental work recorded in this chapter was performed by me at CSU.

5.2.1. DNA fingerprinting

The procedure for RFLP typing of 29 *M. tuberculosis* isolates from Colorado State University was described in Chapter 2

5.2.2. Growth of *M. tuberculosis* and preparation of culture filtrate proteins.

Initially *M. tuberculosis* strains were inoculated from 1ml frozen stocks of approximately 10 CFU/ml onto 7H11 agar and were incubated for three weeks. Bacterial cells were scraped from the plates into 100ml glycerol-alanine salts (GAS) medium and incubated with gentle agitation at 37°C for 14 days. The resultant culture was used to inoculate fresh 400ml GAS media and were grown in bottles by

rolling for 14 days at 37°C. Bacterial cells were harvested by filtration through a 0.22μm filter. The culture filtrate was concentrated by an amicon filtration system.

5.2.3. Two-dimensional PAGE of culture filtrate proteins

Separation of culture filtrate proteins by 2-D PAGE was based on the method described by O' Farrell(1975). After vacuum drying of culture filtrates of *M. tuberculosis* strains, 70 micrograms of dried filtrates were suspended in 30μl of isolectric focusing sample buffer (9M urea, 2% Nonidet P-40, 5% β- mercaptoethanol, 5% Pharmalytes (pH 3 to 10: Pharmacia Biotech, Piscataway, N.J), and incubated for 3 h at 20°C. An aliquot of 25μg of protein was applied to a 6 % polyacrylamide isoelectric focusing tube gel (1.5mm by 6.5 cm) containing 5% Pharmalytes (pH 3 to 10 and 4 to 6.5 in a ratio of 1:4). The proteins were focused for 3 hours at 1kV with 10mM $H_3 PO_4$ and 20 mM NaOH as the catholytes and anolytes, respectively. The tube gels were subsequently immersed in sample transfer buffer (Dunbar *et al.* 1990) for 30 min and placed on preparative SDS-polyacrylamide gel (7.5 cm by 10 cm by 1.5mm) by 0.75 mm) containing a 6% stack over a 15% resolving gel. Electrophoresis in the second dimension was carried out at 20mA per gel for 0.3 h followed by 30mA per gel for 1.8 h. Proteins were visualised by staining with silver nitrate (Morrissey 1981). The MW scale was calibrated with standard proteins (Pharmacia).

5.3. Results

5.3.1. RFLP typing

M. tuberculosis clinical isolates with defined *in vivo* growth characteristics were examined for their IS6110- associated RFLP patterns and protein profiles. Of the 29 clinical isolates subjected to RFLP analysis (Ordway *et al.* 1995), 22, 5, and 2 were previously grouped (in terms of virulence) as rapid, moderate and slow growing strains in mouse lungs, respectively. In all, the number of the IS6110 copies

per isolate ranged from 1 to 20 (Table 5.3.1, Figure 5.1). Among the 22 clinical isolates displaying the rapid *in vivo* growth phenotype, 14 different IS6110 RFLP patterns were observed, while all strains categorised as having slow or moderate *in vivo* growth phenotypes showed different RFLP patterns. Three RFLP clusters (representing 7, 3 and 2 isolates) were identified among the clinical isolates (clusters carrying less than 4 copies excluded).(Table 5.3.1 and Figure 5.1. One isolate, CSU 24 from the 3 membered cluster, CL 3 was categorised as a low virulence strain. The isolates in the largest cluster, CL 1, had hybridisation patterns that were similar to each other and to those referred to as W-strains (CSU 85, CSU 86 and CSU 87, "W" strains were associated with outbreaks of multidrug-resistant tuberculosis in hospital C in New York City, in a State correctional facility, and subsequently in four other New York hospitals (Bifani *et al.* 1996).

Table 5.3.1. Summary of RFLP analysis of *M. tuberculosis* with different virulence levels in mice

STRAIN	SOURCE	VIRULENCE	IS6110 COPIES	CLUSTER	12 kDA protein	MDR
CSU 11	K	High	18	1	Weak	No
CSU 35	NJ C	High	18	1	positive	Yes
CSU 39	NJ C	High	18	1	positive	Yes
CSU 85	PHRI	High	18	1	negative	Yes
CSU 86	PHRI	High	18	1	negative	Yes
CSU 87	PHRI	High	18	1	Weak	Yes
CSU 109	B.Bishai	High	18	1	positive	ND
CSU 92	Shinnick	High	4	2	negative	ND
CSU 93/CDC 1551	Shinnick	High	4	2	negative	No
CSU 22	C	High	13	3	Weak	Yes
CSU 24	C	Low	14	3	Weak	No
CSU 25	C	High	14	3	positive	No
CSU 12	K	High	4		Weak	No
CSU 19	C	High	10		positive	No
CSU 26	C	High	12		positive	No
CSU 28	C	High	11		Weak	No
CSU 31	NJC	High	9		negative	Yes
CSU 32	NJC	High	10		negative	Yes
CSU 37	NJC	High	12		positive	No
CSU 44	NJC	High	15		negative	Yes
CSU 107	B.Kreiswick	High	2		positive	ND
CSU 108	B.Kreiswick	High	4		negative	ND
CSU 15	K	Moderate	13		negative	No
CSU 17	K	Moderate	13		negative	No
CSU 18	K	Moderate	2		negative	No
CSU 21	C	Moderate	3		negative	Yes
CSU 23	C	Moderate	1		negative	No
CSU 20	C	Low	16		negative	No
CSU 27	C	Low	4		negative	No
H37Rv	ATCC 27294	High	14		negative	No

Determined by the ability to grow in the lungs of C57BL/6 mice exposed to a low-dose aerosol of bacilli. Growth was classified relative to that of the type strain. Erdman K. James Kilburn, Centers for Disease Control and prevention, Atlanta, GA; C, Ray Cho, Seoul, Republic of Korea; NJC, National Jewish Center for Immunology and Respiratory Medicine, Denver, CO; PHRI, Public Health Research Institute ; ND, not done.

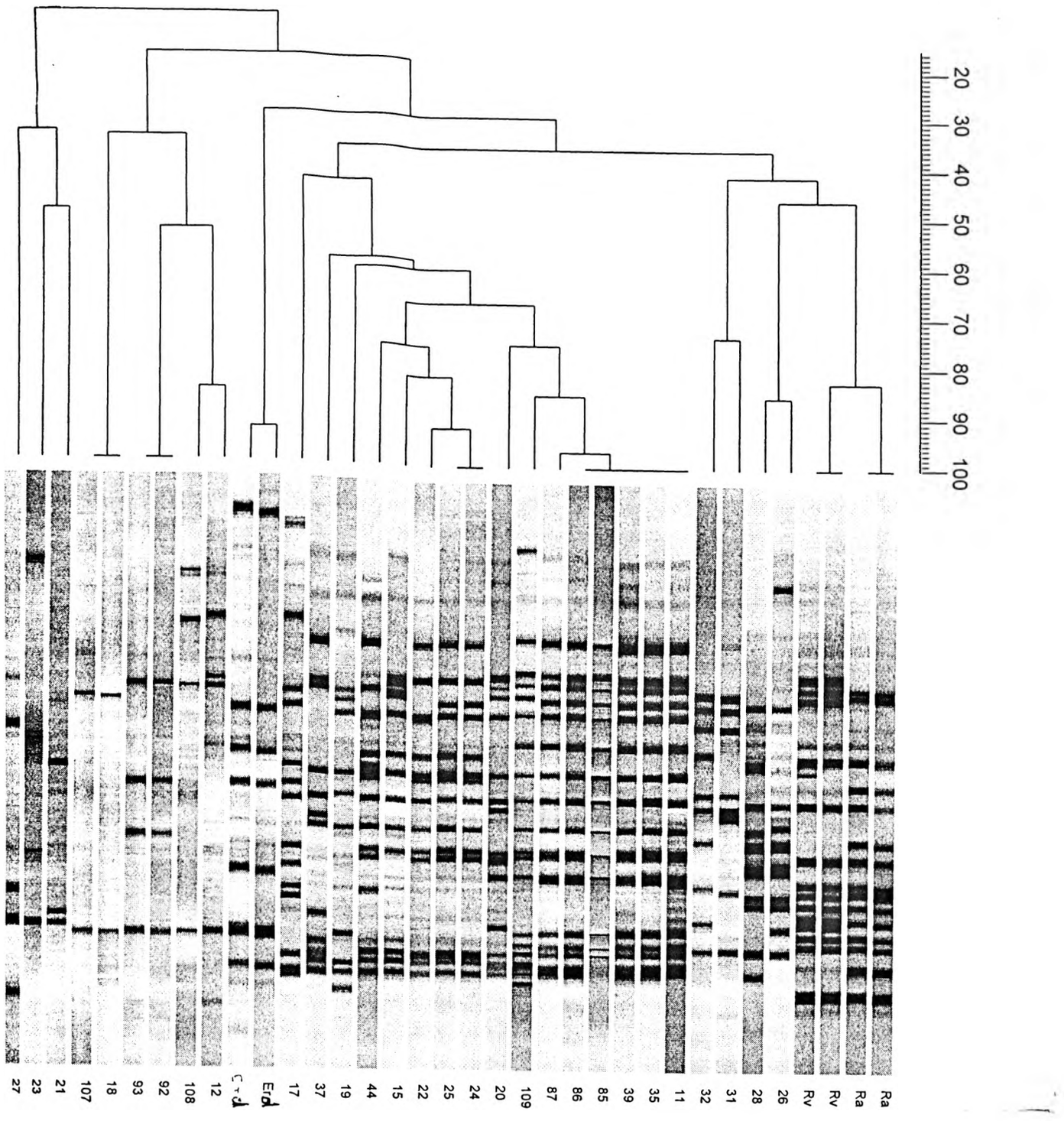


Figure 5.1. Dendrogram based on computer-generated comparison of DNA fingerprints obtained from 29 isolates showing differences in virulence in mice. Abbreviations Rv, Ra and Erd are laboratory *M. tuberculosis* strains H37Rv, H37Ra and Erdman strains respectively.

5.3.2. Culture filtrate proteins profiles of *M. tuberculosis* patient isolates by 2-D PAGE

The 2-D electrophoresis of culture filtrate proteins (CFPs) profile of the reference strain H37Rv was previously studied by Sonnenberg and Belisle (1997). Comparison of the CFPs among clinical isolates and to the reference map of *M. tuberculosis* H37Rv demonstrated several differences not clearly linked to transmissibility or virulence between individual strains. However, one basic protein at 12 kDa (protein spot 7 in Figures 5.2-5.6) was particularly notable in that it was abundant in strains CSU 19, 25, 26, 35, 37, 39, 87 and 109 (all in highly virulence group), weakly expressed in strains CSU 11, 12, 22, 24, 31 and 107 (except for CSU 24, which was categorised as low virulence, all were classified as highly virulent), and was absent from several strains including H37Rv (Sonnenberg and Belisle 1997) (Figures 5.2- 5.6).

Other proteins (protein spots 1, 2, 3,4,5,6 and 8) appeared to be differentially expressed by the clinical isolates (Figures 5.2- 5.6).

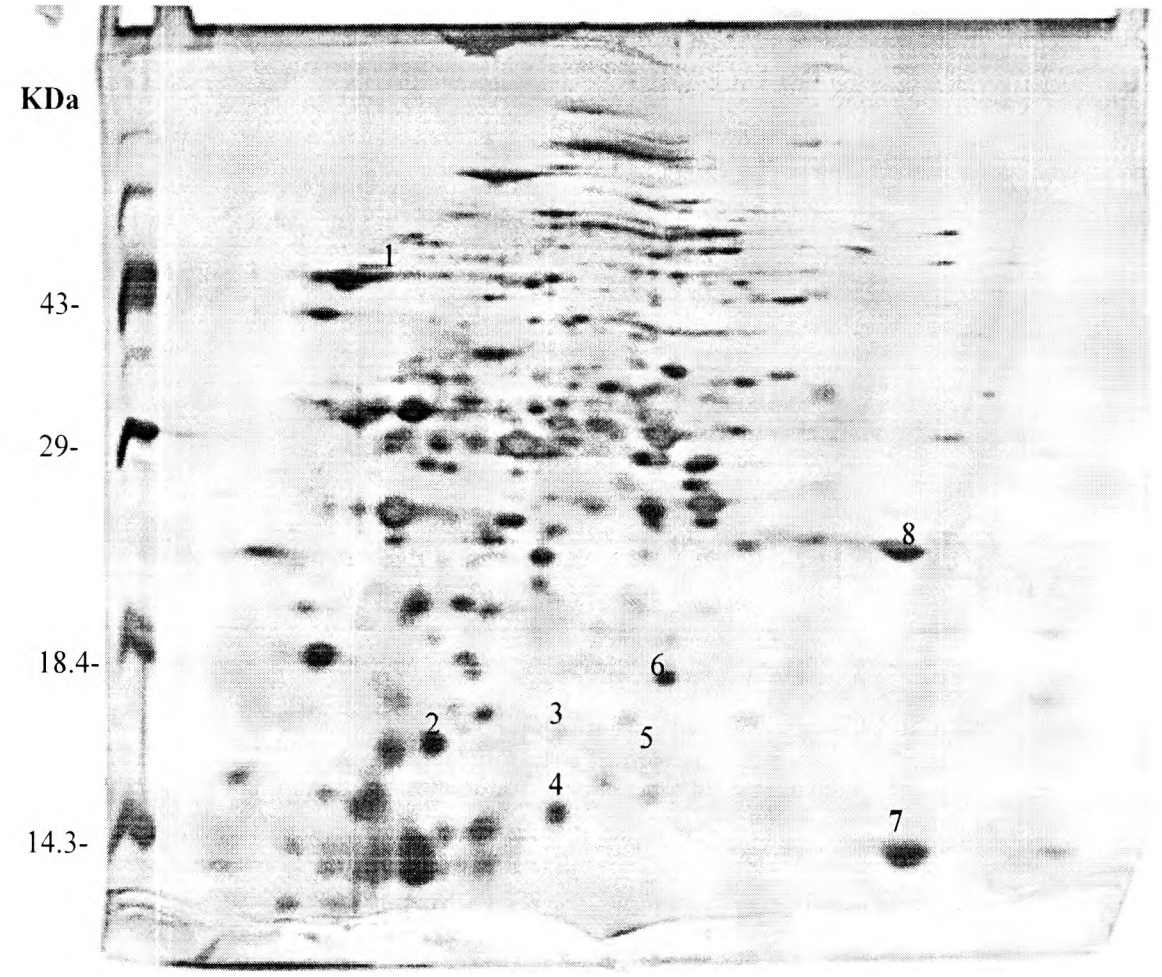


Figure 5.2. 2-D Profile of culture filtrate proteins of *Mycobacterium tuberculosis* strain CSU 35. Protein spots 1-8 are proteins that were cited in the text as being differentially produced

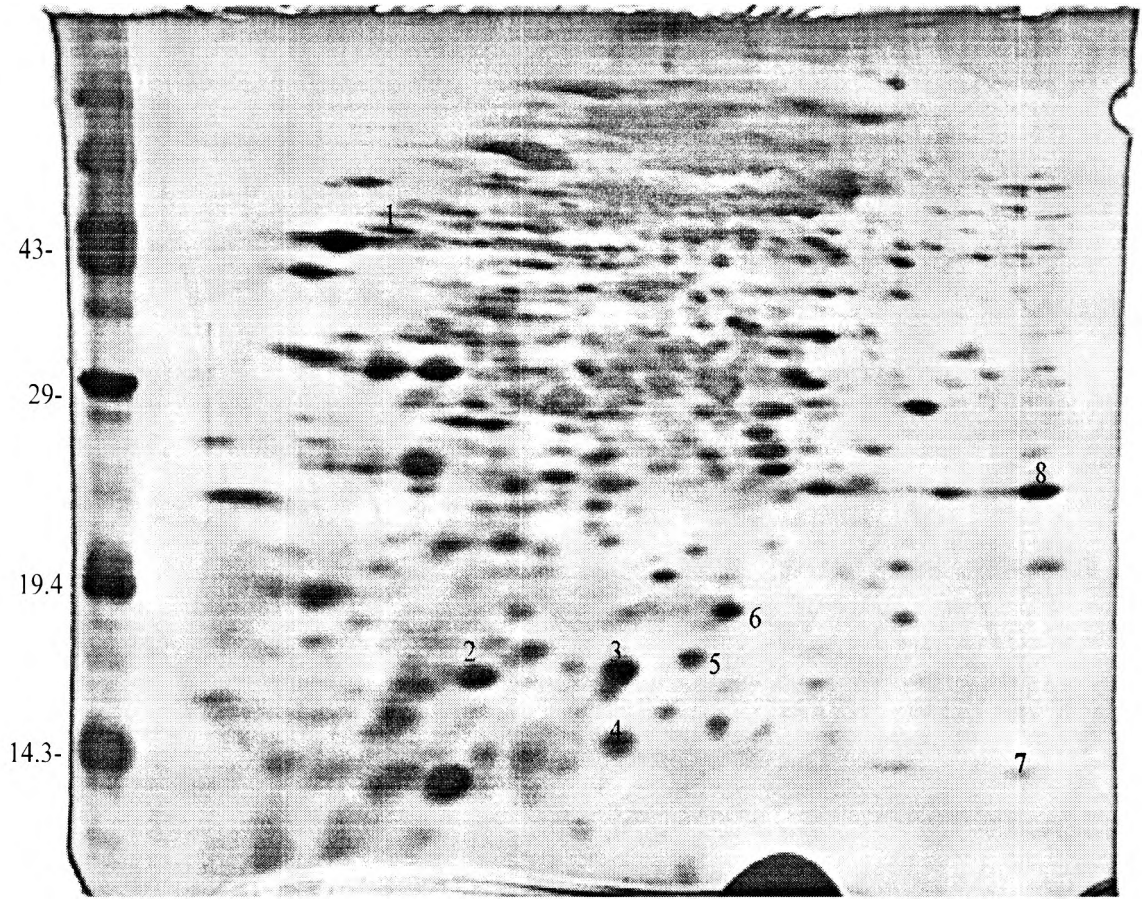


Figure 5.3. 2-D Profile of the culture filtrate protein of *Mycobacterium tuberculosis* strain CSU 22. Protein spots 1-8 are proteins that were cited in the text as being differentially produced.

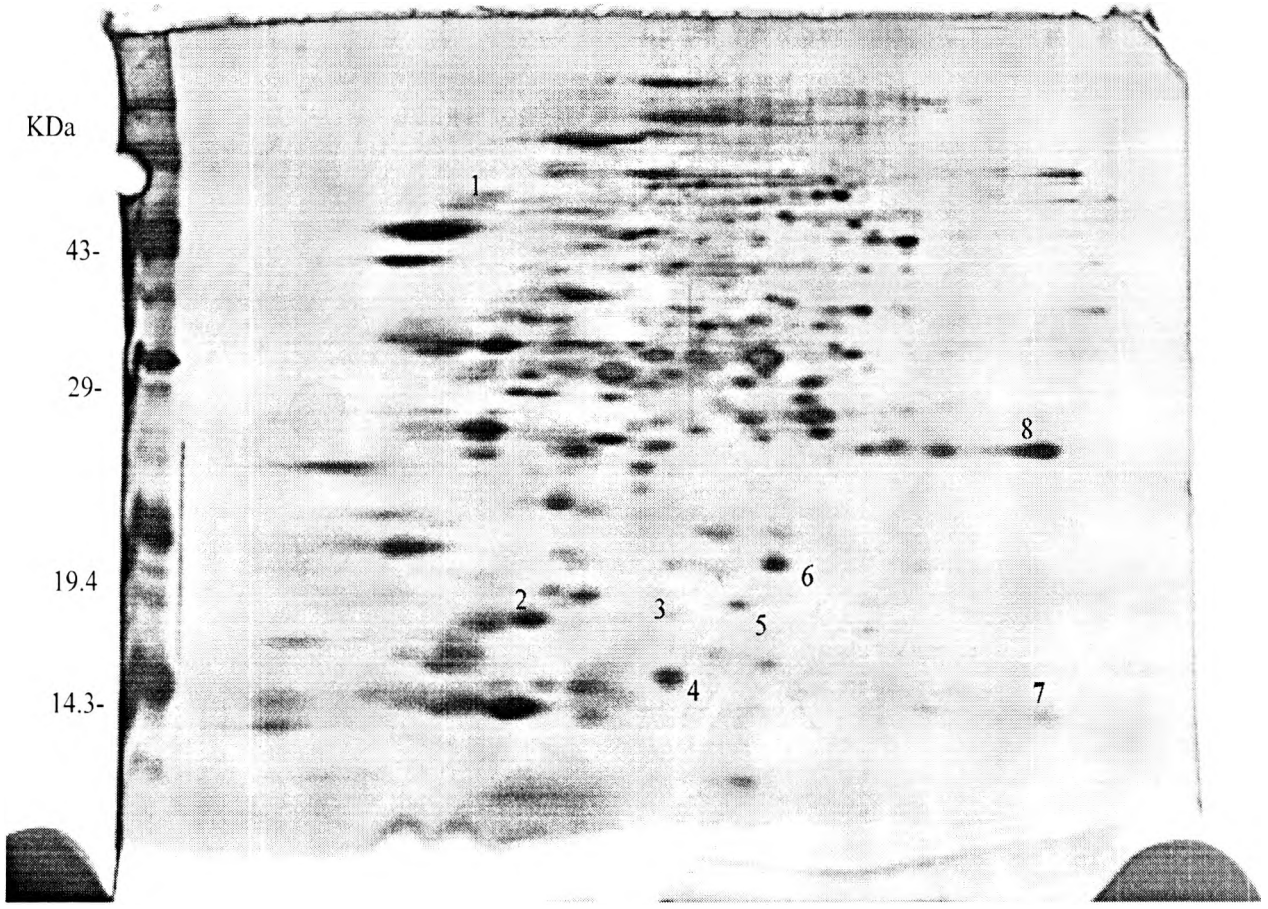


Figure 5.4. 2-D Profile of culture filtrate proteins of *Mycobacterium tuberculosis* strain CSU 24. Protein spots 1-8 are proteins that were cited in the text as being differentially produced

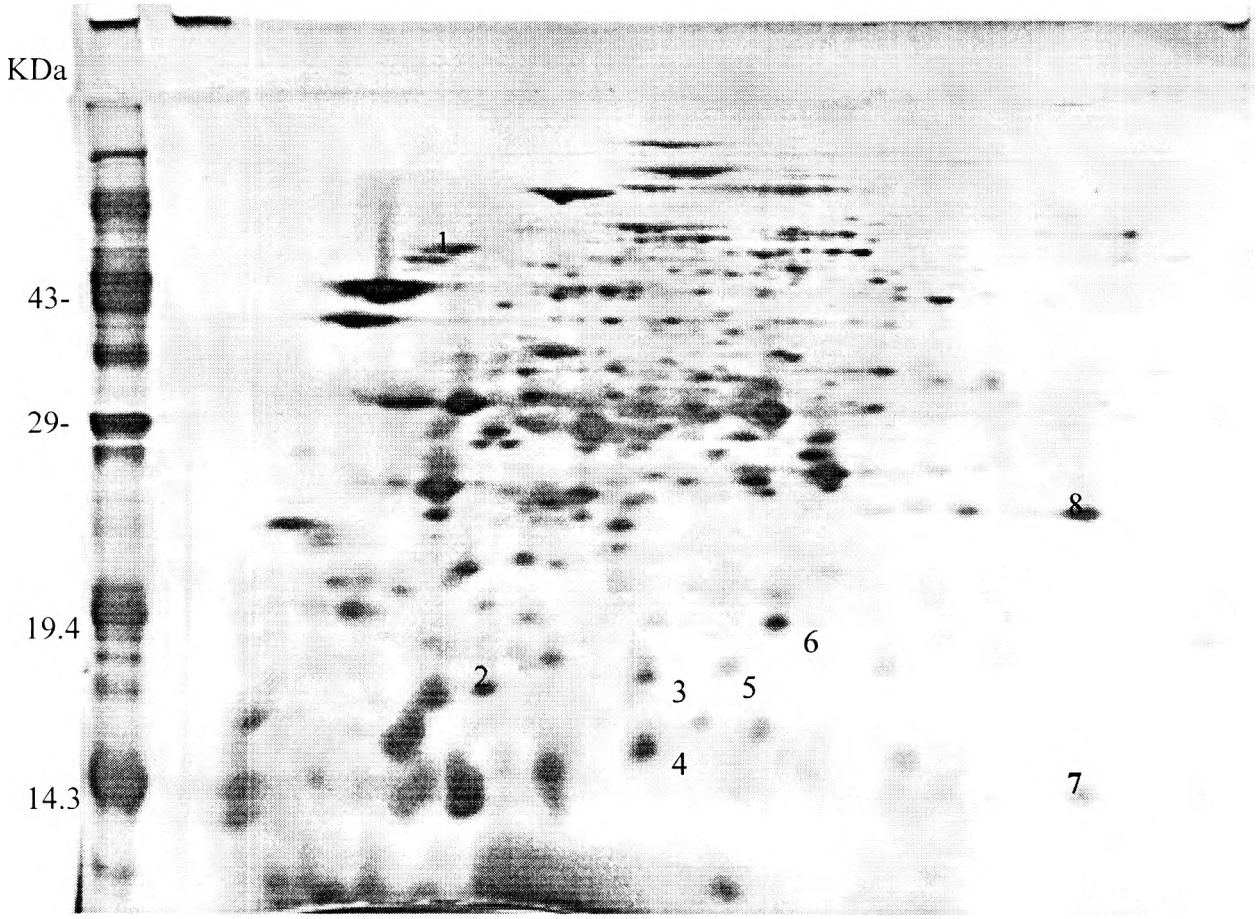


Figure. 5.5. 2-D profile of the culture filtrate of *Mycobacterium tuberculosis* strain CSU 25. Protein spots 1-8 are an examples of proteins that are cited in the text as being differentially produced .

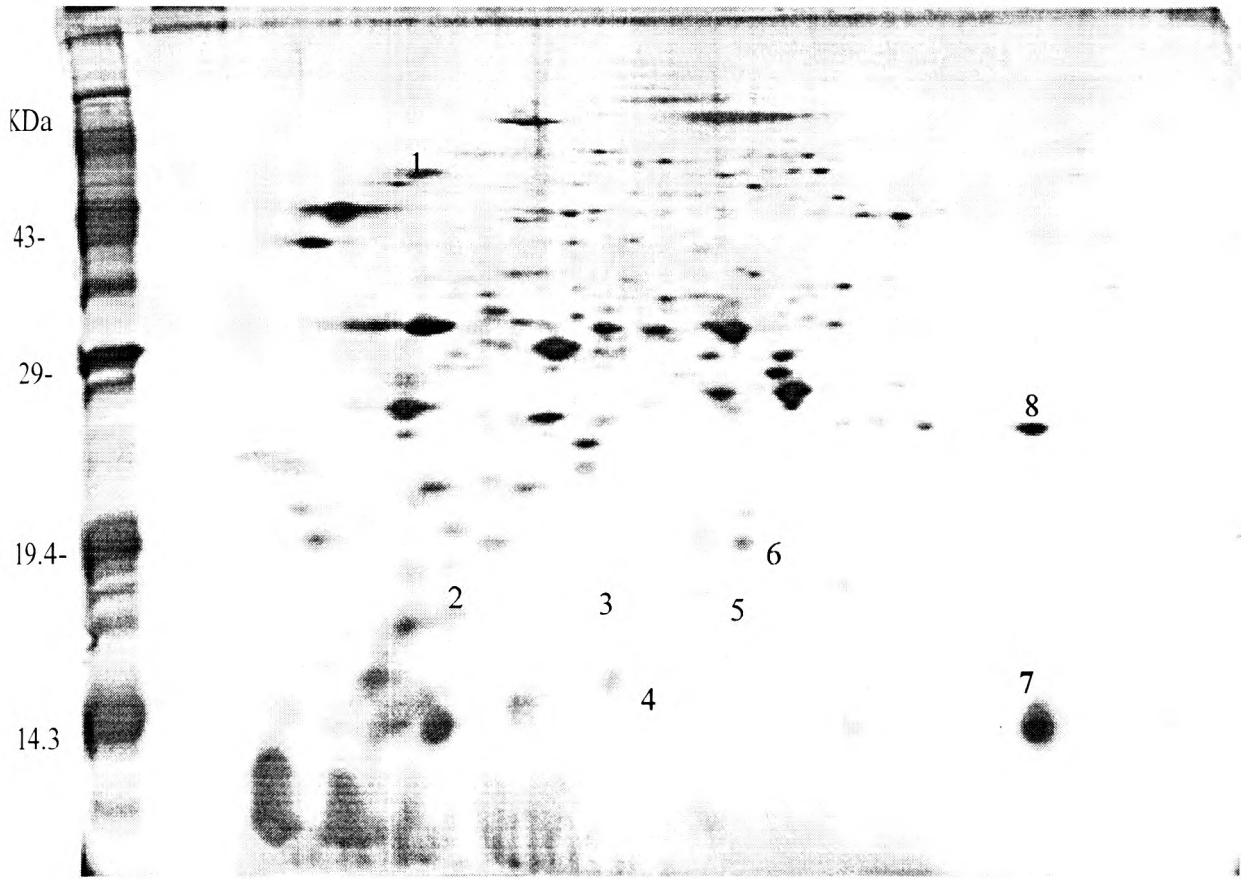


Figure 5.6. 2-D Profile of culture filtrate proteins of *Mycobacterium tuberculosis* strain CSU 109. Protein spot 1-8 are proteins that were cited in the text as being differentially produced

5.4. Discussion

5.4.1. Relationship between RFLP-based DNA fingerprinting and virulence

Strain differentiation by DNA RFLP has been used mainly for epidemiological purposes of *M. tuberculosis* infection. In the present study, an attempt was made to link the molecular and the phenotypic characteristics of *M. tuberculosis* isolates from patients by comparing the DNA fingerprint obtained by RFLP using IS6110 and the *in vivo* growth phenotype in mouse lungs. In addition, early secretory proteins expressed by the different strains were characterised and fingerprint patterns of the strains were matched with protein profile to establish possible links between a distinct protein profile and the presence of strain specific proteins.

RFLP studies based on IS6110 presented in this chapter demonstrated great diversity among strains with a similar virulence phenotype, suggesting that the emergence of a particular virulence phenotypic trait has occurred in multiple clones of *M. tuberculosis*. These results further suggest that more than one factor could be responsible for the growth characteristics of individual strains of *M. tuberculosis* observed in the mouse model.

The basic premise inherent in any typing system is that epidemiologically related isolates are derived from the clonal expansion of a single precursor and, consequently, will share characteristics that differ from those of epidemiologically unrelated isolates. In studies in Europe and the USA, isolates of *M. tuberculosis* with matching fingerprints have always been cultured either from the same individual or from individuals between whom transmission of infection was likely. Even though strains within RFLP families, resembling each other, with only a few band differences are more common in regions where tuberculosis is highly endemic, there is sufficient identity between isolates to make matching fingerprint patterns very rare without an identifiable epidemiological link (Hermans *et al.* 1995). In the present

study, molecular typing demonstrated that the number of IS6110 elements and the RFLP pattern were independent of the *in vivo* growth phenotype. This was best exemplified by CSU 24 and 25, which yielded identical RFLP patterns but have different *in vivo* growth characteristics. Additionally, CSU 35 and 39 had matching RFLP patterns and *in vivo* growth characteristics, but were subsequently shown by Rhoades and Orme (1997) to have differing susceptibilities to reactive nitric oxide intermediates. This phenomenon suggest that insertion sequence-associated polymorphism is accumulating more rapidly than mutations relating to the virulence phenotype.

5.4.2. Candidates for virulence determinants among culture filtrate proteins of *M. tuberculosis*

The proteins of *M. tuberculosis* and other mycobacterial species have been extensively studied in terms of their biochemical, immunological and molecular characteristics (Abou-Zeid *et al.* 1988; Andersen *et al.*1991; Collins *et al.* 1988; Nagai *et al.* 1991; Rosenkrands *et al.* 1998). Sonnenberg and Belisle in 1997 determine the CFPs profile of *M. tuberculosis* H37Rv. However, in the case of *M. tuberculosis*, no report has appeared in the literature on variation in protein profiles among a wide array of different clinical isolates. The present study shows clear evidence of variation in protein profiles. Since *M. tuberculosis* has been one of the most important human pathogens that has long caused disease entities ranging from inapparent to life-threatening, it is to be expected that heterogeneity would exist due to spontaneous mutations among strains over time. Furthermore these results do not come as a surprise, given the recent observation that several *M. tuberculosis* isolates lack the gene encoding MPT 40 (Weil *et al.* 1996), while the loss of *katG* activity in many isoniazid-resistant strains of *M. tuberculosis* is associated with the concurrent over expression of AhpC (Sherman *et al.* 1996).

One basic protein at approximately 12 kDa was particularly notable in that it was differentially expressed among clinical isolates. There was no correlation between the expression of this protein and

DNA fingerprint as the protein was absent in three of the six members of cluster 1. The significance of the expression of this protein in highly virulent strains is unclear, although it must be noted that other highly virulent strains lack this protein. The fact that this protein was not found in any of the eight strains of lesser virulence, suggests that it may be related to one of a number of virulence determinants. Decreased virulence in mutants of parent strains exhibiting this protein may confirm its status as a candidate virulence determinant. Cloning of the gene encoding this protein could form the basis of further genetic studies to establish the nature of the virulence determinants. Studies on the *in vitro* effect of this protein on macrophage interaction compared with low virulence strains may be rewarding as has been shown in models evaluating surface proteins of cell wall components of mycobacteria and other pathogenic bacteria (Bliska *et al.* 1992; Pauls *et al.* 1996). Also, studies on the interaction of this protein with T-cells and the induction of cytokines such as interferon-gamma following T-cell proliferative responses could be performed. Authors of a previous study pointed out that only a few of 12 previously characterised secreted proteins were recognised by T-cells isolated from tuberculosis patients suggesting a number of as yet undefined antigenic targets amongst secreted antigens (Boeson *et al.* 1995)

Using isocitrate dehydrogenase, a cytoplasmic enzyme as an indicator of an autolysis of *M. tuberculosis* in culture, Andersen *et al.*(1991) have shown that autolysis is maximal at 14 days incubation (an early phase of growth decline) and that autolysis increases rapidly during the late logarithmic growth phase. The analysis of the culture filtrate proteins of the clinical isolates *M. tuberculosis* in the present study was performed on 2-week-old cultures and represents the profiles of proteins released into the culture supernatant composing both early secretory and intracellular, including cell wall proteins. Thus, it cannot be claimed that these studies were a true measure of gene expression relating to early secretory proteins which are thought to be important in the initiation of

infection. To ensure that gene expression is evaluated, protein analyses must use proteins metabolically labelled with ^{32}S-methionine or ^{32}S-cystine (Sonnenberg *et al.* 1995).

It should be noted that the culture filtrate represents only a small proportion of the gene products of *M. tuberculosis* and additional analyses focussing specifically on cell wall, cytosol and membrane proteins will most likely yield additional proteins.

Furthermore, protein analysis of a variety of clinical isolates with well-defined clinical history is warranted. This type of basic proteomic analysis will allow the correlation of protein profiles with pathology, geographical distribution, or epidemiological aspects such as outbreaks, including their initiation and termination. Additionally, the continued molecular characterisation of *M. tuberculosis* by 2-D PAGE gel coupled with such powerful techniques as liquid chromatography-mass spectro- mass spectrometry (LC-MS-MS) will supplement the wealth of information that is now being derived from the *M. tuberculosis* genomic sequencing. Suitable animal model able to differentiate transmissibility between strains could be another valuable tool to identify virulence determinates related to transmissibility (see Chapter 4).

6. NEW PERSPECTIVES ON RECENT INFECTION VERSUS REACTIVATION OF TUBERCULOSIS IN FREEGOLD HEALTH REGION MINES

6.1. Introduction.

The findings recorded in this thesis provide strong evidence that a substantial component of the disease load of tuberculosis in the mine environment is due to recent transmission. They also point to transmission cycles in the mine which over time resulted in the establishment of nearly identical strains with DNA fingerprints differing only by a few bands on RFLP-based typing. There was also sufficient heterogeneity of strains indicating infections that were acquired from multiple sources outside the mines and finally found their way in respiratory secretions in miners at the mines following reactivation. In this chapter the author will attempt to place his findings into perspective and to propose further studies and possible control strategies emanating from his investigations

6.2. The case for recent transmission

It is generally accepted that clustered isolates with identical RFLP band patterns (IBPs) provide strong evidence of recent infection by these strains. Close geographical links between patients represented in such clusters, e.g. working in the same mine shaft, lend support to the likelihood of recent infection. In most cases, such infections resulted in disease during the miner's stay in the mine, usually less than 18 months, suggesting a relatively short period between infection and disease. It is therefore likely that the mine environment is conducive to early disease development following infection. Such an enhanced process may either follow of primary infection or reinfection which occurred after a primary infection in the past. In both instances a lowering of cell-mediated immunity (CMI), possibly brought on by physical or mental stress or viral infections which temporarily depress CMI, is required. It is not possible to accurately quantify the prevalence of recent transmission in miners in the FHR mines.

However, the occurrence of 14 cases (21%) with an identical RFLP profiles shared in mine shafts, 6 of whom having worked in only two separate mine shafts during the short study period of six months, provides strong circumstantial evidence of recent transmission in the mines. As miners working in the same shaft also tended to share sleeping quarters, it is likely that transmission in the more confined space of the sleeping quarters may have occurred more frequently than in the mine shafts. If one considers that some of the strains belonging to the nearly identical RFLP families were almost definitely also responsible for recent transmission, one has to conclude that a transmission problem of substantial dimensions exists in the mines. In addition to the miners with proven disease following recent transmission, there are many others who became infected but who did not develop disease during the study period recorded in this thesis.

6.3. Endemicity of RFLP families

As explained elsewhere in this thesis the term "endemicity" as used in this thesis denotes the process whereby strains of *M. tuberculosis* with identical or nearly identical RFLP band patterns become established in the mines as a result of repeated cycles of transmission in miners leading to varying periods of dormancy followed by reactivation. In the process some of the strains within clusters comprising RFLP family genotypes, may fortuitously have been present in the mines at the time of the present investigation and may therefore have been acquired some time in the past rather than recently. It is likely that the initial acquisition of such strains occurred in the mines as there was no evidence of a preponderance of strains within such RFLP families in specific regions from where the migrant miners come. As it is common practice for miners to return to the same mine on being re-employed, it is possible for miners to acquire infection in a mine initially without becoming ill and subsequently experience reactivation when they return to the same mine. It is therefore quite feasible for RFLP family strains to become established in mines. Quantification of endemic strains as opposed to recent infection

based on IBP and RFLP family genotyping is not possible from the data available. However, at least some of the patients who could not be linked by mine shaft/sleeping quarters may fall in the endemic strain category. Prospective studies utilising base-line tuberculin skin testing on arrival in a mine followed by six-month monitoring could be used in future studies to resolve this problem.

6.4. Reactivation tuberculosis in the mine

There is good evidence that reactivation tuberculosis is common in the mines. This is exemplified by the high degree of heterogeneity of RFLP patterns amongst the isolates, while analysis of the fingerprints of drug-resistant strains showing great genetic diversity, again indicates that the patients acquired their infections from diverse sources probably outside the mines. Furthermore the frequency of drug-resistant strains parallel those encountered in rural areas where adherence to treatment is likely to be much more erratic than is the case in the mines where good treatment practices prevail.

The much higher incidence of tuberculosis in the mines can be ascribed to either an increased rate of transmission or an increased propensity of the patients to acquire reactivation tuberculosis under mining conditions or both. Data from the present study suggest that both processes operate simultaneously and also interact with and augment each other: factors depressing CMI will lead to more reactivation cases as well as shorten the period between recent infections and disease, while an increase in transmission rates will lead to more initially asymptomatic cases who may become reactivated in future, affecting tuberculosis in the mines but also the regions feeding the mines with mine workers. Evidence of the major impact of HIV infection/AIDS on this cycle in recent years has recently been reported by Churchyard *et al.* (1999).

6.5. Risk factors associated with transmission.

HIV-infection has been shown to be an important risk factor for transmission of *M. tuberculosis* in hospital-based and other outbreaks (Alland *et al.* 1994; Small *et al.* 1994). In the present, as well as in the Godfrey-Faussett *et al.* (2000) study, HIV-infection was not shown to be a significant risk factor for cluster formation. This may change as the HIV epidemic increases in severity and more miners progress to AIDS.

Of utmost importance was the identification of retreatment of tuberculosis as a risk factor for transmission amongst miners by the Godfrey-Faussett *et al.* (2000) and confirmed by the present study. Retreatment patients are a prolonged source of tubercle bacilli present in large numbers in respiratory secretions and preventive measures should be focussed on this group of miners

6.6. Control measures

Having established that transmission leading to early disease does occur in the FGR mines, probably at a high rate, control measures should focus on a) prevention of transmission and b) identifying and ideally diminishing the risk of CMI depression in the miners.

Early detection of active tuberculosis disease is important to contain the infectious pool in the mines. This could be facilitated by a programme of regular weighing of miners with recording of weight loss, monitoring of viral, especially respiratory infections, as well as occurrence of night sweats and persistent cough. The specific aim of such a program would be the early diagnosis of tuberculosis by smear microscopy or chest radiography. As acute viral infections may compromise CMI, preventive measures against transmission of such infections should be considered.

Mine administrators should also decide on the feasibility of serological monitoring of HIV infection which could assist in identifying candidates for CMI depression. Strongly positive tuberculosis skin test reactors (interpretation suitably adjusted in the case of HIV-positive individuals) or converters would be candidates for preventive treatment, either in the form of INH for six to 12 months or a shorter course such as a combination of RMP and PZA which has recently been shown to be cost-effective under USA conditions (Halsey *et al.* 1998). As the risk of infection is much higher than in the USA, effectiveness of the latter regimen in the mine environment may be inappropriate. Attention to measures aimed at minimising transmission could include improved ventilation in sleeping quarters and mine shafts and other locations in mines where miners are congregated into confined areas.

Dust control is vitally important as dust particles form an essential component of droplet nuclei required for the respiratory spread of tuberculosis. Also long term exposure to dust may lead to varying degrees of silicosis, a condition which constitutes a highly increased risk of acquiring tuberculosis.

The institution and/or maintenance of simple hygienic measures in mines to control transmission by coughing as a result of aerosol formation, e.g. the issuing of absorbent materials (gauze tissues or paper towels) to miners to cover their mouths and noses when they cough and strategically placed paper bags for disposal, and finally, sterilization by incineration of the sputum-soiled absorbent materials may also be an option.

Retreatment of patients who failed initial therapy has been shown in this study and by Godfrey-Faussett *et al.* (2000) to be a risk factor for transmission of tuberculosis in the mines. Early detection of treatment failures and taking preventive measures to interrupt transmission in this group of miners is vitally important and should reduce the incidence of tuberculosis. Such patients should be kept

away from other miners until they become smear-negative for acid-fast bacilli, or preferably when they are culture-negative. As HIV- infection was shown in the FGR mines by other workers to significantly promote recurrent tuberculosis which was also related to treatment regimen (Mallory *et al.* 2000), preventive measures and treatment management of culture-/smear-positive that are HIV-seropositive would be major priority. Early diagnosis of tuberculosis in HIV-seropositive patients would also be very important as these patients often present with atypical clinical features.

The escalating problem of HIV infection/AIDS in the mines poses a major challenge to the mining industry. Management strategies should be adjusted and new measures based on careful studies focussing on cost effectiveness should be implemented promptly. In the process due consideration should be given to ethical aspects of new policies.

6. 7. Future studies

As mention earlier, prospective studies utilising RFLP technology and incorporating monitoring of recent infection by tuberculin skin testing could more accurately define recent infections as opposed to identifying dominant RFLP-based genotypes endemic in the mines (endemic strains) and reactivation cases.

Future studies should extend over a period of at least two years and should cater for critical information on treatment history and possible geographical links which could be identified as possible risk factors.

Virulence determination of strains associated with large IBP clusters to identify strains associated with a high transmission rate should also be considered for future investigation. Valway *et al.* (1998)

described an outbreak of tuberculosis caused by a highly virulent strain, as determined in the mouse lung model which also showed an enhanced capacity for transmission of the disease. The identification of virulence determinants in such strains should be attempted. The 12kDa culture filtrate protein demonstrated in virulent strains in the present study should be investigated further. The absence of this protein in the strains associated with which caused the outbreak described by Valway *et al.* (1998) indicates that other virulence factors are important in human disease. It may also be rewarding to look for mutations in the natural-resistance-associated macrophage protein 1(*Nramp* 1) gene in South Africa using mine workers to identify NRAMP 1 polymorphism associated with host susceptibility to tuberculosis (Bellamy *et al.* 1998)

Epidemiological aspects of future studies should include investigation of transmission in areas carrying a high risk of infection and activities and practices that may promote reactivation. A priority would be to investigate the risk of transmission in sleeping quarters and other geographic sites associated with mining activities.

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