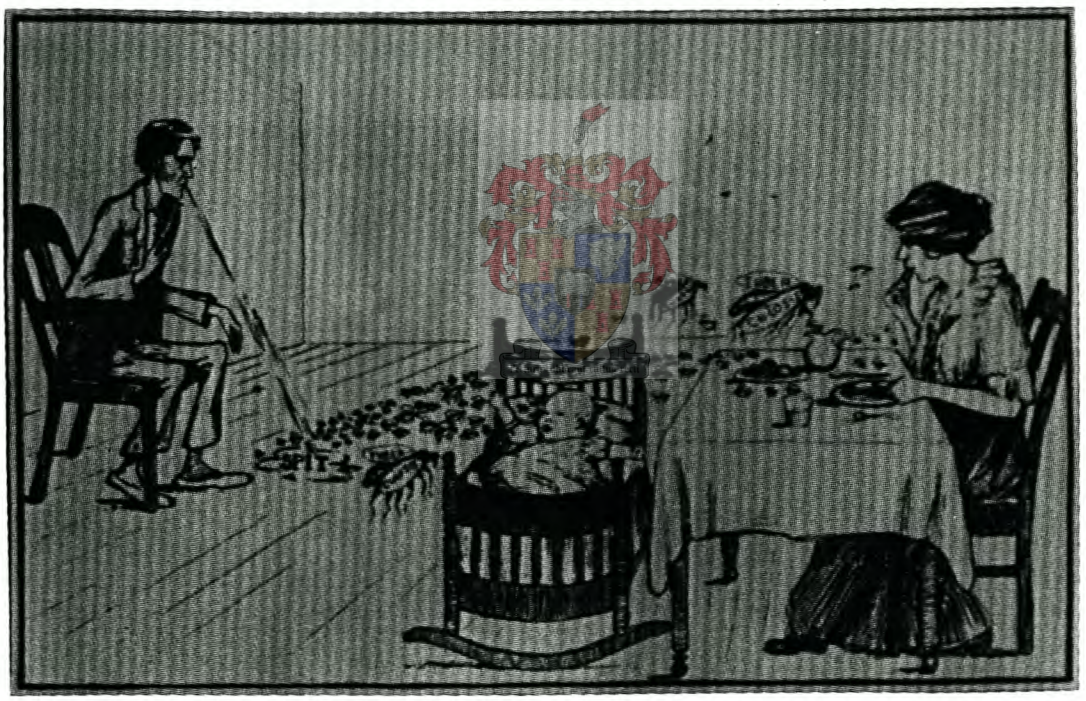
Disease dynamics in patients with drug-resistant tuberculosis residing in a high incidence community



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Promoters: Prof. N. Beyers and Prof. P. van Helden
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"*Declaration*

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature: Date

SUMMARY

Drug-resistant tuberculosis poses a threat to global tuberculosis control by the WHO DOTS strategy. Studies in the United States and Europe have shown (i) that drug-resistant tuberculosis is present in every country; (ii) that, by contrast to previous dogma, drug-resistant bacilli are virulent and can be transmitted, especially in institutional settings and to immunocompromised patients; and (iii) that the majority of cases arise by acquisition of drug resistance due to errors in the management of TB cases. (iv) Furthermore, it has been shown that the extremely high case fatality rates of the 1980s and early 1990s can be reduced by individualized, but costly treatment.

However, the majority of drug-resistant TB cases reside in the developing world. Data on disease epidemics in less developed parts of the world are scarce. The aim of this thesis was to study the disease dynamics of drug-resistant TB in a developing country where TB is endemic.

All cases of drug-resistant TB during a 5-year period in two communities with poor socioeconomic living conditions were included for this observational study. Three different methods were used: restriction fragment length polymorphism (RFLP), mutation detection analysis by dot-blot hybridisation technique and a Geographic Information System. Results of RFLP analysis and mutation detection analysis showed that community outbreaks of drug-resistant *Mycobacterium tuberculosis* strains occur, even without the involvement of immunocomprimised patients. Infection with a drug-resistant strain occurred in new patients (primary drug resistance) as well as in patients treated before (exogenous reinfection). Exogenous reinfection was also shown to be an important mechanism of recurrence after previous cure for drug-sensitive TB. Transmission of drug-resistant strains occurred more frequent in areas with lower socioeconomic living conditions. The relative contribution of transmission differed substantially between the group of multidrug-resistant (two thirds of cases) and single-drug-resistant (no cases) cases, which probably reflects the prolonged infectiousness of multidrug-resistant cases. To stop the growing epidemic of multidrug-resistant TB, prevention of acquisition as well as transmission of drug-resistant tuberculosis will be required. This will only be possible in areas where a DOTS strategy is well functioning and with a modification of central elements of the standard DOTS mechanism: a "DOTS-plus" strategy. Early and

accurate diagnosis of drug resistance is essential for effective management. Diagnosis based on two direct smear tests might have to be replaced by routine drug-susceptibility tests at diagnosis. Because the routine performance of phenotypic drug-susceptibility tests was inferior to the performance of genotypic tests, the development of an affordable commercial kit testing a limited number of mutations conferring resistance could be of great value in the global fight against multidrug-resistant TB. Because of the importance of early diagnosis, selective active contact tracing for multidrug-resistant cases, additional to the routine passive contact tracing, could prove to be cost-effective. Individualized treatment regimens are effective in reducing the failure rate, mortality and probably transmission of multidrug-resistant TB.

Multidrug-resistant tuberculosis is a problem confronting the efforts for global tuberculosis control. Efficient strategies to turn the tide exist, but international political commitment and financial support will be essential.

OPSOMMING

Middel weerstandige tuberkulose hou 'n bedreiging in vir globale tuberkulose kontrole deur die WGO DOTS strategie. Studies in die Verenigde State en Europa het getoon (i) dat middel weerstandige tuberkulose in alle lande voorkom; (ii) dat, in teenstelling met vorige dogma, middel weerstandige bakterieë virulent is en oorgedra kan word, veral in inrigtings en aan immuun-onderdrukte pasiënte; en (iii) dat die meeste gevalle ontstaan deur die verwerwing van middel weerstandigheid a.g.v. die foutiewe hantering van tuberkulose gevalle. (iv) Bykomend is getoon dat die ontsettende hoë mortaliteit syfers van die 1980s verlaag kan word deur ge-individualiseerde, maar duur behandeling.

Die meeste middel weerstandige tuberkulose gevalle woon egter in die ontwikkelende wêreld. Data oor siekte epidemies in minder ontwikkelde dele van die wêreld is skaars. Die doel van hierdie tesis was om die siekte dinamiek van middel weerstandige tuberkulose te bestudeer in 'n ontwikkelende land waar tuberkulose endemies is.

Alle gevalle van middel weerstandige tuberkulose gedurende 'n 5–jaar periode in twee lae sosio-ekonomiese gemeenskappe, is in hierdie studie ingesluit. Drie verskillende metodes is gebruik: restriksie fragment lengte polimorfisme (RFLP), mutasie analise deur dot-blot hibridisasie en 'n Geografiese Inligting Stelsel. Resultate van die RFLP analise het getoon dat uitbrake van middel weerstandige *Mycobacterium tuberculosis* stamme in die gemeenskap voorkom, selfs sonder die aantasting van immuun-onderdrukte pasiënte. Infeksie met middel weerstandige stamme het voorgekom in nuwe pasiënte (primêre middel weerstandigheid) en ook in pasiënte wat reeds voorheen behandel is (eksogene herinfeksie). Daar is ook gevind dat eksogene herinfeksie 'n belangrike meganisme was van herhaalde tuberkulose na vorige genesing van middel sensitiewe tuberkulose. Die oordrag van middel weerstandige stamme het meer dikwels voorgekom in areas met laer sosio-ekonomiese omstandighede. Die relatiewe bydrae van oordrag het merkwaardig verskil tussen multi-middel weerstandigheid (twee derdes van gevalle) en enkel-middel weerstandigheid (geen gevalle). Dit weerspieël waarskynlik die verlengde periode van infektiwiteit van die multi-middel weerstandige gevalle. Die bekamping van die groeiende epidemie van multi-middel weerstandige tuberkulose, vereis die voorkoming van verworwe sowel as oorgedraagde middel weerstandige tuberkulose.

Dit sal slegs moontlik wees in areas waar 'n DOTS strategie reeds goed funksioneer en met 'n aanpassing van die sentrale elemente van die roetine DOTS meganisme: 'n "DOTS-plus" strategie. Vroeë en akkurate diagnose van middel weerstandigheid is essensieël vir effektiewe hantering. Diagnose gebaseer op twee direkte sputum smeer toetse mag moontlik vervang moet word deur roetine middel sensitiwiteit bepalings by diagnose. Die roetine fenotipiese middel sensitiwiteit bepaling is gevind om minderwaardig te wees in vergelyking met die genotipiese toetse. Die ontwikkeling van 'n bekostigbare toetsstelsel wat die mees algemene mutasies vir middel weerstandigheid sal opspoor, kan van groot waarde wees in die stryd teen muti-middel weerstandige tuberkulose. Aangesien vroeë diagnose so belangrik is, kan aktiewe kontak opsporing koste-effektief wees. Ge-individualiseerde behandelingskedules is effektief om die sukses van behandeling en oorlewing te verbeter, en moontlik ook om die oordrag van multi-middel weerstandige tuberkulose te verminder.

Multi-middel weerstandige tuberkulose is 'n probleem vir die globale kontrole van tuberkulose. Effektiewe strategieë om die vloed te stuit, bestaan, maar politieke verbintenis en geldelike ondersteuning sal essensieël wees.

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CHAPTER 1

General Introduction

General Introduction

A. A history of the concept of contagiousness of tuberculosis and the efforts for its control.

The concept of contagiousness of tuberculosis (TB) waxed and waned over the past three millennia. This reflected the chronic nature of TB and the absence of acute dramatic epidemics, as seen with plague, smallpox and cholera. Moreover, the acceptance of the notion contagion was not based on objectivity but reflected the *Zeitgeist* – the spirit of the time[1]. Greek and Roman scholars viewed TB as contagious. Aristotle (384 to 322 B.C.) wrote "*...the breath is bad and heavy, ...one takes the disease because there is in the air something disease producing*"[2]. Galenus (129 to 200 B.C.) stated that phthisis (TB) could easily be caught from a tuberculous patient, especially if they breathed out putrid air. By the end of the 16th century, most physicians and scientists in Southern Europe advocated the contagious theory while empiricism in Northern Europe opposed the notion of contagion in the absence of experimental proof and accepted non-specific factors such as inheritance and environmental factors as the cause of TB[1]. In the 19th century, the established medical profession was influenced by the French Revolution. The year 1865 demarcates the modern era, when experimental medicine began to unravel the nature of TB. Jean-Antoine Villemin reported his success in transmitting TB from man to rabbit by inoculation[3]. Villemin's theory was rejected but stimulated and advanced TB research in Europe. The contagion debate was finally resolved by the discovery of the tubercle bacillus by Robert Koch in 1882[4].

The belief in TB as a disease of a contagious or non-specific nature determined the historic evolution in the combat against TB. In the 15th century, Fracastoro[5] advised that "*When you begin your treatment in the initial stage, pay attention to the germs only, for if these could be destroyed...no more effective remedy could be employed.*" The contagious nature of TB led to the concept of close contact in the transmission of TB. In 1720, Benjamin Marten[6] wrote: "*It may, therefore, be very likely that by habitual lying in the same bed with a consumptive patient, consistently eating and drinking with him or by frequently conversing so nearly as to draw in part of the breath he emits from the lung, consumption may be caught by a sound person....I imagine that slightly*

conversing with consumptive patients is seldom or never sufficient to catch the disease." To prevent transmission, cities such as Naples built a TB hospital and passed stringent laws in 1782 stipulating both precautions during illness (notification of disease by the physician and admission at the hospital) and measures to be taken after death (such as destruction of household goods susceptible to contamination, replastering the house from cellar to garret, burn wooden doors and windows,...) [1]

Hippocrates was the first to recommend environmental measures, such as a change in climate, in the treatment of phthisis.[7] From the mid 17th century, physicians recommended sea voyages or travel to healthy climates for the dreaded phthisis. The Sanatorium Movement, which peaked between mid 1800s and mid 1900s, was an expression of the popular concept of fresh air, diet, rest and exercise as a means of keeping away the miasma[7].

Koch's discovery of the microbial cause of TB did not slow the momentum of the Sanatorium Movement but led to the desire to reinforce natural resistance by a regulated healthy regimen and to prevent spread by isolation and education until a specific cure could be found. It would still be another 60 years after Koch's discovery before the long-hoped-for chemotherapeutic era became reality with the discovery of streptomycin as a potentially reliable cure for TB[8]. The discovery of additional antibiotics such as para-aminosalicycic-acid and isoniazid gave impetus to treatment in general hospitals and outpatient treatment in the community[7]. However, it was only after several controlled studies that the primacy of drug therapy was undeniably established. As stated by Mitchison[9]: " We must conclude that many accepted measures in the treatment of TB are becoming increasingly irrelevant in an age when potent chemotherapy can be given. In particular, treatment by surgery, prolonged rest in bed, and by dietary supplements is usually unnecessary and serves only to prolong the patient's incapacity and to increase the cost of his treatment."

TB is now a disease that can be effectively cured in the vast majority (>90%) of patients. Clinical trials by the British Medical Council and co-workers have elucidated the optimal regimen now incorporated in the World Health Organisation (WHO) directly observed treatment, short-course (DOTS) strategy. The current WHO DOTS strategy includes 5 points: (1) national government commitment, (2) directly observed

standardised treatment, (3) diagnosis by sputum smear microscopy, (4) reliable supply of first-line drugs and (5) standardised recording and reporting system[10].

The close historical relationship between TB and social living conditions stresses the need for attempts to control TB to look beyond medical interventions. Government commitment should thus not only be limited to ensuring a high quality program but also consider efforts to improve the living conditions of the poorest.

B. Tuberculosis in South Africa: history, burden and disease control strategies

Knowledge concerning the prevalence of TB in South Africa prior to the 20th century is limited. It appears that the initial spread was closely related to the degree of contact of native people with European colonizers[11]. In the 19th century, the pool of infection was boosted by the large number of consumptive immigrants seeking the benefits of the South African climate: "*...the Cape which cures consumption; the Cape which is of Good Hope to all weak chests; the Cape which offers life and health...*"[11]. Rapid urbanisation, political policies maintaining unequal living conditions and unequal access to health care have contributed to the extent and racial differences of the South African TB epidemic in the 20th century[12] (Figure 1).

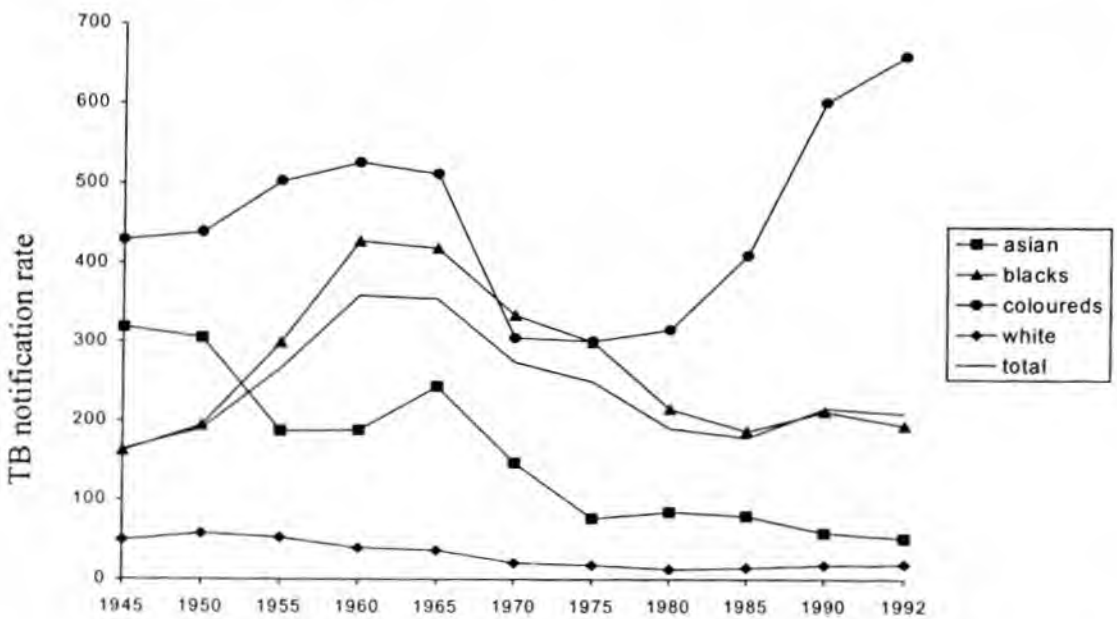

Figure 1. Racial distribution of TB notification rates in South Africa from 1945 - 1992

It has been estimated that 6 to 10 million South Africans are infected resulting in more than 80000 notified cases per year. The highest TB notification rate occurs among persons of the Coloured population[13].

Although TB was a recognised health problem in South Africa, little effort was made to control the disease prior to 1900. In 1919, a commission made numerous recommendations for the implementation of various control measures: improvement in housing and sanitation, provision of diagnosis facilities, TB dispensaries and hospitals. However, few of these recommendations were acted upon[12]. The failure of early efforts to control the spread of TB is dramatically highlighted in the 1940s. Armed for the first time with a 'silver bullet', in the form of streptomycin and isoniazid, South African medical authorities launched mass chest x-ray surveys and educational campaigns during the 1950s. Because of lack of hospital accommodation, cases were treated on an outpatient basis. In the absence of adequate staff to supervise therapy, this often resulted in patients failing to complete their therapy[12]. By the end of the 1980s, shorter regimens and full supervision could improve the situation. In 1996, the government committed itself to the implementation of the WHO DOTS program[14].

C. Drug-resistant tuberculosis

Pioneering studies recognised the problem of drug resistance in TB almost immediately after the introduction of streptomycin. In 1946, Youmans observed that a large number of patients treated with streptomycin relapsed after a period of clinical improvement. The bacilli isolated from these patients were resistant to streptomycin[15]. A study in 1947 showed that during treatment, the proportion of bacilli resistant to streptomycin increased progressively[16]. Addition of para-aminosalicyclic acid reduced the prevalence of streptomycin resistance significantly[17] and introduced the principle of multidrug regimens, central in modern TB treatment. Another landmark in the study of drug resistance in TB was the concept of spontaneous mutants, defined as those bacilli resistant to antituberculous drugs present in large bacterial populations prior to any contact with antituberculous drugs[18]. These resistant mutants occur at a low, but fixed rate for each drug: 1 in 10^6 for isoniazid and streptomycin and 1 in 10^8 for rifampin[18-20]. Drug-resistant *M. tuberculosis* mutants have a selective advantage over susceptible

bacilli under circumstances of inadequate treatment management, such as erratic drug supply, prescription of monotherapy and patient non-compliance. Clinical drug resistance occurs once these mutants comprise a substantial proportion of the bacterial population. This type of drug resistance is classified as acquired drug resistance and defined by the WHO as TB caused by bacilli resistant to one or more drugs in a patient previously treated for 1 month or longer[21]. Early data from animal models suggested that drug-resistant strains were less infective and less pathogenic[22,23]. Primary drug resistance, reflecting the transmission of a drug-resistant strain, and defined by the WHO as TB caused by bacilli resistant to one or more drugs in a patient never treated before[21], was therefore assumed to be rare. The rare occurrence of outbreaks of drug-resistant TB before 1990 supported this dogma.

As Canetti noted 30 years ago: "*The basic aspects of this (drug-resistant TB) was gained within the first 15 years after the introduction of chemotherapy for TB treatment.... And then the research impetus fell away...Any disease-linked phenomenon loses much of its attraction for research once the ways for its prevention have been found. ...The general feeling tends to prevail that resistance has slipped into history.... To make a long story short, plenty of work is waiting for microbial geneticists in the field of TB... Drug resistance is a field of admirable diversity. The field has something to offer to almost everybody: the clinician, the pathologist, the pharmacologist, the biochemist, the epidemiologist, and the geneticist.*"[24]

When there was a resurgence of TB in the developed world in the mid-1980s after decades of steady decline, and outbreaks of drug-resistant TB occurred, it was clear that drug-resistant TB had not slipped into history. Research into drug-resistant TB revived. A global survey on anti-tuberculosis drug resistance determined that drug resistance is ubiquitous, with several hotspots of multidrug-resistant TB around the world[25]. Microbial geneticists elucidated the genetic basis of resistance. Point mutations in specific genes are known to confer resistance to the 5 first-line drugs. The most important genes are the *rpoB* gene[26] (rifampin resistance), *katG* gene[27] (isoniazid resistance), *rpsL* gene[28] (streptomycin resistance), *embB* gene[29] (ethambutol resistance) and *pncA* gene[30] (pyrazinamide resistance). This knowledge opened possibilities for the development of rapid diagnostic tests. Traditional epidemiological and molecular

epidemiological research clearly showed that drug-resistant strains are transmissible and pathogenic[31-34]. Clinical research has shown that the outcome of patients with drug-resistant TB is poor, especially among HIV positive patients, but can be improved with rapid initiation of appropriate treatment.

During the last decade, our knowledge about drug-resistant TB has broadened, but the management of these patients has changed little. Routine clinical diagnosis is still based on 'slow' culture-based drug-susceptibility techniques, requiring 6 to 8 weeks. The optimal treatment for multidrug-resistant TB cases is not known because regimens have not been investigated in large clinical trials. Furthermore, TB control programmes are directed towards the prevention of the acquisition of drug resistance and, especially in developing countries, ignore the occurrence of transmission of drug-resistant strains.

D. Molecular epidemiology of drug-sensitive and drug-resistant tuberculosis

Understanding the disease dynamics of TB is complicated because only a small proportion (approximately 10 %) of persons infected with *M. tuberculosis* ever develop clinical TB, and this only after an incubation period ranging from days to decades. Furthermore, in areas with a high incidence of TB, multiple transmission events can occur as people are often in contact with more than one TB case. This makes it extremely difficult to identify the source of infection and site of transmission by conventional epidemiological research using case history, contact investigation and the tuberculin skin test.

Laboratory-based tests were investigated to improve data gathering. Since 1971, phage typing was used to differentiate between different *M. tuberculosis* strains. However, the possible application of phage typing was limited: the method was labor-intensive, the interlaboratory reproducibility of the method poor and the discriminatory ability of the scheme limited, as only three types could be distinguished reliably[35]. The first application of modern molecular biology to identify strains of *M. tuberculosis* was reported in 1984[36]. The finding that two isolates with the same phage type possessed different restriction-fragment-length polymorphism (RFLP) patterns when the mycobacterial DNA was hybridised with the entire *M. tuberculosis* chromosome gave an indication of the potential of the RFLP technique[37]. The complexity of the observed

banding patterns obtained by this method resulted in poor differentiation between strains. The discovery that repetitive DNA elements are located at different chromosomal sites of *M. tuberculosis* has revolutionised the DNA fingerprinting or RFLP technique of *M. tuberculosis*[38-43]. The repetitive DNA elements investigated are insertion sequences (IS*6110*, IS*986* and IS*1081*) and short repetitive DNA sequences (direct repeat, major polymorphic tandem repeat, and polymorphic GC-rich repetitive sequences)[35]. Southern blotting of genomic DNA with IS*6110* as a probe has become the most widely used and internationally standardised technique for strain typing of *M. tuberculosis*[44]. Multiple studies have demonstrated that the degree of polymorphism generated by this technique appears well suited for epidemiological investigations in areas with a high as well as areas with a low incidence of TB[45-48]. There is sufficient data indicating that epidemiological independent isolates of *M. tuberculosis* can have a high degree of RFLP polymorphism while epidemiological related isolates show identical banding patterns[32,45, 49-51].

Since 1990, RFLP analysis has been applied to multiple aspects of the epidemiology of TB. RFLP analysis has been used to investigate outbreaks in communities[52], find unexpected chains of transmission[53], track nosocomial transmission of strains[31], differentiate between disease originating from reactivation of latent infection or recent infection [47,54], document exogenous reinfection and relapse in individual patients[55], prove laboratory contamination[56] and document characteristics of TB in specific populations, such as HIV positive patients[51]. RFLP analysis has also been used in the study of drug-resistant TB. Many studies have investigated outbreaks of drug-resistant TB and were able to demonstrate the occurrence of transmission of drug-resistant strains from an infectious case to another person[31,32,46,57-63]. Studies on individual patients have documented that the RFLP pattern does not change with the development of drug resistance[64]. Exogenous reinfection with a drug-resistant strain has been proven to occur in an HIV positive patient[55]. Population-based studies in low incidence areas have demonstrated that the proportion of drug-resistant TB due to transmission of drug-resistant strains is low, since the overwhelming majority of drug-resistant stains retrieved from patients residing within a specific geographic area had distinct RFLP patterns[65].

Even though the application of DNA fingerprinting has increased insight into the epidemiology of TB, some key assumptions have not been rigorously evaluated, thereby leading to possible misinterpretation of data.

The first assumption is that IS*6110* RFLP analysis accurately identifies clones of *M. tuberculosis*. The use of insertion elements may seem paradoxical, as they are genetically unstable. The pace at which changes occur determines the number of different strains involved in a group of isolates with a common ancestor. To be useful for epidemiologic studies, a certain rate of genetic alteration is necessary. If genetic rearrangements occur considerably faster than the epidemiological event studied, epidemiological related strains will appear unrelated. If, however, genetic events occur slower than the epidemiological event, unrelated strains will appear related[49]. Animal models and in vitro studies have suggested that IS*6110* patterns are stable, for example, there was no observable change in IS*6110* patterns after 2 months of passage through guinea pigs[50], after 4 weeks passage in monolayers of in vitro cultivated macrophages or after 6 months passage in liquid medium culture[66]. However, minor changes in RFLP patterns (addition or loss of one band) have been documented in serial isolates from one patient and in isolates from patients with clear epidemiological links[45,67-69]. For population-based studies, the interpretation of strains with minor differences remains controversial. The best DNA fingerprinting technique will therefore be the one that is based on repetitive DNA elements with a rate of genetic rearrangement that occurs in synchrony with the time frame of the epidemiologic situation studied[49].

The molecular clock of an insertion element is not the only determinant for validity of the assumption that identical fingerprints indicate clonal dissemination. The degree of *M. tuberculosis* heterogeneity in the population studied is another crucial component in limiting the possibility that identical RFLP fingerprints might occur by chance among unrelated individuals. Clonality is therefore only convincingly demonstrated when considerable background diversity exists in the population, such that isolates with identical patterns imply infection by a common source[49]. Furthermore, the accuracy of strain classification has been shown to be dependent on the number and location of IS*6110* insertions because of the existence of preferential sites or hotspots for integration of IS*6110,* such as the direct repeat locus[70]. It is now generally accepted that

additional genetic markers are necessary for the unambiguous confirmation of identity of strains with a low (less or equal to 5) number of IS*6110* copies[71-73]. No consensus has been reached whether additional RFLP analysis is valuable for strains with ≥ 6 IS*6110* insertion elements[73].

The second key assumption that might interfere with correct interpretation of RFLP analysis is that isolates with identical patterns indicate recent transmission from an infectious patient to another person. It is possible that interpretation of clustering may vary in different communities, depending on the TB incidence in the community[53]. In areas with a high TB incidence, clusters may not always represent a single chain of recent transmission but may be formed by repeated generations of recent transmission[74]. In contrast, clusters of *M. tuberculosis* isolates occurring in areas with a low TB incidence may result from transmission that occurred in the distant past and reactivation at the same time in different persons[53,75].

In conclusion, the value of molecular epidemiology will depend on the integration of a multi-probe RFLP technique with conventional epidemiological approaches and requires knowledge about the demographic characteristics of the population studied. Only then will molecular epidemiology improve our understanding of disease dynamics of TB and subsequently permit the design of more efficient TB control strategies.

E. Aim, setting and structure of this study

Drug-resistant TB is recognised as a threat for global TB control. Data on the epidemiology of drug-resistant TB in the developed world is available from surveillance studies, nosocomial outbreaks and a few studies on community outbreaks of drug-resistant TB. The most important results of these studies are fourfold. First, data from the recent WHO-IUATLD survey on drug resistance in TB suggest that drug-resistant TB is a global problem with several hotspot locations of multidrug-resistant TB[25]. Second, data from outbreak studies indicate that, in contrast to the prior dogma, drug-resistant *M. tuberculosis* strains are virulent, pathogenic and can be transmitted from one patient to another[31-34]. Third, the majority of cases of drug-resistant TB are acquired due to treatment errors and not through transmission of a drug-resistant strain[65]. Fourth,

outcome-based research has shown that morbidity and mortality of drug-resistant TB is much higher compared to morbidity and mortality of drug-sensitive TB[31,32].

Even though the majority of drug-resistant TB cases occur in the developing world, our knowledge concerning the epidemiology of drug-resistant TB in the developing world is extremely limited, except for some data gained from surveillance studies. The aim of this study was therefore to primarily evaluate the disease dynamics of drug-resistant TB in a developing country where TB is endemic.

The work for this thesis was performed in a well-defined geographic area consisting of two communities within the Cape Town Metropolitan area, South Africa. This area was selected in 1992 as the principal research site for the TB research team of the University of Stellenbosch and the Glaxo Wellcome Action TB Programme[76]. The area shows many characteristics of an urban area in a developing country (a detailed demographic and socio-economic description is included chapter 7). The area is further characterised by an extremely high incidence of TB: a notification rate of more then 1000/100000/year in 1991[47] and an incidence rate of bacteriological positive cases of 225/100000/year during the period 1996-1998[77]. The discrepancy between these two rates can be explained by 3 factors: (1) the relatively important contribution of smear and culture negative childhood cases (39% of all notified cases); (2) the high rate of retreatment cases (approximately 30 %) and (3) the fact that only 70% of notified adults are bacteriological positive.

Since 1992, the epidemiology of TB in this area has been studied prospectively by collecting clinical data and cultures positive for *M. tuberculosis* from all patients notified as TB cases. The research team has not interfered with patient treatment, which is prescribed and provided by the primary health care clinics according to the guidelines of the National Tuberculosis control Program. Treatment facilities and treatment guidelines are outlined in chapter 2. Diagnostic procedures were extended for research purposes to ensure that all patients were interviewed by a study nurse and investigated by chest radiography, that all sputa obtained were cultured and all cultures positive for *M. tuberculosis* were analysed by RFLP method. A computerised database has been created. This database consists of clinical data on all (more than 900) patients included

and results of RFLP analysis of more than 2000 *M. tuberculosis* cultures. Furthermore, all *M. tuberculosis* cultures are stored at minus 20 degrees Celsius for further analysis.

My Ph.D. study was nested in this large prospective epidemiologic study on TB. All 70 residents identified as cases of drug-resistant TB during a 5 year period (1 April 1992 until 31 March 1997) were included. Three different methods were used to study the disease dynamics of drug-resistant TB in the selected study site: RFLP analysis, also known as DNA fingerprinting, mutation detection analysis by dot-blot hybridisation method and spatiotemporal analysis by the use of a geographic information system (GIS). The molecular epidemiologic method of RFLP analysis is described in detail in chapter 2. Chapter 2 also includes a description of the methods used for microscopy and culture of *M. tuberculosis*. The dot-blot hybridisation method used for mutation detection analysis is described in detail in the chapter 3. The Geographic Information System is described in detail in chapter 8. All analysis by RFLP method and dot-blot hybridization method of *M. tuberculosis* drug-resistant isolates has been performed by myself. The GIS analysis was performed in collaboration with M. Kunneke, under supervision of Prof. L. Zietsman.

Each chapter describes a clinical application of molecular biology and/or GIS and includes the literature review relevant to the specific topic. In chapter 2, I have investigated the possibilities and limits of the RFLP method for the epidemiologic study of drug-resistant TB. In chapter 4, the classification of patients into different categories by clinical and molecular epidemiologic methods is compared. In chapter 6, mutation detection analysis is applied to study the frequency distribution of the different mutations within the area and to evaluate the clinical use of mutation detection analysis. In chapter 7, an outbreak of drug-resistant TB is described by the combined use of RFLP and mutation detection analysis. In chapter 8, a GIS is applied to characterise the socio-economic risk factors for the occurrence of drug-resistant TB and the transmission thereof. Chapter 9 reports on the outcome analysis of patients treated for drug-resistant TB. All patients included in these studies form part of the 70 cases of drug-resistant TB identified in the area during the 5-year study period.

An additional study on a selected population of cases of drug-sensitive TB was included to support one of the major findings of this Ph.D. study, namely that most

cases of drug-resistant TB were caused by exogenous (re)infection with a drug-resistant strain. The study on the relative importance of exogenous reinfection after curative treatment for drug-sensitive TB is reported in chapter 5.

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CHAPTER 2

Use of DNA fingerprinting in evaluating the epidemiology of drug-resistant tuberculosis in a high incidence area

Use of DNA fingerprinting in evaluating the epidemiology of drug-resistant tuberculosis in a high incidence area.

Background: Restriction Fragment Length Polymorphism (RFLP) analysis has been validated as a useful tool for the epidemiological study of tuberculosis (TB). Reports on drug-resistant TB outbreaks have shown the importance of nosocomial transmission. Population-based studies in low incidence areas have shown a high diversity of drug-resistant *Mycobacterium tuberculosis* strains. The aim of this study was to evaluate the use of RFLP in the epidemiologic study of drug-resistant TB in an area endemic for TB. Method: All patients residing in two Cape Town communities and diagnosed with culture-positive drug-resistant TB during a 5year period were included. Patients were interviewed in a search for epidemiological connections. RFLP analysis was performed by an internationally accepted method (IS*6110*-3') and by additional genetic markers (IS*6110*-5', DRr, MTB484(1)). Cluster analysis was performed to identify strains with identical RFLP patterns, but retrieved from patients not diagnosed as drug-resistant TB cases. Results: RFLP analysis showed a high degree of strain diversity among single drug resistant TB cases but a low degree of strain diversity among multidrug-resistant TB (MDR-TB) cases. An epidemiologic link could be identified in 68% of clustered patients (IS*6110*-3' method) and in 74% by the use of multiple genetic markers. Cluster analysis could identify drug -resistant strains in patients not diagnosed as drug-resistant TB cases. Conclusion: RFLP analysis improved the understanding of the epidemiology of drug-resistant TB in an endemic area. Strain diversification among MDR-TB cases was unexpectedly low, indicating substantial community transmission. RFLP analysis could identify transmission among patients without conventional epidemiological links. RFLP analysis could also show that drug-resistant TB case finding by the National Tuberculosis Control Program was incomplete.

INTRODUCTION

Drug-resistant tuberculosis can result from acquisition of resistance in a patient's original *M. tuberculosis* strain due to treatment errors or from transmission of a drug-resistant strain from an infectious case to another person[1]. Based on the current general belief that drug-resistant TB is predominantly acquired through treatment errors, TB control programmes are primarily directed at the prevention of acquisition of drug-resistance. This strategy, or a failure to apply this strategy correctly, failed to contain the problem of drug resistance. This suggests the need for a re-evaluation of the problem.

Understanding the disease dynamics of TB is complicated, because infection is followed by disease in only a minority of infected persons (approximately 10 %) and this after an incubation period ranging from days to decades. Furthermore, in areas with a high TB incidence, multiple transmission events can occur as people are often in contact with more than one TB case. Without accurate strain typing, it is difficult to distinguish the origin of different isolates[2]. Modern molecular epidemiology is based on the integration of molecular biology techniques with conventional epidemiologic approaches to track the spread of specific *Mycobacterium tuberculosis* strains and understand the distribution of disease in a population. DNA fingerprinting or Restriction Fragment Length Polymorphism (RFLP) can detect a high degree of genetic polymorphism among *M. tuberculosis* and has been validated as a useful tool in the epidemiologic study of TB[3-8]. Studies have shown that an identical RFLP pattern may imply infection by a common source and therefore an epidemiologic connection while epidemiologic unrelated strains have different RFLP patterns[6-13].

Many studies have investigated outbreaks of drug-resistant TB by the use of RFLP analysis to and showed the occurrence of clonal dissemination of drug-resistant strains[14-21]. Population-based studies can determine the relative proportion of drug-resistant TB due to transmission. Demonstrating a high degree of RFLP polymorphism would imply that drug-resistant TB is predominantly emerging from treatment errors. In contrast, limited RFLP diversity would suggest a high degree of transmission with clonal dissemination of drug-resistant strains. Population-based studies in low incidence areas have shown that the majority of drug resistant stains have distinct RFLP patterns

implying acquisition of drug resistance[22]. There is no data on the degree of RFLP polymorphism among drug-resistant stains in an area where TB is endemic.

The aim of this study was to evaluate the use of RFLP analysis in the epidemiological study of drug-resistant TB in two communities with a high TB incidence. The improved insight in the nature of transmission of drug-resistant TB in the community may subsequently permit more precise targeting of disease-control strategies.

MATERIAL AND METHODS

Setting

The area studied consists of two neighbouring Cape Town communities (Ravensmead and Uitsig) with a total residential surface area of 2.42 km^2 and a population of 34294 (1991 census data). Two primary health care clinics and a tertiary hospital (Tygerberg Hospital, University of Stellenbosch) serve the area. All treatment received from the clinics is free of charge. TB patients and malnourished children receive a daily basic meal from the nutrition kitchen in part supported by the Department of Health. Despite the easy access to health care, many problems remain. The infant mortality rate was still very high in 1991 (38 per 1000 life births) but has dropped to 29 per 1000 life births in 1996. The HIV prevalence in the Western Cape Province, estimated by a survey on pregnant women conducted by the Department of Health and Population Development, has been rising steadily from 0.37% in 1991 to 3.1% in 1996 (data from the National HIV surveys on women attending antenatal clinics, conducted by the Department of Health and Population Development, South Africa).

TB is one of the top 3 health problems of South Africa. The Cape Town Metropolitan area, with a TB notification rate of 560/100 000/year has the dubious distinction of possibly being the world's TB capital. Within the province, the Coloured ethnic group is the population most affected by TB. The TB notification rate in the communities studied has been estimated to be more than 1000/100 000/year in 1991[23].

The notification rate of all (new and retreatment) bacteriologic positive cases is 363/100.000/year during the period 1996-98. The incidence of new bacteriologic positive TB cases in the communities is 225/100 000/year (period 1996-1998)[24]. A survey of drug resistant TB in the Western Cape Province conducted in 1992-93 found a rate of 8.6% acquired and 3.2 % initial resistance in the West Cape region[25].

The National Tuberculosis Control Programme (NTP) guidelines for TB management have changed with time. Before 1996, diagnosis was based on direct smear stain for acid-fast bacilli, culture for *M. tuberculosis* and chest radiography. Drug sensitivity testing was requested for clinical indications such as retreatment cases and patients who failed to respond to treatment. All patients were treated under direct supervision for 6 months with a combination tablet consisting of isoniazid, rifampin and pyrazinamide. Ethambutol was added for retreatment cases. In 1996, the Directly Observed Treatment, Short-course (DOTS) strategy of the World Health Organisation (WHO) was implemented. Since then, diagnosis of new patients is based on two direct smear stains for acid fast bacilli. Chest radiography and *M. tuberculosis* culture is reserved for retreatment cases and new patients with symptoms compatible with TB but negative acid fast smears. Drug sensitivity testing is performed in retreatment cases and patients who fail to respond to treatment. The intensive treatment phase for new patients consists of 4 drugs (isoniazid, rifampin, pyrazinamide and ethambutol) for 2 months; supplemented by streptomycin for retreatment cases. The continuation phase consists of isoniazid and rifampin for 4 months for new patients. For retreatment cases, the intensive phase consists of isoniazid, rifampin, ethambutol and pyrazinamide for 1 month followed by 3 drugs isoniazid, rifampin and ethambutol for 5 months.

Official NTP guidelines for treatment of multidrug-resistant TB (MDR-TB), defined as resistant against at least isoniazid and rifampin, were not available during the study period. In the Western Cape Province, however, special treatment units for MDR-TB patients have been developed. Patients had the option to be treated at the primary health care clinic or at a specialised MDR-TB treatment unit which provides inpatient treatment as well as outpatient treatment advice and outpatient follow-up.

Data collection

In 1992, the Tuberculosis Research Group of the University of Stellenbosch started a prospective study for which positive *M. tuberculosis* cultures from all patients notified with TB were collected from the routine laboratory (South African Institute for Medical Research, SAIMR) for RFLP analysis. The retrieval rate from this study has been approximately 80%[24]. From the clinical and RFLP database created by this study, all patients with a positive culture for *M. tuberculosis* resistant to at least one antituberculosis drug between 1 April 1992 and 31 March 1997 were identified. Medical files and compliance charts were reviewed and detailed information on medical history, HIV status, history of prior TB treatment, results of microscopy, culture and drug susceptibility testing for *M. tuberculosis* and details of treatment for drug-resistant TB was collected. Furthermore, patients were interviewed by a medical anthropologist to determine epidemiological links.

Laboratory methods

1. Microscopy and culture for M. tuberculosis

Sputum samples are sent directly from the clinics to the state SAIMR laboratory. Samples are mucolyzed and decontaminated by the addition of 1 % N-acetyl-L-cysteine, 3 % tri-Sodium-Citrate and 4 % Sodium Hydroxide. A phosphate buffer is added to 15 ml of specimen to reach a pH of 6.7. Samples are centrifuged at 3000 g for 20 minutes. The sediment obtained is used for acid fast smear and culture. The staining technique used for the acid-fast smear test is the auramine O fluorochrome dye.

M. tuberculosis is cultured in the BACTEC system. If the sputum sample was positive for acid fast bacilli, the growth index is checked on alternate days for 4 weeks. Cultures from smear negative samples are checked weekly for 4 weeks. When growth is obtained, confirmation of acid fast bacilli is performed by a Ziehl-Neelsen stain. A blood agar procedure is used to check for contamination. Mycobacterial, non-contaminated BACTEC growths are transferred to a Middelbrook 7H10 broth to allow growth for 3 weeks. The confirmation of *M. tuberculosis* is then performed by the

niacin production test using commercially available strips containing cyanogen bromide.

Drug susceptibility testing is performed by the economic variation of the indirect proportion method[26]. This entails incorporation of the required drug concentration into Löwenstein-Jensen medium before slants are inoculated with a standardised inoculum retrieved from the initial BACTEC growth. Drugs were tested at the critical concentrations of 0.2 µg/ml isoniazid, 30 µg/ml rifampin, 2 µg/ml ethambutol, 5µg/ml streptomycin and 20µg/ml ethionamide. Kanamycin and thiacetazone are tested on Middlebrook 7H10 agar containing critical concentrations of respectively 5 and 2 µg/ml. Resistance was defined as 1% or more bacterial growth in comparison with a control using international criteria. Initially, cultures are tested for resistance to isoniazid and rifampin. If resistance to these drugs was detected, cultures were subsequently tested for resistance to the other antituberculous drugs. Resistance to pyrazinamide was not tested. Laboratory proficiency testing is performed internally (a susceptible HR37v strain and a *Mycobacterium avium* strain resistant to all drugs is added to each batch) as well as externally every 6 months.

2. RFLP analysis

Cultures received from SAIMR are subcultured on Löwenstein-Jensen medium by scraping of the slant in order to prevent selection of dominant colonies. Once sufficient growth is achieved, one subculture together with the original culture (from SAIMR) is stored at minus 20° Celsius. The other subculture is heated at 80° Celsius for 1 hour to kill all mycobacteria. All colonies are scraped of the slant after addition of extraction buffer (50 mM Tris-HCl, 25 mM EDTA, 5% Mono-sodium Glutamate). Samples are vortexed with glass balls to disrupt all colonies and 450 µl of lysozyme, 10 µl of RNA-se and 150 µl Proteinase K (plus buffer) are added successively leaving enough incubation time for each enzyme. The DNA is extracted by treating the sample successively with Phenol:Chloroform:Isoamylalcohol (25:24:1, pH 7.6), Chloroform:Isoamylalcohol (24:1), 3M Sodium Acetate (pH 5.2) and ice-cold Isopropanol. DNA is precipitated by centrifusion in ethanol and dissolved in TE buffer. To determine the concentration of DNA and the purity of each sample, optical density is

read at a wavelength of 260 nm (DNA) and 280 nm (protein). Six µg of mycobacterial DNA is digested with P*vu*II; the rest of the extracted mycobacterial genomic DNA is stored at - 20°C. Digestion is confirmed by electrophoresis on a agarose gel. Digested DNA is precipitated with 10 µl tRNA, 10 µl Sodium Acetate 3M, and ethanol after which the DNA pellet is redissolved in TE buffer. DNA fragments are separated by electrophoresis on a 0.8% agarose gel in TBE buffer. After denaturation of the DNA fragments with 0.4 M Sodium Hydroxide, the DNA fragments are transferred to a Hybond membrane (Amersham, United Kingdom) in SSPE buffer by Southern transfer method. The membrane is washed in 2 x SSC for 10 min and baked at 80° Celsius for 2 hours. The IS*6110* probe is prepared by denaturing 2.5 µl of IS*6110* probe (10 ng/µl), adding labelling reagent to visualise the probe during ECL procedure and gluteraldehyde to fix the labelling agent firmly to the probe. The DNA on the membranes is hybridised with the labelled IS*6110* probe at 42° Celsius. The membrane is washed twice with primary wash to remove all excess probe and twice with 2 x SSC to eliminate the urea. The RFLP patterns are visualised by ECL procedure and autoradiography.

Since late 1992, as part of a prospective study, positive cultures for *M. tuberculosis* from patients residing in the 2 communities studied have been genotyped by RFLP analysis (IS*6110*) and analysed using Gel Compar 4.0 software (Applied Maths BVBA, Kortrijk, Belgium). The Dice coefficient and UPGMA clustering formula was applied to identify possible clusters of transmission.

For the purpose of my study, drug-resistant samples were run on separate gels. To enhance accuracy, clusters of identical drug-resistant isolates were subjected to further genotyping. The P*vu*II digested DNA was sequentially hybridised with ECL labelled IS*6110*-5' and ^{32}P labelled DRr probe[27]. The membrane was stripped after each hybridisation process with a neutralising reagent (0.2 M TRIS, 0.1% SDS and 0.1% SSC) to remove all present probe. In addition, 6 µg of DNA was also digested by H*inf*I and hybridised with ^{32}P labelled MTB*484* (1) probe[28]. The RFLP patterns were visualised by autoradiography.

3. Mutation detection analysis by dot-blot hybridisation method.

A detailed description of the detection of mutations in drug resistance genes *of M. tuberculosis* by a dot-blot hybridisation method is attached as an addendum to this chapter. In short, extracted DNA of drug resistant *M. tuberculosis* isolates was amplified by PCR technique with specific primers directed at amplifying regions of the *kat*G, *kas*A, *rpo*B, *rps*L, *rrs* and *emb*B genes, directed towards genes which are most frequently associated with resistance to antituberculous drugs. Efficient amplification was confirmed by gel electrophoresis. An aliquot of each PCR product was fixed onto a Hybond-N^+ membrane. The membranes were then hybridised with radiolabelled wild type and mutant-specific oligonucleotide probes. Hybridisation results were visualised by autoradiography.

Definitions

1. Clinical definitions

Drug resistance is defined as a decrease in the in-vitro susceptibility of *M. tuberculosis* of sufficient degree to be reasonably certain that the strain concerned is different from a strain that has never been in contact with the drug[1]. Clinical drug resistance was determined according to the results of bacteriological testing (indirect proportion method). *Single-drug resistance* is defined as a strain resistant to only one antituberculous drug. *Multidrug resistance* (MDR) is defined by the WHO as resistance against at least isoniazid and rifampin[1]. *Multiple-drug resistance* is defined as a strain resistant to more than one antituberculous drug but not the combination of isoniazid and rifampin.

Acquired drug resistance is defined as resistant bacilli retrieved from a patient who had previously received one month or more of antituberculosis treatment, as documented in the tuberculosis registry, in medical records or by the patient's account[1]. *Primary drug resistance* is defined as the presence of drug-resistant strains of *M. tuberculosis* in a patient who denies having had anti-tuberculosis treatment for more than one month and has no evidence of such a treatment in the tuberculosis registry or medical records[1].

2. Molecular epidemiology definitions

A *cluster* of drug resistant isolates was defined as a group of two or more drug-resistant isolates originating from different patients, with RFLP fingerprint patterns identical with respect to both the number and molecular size of all bands[12] on all probes used (IS*6110*-3', IS*6110*-5', DRr and MTB*484* (1)). A drug-resistant strain was classified as *unique* if the RFLP pattern was unique (i.e. did not match with any other drug resistant isolate) with respect to both the number and molecular size of all bands among all drug-resistant strains retrieved from the communities. To calculate the extent of *transmission* of drug-resistant isolates, the formula n - 1 was used, assuming that a typical cluster of n persons comprises one index case[29]. The first patient in a cluster presenting with drug-resistant TB was assumed to be the *index case*.

RESULTS

Strain diversity among patients with drug resistant tuberculosis based on RFLP analysis with IS*6110*-3' probe

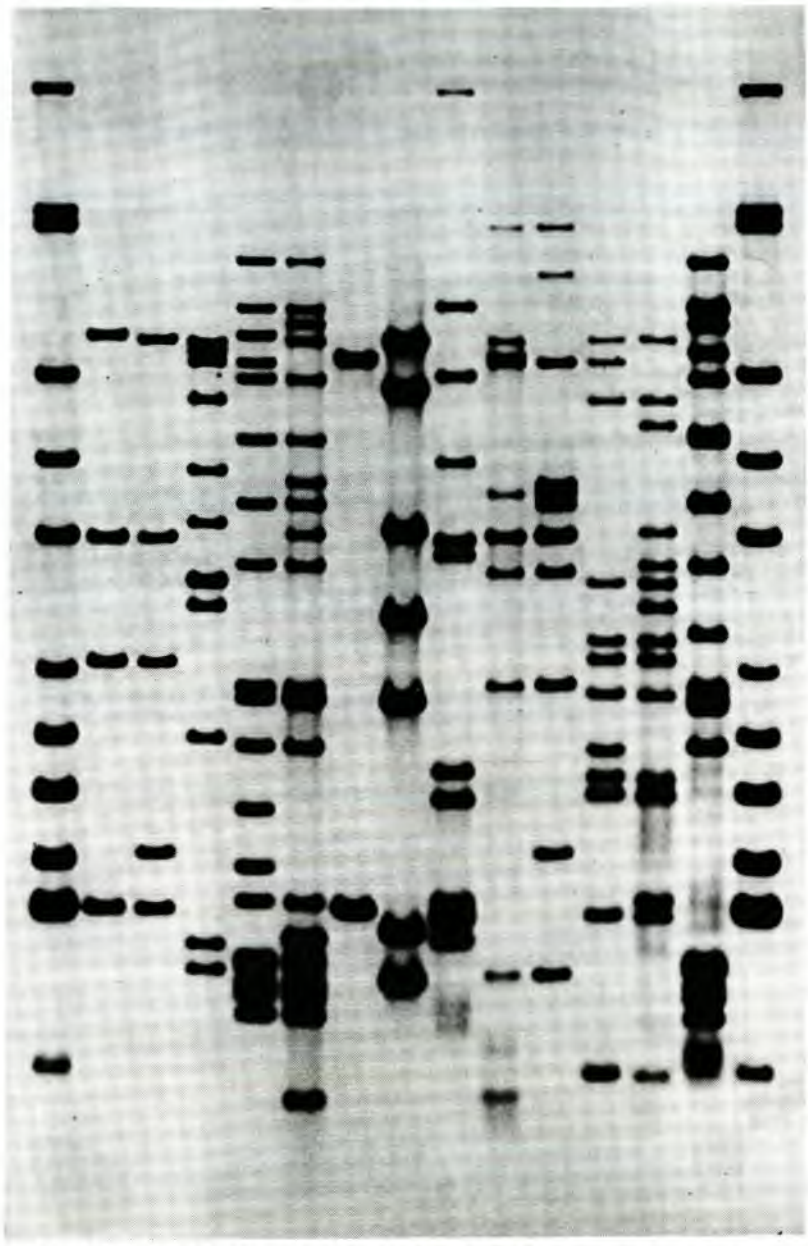
During the 5-year period, 70 patients were treated for drug-resistant TB. The 63 (90%) patients of whom at least one *M. tuberculosis* isolate was available for RFLP analysis constitute the study population. According to the drug-susceptibility results, the majority of patients (71%) had MDR-TB, 12 (19%) had single-drug-resistant TB (11 resistant to isoniazid, 1 resistant to rifampin) and the remaining 6 (10%) patients had multiple-drug-resistant TB (table 1).

According to the internationally recommended RFLP analysis (digestion with the *PvuII* restriction enzyme and IS*6110*-3' hybridisation [30]), 26 different drug-resistant strains were retrieved from the 63 patients. The number of IS*6110* copies per isolate of *M. tuberculosis* varied from 2 to 23. Twenty-one strains represented unique strains as they were retrieved from only one patient with drug-resistant TB (table 1). The remaining 7 strains represented clustered strains as each of these strains was retrieved from at least 2 different patients. The size of the outbreaks, caused by each cluster varied from 2 to 16 patients.

Table 1: Classification of 63 drug-resistant patients by clinical method and Restriction Fragment Length Polymorphism (RFLP) analysis

	All patients	**MDR-TB**	**Single-drug-resistant TB**	**Multiple drug-resistant TB**
		No. (%)	**No. (%)**	**No. (%)**
No. of patients	63 (100)	45 (71)	12 (19)	6 (10)
RFLP classification				
IS6110-3'probe				
Unique	21(33)	10 (22)	9 (75)	2 (33)
Cluster	42 (67)	35 (77)	3 (25)	4 (67)
Transmission *	35 (55)	30 (67)	1 (8)	4 (67)
All 4 probes				
Unique	24 (38)	11 (24)	11(92)	2 (33)
Cluster	39 (62)	34 (76)	1 (8)	4 (67)
Transmission *	33 (52)	29 (64)	0 (0)	4 (67)

Overall, 42 (67%) patients with drug-resistant TB were infected with an isolate belonging to one of the 7 clusters of drug-resistant isolates (table 1). Four clusters included only MDR-TB patients; one cluster included only single-drug-resistant TB patients. The 2 other clusters consisted of a mix between either MDR-TB and multiple drug-resistant TB, or single-drug-resistant TB and multiple drug-resistant TB. The diversity of RFLP patterns *of M. tuberculosis* strains among patients with MDR-TB is presented in figure 1. This figure shows that the majority of the MDR-TB patients (35/45 or 77%) were infected with a *M. tuberculosis* strain belonging to a cluster. The same is true for patients with multiple drug-resistant TB (67%) (table 1). In contrast, 75% of patients with single-drug-resistant TB were infected with a unique *M. tuberculosis* strain (table 1). According the n-1 formula[29], 55% of all drug-resistant TB patients, 67% of MDR-TB and multiple-drug-resistant TB patients but only 8% of the single-drug-resistant TB patients were classified as infected with a drug-resistant strain due to transmission of the strain from an infectious drug-resistant TB case (table 1).



	R	C_1	C_2	C_3	C_4	C_5	U_1	U_2	U_3	U_4	U_5	U_6	U_7	U_8	R
Strain classification															
No. of patients with identical strain		3	11	3	16	2	1	1	1	1	1	1	1	1	

Figure 1 Diversity of Restriction Fragment Length Polymorphism patterns of *Mycobacterium tuberculosis* strains among patients with multidrug-resistant tuberculosis. Lanes R are control strains (MTB 14323)[30]; lanes C1 to C5 represent clustered *M. tuberculosis* strains (C1=cluster 1 etc.); lanes U1 to U8 represent unique *M. tuberculosis* strains. Two of the 10 unique strains are not represented.

These results demonstrate a relatively low rate of IS*6110* strain diversity among drug-resistant isolates (28 different strain among 63 patients) retrieved from a community where TB is endemic. The lowest degree of strain diversity was observed among patients with MDR-TB (15 strains/45 patients) while strains infecting patients with single-drug-resistant TB showed a high degree of genomic polymorphism (11 strains/12 patients).

Use of multiple genetic markers in the differentiation of drug-resistant *M. tuberculosis* strains.

To improve the accuracy of strain typing, clusters of identical (with IS*6110*-3' probe) strains were subjected to further analysis with additional probes: (IS*6110*-5', DRr, MTB484(1)). One of the 11 strains with 5 IS*6110*-3' elements and both strains with 3 IS*6110*-3' elements, initially classified as clustered were now found to be unique (figure 2). This reduced the rate of transmission among drug-resistant TB patients, from 55 to 52% (table 1). For MDR-TB, the rate of transmission was reduced from 67 to 64%. When multiple RFLP probes were used, there was not a single case of transmission among single-drug-resistant TB cases.

Correlation between conventional and molecular epidemiological data.

A medical anthropologist could conduct interviews with 38 of the 42 patients infected with a drug resistant isolate that belonged to a (IS*6110*) cluster. Three of patients (7%) could not be interviewed as they had died. One patient could not be interviewed, as he was lost to follow-up after moving away from the study area. Epidemiologic connections among patients infected with an identical strain were identified for 26 of the 38 patients (68%), ranging from 50 to 75 of epidemiological connections among patients within each cluster. The most frequent places of contact were either house of residence or social drinking places.

The use of RFLP analysis with multiple probes marginally improved the correlation between conventional and molecular epidemiologic data: the percentage of social links that could be identified among patients infected with clustered isolates increased from 68% (26/38 patients) to 74% (26/35patients).

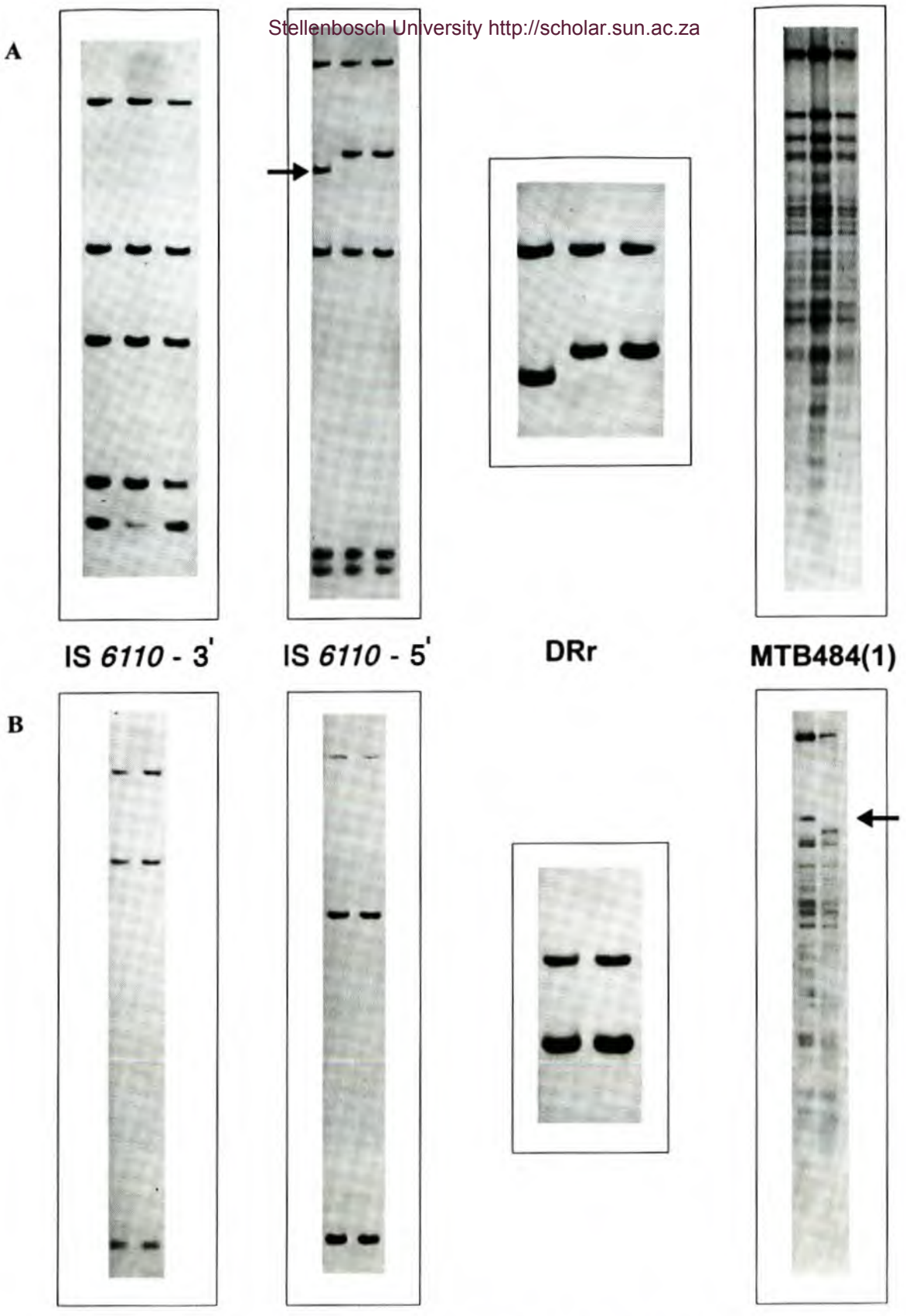


Figure 2. Restriction Fragment Length Polymorphism analysis with four probes (IS*6110*-3', IS*6110*-5', DRr and MTB484(1)) of two clusters with low IS-6110-3' copy numbers.

A. Cluster of 11 isolates (3 represented) with 5 IS*6110*-3' elements. Arrows indicate where one isolate has a different RFLP pattern with the IS*6110*-5' and DR probe.

B. Cluster of 2 isolates with 3 IS*6110*-3' elements. Arrow indicates where the isolates have a different RFLP pattern with the MTB484(1) probe

Diagnosis of unsuspected cases of drug-resistant TB by the application of DNA fingerprinting

Cluster analysis was applied to the complete RFLP database (consisting of more then 2000 isolates of approximately 900 patients with drug sensitive or drug resistant TB included in the prospective epidemiology study of TB) in order to identify strains classified as 'drug-sensitive' or 'drug resistance not tested', with RFLP patterns identical to those of drug-resistant strains. One or more matching isolate could be identified for 16 of the 24 unique and 2 of the 6 clusters of drug-resistant isolates. One of these clusters was studied in detail. Four isolates had an RFLP pattern that matched the IS*6110*-3' pattern of the cluster of drug-resistant isolates with 5 IS*6110*-3' elements (fig3). Further RFLP analysis of these 4 isolates showed that the position of the top band on MTB*484(1)* hybridisation differed for two of those 4 strains (lane 4 and 5 of fig 3). Mutation detection analysis identified the same mutations in *katG*315, *rpoB*513 and *rpsL*43 in the drug resistant isolate (lane 1, fig 3) and in the two genetically identical strains (lane 2 and 3, fig 3) but no mutations in the genetically different isolates (lane 4 and 5, fig 3). Phenotypic drug resistance was never detected in these isolates, as the infecting isolates were never tested for drug resistance. Epidemiological investigation by contact tracing demonstrated that one of the patients had been in close contact with a patient belonging to the outbreak as originally defined. This patient had been 'cured' (lost to follow-up one year after diagnosis of TB) with standard treatment. The other patient could not be interviewed as she had died after interruption of treatment.

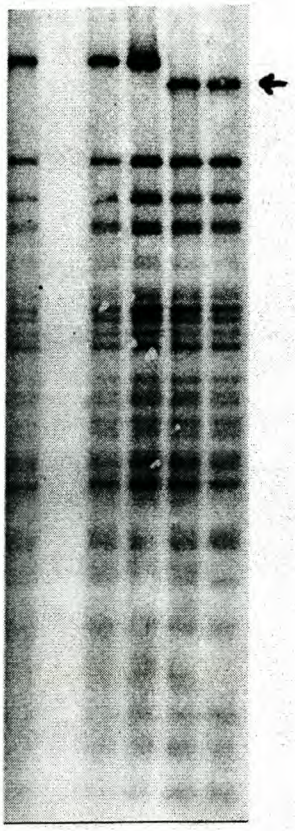


Fig.3 Restriction fragment length polymorphism pattern by MTB484(1) probe of strains with 5 IS6110-3' copies. First lane is a drug resistant strain; lanes 2 to 5 are 'drug sensitive' or 'resistance not tested strains'. Arrow indicates the difference in RFLP pattern of isolates in lane 4 and 5

DISCUSSION

We used RFLP analysis and traditional epidemiologic methods to investigate the disease dynamics of drug-resistant TB in an area characterised by a high TB incidence. Our results show, by contrast to the high degree of strain diversity found by population-based studies in low incidence areas[22], a relatively low degree of genomic polymorphism among drug-resistant *M. tuberculosis* strains. The lowest degree of strain diversity was found among MDR-TB patients. This can probably be explained by the prolonged infectiousness of MDR-TB patients facilitating transmission of MDR-TB strains.

The retrieval of drug-resistant isolates from patients not diagnosed as cases of drug-resistant TB indicates a shortcoming of the current NTP strategy. Guidelines state that drug resistance should only be tested for in patients with a history of previous TB treatment and in patients with current treatment failure. Diagnosis of patients with primary drug-resistant TB (no history of TB treatment) will be delayed substantially. A first and essential step to improve the yield of case finding of drug-resistant TB could be a more thorough contact tracing procedure for cases with drug-resistant TB.

Several studies have combined molecular and conventional epidemiologic data and found that there is never a 100 % correlation, especially outside small outbreak situations [29,31,32]. The explanation for this is threefold. First, a detailed interview and record review might not be sensitive enough to identify all potential epidemiologic connections[11,31-33]. Second, patients infected with identical strains are not always epidemiological connected. The predictive value of RFLP clustering will be higher in crowded, urban populations where TB is still endemic then in scarcely populated rural areas with a low TB incidence[30]. In areas with a high TB incidence, there may be multiple index cases with identical strains at the same time. In areas with a low TB incidence, identical RFLP pattern may not point to recent transmission but to contact in the past. Third, not all strains with an identical IS*6110* pattern are necessarily part of one cluster, especially when a strain contains only a few IS*6110* copies. In one study, the proportion of patients infected with identical strains and identified epidemiological connections increased with the number of IS*6110* copies from 14% for clusters with less

than 5 IS*6110* copies to 60% for clusters with more than 12 copies[30]. In that study, the overall percentage of patients in clusters with an epidemiologic connection increased from 34 to 42% when RFLP by multiple probes was analysed. In our study, the proportion of patients infected with identical strains and identified epidemiological connections increased from 68 to 74% by the use of multiple-probe RFLP analysis. It is now generally accepted that the RFLP method based on a single probe (IS*6110*) is not accurate enough to distinguish all strains, in particular strains with a low number of IS*6110* copies[30,34-36]. Accurate interpretation of strain identification by RFLP analysis therefore requires knowledge on the demographic characteristics of the population, a combination of clinical and molecular data and the use of multiple probes. Only then will molecular epidemiology improve our understanding of disease dynamics and aid to the formulation of more efficient disease control strategies.

In this study, RFLP analysis could refine the knowledge on drug-resistant TB in an area with a high TB incidence. First, RFLP analysis showed that the current control strategy is insufficient in case finding of drug-resistant TB cases because cases of primary drug resistance are missed. Second, chains of transmission could be identified among patients without known social links, indicating the possibility of transmission of drug-resistant strains by casual or unidentified contact. Third, the limited strain diversity among drug-resistant strains, especially among MDR strains, implies substantial transmission of drug-resistant strains in an endemic area. The focus of the TB control strategy in an area endemic for TB should therefore not only focus on the extremely important prevention of acquisition of drug resistance but also limit transmission of drug-resistant strains by improved case finding and accurate treatment of cases of drug-resistant TB.

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CHAPTER 3

Detection of mutations in drug resistance genes of Mycobacterium tuberculosis by a dot-blot hybridization strategy

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Detection of mutations in drug resistance genes of Mycobacterium tuberculosis by a dot-blot hybridization strategy

Setting: *Mycobacterium tuberculosis* isolates from patients in communities endemic for tuberculosis in South Africa. Objective: To develop a reliable PCR-based dot-blot hybridization strategy to detect mutations conferring drug resistance. Design: Different loci in six genes associated with drug resistance to isoniazid, rifampin, streptomycin and ethambutol were selected to develop the PCR-based dot-blot hybridization strategy. Results: Primers and probes to detect mutations at codons 315, 463 (*katG*) 269 (*kas*A) 531, 526 (*rpo*B) 43 (*rps*L) 513 (*rrs*) and 306 (*emb*B) were designed and used to develop a PCR-based dot-blot hybridization strategy. The dot-blot hybridization strategy with wild-type probes can efficiently be used to detect drug resistant mutations since these do not hybridize to mutant loci. Stripped blots and mutant probes can be used to identify the precise mutation. The *emb*B gene (ethambutol resistance) was used to show how the dot-blot strategy can assist with the prediction of drug resistance more accurately. The method is rapid, reproducible, not technically demanding and samples can be done in batches. Additional loci can easily be incorporated. Conclusions: A PCR-based dot-blot hybridization strategy is described which can accurately identify drug resistant strains and the method is useful to patients at risk and in areas endemic for tuberculosis.

INTRODUCTION

Tuberculosis remains one of the world's most serious afflictions, leading to 3 million deaths per annum. This epidemic is further complicated by the emergence of drug resistant strains of *M. tuberculosis*, which pose a threat to tuberculosis control programmes and often result in high-level mortality [1,2]. Traditional susceptibility testing depends on a positive culture for diagnosis and subcultures for susceptibility testing. Usually a minimum of 3 - 6 weeks is required for a positive culture diagnosis and at least a further 3 weeks for susceptibility testing on solid media using conventional techniques, or 15 days when radiometric methods with the BACTEC system are used [3-6]. Susceptibility testing is not only time-consuming, but there are significant technical problems in the standardization of these tests, such as difficulties to establish an appropriate inoculum size, the stability of the compounds in different culture media and the reliability of results when testing the drugs [7,8]. Rapid diagnosis of drug resistance may help to stop chains of transmission of multidrug resistant tuberculosis, which is a threat in many societies.

The molecular basis of drug resistence to all the front-line drugs is due to mutations in specific genes of *M. tuberculosis.* To date, approximately 12 genes are known to be linked to resistance [9]. Molecular results indicated that the genome of *M. tuberculosis*, including mutations in the drug resistant genes, is stable in follow-up samples from multidrug resistant (MDR) patients [7]. This favours the use of molecular techniques to predict drug resistance by mutational analysis. Many PCR-based strategies have been used to detect mutations in genes of interest, which include DNA sequencing [7,9], dideoxy-Fingerprinting [10], heteroduplex analysis [11] , RFLP [7,12], SSCP [7,13] and the use of probes [14,15]. Each system has inherent problems: for example, not all mutations result in the gain or loss of a restriction enzyme site, therefore limiting the use of RFLP as a general method to screen for (drug resistant) mutations. Other screening procedures which depend on DNA mobility shifts (eg. PCR-SSCP) are often used, but are technically demanding and mutations are not always detected by these procedures. PCR amplification followed by DNA sequencing is possibly the most widely used technique for the identification of mutations, but is not readily available in routine laboratories.

Furthermore, in countries most severely affected by tuberculosis there is often a shortage of expertise and funding for sequencing.

It is for these reasons that additional techniques for the efficient detection of drug resistant mutations are needed. Ideally, a simple technique which can detect any known or newly described mutation and which fullfills the criteria of accuracy, speed an simplicity is required. In the present study, a dot-blot strategy is described which can be used to analyse any of the drug resistant mutations. The results show that hybridization with wild-type probes can be used to efficiently screen for all mutations, conferring drug resistance.

MATERIALS AND METHODS

Clinical isolates

The clinical isolates included in this study originate from patients with culture-tested susceptible and drug resistant tuberculosis. Patients were from two communities of Cape Town, South Africa, with a tuberculosis notification rate of over 1000/100 000 p.a.[16]. Antibiotic susceptibility testing was done as described previously [7]. The reference strain $H_{37}Rv$, together with susceptible isolates and molecularly well-characterized clinical isolates of *M. tuberculosis* [7] were used as controls.

PCR amplification

Primers to amplify regions of the *kat*G, *kas*A, *rpo*B, *rpsL*, *rrs* and *embB* genes were designed (Table 1). Five microlitres (300ng) of genomic DNA were used as template for amplification in a 100 µl reaction mixture consisting of Magnesium Chloride, 2.25 mM; dNTP's (dATP, dGTP, dCTP and TTP) [Boehringer Mannheim], 200 µM each; 5' primer and 3' primer, 0.2µM each and 1.5 U of Taq Polymerase (Promega). Reaction mixtures were then heated in a thermal cycler (Techne Gene E) as follows: 93°C for 3 min followed by 35 cycles at 93°C for 1 min, annealing at Tm for 1 min and an extension step at 72°C for 2 min. Final extension was done at 72°C for 10 min. The annealing temperature for each primer set was calculated as follows: Tm = 4(G+C) +

2(A+T) and the optimal annealing temperature is given in Table 1. Efficient amplification was confirmed by gel electrophoresis on 12% polyacrylamide mini-gels (Biorad, Protean II). Care was taken to prevent false results due to amplicon contamination [18].

Table 1: Primers for PCR amplification

Primer	Sequence	Tm	Fragment	Reference
***katG* gene**				
RTB 59	GCTGGTGATCGCGTCCTTAC			
RTB 36	TCGGGGTCGTTGACCTCCCA	66°C	804 bp	Ref. 17
***kasA* gene**				
kasA 51	ATTGAGTCGGAGAACCCCGA			
kasA 31	CCTTCCATATCGGTCCGACT	56°C	1389 bp	Current
***rpoB* gene**				
TR 8	TGCACGTCGCGGACCTCCA			
TR 9	TCGCCGCGATCAAGGAGT	58°C	157 bp	Ref. 7
***rpsL* gene**				
STR 52	GTCAAGACCGCGGCTCTGAA			
STR 34	TTCTTGACACCCTGCGTATC	60°C	272 BP	Ref. 7
***rrs* gene**				
STR 53	TCACCATCGACGAAGCTCCG			
STR 31	CTAGACGCGTCCTGTGCATG	64°C	570 bp	Ref. 7
***embB* gene**				
emb 151	CGGCATGCGCCGGCTGATTC			
emb 131	TCCACAGACTGGCGTCGCTG	65°C	260 bp	Current

Dot-blot Hybridization

Wild-type and mutant-specific oligonucleotides to screen for the presence or absence of specific mutations in six of the genes associated with resistance to antituberculosis drugs, were designed according to published sequences (X68081, *kat*G; RV2245, *kas*A; L05910, *rpo*B; L08011, *rpsL*; X52917, *rrs* and U68480, e*mb*B). Oligonucleotide sequences are given in Table 2. Oligonucleotides were 5' end-labelled by phosphorylation with [γ-^{32}P]-ATP (Amersham) as described previously (19) or 3' end-labelled with digoxigenin by terminal transferase (Boehringer) according to the specifications of the manufacturer.

Table 2: Probes for dot-blot hybridization

Probe	Sequence
katG315 wt	GATCACCAGCGGCATCGAGG
katG315 mu	GATCACCACCGGCATCGAGG
katG315 dmu	GCGATCACCACAGGCATCGA
katG463 mu	AGCCAGATCCTGGCATCGGG
katG463 wt	AGCCAGATCCGGGCATCGGG
kasA 269wt	GGTGCCGGTATCACCTCGGA
rpoB531 wt	AGCGCCGACTGTCGGCGCTG
rpoB531 mu	AGCGCCGACTGTTGGCGCTG
rpoB526 wt	GGGTTGACCCACAAGCGC
rpsL43 wt	ACCACTCCGAAGAAGCCGAA
rrs512/3 wt	ACGTGCCAGCAGCCGCG
embB306 wt	CCTGGGCATGGCCCGAGTCG

An aliquot of each PCR product was adjusted to 0.4 N NaOH, 25mM EDTA in 100 µl, heat-denatured at 95°C for 10 min and applied under vacuum to a Hybond-N^+ nylon filter (Amersham) in a dot-blot apparatus (Biorad). The DNA was fixed onto the membrane by baking at 80°C for 1 hour. For radioisotopic detection, each filter was hybridised in 5xSSPE and finally washed in 1.5xSSPE for 10 min at 74°C as described previously (19). Autoradiography was done at room temperature for 2-3 hours. Non-radioactive hybridization was done in 5xSSPE solution and the final wash and detection was done with anti-DIG alkaline phosphatase according to the specifications of the manufacturer (Boehringer). Although it is important to confirm DNA amplification by gel electrophoresis, the results indicate that small differences in efficiency of amplifcation do not have a significant effect on the outcome of the hybridization results. To reprobe, the membranes were stripped by incubation at 46°C for 30 min in 25 ml 0.4 M NaOH with constant shaking. The NaOH was neutralized by a further 15 min of incubation at 42°C in 25 ml of neutralizing solution containing 0.2M Tris-HCL (pH 7.5), 0.1% SDS, 0.1x SSC (20xSSC = 175.3g NaCL, 88.2g $C_6H_5Na_3O_7.2H_2O$ dissolved in H_2O to a final volume of 1 litre, pH 7.0). Although radioactive and non-radioactive detection procedures gave similar results, the radioactive procedure was favoured to empirically evaluate stringent hybridization washes during the developmental phase of this strategy. The following experimental flow is recommended: PCR amplification, confirmation of amplification by gel electrophoresis; label of probe for region of

interest; dot-blot hybridization; overexposure of first time autoradiography to ensure that samples were sufficiently blotted; final autoradiography.

Sequence analysis

Direct sequencing of amplified PCR products was done with an ABI PRISM (model 377, Perkin Elmer).

RESULTS

A dot-blot hybridization strategy was developed to test the hypothesis that mutant and wild-type allele specific probes can detect drug resistant conferring mutations in *M. tuberculosis*. Results for codon 315 in the *kat*G gene and codon 531 in the *rpo*B gene are given in Figures 1 and 2 respectively. Firstly, a 804 bp region of the *kat*G gene containing the codon 315 mutation associated with isoniazid (INH) resistance, was PCR amplified from MDR and drug susceptible clinical isolates. Autoradiography after hybridization correctly identified the control samples on the blot and demonstrated *kat*G315 mutant alleles for 16 of the clinical isolates. To test the accuracy of the technique, one of the INH resistant clinical isolates (sample A3) was subjected to sequence analysis which then confirmed the presence of a G-C mutation at codon 315. Secondly, the example in Figure 2 for codon 513 of the *rpo*B gene (RMP resistance) does not only confirm the accuracy of the technique, but also show how efficiently allele specific wild type probes can detect drug resistant conferring mutations. Good discrimination was obtained for control samples and confirms that the wild-type probe hybridizes specifically to the amplified region of susceptible isolates, while no hybridization was detected after hybridization to amplified regions containing mutations. Gel electrophoresis and the combined strategy with wild-type and mutant probes also showed that the absence of a signal was not due to poor amplification. The codon 531 mutation (C-T) in clinical isolate B6 was confirmed by sequence analysis. Both wild-type and mutant probes clearly indicated which of the other clinical samples loaded onto the filter have a mutation at codon 531.

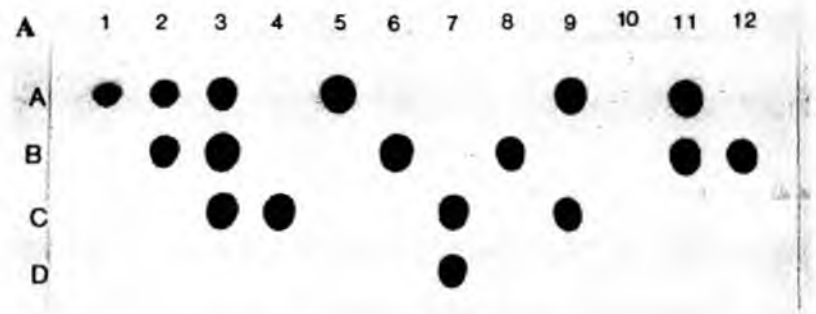

Figure 1. A 804 bp region of the *kat*G gene was amplified with primers RTB59 and RTB36 from drug resistant and drug susceptible isolates. The dot-blot hybridization strategy with radiolabelled probe katG315 mu was subsequently used to screen for mutations at codon 315. The *M. tuberculosis* reference strain H37Rv was included as a negative control (D9) and amplified genomic DNA from two previously well-defined isolates (ref 7) were included as additional controls. Good discrimination was obtained for samples D7 (known G-C mutant for *kat*G315) and D8 (known wild-type sequence at codon 315).

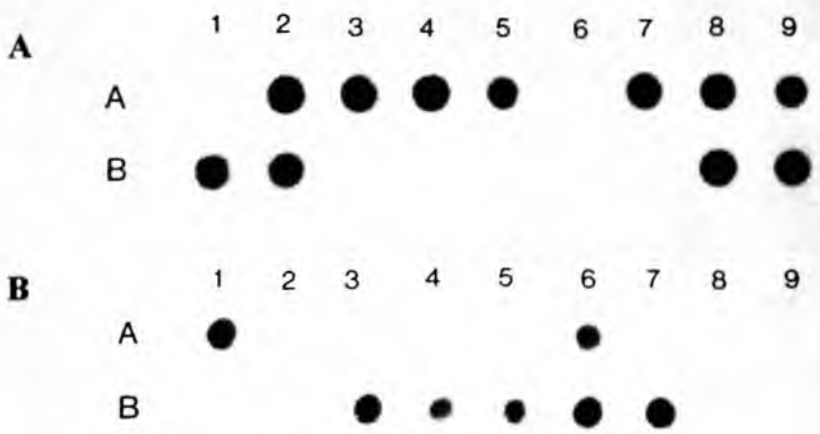

Figure 2. Dot-blot hybridization of codon 531 in the *rpoB* gene. DNA (n = 18) from drug resistant and susceptible isolates of *M.tuberculosis* were amplified with primers TR 8 and TR 9 and hybridized in a dot-blot format with radiolabelled probes rpoB531 wt (filter A) and rpoB531 mu (filter B; same filter as A, but were stripped and reprobed with rpoB531 mu). Good discrimination was obtained for samples B7 and B8 which are a known mutant (C-T) and wild-type for codon 531 respectively, as characterized previously (Ref 7). Amplified DNA from the control H37Tv was loaded in B9.

Similar results were obtained for codons 463 (*kat*G gene), 269, (*kas*A gene), 526, (*rpoB* gene), 43, (*rpsL* gene), 306 (*emb*B gene) and position 513 (*rrs* gene). Comparison of genotype and phenotype results in Table 3 are examples of results obtained from clinical isolates with the described dot-blot hybridization strategy.

Table 3: Genotype and phenotype resistance data

Isolate	Drug pattern	Mutations
1	INH, RMP, SM,	*kat*G315, *kat*G463, *rpo*B531, *rrs*513,
2	INH, RMP SM	*kat*G315, *rpo*B531, *rps*L43
3	INH	*kat*G315
4	INH, RMP, SM,	*kat*G315, *kat*G463, *rpo*B531, *rrs*513,
5	INH	*kas*A269
6	RMP	*rpo*B531
7	INH, RMP	*kat*G315, *rpo*B531
8	INH, RMP, SM,	*kat*G315, *kat*G463, *rpo*B531, *rrs*513,
9	INH, RMP, SM	*kat*G315, *rpo*B531, *rps*L43
10	INH	*kat*G315
11	INH, RMP, SM	*kat*G315, *rpo*B526, *rps*L43
12	S	-
13	S	-
14	S	-

INH = isoniazid, RMP = rifampin, SM = streptomycin, EMB = ethambutol,S = susceptible

However, drug resistance testing in *M. tuberculosis* is difficult and can sometimes give inconsistent results [7]. Table 4 shows how mutational analysis, using the wild-type probe strategy, can assist with the correct diagnosis of EMB resistance in follow-up MDR clinical isolates. A wild-type probe for codon 306 (embB306 wt) was designed and used to analyze follow-up samples from a patient diagnosed with EMB resistant tuberculosis. None of the samples hybridised to the wild-type probe and therefore predicts codon 306 mutations [20] for samples V121-V123. A mutation (ATG→ATA) was subsequently confirmed for all the samples by sequence analysis (Table 4). However, only one of these were phenotypically classified as EMB resistant. This strongly suggests that samples V122 and V123 were misdiagnosed by the routine (culture) diagnostic laboratory. Subsequent re-testing by culture-confirmed initial misdiagnosis.

Table 4: Mutational analysis of ethambutol resistance

Sample	Drug pattern	Probe*	Sequence
V121	INH, RMP, SM, **EMB**	+	ATG→ATA
V122	INH, RMP, SM, KM, TH	+	ATG→ATA
V123	INH, RMP, SM, KM, TH	+	ATG→ATA

INH, Isoniazid; RMP, Rifampin; SM, Streptomycin; EMB, Ethambutol;
Probe*: positive in this column indicates presence of a mutation at codon 306 of the embB gene.

DISCUSSION

Mutations in clinical isolates of *M. tuberculosis* have been the focus of many investigations in order to determine their association with drug resistance and more recently as markers to study the evolution of drug resistance in this organism [21]. However, the complexity of molecular techniques used to study these mutations make them unsuitable as routine diagnostic tools throughout the world. This study has shown how a dot-blot hybridization strategy with wild-type probes can be used efficiently to detect mutations (in multiple isolates) at different loci in six genes associated with INH, RMP, SM, and EMB resistance. Although the current study focussed on selected regions in some of the most important target genes, the flexibility of the dot-blot strategy ensures that additional loci can easily be incorporated into the screening strategy as described (e.g. recently described *kas*A gene, [22]).

The dot-blot procedure described may be specifically useful in countries with a high incidence of tuberculosis and where procedures such as automated DNA sequencing are not readily available. As the predictive value of any test is dependent on prevalence, a mutational screening strategy should initially focus on the mutations most frequently diagnosed in the geographic area studied. Codons 315, 463 (*kat*G), 531, 526, 516 (*rpo*B), 43 (*rps*L), 491, 513 (*rrs*) and 306 (*emb*B) are frequently altered in clinical isolates from other studies and these were the initial focus for isolates originating from the Cape Town communities. Results in Table 3 are examples of drug resistant strains from the local communities where the described dot-blot strategy was used. This forms part of an ongoing study where mutational and DNA fingerprint analysis forms the basis of a molecular approach to understand the dynamics of drug resistant tuberculosis in a high incidence community. Molecular studies are needed to determine the most important mutations associated with drug resistance in different geographical regions, since it is known that drug resistant mutations may vary with the geographical origin of the samples [23]. The wild-type probe strategy is unable to provide a precise understanding of the different mutations occurring at a specific codon, however, it is known that >99% of mutations within these loci confer resistance and therefore the absence of a hybridization signal has been interpreted to directly reflect drug resistance.

These results are confirmed by phenotyping. The application of specific mutant probes can be applied to identify or confirm the nature of this mutational event, although it may be argued that it would not be possible to identify cases where both the resistant and sensitive bacilli are present in the host. This may also be true for all other molecular based methods. The dot-blot hybridization strategy with mutant probes can detect drug resistant alleles when they represent 1-2% of the overall population (results not shown). However, a review of the literature indicates that mixed infections in active tuberculosis cases are rare. Telenti *et al.*, reported a mixed population of susceptible and resistant strains in 1 out of 42 RMP resistant isolates [24]. Furthermore, while spontaneous resistant isolates clearly arise, the rate is low ($<10^{-5}$) and is usually confined to mono-resistance, which is effectively dealt with under compliant conditions with the multiple drug regimen. Thus, under normal conditions, a resistance level of 1-2% may not be seen. In cases of transmission of drug resistant isolates, the resistant alleles will be 100%. In those cases when resistance is acquired, one may assume that a 100% sensitive population will become superceded by a resistant mutant and that at a certain stage of disease, the resistant population will be <2% of the total. However, these cases are relatively rare and furthermore, detection will be problematical at certain stages only. In practice, there will usually be other indicators of problems to come, for example, non-compliance. When possible, these individuals will be managed appropriately.

The screening strategy is adaptable and lends itself to the easy inclusion of any other previously untested or newly discovered loci. This methodology demonstrates how the wild-type probe hybridization strategy can effectively be used to detect the presence of mutations in genes conferring drug resistance in clinical isolates of *M. tuberculosis*. The method is reproducible, not technically demanding and it takes about two normal working days to obtain results from the start of amplification (done in batches of 30-40 samples) to the final autoradiography step of the dot-blot hybridization (done in batches of up to 150 samples, including controls). This technique could be adapted to amplify and detect drug resistant mutations directly from sputum samples or microscopy stained slides. The high daily output possible with this strategy would enable the focus of drug resistance control strategies to include all smear-positive samples.

Drug susceptibility testing is usually performed in order to provide information for the treatment of individual patients. In addition, resistance surveillance gives a good measure of the efficacy of regional control programmes. Apart from time, the other limiting factor associated with susceptibility testing is the technical complexity [7,8], leading to problems such as intermittent resistance patterns, which have been reported previously [7] and in this study. An alternative means of testing would be particularly useful for EMB and pyrazinamide (PZA), since susceptibility testing to these drugs remains suboptimally standardized because of the poor stability of the agent in growth medium and discrepancies encountered when performing the analysis in solid versus liquid medium [25]. Misdiagnosis of drug resistance by traditional culture methods can be a problem at times as indicated for EMB resistance in this study. The same may be true for other drugs, specifically in high incidence areas with a high throughput of samples. Mutational analysis such as described in this study may help to investigate the extend of the problem. Furthermore, molecular epidemiological studies based on the dot-blot method could determine the frequency of specific mutations in a geographical area and monitor the spread of a cluster of drug resistant strains within a community or institution.

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CHAPTER 4

Classification of drug-resistant tuberculosis in an epidemic area

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Classification of drug-resistant tuberculosis in an epidemic area

Background: Traditionally, patients with drug-resistant tuberculosis are classified as having acquired drug-resistant or primary drug-resistant disease on the basis of a history of previous tuberculosis treatment. Only cases of primary drug resistance are assumed to be due to transmission of drug-resistant strains. Methods: This descriptive study of 63 patients with drug-resistant tuberculosis assessed the relative contribution of transmission of drug-resistant strains in a high incidence community of Cape Town, South Africa, by restriction fragment length polymorphism (RFLP). The RFLP results were compared with the results obtained by traditional classification methods. Findings: According to RFLP definitions, 52% (33 cases) of drug-resistant tuberculosis was caused by transmission of a drug-resistant strain. The proportion of cases due to transmission was higher for multidrug-resistant (64%; 29 cases) than for single-drug-resistant (no cases) tuberculosis. By the clinical classification, only 18 (29%) patients were classified as having primary drug-resistant tuberculosis (implying transmission). The clinical classification was thus misleading in 25 patients. Interpretation: The term acquired drug-resistance includes patients infected with strains that truly acquired drug resistance during treatment and patients who were initially infected with or reinfected with a drug-resistant strain. This definition could lead to misinterpretation of surveillance studies, incorrect evaluation of tuberculosis programmes and delayed diagnosis and treatment of patients with multidrug-resistant disease. The clinical term acquired drug resistance should therefore be replaced with the term "drug resistance in previously treated cases", which includes cases with drug resistance due to true acquisition as well as that due to transmitted drug-resistant strains.

INTRODUCTION

A 1994-97 survey by the WHO and International Union Against Tuberculosis and Lung Disease (IUATLD) found that multidrug-resistant tuberculosis has become established worldwide.[1] In 1995, the WHO estimated that 50 million people were infected with drug-resistant strains of *Mycobacterium tuberculosis*.[2] In Sub-Saharan Africa, 200 000 cases of multidrug-resistant tuberculosis can be expected by the year 2000.[3]

Drug-resistant tuberculosis is a man-made problem.[4] Poor management, including the prescription of incorrect regimens and non-compliance with treatment, can lead to the selection of *M. tuberculosis* with mutations conferring resistance to antituberculosis drugs. Resistant strains can then be transmitted from an infectious patient to another person. We need to know the relative contribution of acquisition and transmission in different settings so that tuberculosis control strategies can be evaluated and new strategies for the control of multidrug-resistant tuberculosis can be developed.

Traditionally, patients with drug-resistant tuberculosis are classified as having acquired or primary drug resistance on the basis of a history of previous treatment. WHO criteria define acquired drug resistance as the isolation of a drug resistant *M. tuberculosis* from a patient who has been treated for tuberculosis for 1 month or longer,[5] and primary drug resistance as the isolation of a drug-resistant strain from a patient without a history of previous treatment. In surveys, primary resistance has been consistently less common than the acquired resistance.[6] In the recent WHO/IUATLD survey in 35 countries,[1] the median prevalence of primary resistance to any antituberculosis drug was 9·9% (range 2 - 41%) and that of acquired resistance was 36% (range 5 - 100%).

Analysis of restriction fragment length polymorphism (RFLP) is useful in the study of the epidemiology of tuberculosis, because different strains of *M. tuberculosis* can be reliably distinguished. Patients infected with *M. tuberculosis* strains that have the same RFLP pattern are classified as part of a chain of recent transmission, whereas strains with a unique RFLP pattern are assumed to represent reactivation of a latent infection.[7,8] RFLP analysis has been extensively used to document the disease dynamics of

multidrug-resistant tuberculosis in institutions such as hospitals and prisons.[9-11] Most of the cases involved in nosocomial outbreaks were the result of transmitted infection.[10] RFLP analysis has been seldom used to investigate multidrug-resistant tuberculosis in community settings. A study in Scotland found that *M. tuberculosis* strains isolated from patients with multidrug-resistant tuberculosis were genetically diverse and unrelated to each other, suggesting acquisition of drug resistance as the predominant mechanism.[12] No data are available on the characteristics of multidrug-resistant tuberculosis in less developed countries, where the burden of tuberculosis and drug-resistant disease is the largest.

The aim of this study was to find out by RFLP analysis the contribution of transmission of drug-resistant strains within a community with a high incidence of tuberculosis. The results were compared with those obtained by traditional classification method based on tuberculosis treatment history.

METHODS

Participants

The patients were identified between April 1, 1992; and March 31,1997. All lived and were treated in two neighbouring communities of Cape Town, South Africa. This area (2·5 km^2) has a population of roughly 34000 people living in poor socio-economic conditions. The annual incidence of new (never previously treated) patients with bacteriologically positive tuberculosis in the area is 225/100 000, [13] and the annual notification rate of bacteriologically positive patients (new and retreatment cases) is 363/100 000. A survey of drug-resistant tuberculosis in the Western Cape province conducted in 1992-93 found a rate of 8·6% acquired and 3·2% initial drug resistance.[14] The reported prevalence of HIV infection in the region ranged from 0·25% in 1992 to 3.0% in 1996 (data from the National HIV surveys on women attending antenatal clinics, conducted by the Department of Health and Population Development, South Africa).

Procedures and definitions

Information on age, sex, medical history, HIV status, previous tuberculosis treatment, sputum microscopy and cultures and susceptibility testing was obtained by review of medical files and compliance charts.

In patients who had not previously received antituberculous drugs, the bacterial resistance was classified as primary. In patients with a record of previous treatment for 1 month or longer, the bacterial resistance was as classified acquired. Multidrug-resistant tuberculosis was defined as resistance to at least isoniazid and rifampin. Multiple-drug resistance was defined as resistance to more than one first-line antituberculous drug but not the combination of isoniazid and rifampin. Single-drug resistant tuberculosis was defined as resistance to only one antituberculous agent.

Sputum samples were sent to the routine laboratory for microscopy and culture. Drug-susceptibility testing was done by the indirect proportion method on Löwenstein-Jensen medium containing critical concentrations of 0·2 mg/L isoniazid, 30 mg/L rifampin, 2 mg/L ethambutol, 5 mg/L streptomycin and 20 mg/L ethionamide. Kanamycin and thiacetazone resistance was tested on Middlebrook 7H10 agar containing critical concentrations of 5 and 2 mg/L, respectively. Resistance was defined as 1% or more bacterial growth in comparison with a control according to international criteria. We did not test for resistance to pyrazinamide. As an internal control, a susceptible H37Rv strain as well as a *Mycobacterium avium* strain resistant to all drugs were included in every batch of drug-resistance testing. Furthermore, external quality control is done every 6 months.

A prospective study aimed at genotyping (RFLP analysis) all positive *M. tuberculosis* cultures from patients in these communities was started in 1992.[15,16] This study has had a recovery rate of about 80% of positive cultures [13] and resulted in an RFLP database consisting of 1272 isolates from 571 patients. The Dice coefficient and UPGMA clustering formula were used to analyse the results of the IS*6110*-3' (complementary to the IS*6110* domain between nucleotides 631 and 875) RFLP data to identify possible clusters of transmission. To improve accuracy, clusters of identical drug-resistant isolates were subjected to further genotyping: genomic DNA digested

with *Pvu*II was sequentially hybridized with Enhanced-chemiluminescence-labelled IS*6110*-5' probe (complementary to the 5' IS*6110* domain between nucleotides 77 and 462) and phosphorus-32-labelled DRr probe.[17] In addition, the DNA was digested by *Hin*fI and hybridised with ^{32}P-labelled MTB484(1) probe.[18] RFLP patterns were visualised by autoradiography.

The extent of accumulated laboratory error for the complete RFLP database was calculated for serial isolates collected during the first two months of treatment, as the number of isolates with results that did not match the result of subsequent isolates divided by the total number of serial isolates analysed. The extent of accumulated laboratory error was 3·4 % (17 discordant results/ 499 serial isolates).[13]

A cluster was defined as a group of two or more drug-resistant isolates originating from different patients for which RFLP fingerprint patterns were identical in terms of both the number and molecular size of all bands[8] with all four probes. *M. tuberculosis* strains with an RFLP banding pattern that did not match the RFLP pattern of any other drug-resistant isolate (in terms of number and molecular size of all bands) were classified as non-clustered or unique. To calculate the extent of transmission of drug-resistant isolates, we used the formula n - 1 for each cluster, with the assumption that a typical cluster of n individuals includes one index case.[7] The first patient in a cluster presenting with drug-resistant tuberculosis was taken to be the index case.

RESULTS

Clinical data

During the 5-year period, 70 patients were treated for drug-resistant tuberculosis. In 63 patients (90%), at least one *M. tuberculosis* isolate was available for RFLP analysis, and these patients formed the study population.

All patients had pulmonary tuberculosis. 84% were smear positive. 33 (52%) patients were female. The mean age at diagnosis of drug-resistant tuberculosis was 33 years (range 11 to 55 years). 54 (86%) patients were tested for HIV and all were seronegative. In one patient, an undifferentiated carcinoma of the lung was diagnosed 5

months after the diagnosis of isoniazid resistance. No patient was found to have diabetes or end-stage renal disease, and none was given or immunosuppressive therapy. Only one patient had been admitted to hospital for tuberculosis treatment before the diagnosis of drug-resistant tuberculosis.

Clinical classification

According to the drug-susceptibility results of the entire treatment episode, most of the patients (45 [71%]) had multidrug-resistant tuberculosis (table 1). In 12 (19%) cases, the isolate was resistant to a single drug (11 isoniazid, one rifampin). The remaining six (10%) had multiple-drug resistance; because this number was small, comparisons are restricted to the other categories.

By the clinical definitions of drug resistance, 45 patients were classified as having acquired drug resistance and 18 as having primary drug resistance (table1) because of a history of prior treatment with antituberculous medications (table 1). The majority of both multidrug-resistant and single-drug-resistant cases had acquired resistance.

Table 1. Classification of 63 patients with drug-resistant tuberculosis by clinical method and by RFLP analysis

	All patients (n=63)	Multidrug resistant* (n=45)	Single-drug resistant (n=12)	Multiple-drug resistant (n=6)
Clinical classification				
Acquired	45 (71%)	32 (71%)	8 (67%)	5 (83%)
Primary	18 (29%)	13 (29%)	4 (33%)	1 (17%)
RFLP classification				
Unique	24 (38%)	11 (24%)	11(92%)	2 (33%)
Cluster	39 (62%)	34 (76%)	1 (8%)	4 (67%)
Transmission °	33 (52%)	29 (64%)	0	4 (67%)

*Resistance against isoniazid and rifampin
° n – 1

Molecular classification

In 24 (38%) patients the *M. tuberculosis* strain had a unique RFLP pattern; 39 (62%) were infected with an isolate belonging to one of six clusters of drug-resistant isolates (table 1). 34(76%) of the patients with multidrug-resistant tuberculosis were infected with a strain belonging to a cluster. By contrast, 11 of the 12 patients with

single-drug-resistant disease were infected with a unique strain (Table 1).Transmission of a drug-resistant strain was calculated to have occurred in 52% of the whole group, 64% of the multidrug-resistant group but none of the single-drug-resistant group.

Comparison of clinical and RFLP classification

According to the clinical classification, 45 of the patients had acquired drug-resistant tuberculosis. On RFLP classification, only 17 of these 45 patients had a unique strain and 28 were infected with a strain belonging to a cluster of drug-resistant isolates. The majority (25 out of 45 [56%]) of cases therefore had drug-resistant tuberculosis caused by transmission of an already drug-resistant strain as calculated by the formula suggested by Small and colleagues.[7] Of the 32 patients with acquired multidrug-resistant tuberculosis, 21 (66%) were classified as having transmitted drug-resistant tuberculosis on the basis of the RFLP definitions. Only nine of these 32 patients were infected with a unique strain. The history of previous antituberculous treatment was thus associated with the presence of drug resistance in only 28% of multidrug resistant cases.

Table 2. Comparison of the clinical and RFLP classification

	All patients		Multidrug resistant		Single-drug resistant	
	Acquired (n=45)	Primary (n=18)	Acquired (n=32)	Primary (n=13)	Acquired (n=8)	Primary (n=4)
RFLP classification						
Unique	17 (38%)	7 (44%)	9 (28%)	2 (15%)	7 (88%)	(100%)
Cluster	28 (62%)	11(56%)	23(72%)	11(85%)	1 (12%)	0
Transmission °	25 (56%)	8 (44%)	21(66%)	8 (62%)	0	0

DISCUSSION

In the community studied, we recorded a higher proportion of cases of acquired resistant tuberculosis than primary resistant tuberculosis cases (29%) by clinical definitions. We confirmed this finding in multidrug-resistant tuberculosis and in cases of single-drug-resistance. This finding accords with results of surveillance studies in the region[15] and worldwide.[1,6]

By contrast, the use of RFLP analysis in the same population showed that a high proportion of transmission of drug resistant strains in this area were transmitted from another person infected with a drug-resistant strain. The history of previous tuberculosis treatment was misleading in the majority of patients, because the history implied acquisition of drug resistance whereas what had really happened was transmission resulting in infection with or reinfection with a drug-resistant strain. The contribution of transmission differed substantially between the multidrug (nearly two-thirds of cases) and single-drug resistant (no cases) categories. The low rate of transmission of singly resistant strains probably reflects the fact that patients with these strains, in contrast to those infected with multidrug-resistant strains, are not infectious for long periods of time because a standard treatment regimen will provide drugs to which the bacterium is sensitive. Transmission was thus an important mechanism in the creation of multidrug-resistant tuberculosis in this community, and acquisition was the predominant mechanism for single-drug-resistant tuberculosis. 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 *M. tuberculosis* organisms.[4] Furthermore, primary drug resistance is believed to be less severe because it is common in single-drug-resistant cases and rare in multidrug-resistant cases.[4] RFLP analysis indicates the opposite. Of the patients classified as having transmitted drug resistance, none had single-drug-resistant tuberculosis, 12 % had multiple drug resistance, and 88 % had multidrug-resistant tuberculosis. Thus, transmitted drug resistance can be severe, because it is common in multidrug-resistant tuberculosis.

The importance of transmission of drug-resistant mycobacteria in this study may have been overestimated for two reasons. First, clustering can be the consequence of laboratory contamination. We believe that this explanation is unlikely because the accumulated laboratory error rate for the complete RFLP database is 3·4 %, and more than one RFLP result was available in 28 of the 39 patients with cluster infections. Second, in theory, *M. tuberculosis* could be transmitted as a drug-susceptible organism, and patients could acquire drug resistance independently. However, in this case, drug-sensitive isolates with similar RFLP patterns to drug-resistant isolates should be found. We found matching drug-sensitive isolates for only 9% of isolates classified as

transmitted drug-resistant strains by RFLP. Furthermore, in all the clusters (except one cluster of four isolates), the resistance pattern of the isolates within a cluster was similar.

We believe, instead, that the importance of transmission in this study may have been underestimated. First, because the study was confined to a specific period, patients could have been infected by an infectious case before the start of the study. Second, index cases could have been missed because retrieval of drug-resistant strains in the community was 90 % (63/70 patients). Third, patients spent time in other communities where they could have been infected and transmitted infection to others.

The classification of patients as primary or acquired is used as an indicator of the efficiency of national tuberculosis programmes and in the adjustment and development of these programmes. The rate of primary drug resistance is interpreted as an epidemiological indicator for long-term surveillance of the quality of tuberculosis treatment in the community. The rate of acquired drug resistance reflects the efficacy of management of individual patients.[19] The evaluation of the South African national tuberculosis programme on the basis of the rates of multidrug-resistant tuberculosis in the community would give different results depending on the classification method used. By the traditional clinical classification, the predominance of acquired multidrug-resistant tuberculosis indicates poor management of individual patients before of drug-resistant tuberculosis is diagnosed. By contrast, with the RFLP classification, the predominance of transmitted multidrug-resistant tuberculosis cases points to a problem of continuing transmission of multidrug-resistant tuberculosis within the community. To reduce the rate of transmission of multidrug-resistant tuberculosis in an epidemic area, greater awareness of this problem is essential. Effective treatment of infectious cases and the use of rapid diagnostic tests might be helpful.

In the study population described, the clinical classification was not a valid representation of the mechanisms leading to drug resistance. The study showed that the category acquired drug resistance includes patients in whom *M. tuberculosis* truly acquired drug resistance during treatment as well as patients initially infected with or reinfected with a drug-resistant strain.[1] We therefore suggest that the current terminology should be replaced by the terms "drug resistance in previously treated

cases" and "drug resistance in new cases". To improve our understanding of the disease dynamics of drug-resistant tuberculosis, further studies should investigate stratification of cases classified as drug resistance in previously treated cases into transmitted and truly acquired drug-resistant tuberculosis in areas with a high and low rates of tuberculosis. Furthermore, the effect of HIV coinfection on the disease dynamics of drug-resistant tuberculosis within communities needs to be investigated. Design and interpretation of surveillance studies in epidemic areas should take into account that the traditional clinical classification based on a history of previous treatment may result in misleading interpretations and the underestimation of transmission. This inaccuracy may in turn lead to inappropriate and ineffective clinical management. If, as shown in this study, most cases of multidrug-resistant tuberculosis occur in clusters and are due to transmission, the prevention of transmission by early detection and appropriate treatment needs urgent attention.

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CHAPTER 5

Exogenous Reinfection as a Cause of Recurrent Tuberculosis after Curative Treatment

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Exogenous Reinfection as a Cause of Recurrent Tuberculosis after Curative Treatment

Background. For decades it has been assumed that postprimary tuberculosis is usually caused by reactivation of endogenous infection rather than by a new, exogenous infection. *Methods.* We performed DNA fingerprinting with restriction-fragment-length polymorphism analysis on pairs of isolates of *Mycobacterium tuberculosis* from 16 compliant patients who had a relapse of pulmonary tuberculosis curative treatment of postprimary tuberculosis. The patients lived in areas of South Africa where tuberculosis is endemic. Medical records were reviewed for clinical data. *Results.* For 12 of the 16 patients, the restriction fragment-length polymorphism banding patterns for the isolates obtained after the relapse were different from those for the isolates from the initial tuberculosis disease. This finding indicates that reinfection was the cause of the recurrence of tuberculosis after curative treatment. Two patients had reinfections with a multidrug-resistant strain. All 15 patients who were tested for the human immunodeficiency virus were seronegative. *Conclusions.* Exogenous reinfection appears to be a major cause of postprimary tuberculosis after a previous cure in an area with a high incidence of this disease. This finding emphasizes the importance of achieving cures and of preventing anyone with infectious tuberculosis from exposing others to the disease.

INTRODUCTION

Postprimary tuberculosis, which occurs many years after a primary infection, may develop as the result of reactivation of the exogenous, primary infection or as a result of a recent exogenous infection. Models developed by Sutherland and colleagues[1] and more recently Vynnycky and Fine[2], based on estimates of the annual risk of infection and the incidence of tuberculosis, have suggested that the relative contribution of exogenous reinfection increases in parallel with the incidence of the disease. However, data for use in evaluating these statistical models have been difficult to obtain. In only a few patients has there been reasonable proof of reinfection by a different organism after known previous infection.

Before the introduction of antituberculous medication, there was little recognition of the distinction between endogenous reactivation and exogenous reinfection in patients who had multiple episodes, since untreated established tuberculous lesions may be alternately active and dormant.[3] Effective treatment regimens made possible the sterilization of pulmonary lesions, but it was accepted that subsequent episodes of tuberculosis were almost invariably caused by endogenous reactivation[4]. The complete sterilization of a lesion became possible with improved treatment regimens, especially with the introduction of rifampin, a drug with a potent sterilizing effect. With short-course combination therapy consisting of isoniazid, rifampin and pyrazinamide, the relapse rate dropped from 21 percent to 1 to 2 percent[5-7]. In this era of effective treatment regimens, the notion that multiple episodes of tuberculosis in one patient are almost always caused by endogenous reactivation may be questioned. It is now possible to characterise the genotype of *Mycobacterium tuberculosis* by DNA fingerprinting, which can show whether a new episode of the disease is caused by infection with the same strain that caused a previous episode by a different strain.

In this study we used DNA fingerprinting to determine the relative frequency of endogenous reactivation and exogenous reinfection in patients with multiple episodes of postprimary tuberculosis. We aimed to determine the importance of this distinction in terms of the definition of cure, the efficacy of current treatment regimens, and the control of tuberculosis.

MATERIALS AND METHODS

All patients described in this report were treated in two neighbouring suburban communities of metropolitan Cape Town, South Africa. These communities have a geographic area of 2.42 km^2 and a population of 34,294 people of whom 99.7 percent are of the mixed race. The number of reported cases of tuberculosis per year in these two is very high (1000 cases per 100,000 population per year)[8]. The birth rate is 29.3 per 1000 population and the infant mortality rate is 38 per 1000 live births. In general, people live in poor socio-economic conditions, although most live in houses with running water and electricity. Two primary care clinics and an adjacent tertiary hospital serve the area.

Before 1996, when all the elements of the World Health Organisation (WHO) strategy of directly observed short-course treatment (DOTS) were implemented, all patients were treated at one of the primary care clinics by directly observed therapy with three drugs (patients needing treatment for a first episode [new patients]) or four drugs (patients needing treatment for a subsequent episode [returning patients]). Until 1996, there was no systematic surveillance for treatment failure, although for most patients a sputum sample was examined (directly and by culture) between the fourth and sixth months. With its implementation in 1996, the WHO DOTS strategy required an accurate recording of cases in clinic registers, the administration of directly observed therapy (four drugs for new patients and five drugs for returning patients), and surveillance for treatment outcome, including treatment failure. Except for multidrug-resistant infections, there was no surveillance for relapse.

Patients

Patients included in this study have had at least two episodes of postprimary pulmonary tuberculosis within the study period (between September 1992 and May 1998), with the cultures of the last two most recent successive episodes (referred to as first and second episode) available for restriction-fragment-length polymorphism (RFLP) analysis and with cure as the outcome of the first episode. Extensive clinical histories of the included patients were obtained and included data on age, sex, medical history, status with regard to infection with the human immunodeficiency virus (HIV),

findings on chest radiography, the results of sputum staining and cultures, drug sensitivities, treatment, and outcome. According to WHO criteria, cure was defined as the completion of a course of six to eight months of directly observed combination therapy (with isoniazid, rifampin, and pyrazinamide in a single tablet), compliance (attendance for the course of therapy, with at least 80 percent of prescribed doses taken), and sputum culture positive for *M. tuberculosis* at diagnosis and at least one negative sputum culture at the end of treatment. All patients who needed treatment for a subsequent episode but who did not meet the criteria for previous cure were excluded from the study.

M.tuberculosis Isolates

Sputum samples were stained and cultured at the laboratory that routinely served the clinics. Drug-susceptibility testing was performed by the indirect-proportion method in accordance with guidelines of the South African National Tuberculosis Program. In September 1992, in the Cape Town communities described above, we initiated a prospective study in which all cultures positive for *M. tuberculosis* from patients residing in these communities were genotyped by RFLP and a data base of the results was established. To evaluate the representatives of the patients included in the RFLP data base, they were compared with patients who had positive cultures in the district tuberculosis register from January 1996 through May 1998 with regard to the clinical classification. The percentage of cultures obtained for RFLP analysis was calculated for both new and returning patients. These data were also used to calculate the annual number of cases of culture-positive pulmonary tuberculosis per 100,000 population.

Isolates of *M. tuberculosis* were genotyped by RFLP according to an internationally standardized method,[9] and the results were analyzed with Gel Compar software (version 4.0 Applied Maths BVBA, Kortrijk, Belgium). For the RFLP data base, the extent of accumulated laboratory error was calculated by comparing successive DNA fingerprints for serial isolates collected during the first two months of treatment, according to the following formula: laboratory error rate equals the number of isolates with DNA fingerprints that failed to match the DNA fingerprints of the subsequent isolate, divided by the total number of serial isolates analyzed. To exclude the possibility of mixed

infection, DNA was isolated from the entire culture, and all RFLP patterns were carefully analyzed to identify any background bands reflecting different strains. Analysis of the complete RFLP data base identified only five isolates with background bands. The isolates were excluded from further analysis.

The isolates included in this study were analyzed as follows. Genomic DNA was digested with *Pvu*II or *Hin*fI, electrophoretically fractionated, and transferred to Hybond N^+ membranes (Amersham, Buckinhamshire, United Kingdom). The *Pvu*II digests were hydridized with an IS*6110* 3' probe (complementary to the IS*6110* domain between nucleotides 631 and 875) that has been labelled by enhanced chemiluminescence. The *Hin*fI digests were hybridized with a ^{32}P-labeled MTB484(1) probe.[10] The respective RFLP patterns were visualized by autoradiography. The IS*6110* 3' DNA fingerprints from each patient's isolated were compared with those in the complete RFLP data base (with data gathered from September 1992 through May 1998) by means of the clustering formula known as the "unweighted pair-group method using arithmetic averages" and the Dice coefficient to determine whether the isolates belonged to a cluster (suggesting infection by recent transmission) or were unique (suggesting reactivation of a latent infection) within the communities studied.

A patients whose isolates of *M. tuberculosis* from the first and second episodes of postprimary tuberculosis were identical on RFLP analysis with each DNA probe was considered to have tuberculosis due to reactivation of endogenous infection. A patient whose isolates from the first and second episodes of postprimary tuberculosis were different was considered to have tuberculosis due to a new, exogenous infection.

RESULTS

The rate of reported cases of culture-positive pulmonary tuberculosis from January 1996 through May 1998 was estimated to be very high (225 cases per 100,000 per year for all true incidence cases) (Table 1).

During the study period (September 1992 through May 1998), DNA from cultures of *M. tuberculosis* was available for al least one RFLP analysis for 698 patients. For the period from January 1996 (when reliable information became available for case

notification as a result of the implementation of the DOTS strategy) through May 1998, the average rate of recovery of cultures of *M. tuberculosis* for RFLP analysis was estimated to be 82 percent (83 percent for new patients, 67 percent for patients needing retreatment after cure and 84 percent for other patients needing retreatment) (Table 1).

Table 1: Distribution of patients with tuberculosis for whom cultures were available for analysis, according to source of information*.

Patients	RFLP data Base		District Register	Rate of culture recovery for RFLP†	Incidence of Culture-positiveCases
	September 1992 through May 1998	January 1996 through May 1998	January 1996 through May 1998		
	No. of patients			percent	no./100,000/yr‡
Total	770§	279	339	82	
New patients	461	179	210	85	225
Patients requiring retreatment after previous cure	48¶	16	24	67	26
Others requiring retreatment	261	88	101	84	

* RFLP denotes restriction-fragment-length polymorphism, and ND not determined.

† The rate of recovery of *M. tuberculosis* for RFLP analysis was estimated as the number of patients with results in the RFLP data base from January 1996 through May 1998 divided by the number of patients with episodes in the register for the same period.

‡ These rates were calculated only for new patients and patients needing retreatment after cure, since other patients needing retreatment do not represent true incidence rates.

§ This value includes 698 individual cases plus 72 samples from 55 patients from whom DNA was available for two or more treated episodes.

¶ This value includes patients from whom an RFLP analysis was available for an episode requiring treatment after a previous cure. For 16 of these cases, the RFLP result of the first episode was also available, and these patients thus constituted the study population.

DNA from at least one episode was available for RFLP analysis for 48 patients considered to have tuberculosis requiring retreatment after cure. However, in only 16 of these 48 patients were the results of the RFLP analysis for two episodes available, and these 16 patients thus met the requirements for inclusion in the study: two episodes of postprimary pulmonary tuberculosis within the study period and cure as the outcome of the first of those two episodes.

The median age of these 16 patients was 35 years; 9 were women and 7 were men (Table 2). The median interval between cure and subsequent diagnosis (isolation of a subsequent culture-positive specimen) was 25.5 months. Fifteen patients (94 percent) were tested for HIV infection, and all 15 tested negative. None of the patients had a medical history of diabetes, end-stage renal disease, or cancer or had been treated with immunosuppressive drugs. Chest radiographs revealed evidence of cavitary disease in 11 patients during the first episode of tuberculosis and in 12 during the second episode of tuberculosis.

Table 2: Epidemiologic and clinical characteristics of 16 patients with postprimary pulmonary tuberculosis after previous cure.*

Patient no.	Age (yr) /sex	Interval Between episodes†	Negative smear, Single positive culture		Exogenous reinfection
			Previous episode	subsequent episode	
1	25/F	28	yes	no	Yes
2	29/M	26	yes	no	yes
3	34/F	38	yes	no	yes
4	42/M	32	no	no	yes
5	36/M	19	yes	no	No (reactivation)
6	40/M	8	no	no	yes
7	47/F	7	no	yes	yes
8	50/M	21	no	no	yes
9	45/M	12	no	yes	yes
10	30/F	35	no	no	yes
11	39/F	31	no	no	No (reactivation)
12	30/F	52	yes	no	yes
13	28/F	35	yes	no	yes
14	54/M	25	no	no	yes
15	24/F	13	no	no	No (reactivation)
16‡	33/F	11	no	no	No (reactivation)

* For patients one through 14, RFLP patterns are shown in figure 1 For patients 13 and 14, the subsequent episode was diagnosed as multidrug-resistant tuberculosis, for patients 15 and 16, the subsequent episode was diagnosed as tuberculosis resistant only to isoniazid.

† His interval is the time between the cure of the previous episode and the diagnosis of the subsequent episode.

‡ patient not tested for HIV

For 12 of the 16 patients, the RFLP patterns of the strains of *M. tuberculosis* responsible for the disease differed between the two episodes, indicating exogenous reinfection (Table 2 and Fig. 1). For the other four patients the RFLP patterns of the *M. tuberculosis* strains were identical for the two episodes, indicating endogenous reactivation. Of the 16 patients, 4 had drug-resistant tuberculosis during the second episode (Table 2), which in 2 patients was caused by exogenous reactivation with a multidrug-resistant strain and in the other 2 patients (both resistant to only isoniazid) was caused by endogenous reactivation. In one of the latter two patients, drug-susceptibility testing performed during the first episode identified a fully drug-sensitive organism. However, during an episode before the two described in this study, resistance to isoniazid had been diagnosed. During the episode after cure, drug resistance was proved in only one of five cultures obtained, indicating that the resistance to isoniazid was borderline. In the other patient, no drug-susceptibility testing was performed on the isolate from the first episode.

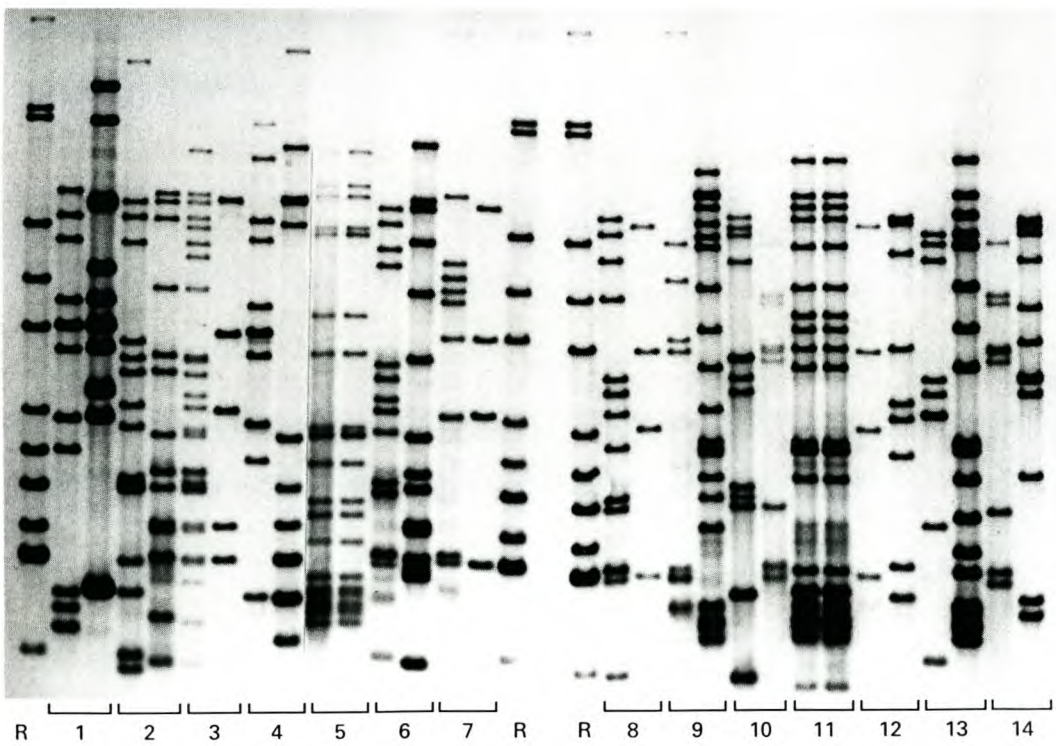

Figure 1. RFLP patterns of 14 pairs of *M. tuberculosis* isolates obtained from patients with recurrence of tuberculosis after cure. Lane 1 to 14 correspond to patient 1 to 14 respectively, lanes R are control strains (MTB 14323)

Because exogenous reinfection most likely results from close contact with an adult who has active infection, isolates from the 12 patients with reinfection were studied in relation to the complete RFLP data base for the two communities (covering September 1992 through May 1998 and including 698 patients). It was found that 11 of the 12 isolates obtained during the second episode of tuberculosis from patients who had exogenous reinfection belonged to a cluster of strains present in the communities; for the remaining isolate no matching strain was identified in the data base.

Of the four patients with tuberculosis due to reactivation, two were older than the median age and two younger. The interval from cure to reactivation was longer than the median interval in one patient and less than the median in the other three. All four patients were infected with a strain that belonged to a cluster circulating in the community during their respective disease-free intervals.

DISCUSSION

Using DNA fingerprinting, we found evidence that exogenous reinfection can have a dominant role in the pathogenesis of postprimary tuberculosis in adults in an area with a high incidence of the disease.[1,2,11] To our knowledge, there have been only two other reports of RFLP analysis of isolates from patients with repeated episodes of tuberculosis who were living in a high-incidence area.[12,13] These studies suggested that under conditions of endemic disease, the rate of endogenous reactivation far exceeds the rate of exogenous reinfection. However, these reports do not include a definition of cure or detailed information on the patients' drug regimens, compliance, or immune status. Furthermore, 15 patients (34 percent)[12] and 40 patients (49 percent)[13] were excluded because only a single culture was positive and the possibility of laboratory contamination was raised. If they had not been excluded, 90 percent of these patients would have been classified as having disease due to exogenous reinfection. Their exclusion might therefore have biased the results toward the importance of exogenous reactivation.

In our study, the rate of recovery of cultures of *M. tuberculosis* for RFLP analysis was lower for patients needing retreatment after cure than for other patients. The reason

for this difference is not clear; it was not the result of any deliberate policy. The results of the study must, however, be viewed with caution because of the possibility of some unintentional bias. Nevertheless, there is no reason to suppose that strains from cases due to reinfection would be preferentially recovered as compared with those from cases due to reactivation, a condition necessary for such bias.

In our study, we did not exclude patients with only a single positive culture, for the following reasons. It has been shown that tuberculosis lesions can yield an isolated positive culture.[14] There was no evidence of false positive culture: all the patients received a diagnosis of active tuberculosis for both episodes on the basis of positive sputum-test results, the findings on chest radiographs, and the clinical history. The complete RFLP data base was analyzed to assess the extent of laboratory contamination, and the rate of accumulated error was found to be only 3,4 percent (17 discordant results among 499 serial isolates). On the basis of this error rate, it is possible that results for 1 isolate (3.4 percent of 32 isolates) may have been erroneous. Therefore, we believe it is unlikely that the classification of disease as due to exogenous reinfection reflects extensive laboratory error, such as error due to cross-contamination or mixed infection at the first episode. This argument is further supported by the observation that in 11 of the 12 cases of exogenous reinfection, the strain responsible for reinfection was identified as one of a cluster of strains present in the communities. Only one isolate was retrieved from a patient with reinfection for whom there was no matching isolate in the entire data base. This patient appears to have been reinfected outside the communities or by an undetected source within the communities.

All four patients who were found to have disease due to reactivation were infected with individual strains that belonged to a cluster circulating within the communities during these patients' respective disease-free intervals. The molecular definition of exogenous reinfection used in this study excludes the possibility of exogenous reinfection with the same strain. Therefore, it is possible that some of the patients considered to have reactivation actually had new, exogenous infection with the same strain. Our results may thus underestimate the extent of exogenous reinfection.

It has generally been believed that reinfection is more difficult to confirm than primary infection, because of the immune response to *M. tuberculosis* antigens that

develops after primary infection. There are no empirical data on the changes in the level of immunity over time, but it is assumed that in immunocompetent persons, reinfection is rare during the first two to five years after a first infection.[2] In our study, cases of reactivation tended to occur soon after the previous episode was cured, but many of the cases due to reinfection also occurred early after a previous cure. It has been shown in persons infected with HIV that reinfection can occur not only years after a previous infection (or episode of disease) but even during treatment for active tuberculosis.[15] In our study, in which all the patients tested for HIV infection were negative, reinfection occurred as little as seven and eight months after a previous cure. These results suggest that in immunocompetent persons living in an area where tuberculosis is endemic, reinfection and progression to active disease may occur at any time after treatment has been discontinued.

In patients previously treated and cured, a subsequent episode would be expected to represent endogenous reactivation. We found that exogenous reinfection has a predominant role in this population of patients, who have multiple episodes of active tuberculosis. In areas with a high incidence of tuberculosis, exogenous reinfection might also be a cause of the first episode of postprimary tuberculosis, since the immunity that develops after primary infection followed by a period of latency cannot be expected to confer more protection against exogenous reinfection than the immunity that develops after an episode of active disease.

The controversy with regard to endogenous as opposed to exogenous pathogenesis of tuberculosis is of importance in the planning of clinical trials and national tuberculosis-control programs. If, in areas with a high incidence of the disease postprimary episodes of pulmonary tuberculosis after previous cure result predominantly from exogenous reinfection, as indicated by our results, the effectiveness of a drug regimen cannot be evaluated on the basis of the relapse rate without the additional information provided by RFLP analysis of bacterial isolates. In the evaluation of national tuberculosis-control programs for areas in which the disease is endemic, RFLP analysis can prove the effectiveness of the currently used treatment regimens. "Cure" in a patient who later has another episode of tuberculosis is not necessarily an incorrect concept. The more likely possibility is that he or she has a new infection after

the cure. The emphasis should thus be placed on achieving cure in patients and on prompt case detection to prevent re-exposure to sources of active tuberculosis.

ACKNOWLEDGEMENTS

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CHAPTER 5 ADDENDUM

EDITORIAL

Exogenous Reinfection in Tuberculosis

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Exogenous Reinfection In Tuberculosis

Tuberculosis, like all infectious diseases, involves exposure to a pathogen resulting in an asymptomatic period of incubation or latency that may progress to active disease. Unlike most other infectious diseases, tuberculosis involves a delay between infection and disease that is extremely variable, ranging from a few weeks to a lifetime. Therefore, the development of active tuberculosis in someone known to have been previously infected raises the question whether this represents a recrudescence of the initially infecting organism (endogenous reactivation) or a new strain of *Mycobacterium tuberculosis* (exogenous reinfection).

For decades, this question has been central to a debate in which the intensity of the sentiments seems to be inversely proportional to the quality of the supporting data. Publications dating to the 1920s reflect highly divergent views, from the assertion that reinfection "for all practical purposes... can be ignored"[1] to the contention that "the adult type of tuberculosis is, with few exceptions, the result of exogenous reinfection."[1] Currently, most medical textbooks refer to the unitary concept of pathogenesis, which states that the majority of cases of tuberculosis are a consequence of endogenous reactivation.[2] Yet there is persistent evidence that exogenous reinfection may also be an important contributor to the total tuberculosis burden.[3]

This question is of more than academic interest. If exogenous reinfection is common, the use of antituberculosis chemoprophylaxis in people who have recently been exposed to infectious tuberculosis may be prudent, regardless of whether they have evidence of prior infection. It would be dangerous to assume that elderly patients with tuberculosis have disease caused by infection they acquired before the widespread emergence of drug-resistant organisms. If exogenous reinfection, which is clinically indistinguishable from relapse, is common, then new regimens that effectively eliminate infection or treat disease will be unfairly judged in clinical trials. If natural infection does not confer protective immunity, the development of improved vaccines against tuberculosis will be especially challenging and the need for better understanding of host defenses against tuberculosis particularly critical. Perhaps most important, the achievable rate of decline in tuberculosis worldwide would be much faster if most cases

are due to recently acquired infection rather than to continual reactivation throughout the lifetime of the more than 1.5 billion persons already infected.

In elegant animal models, the presence of acquired immunity makes it more difficult to establish a new infection.[4] However, these types of data cannot tell us whether exogenous reinfection occurs under natural conditions in humans. Rapid advances in immunology and techniques such as the use of genetically altered animals now hold the promise of elucidating the biology of antituberculosis immunity -- information that is crucial for the development of improved vaccines.

There is anecdotal evidence that exogenous reinfection does occur in humans. For example, during an outbreak in a homeless shelter, there were four cases of exogenous reinfection in persons with previously documented tuberculosis infection or disease.[5] More recently, fingerprinting techniques for analysis of bacterial DNA have proved that reinfection can occur in both immunocompromised and apparently immunocompetent persons.[6,7] However, such anecdotal evidence cannot be used to answer the more important question about the relative contribution of exogenous reinfection within populations. More important, we have not been able to quantify the prevalence of reinfection in high-risk, resource-poor communities -- where the majority of cases are found today.

A report by van Rie et al. in this issue of the *Journal* provides the most dramatic data to date on the extent to which tuberculosis is attributable to reinfection.[8] The authors used DNA fingerprinting to examine isolates of *M. tuberculosis* obtained over a period of almost six years from 698 patients from a metropolitan area in South Africa that has one of the highest rates of tuberculosis in the world. They identified 16 patients with recurrent tuberculosis after curative therapy for whom complete data were available. Fifteen of these patients were known to be seronegative for the human immunodeficiency virus. After comparing the DNA fingerprints of bacilli isolated during the initial episode with those of bacilli isolated during the subsequent episode, the authors concluded that 12 patients had been exogenously reinfected with a different strain. This finding suggests that 75 percent (95 percent confidence interval, 50 to 94 percent) of the cases of postprimary disease were attributable to exogenous reinfection. This study is a model of the way in which population-based research, when integrated

with detailed information about patients and coordinated with a tuberculosis-control program, can provide basic insights and have important public health implications.

Some of the most important challenges in this type of research are to eliminate both sampling bias and the risk of cross-contamination in the laboratory. Although van Rie et al.[8] could identify no bias in their selection process, their analysis included only 16 of the 698 patients with a culture available for analysis during the study period and their results must therefore be interpreted cautiously. For example, if infections are transmitted at disproportionate rates in various health care settings, patients with exogenous reinfection may be overrepresented in an analysis that includes only patients known to have regularly attended clinics to receive observed therapy. The low overall rate of contamination (3.4 percent) in the study by van Rie et al. strongly supports their contention that laboratory error did not contribute to their results.

This study provides an important clue to resolving this age-old debate, but it is not the final word. The relative contributions of reinfection and reactivation are likely to depend on the epidemiologic context.[9] In populations at high risk for infection, people may have an appreciable chance of repeated infection, and thus reinfection may be a major contributor to the overall rate of tuberculosis in adults. However, in populations with a low risk of infection, such as those in developed countries, the likelihood of reexposure, let alone successful reinfection, is small, and thus most cases of postprimary disease in adults probably result from reactivation.

The realization that exogenous reinfection may account for much of the tuberculosis in a given region has implications for control of the disease. It is widely acknowledged that the most important recent advance in tuberculosis control has been the implementation of the strategy of directly observed short-course treatment (DOTS). Although the DOTS strategy is frequently assumed to consist simply of direct observation of treatment, it actually contains the essential elements for national control of tuberculosis. The program includes governmental commitment, the use of sputum smears for diagnosis, careful tracking of each patient, and measures to ensure an uninterrupted drug supply.[10] When successfully implemented, the DOTS strategy dramatically improves patient outcomes. By reducing the transmission of disease, this

approach should also decrease reinfection and thus will be the essential first step toward controlling tuberculosis in areas of high prevalence.

Much of the success and appeal of the DOTS strategy is attributable to the simplicity and broad applicability of its principles. However, a hallmark of the epidemiology of tuberculosis is its variability among populations, which may be reflected as differences in the proportions of disease caused by reinfection. Attempts to go beyond the DOTS strategy will require a more open-minded approach. Given the persistent toll of tuberculosis, the formulation of an array of specific interventions tailored to the diverse settings in which patients suffer will be one of the major public health challenges of the coming millennium.

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CHAPTER 6

Analysis of a limited number of gene codons can predict the majority of drug resistance of *Mycobacterium tuberculosis* in a high incidence community.

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Journal of Clinical Microbiology (*in press*)

Comparison of phenotypic and genotypic drug-resistance testing of *mycobacterium tuberculosis* in a middle income country

Correct and rapid diagnosis is essential in the management of multidrug-resistant tuberculosis (MDR-TB). This population based study of 61 patients with drug-resistant tuberculosis evaluated the frequency of mutations and compared the performance of a genotypic (mutation analysis by dot blot hybridisation) to a phenotypic (indirect proportion method) drug-resistance test. Three selected codons (rpoB531, rpoB526 and katG315) allowed identification of 90 % of MDR-TB cases. Ninety percent of rifampin, streptomycin and ethambutol resistance and 75% of isoniazid resistance was detected by screening for 6 codons: rpoB531, rpoB526, rrs513, rpsL43, embB306 and katG31. The performance (repeatability, sensitivity and specificity) of the genotypic method was superior to the routine phenotypic test with the exception of sensitivity for isoniazid resistance. A commercialised molecular genetic test for a limited number of target loci might be a good alternative for a drug-resistance screening test in the context of an MDR DOTS-plus strategy

INTRODUCTION

The emergence of drug-resistant strains of *Mycobacterium tuberculosis*, especially multidrug-resistant (MDR) strains, defined as resistant to at least isoniazid (INH) and rifampin (RIF)[1], poses a threat to the success of tuberculosis control programs. As a consequence of the increase in multidrug resistant tuberculosis (MDR-TB) and the relatively restricted number of therapeutic agents, there has been a renewed effort during the last decade to define the molecular basis of drug resistance in *M. tuberculosis*. Resistance to drugs is due to particular genomic mutations in specific genes of *M. tuberculosis*[2]. To date 9 genes are known to be linked to resistance to the first line antituberculous drugs: *katG*, *inhA*, *aphC*, *kasA* for INH resistance; *rpoB* for RIF resistance; *rpsL* and *rrs* for streptomycin(SM); *embB* for ethambutol (EMB)and *pncA* for pyrazinamide (PZA) resistance. Multiple drug-resistance is the consequence of an accumulation of mutations[3,4].

Under the current WHO guidelines for TB control in low and middle-income countries[5], diagnosis of new TB patients is based on examination of sputum smears by microscopy for the presence of acid-fast organisms. Cases of primary drug-resistant TB will thus be missed with consequent prolonged infectivity and further spread of drug-resistant tuberculosis. A new strategy, "DOTS plus"[6,7], which reinstalls *M. tuberculosis* culture and sensitivity testing at diagnosis, has been introduced in a few pilot projects. However, when drug-susceptibility testing is culture-based, detection will still take 2 to 9 weeks[6]. The molecular basis of drug resistance in *M. tuberculosis* makes it possible to create new, rapid diagnostic tests. Rapid detection of drug resistance could not only optimise the treatment and improve the outcome of patients with drug-resistant TB, but is especially important in the prevention of transmission of drug-resistant TB. When the first study on detection of mutations in clinical isolates was published, it was hoped that early detection of resistance in *M. tuberculosis* would soon be routine clinical practice[8]. Seven years later, mutation detection analysis is still not part of clinical practice. To be cost-effective in resource-poor countries where most MDR-TB patients reside, it will be crucial that molecular genetic tests fulfil the criteria of accuracy, speed and simplicity. The evaluation of the frequency distribution of various mutations in clinical isolates

originating from different geographical regions will be essential for the selection of a limited number of target mutations which enable the detection of the majority of drug resistance[9,10].

This study investigates the frequency of gene mutations in clinical isolates of *M. tuberculosis* originating from two communities of Metropolitan Cape Town (South Africa) with a high incidence of TB and documented outbreaks of MDR-TB within the studied communities[11]. This site, a high TB incidence area of a middle income country, provides the possibility of evaluating the clinical usefulness of a genotypic method compared to the culture-based phenotypic drug susceptibility test under routine conditions.

MATERIALS AND METHODS

Setting

The patients described in this paper were identified as active cases of drug resistant tuberculosis (on the basis of culture based drug susceptibility tests) between 1 April 1992 and 31 March 1997. All patients resided in two neighbouring communities of metropolitan Cape Town (Western Cape Province, South Africa), a 2.4 km^2 area with a population of approximately 34,000 people living in poor socio-economic conditions. The rate of new bacteriologically confirmed cases in these communities is 225/100.000/year[12]. A survey of drug-resistant tuberculosis in the Western Cape province conducted in 1992-1993 found a rate of 8.6% acquired and 3.2% initial drug resistance in the West Cape region[13]. The reported prevalence of HIV infection in the region ranged from 0.25% in 1992 to 3 % in 1996 (data from the National HIV surveys on women attending antenatal clinics, conducted by the Department of Health and Population Development, South Africa).

All patients were treated by direct observation at the local primary health care clinics, 62% also received inpatient care. Compliance during treatment was defined as the intake of > 80% of prescribed dosages before interruption or completion of treatment.

Laboratory procedures

Sputum samples were sent for microscopy and culture to the routine laboratory, which is the provincial reference laboratory for drug susceptibility testing. Phenotypic drug susceptibility testing was performed by the economic version of the indirect proportion method[14] on Löwenstein-Jensen medium containing critical concentrations of 0.2 μg/ml isoniazid, 30 μg/ml rifampin, 2 μg/ml ethambutol, 5μg/ml streptomycin and 20μg/ml ethionamide. Resistance was defined as 1% or more bacterial growth in comparison with a control using international criteria.

Genotypic drug resistance testing was performed by mutation analysis according to a recently described PCR based dot-blot method[15]. The probes and primers used in this study were directed towards mutations most frequently described in the literature. All samples were tested for mutations at the following loci: *katG* 315, *kasA*269, *inhA* -10 and -34, *rpoB* 531,526 and 516, r*psL* 43 and 88, *rrs* 513, 491 and *embB*. The reference strain H37Rv, 10 fully susceptible isolates and isolates characterised by gene sequencing as mutant (resistant) or wild type (susceptible) for specific gene loci were used as negative and positive controls. In cases where resistance could not be explained by the identification of mutations in the above gene codons, samples were also tested for additional codons (*katG* 275, 409; *kasA* 66, 312, 413; *inhA*-15, *rpoB* 533, 513 and *rrs* 904). Direct sequencing of selected PCR products was performed with the Sequenase PCR Product sequencing kit (United States Biochemical, Cleveland, USA) according to the manufacturers' instructions.

M. tuberculosis isolates were also genotyped by RFLP analysis[16]. A cluster was defined as a group of two or more isolates originating from different patients whose RFLP fingerprint patterns were identical with respect to both the number and molecular size of all bands[17].

For each patient, the first and last available isolates were defined as the first and last isolate for which DNA was available for mutation detection and RFLP analysis. The results of the first available isolates were used to evaluate the frequency of mutations and thereby compare the clinical usefulness of phenotypic and genotypic methods. The

result of the last available isolate was used to determine the acquisition of additional mutations during treatment.

A discrepancy between the phenotypic and genotypic drug-resistance test of the first available isolate was defined as a false positive result of the genotypic method if the isolate was predicted to be resistant by mutation analysis but phenotypically drug susceptible. A false negative result of the genotypic method was defined as a phenotypically resistant isolate in which no mutations conferring resistance were detected.

In cases of discrepancies, the infecting *M. tuberculosis* isolate was retested by phenotypic and genotypic method if no follow-up isolate was available. Retesting by phenotypic method was performed using a different method (BACTEC) at a different laboratory (Department of Biochemistry, University of Stellenbosch). In cases of discrepancies where at least one follow-up isolate was available, the result of the follow-up isolate(s) was used to re-evaluate the discrepancy of the first isolate.

After investigation of all discrepancies, the "corrected pattern" for each of the first available isolates was determined using a method similar to the method used by Telenti et al.[18]. This "corrected pattern" was used as the "gold standard" to evaluate and compare the intrinsic characteristics such as the sensitivity and specificity of both phenotypic and genotypic drug-resistance testing.

Results of drug-susceptibility tests of consecutive isolates of individual patients were used to determine the repeatability of both phenotypic and genotypic tests by calculating the Kappa coefficient[19]. Pairs of isolates retrieved from either a single clinical episode or from different clinical episodes but caused by the same *M. tuberculosis* strain as determined by RFLP analysis were included in this analysis. Pairs of isolates where the first isolate was drug susceptible and the following isolate drug resistant were discarded for this analysis as this might not represent a lack of intra-subject variation but acquisition of resistance during treatment.

RESULTS

Patient Characteristics:

Between April 1992 and March 1997, 70 patients were identified with isolates phenotypically resistant to one or more antituberculous drugs. In 61 of these, a minimum of one culture was available for molecular genetic analysis. These 61 patients constitute the study population. All patients had pulmonary tuberculosis and 84% were smear positive. Half of the patients were female (31 or 51%). The mean age at diagnosis of drug-resistant tuberculosis was 33 years (range 11 to 55 years). Fifty-two patients (85%) were tested for HIV and all were seronegative. Thirty-five patients (57%) were compliant during their MDR-TB treatment.

Phenotypic resistance pattern of first available *M. tuberculosis* isolates

The resistance pattern was determined by phenotypic drug susceptibility tests (table 1 and 2). In 34 (56%) patients, the first isolate available for this study was the isolate on which the diagnosis of drug resistance was based. In 2 patients (case 41 and 42), resistance was detected by mutation analysis on an isolate predating the isolate on which the diagnosis of drug resistance was made by phenotypic method. Resistance was detected in 57 (93%) cases for INH, in 34 (56%) cases for RIF, in 25 (41%) cases for SM and in 11 (18%) for EMB. Thirty-two (52%) cases were diagnosed as multidrug-resistant TB.

Additional resistance acquired during treatment was diagnosed as in 40% (n= 25) of the patients (table 1). The additional resistance acquired was for INH (n=2), EMB (n=8), RIF (n=10) and SM (n=14).

RFLP data

Six clusters of drug-resistant strains and 23 unique strains were identified. Clusters 1 (19 *IS6110* insertion elements with a pattern resembling "strain W"[20]) and cluster 2 (5 *IS6110* insertion elements) represent the predominant type of drug-resistant strains in these communities.

Table 1: RFLP classification, phenotypic, genotypic and corrected drug-resistance pattern in 61 patients

Patient	RFLP pattern	FIRST AVAILABLE ISOLATE						Interval to last isolate (days)	LAST ISOLATE		Patient compliant
		Phenotypic resistance	mutation analysis				Correct pattern		Additional resistance	Additional Mutations	
			isoniazid	rifampin	Strepto-mycin	ethamutol					
1	cluster 1	H, R	*katG315*	*rpoB531*	*rrs513*	*embB306*	H, R, S, E	323	S, E		yes
2	cluster 1	H,R,E	*katG315*	*rpoB531*	*rrs513*	*embB306*	H, R, S, E	176	S		yes
3	cluster 1	H, R, S, E	*katG315*	*rpoB531*	*rrs513*	*embB306*	H, R, S, E	399			no
4	cluster 1	H, S	*katG315*	*rpoB531*	*rrs513*	*embB306*	H, R, S, E	NA	R, E		yes
5	cluster 1	H, R, S, E	*katG315*	*rpoB531*	*rrs513*	*embB306*	H, R, S, E	NA			yes
6	cluster 1	H, R, S, E	*katG315*	*rpoB531*	*rrs513**	*embB306*	H, R, S, E	302			no
7	cluster 1	H, R, S, E	*katG315*	*rpoB531*	*rrs513*	*embB306*	H, R, S, E	175			no
8	cluster 1	H, R, S, E	*katG315*	*rpoB531*	*rrs513*	*embB306*	H, R, S, E	77			no
9	cluster 1	H, R, S	*katG315*	*rpoB531*	*rrs513*	*embB306*	H, R, S, E	346	E		yes
10	cluster 1	H, R	*katG315*	*rpoB531*	*rrs513*	*embB306*	H, R, S, E	NA	S		yes
11	cluster 1	H, R, S, E	*katG315*	*rpoB531*	*rrs513*	*embB306*	H, R, S, E	277			yes
12	cluster 1	H, R, S	*katG315*	*rpoB531*	*rrs513*	*embB306*	H, R, S, E	NA	E		yes
13	cluster 1	H, R, S, E	*katG315*	*rpoB531*	*rrs513*	*embB306*	H, R, S, E	NA			yes
14	cluster 1	H, R	*katG315*	*rpoB531*	*rrs513*	*embB306*	H, R, S, E	269	S, E		no
15	cluster 1	H, R,S, E	*katG315*	*rpoB531*	*rrs513*	*embB306*	H, R, S, E	NA			no
16	cluster 2	H	*katG315d*	*rpoB531**	*rpsL43*		H, R, S	363	R, S		yes
17	cluster 2	H, R, S	*katG315d*	*rpoB531*	*rpsL43*		H, R, S	NA			yes
18	cluster 2	H, R, S	*katG315d*	*rpoB531*	*rpsL43*		H, R, S	394			no
19	cluster 2	H, R, S	*katG315d*	*rpoB531*	*rpsL43*		H, R, S	215			yes
20	unique	H, S	*katG315d*	*rpoB516**	*rpsL43*		H, R, S	1026	R		no
21	cluster 2	H, R, S	*katG315d*	*rpoB531*	*rpsL43*		H, R, S	NA			yes
22	cluster 2	H, R, S	*katG315d*	*rpoB531*	*rpsL43*		H, R, S	660	E		no
23	cluster 2	H, S	*katG315d*	*rpoB533**	*rpsL43*		H, S	201			no
24	cluster 2	H, R, S	*katG315d*	*rpoB531*	*rpsL43*		H, R, S	NA			yes
25	cluster 2	H, R, S	*katG315d*	*rpoB531*	*rpsL43*		H, R, S	NA			yes
26	cluster 2	H, R	*katG315d*	*rpoB531*	*rpsL43*		H, R, S	444	S		no
27	cluster 3	H, R	*katG315*	*rpoB531*			H, R	NA			yes
28	cluster 3	H, R	*katG315*	*rpoB531*			H, R	1450	S		yes
29	cluster 3	H, R	*katG315*	*rpoB531*			H, R	14			yes
30	cluster 4	H		*			none	124	R	*rpoB526**	no
31	cluster 4	H					H	493			yes
32	cluster 4	H, R		*rpoB531*	*rpsL43*		H, R, S	NA			no
33	cluster 4	R		*rpoB531*			R	1188	H	*inhA-10*	no
34	cluster 5	H, R, S	*katG315*	*rpoB531*	*rpsL43*	*embB306**	H, R S, E	NA			yes
35	cluster 5	H, R	*katG315*	*rpoB526*	*rpsL43*		H, R, S	NA	S		yes
36	cluster 5	H, R,S	*katG315*	*rpoB526*	*rpsL43*		H, R, S	103			no
37	cluster 6	H	*katG315*				H	NA			yes
38	cluster 6	H	*katG315*		*rrs491*		H, S	332	S		yes
39	unique	H	*	*rpoB518d**			H	75			yes
40	unique	H					H	NA			no
41	unique	none	*katG315d*	*rpoB531*			H, R	2154	H, R, S		no
42	unique	none		*rpoB531**			R	NA	R		no
43	unique	H	*katG315*				H	NA			yes
44	unique	H, R, E				*embB306*	H, R, E	92	S	*rpoB526*	no
45	unique	R	*InhA-15**	*rpoB531*			R	NA			yes
46	unique	H, S	*katG315*				H, S	253	E		yes
47	unique	H					none	268		*rpoB516*	no
48	unique	H					H	613	R, S	*rpoB531*	no
49	unique	H					H	NA			no

Table 1: continued

Patient	RFLP pattern	FIRST AVAILABLE ISOLATE						Interval to last isolate	LAST ISOLATE		Patient compliant
		Phenotypic resistance	mutation analysis				Correct pattern		Additional resistance	Additional Mutations	
			isoniazid	rifampin	Strepto-mycin	ethambutol					
50	unique	H, R, E		*rpoB516**		*embB306*	H, R, E	683	S	*inhA-34*	no
51	unique	H					H	NA			yes
52	unique	H, S	*katG315*	*			H,S	131	R	*rpoB526*	yes
53	unique	H					H	NA			yes
54	unique	H		*rpoB531*			H, R	307	R	*rrs491*	no
55	unique	H			*rrs491*		H	669			yes
56	unique	H	*kasA269**				H	NA			no
57	unique	H,R			*rrs491*		none	NA			
58	unique	H					H	NA			yes
59	unique	H	*katG315*	*rpoB526*	*rpsL43*	*embB306*	H, R, S, E	1056	S, E, R		yes
60	unique	H	*katG315*				H	NA			yes
61	unique	H, S	*katG315d*		*rrs513*	*	H, S	NA			no

Legend:

Definition of abbreviations: H and INH = isoniazid, R and RIF = rifampin, S and SM = streptomycin, E and EMB = ethambutol.

katG315=AGC to ACC mutation, katG315d=AGC to ACA mutation, katG315d*=katG315 + inhA-10 mutation

* indicates that the presence or absence of a specific mutation has been confirmed by gene sequencing.

Table 2: Number (%) of resistant first isolates as determined by different methods

	isoniazid	rifampin	streptomycin	ethambutol	isoniazid + rifampin
Phenotypic test	57 (93)	34 (56)	25 (41)	11 (18)	32 (52)
Genotypic test	43 (70)	41 (62)	35 (57)	19 (31)	34 (56)
Corrected	55 (90)	40 (66)	35 (57)	19 (31)	37 (61)

Genotypic analysis of first available *M. tuberculosis* isolates (table 1 and 2).

Mutations in genes conferring resistance to INH were detected in 43 (70%) cases. The most frequent mutation associated with INH resistance was codon 315 in the *katG* gene (41/43, 95%). One isolate (2%) had a mutation in *inhA*-10 and one isolate had a *kasA*269 mutation. A *katG*315 mutation was present in 1 of 4 (25%) isolates classified as phenotypically susceptible to INH. No mutations could be detected in 16 of 57 (28%) isolates phenotypically resistant to INH.

Mutations in genes conferring resistance to RIF were identified in 41 (62%) cases. Codon 531 of the *rpoB* gene (34/41, 83%) was the most frequent mutation associated with RIF resistance. Other mutations detected were *rpoB*526 (3/41, 7%), *rpoB*516 (2/41, 5%), *rpoB*533 (1/41, 2%) and *rpoB*518 (1/41, 2%). Nine of the 41 (22%) isolates with *rpoB* mutations were phenotypically classified as susceptible to RIF. No *rpoB* mutations could be detected in 2 of 34 (6%) isolates phenotypically classified as RIF resistant.

A total of 34 of the 61 isolates (56%) demonstrated mutations of both *katG* and *rpoB* genes. Seven of these isolates were phenotypically not classified as multidrug resistant. Mutations conferring resistance to both isoniazid and rifampin were absent in 4 of 32 (12%) isolates phenotypically classified as multidrug-resistant.

Mutations in genes conferring resistance to SM were detected in 35 (57%) cases. The most frequent mutations conferring resistance to SM were found at position 513 of the *rrs* gene (16/35, 46%) and codon 43 of the *rpsL* gene (16/35, 46%). Three isolates (8%) had a mutation in at position 491 of the *rrs* gene. Mutations were present in 12 of the 36 (33%) isolates phenotypically classified as susceptible to SM. Gene mutations could not be detected in 2 of 25 (8%) phenotypically SM-resistant isolates.

Codon 306 in the *embB* gene was the only codon screened for EMB resistance. A mutation was detected in 19 (31%) cases. Mutations were present in 8 of 50 (16%) isolates phenotypically susceptible to EMB. *EmbB*306 mutations were detected in all 11 isolates phenotypically resistant to EMB.

Except for one drug susceptible control isolate with a *rrs* 491 mutation, none of the other 9 susceptible isolates or the H37Rv control strain had mutations in the screened genes.

Investigation of discrepancies between phenotypic and genotypic tests of the first available *M. tuberculosis* isolates

Twenty discrepancies (INH n=16, SM n=2, RIF n=2) represented potential false negative results of mutation analysis, according to the definition outlined in the methods section. Retesting by genotypic method confirmed prior results in all cases. Ten of these cases were confirmed resistant according to the result of a phenotypic test of follow-up

isolates (INH n=7, SM n=2, RIF n=1). Upon retesting of the remaining 10 isolates by phenotypic method, 4 isolates were confirmed resistant to INH. Two isolates could not be retested as they had lost viability. For further analysis, the original phenotypical result of these 2 isolates was considered correct. Four isolates were found to be drug susceptible on retesting by phenotypic method (INH n=3, RIF n=1). In conclusion, after investigation of 20 discrepancies, 16 cases of false negative results of mutation analysis remained. False negative results occurred predominantly in cases of INH resistance.

There were 31 (SM n=12, RIF n=9, EMB n=8, INH n=2) potential false positive results of mutation analysis, according to the definition proposed in the methods section). The presence of a mutation was confirmed in follow-up isolates (n=21) or upon retesting of the first isolate (n=10). Phenotypic resistance was detected on the next available follow-up isolate in 23 cases. The genotypic classification of the first isolate as resistant was considered correct in the cases (n=23) where the mutation present in the first isolate conferred for the resistance phenotypically detected in the follow-up isolate. Five cases were classified as drug suceptible because follow-up isolates remained drug susceptible. The three remaining cases were phenotypically retested. Case 10 was found to be resistant to EMB. Case 32 was found to be resistant to 2 and 4 μg of SM but susceptible to 8 μg of SM. Case 34 was found to be resistant to 2.5 μg of EMB but susceptible to 5 and 10 μg of EMB.

In conclusion, after investigation of the 31 discrepancies, 5 cases of false positive results of mutation analysis remained. False positive results occurred in cases with rare mutations such as *rpoB*533, *rpoB*518d, *rrs*491 and *inhA*-15.

Additional resistance acquired during treatment

Additional resistance acquired during treatment (as determined by phenotypic method) was present in 25 cases (40%) (Table 1). Of these patients, 46% (n=12) were non-compliant during treatment. The mutation conferring the "additional acquired" resistance was already present in the first available isolate in 9 of 14 cases (64%) of additional resistance to SM, 7 of 11 cases (64%) of additional resistance to RIF, 6 of 8

cases (75%) of additional resistance against EMB and in 3 of 4 cases (75%) of additional resistance to INH.

In 35 patients, a follow up isolate was available for genotypic analysis (Table 1). The time between the first and last isolate was on average 485 days (range 14-2145) days. First and last available isolate possessed the same RFLP pattern in all cases. An additional mutation was detected in 8 of the 35 (23%) cases. Seven of these 8 cases (88%) with an additional mutation on follow-up were non-compliant.

Performance of phenotypic and genotypic test

After correction for errors in phenotype assessment and mutation analysis, the resistance pattern of each isolate was re-classified (Table 1 and 2).

Isolates were correctly classified as MDR in 90 % of cases by genotypic method and in 84% of cases by phenotypic method. Phenotypic resistance was detected in 98%, 83%, 71% and 58% of all isolates resistant to respectively INH, RIF, SM and EMB according to the correct classification. The diagnosis of resistance of the first isolate by phenotypic method was thus missed in 2% (n=1) of cases resistant to INH, 17% (n=7) of cases resistant to RIF, 29% (n=10) of cases resistant to SM and 42% (n=8) of cases resistant to EMB. Genotypic resistance was detected in 76%, 98%, 94% and 100% of all isolates resistant to respectively INH, RIF, SM and EMB according to the correct classification. Detection of resistance was thus missed in 24% (n=13) of cases resistant to INH, 2 % (n=1) of cases resistant to RIF and 6% (n=2) of cases resistant to SM. The lowest yield of mutation detection (31%) was for isolates mono-resistant to INH.

Repeatability, sensitivity and specificity of each method were evaluated for each drug using the corrected classification as the "gold standard" (Table 3). Genotypic analysis had a repeatability of 100 % (Kappa 1.0) for all drugs tested (no discrepancies between 151 follow-up isolates of 35 individual patients). Repeatability of the phenotypic method was evaluated in 246 follow-up isolates of 54 patients and found to be fair for INH and EMB (Kappa 0.43 and 0.49) and good for RIF and SM (Kappa 0.68 and 0.6). Except for INH, sensitivity (ability to detect true drug resistance) was lower

for phenotypic test while the specificity (ability to detect true drug susceptibility) was lower for the genotypic tests.

Table 3: Repeatability and validity of phenotypic and genotypic drug resistance testing

	isoniazid		**rifampin**		**streptomycin**		**ethambutol**	
	P	G	P	G	P	G	P	G
Repeatability (Kappa coefficient)	0.43	1	0.68	1	0.6	1	0.49	1
Sensitivity (%)	98	76	83	98	71	94	58	100
Specificity (%)	50*	83*	95	91	100	92	100	100

P: phenotypic test (economic variation of the indirect proportion method)

G: genotypic test (mutation analysis)

* Results cannot be interpreted correctly because of the high prevalence (90%) of INH resistance in the study population

DISCUSSION

This population-based study investigated 61 patients diagnosed with drug-resistant TB by conventional drug susceptibility methods. The analysis of the results focussed on the first available isolate of each patient, as this is the most important isolate for patient management. We did not correct for the number of strains involved in an outbreak because the detection of resistance is important for clinical management, independent of the classification of an isolate as clustered or unique. In contrast to most studies, where phenotypic tests are performed in a high-quality national, supranational or research laboratory, the phenotypic tests for this study were performed in a routine provincial laboratory to approximate everyday reality of *M. tuberculosis* drug-resistance testing in a middle-income country. As done previously by Telenti et al[18], we did not use the phenotypic test as the gold standard but the "corrected" version of resistance patterns obtained by investigation of discrepancies between phenotypic and genotypic tests. In this way, the performance of both phenotypic and genotypic test could be evaluated and compared.

In this study, genotypic tests for only 3 selected codons (*rpoB*531, *rpoB*526 and *katG*315) allowed correct identification of 90 % of all MDR-TB cases from the studied

communities. Furthermore, more then 90% of resistance to RIF, SM and EMB and 75% of resistance to INH could be detected by screening for only 6 gene codons: *rpoB*531, *rpoB*526, *rrs*513, *rpsL*43, *embB*306 and *katG*315. Except for position *rrs513*, this array of loci is reported in the literature as the most frequently mutated loci[2,10,21-24]. The only category with a low yield of mutation analysis (31%) was for isolates mono-resistant to INH. The genotypic method was superior to the phenotypic method in the correct identification of resistance to RIF, streptomycin and EMB while the phenotypic method was superior in the correct identification of INH resistance.

An important question in the evaluation of a new diagnostic test is the advantages and disadvantages as compared to the existing tests. Sensitivity, specificity and repeatability are important elements in this comparison, besides complexity, labour intensiveness, and turnaround time for results and cost. The repeatability was excellent for the genotypic tests while fair to good for the phenotypic tests. Under such conditions, phenotypic tests will be evaluated as relatively unreliable by the health professional and repeated several times to ensure correct clinical management while one can rely on a single positive molecular genetic test. This reduces the total time to diagnosis of molecular genetic test even further as compared to phenotypic tests. In this study, resistance would have been detected by molecular genetic tests on an isolate prior to the phenotypic test in 2% of INH-resistant cases, 17% of RIF-resistant cases, 29% of SM-resistant cases and 42% of EMB-resistant cases.

The poorer performance of phenotypic tests could serve as an explanation for the discrepancy between the acquisition of phenotypical resistance during therapy (25 cases) compared to the acquisition of an additional mutation (8 cases). This speculation is supported by the fact that 88% of patients acquiring an additional mutation while on treatment were non-compliant compared to only 46% of non-compliance among patients who phenotypically acquired additional resistance.

The specificity of both methods was high for RIF, SM and EMB. The specificity for INH-resistance could not be interpreted because of the extremely high prevalence (90%) of INH-resistance in the population studied. The sensitivity (detection of true drug resistance) of the molecular genetic test was superior to the phenotypic test for

detection of resistance to RIF, SM and EMB while the sensitivity of the phenotypic test was superior for detection of INH resistance.

There remain, however, major limitations to the molecular genetic detection of drug resistance[25]. (i) Molecular genetic tests only detect the mutations that are screened for while phenotypic test detects resistance independent of the underlying mechanism. (ii) Not all mutations conferring resistance to antituberculous drugs are known. This is especially a problem in the detection of INH resistance and explains the low sensitivity of the genotypic method for INH-resistance testing. (iii) Only a few mutations conferring resistance to second-line drugs are known. (iv) The causal relationship between the presence of a mutation and the occurrence of resistance has been shown for some mutations, for example codon 315 in the *katG* gene[26], but a causal relationship has not been reported for all mutations currently believed to confer resistance. In our study, false positive results were obtained with *rpoB*533, *rpoB*518, *rrs*419 and *inhA*-15, mutations that are infrequently described. It is possible that these are silent mutations. Analysis of the rrs491 mutation at our laboratory showed that the *rrs*491 mutation was also detected in several pansusceptible isolates. The mutation responsible for a low-grade resistance to EMB in case 34 was explained by a rare embB306 mutation (ATG to GTG). Further research should therefore be directed at establishing causal relationships between specific mutations and drug resistance.

The high sensitivity in combination with rapid diagnosis and high reliability of genotypic drug-resistance testing are important as it will allow correct patient management within days of TB diagnosis. The detection of 90 % of cases of MDR-TB by screening only 3 codons (*rpoB*531, *rpoB*526 and *katG*315), the correct identification of more then 90% of resistance to RIF, SM and EMB by screening 5 gene loci (*rpoB*531, *rpoB*526, *rrs*513, *rpsL*43, *embB*306) and correct identification of 75 % of isoniazid resistance by screening for 1 locus (*katG*315) is promising for the development of a cost-effective commercial screening test. Although molecular genetic testing cannot as yet (and probably will never) fully replace the traditional phenotypic susceptibility tests, a commercialized molecular genetic test for a limited number of target codons might be a good alternative for a drug-resistance screening test in the context of a MDR DOTS-plus strategy.

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CHAPTER 7

Transmission of a multidrug-resistant *Mycobacterium tuberculosis* strain resembling "Strain W" among non-institutional, HIV seronegative patients

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Transmission of a Multidrug-Resistant *Mycobacterium Tuberculosis* Strain Resembling "Strain W" Among Non-Institutional, HIV Seronegative Patients

Since 1990, several outbreaks of multidrug-resistant tuberculosis (MDR-TB) have been described among institutionalised patients infected with human immunodeficiency virus (HIV). We describe a community MDR-TB outbreak among HIV-seronegative patients in Cape Town, South Africa. Isolates were characterised by restriction length polymorphism (RFLP) analysis and dot-blot hybridisation analysis of mutations conferring resistance for isoniazid, rifampin, streptomycin and ethambutol. The outbreak involved 16 patients of which twelve could be linked epidemiologically. All isolates were identical on RFLP analysis. In 2 patients, RFLP analysis showed exogenous reinfection during or after treatment for drug susceptible tuberculosis. Mutation analysis confirmed the genotypic identity of the isolates. The infecting strain was genotypically related to strain W, which was responsible for the majority of MDR-TB outbreaks in New York City. Transmission of MDR-TB is thus not limited to HIV seropositive patients in an institutional setting but occurs within a community.

INTRODUCTION

Clinical drug resistance in tuberculosis can be classified as acquired resistance, when drug-resistant mutants are selected as a result of ineffective treatment, or as primary resistance, when a patient is infected with a resistant strain. Mutations in the genome of *Mycobacterium tuberculosis* that can confer resistance to antituberculous drugs occur spontaneously, with an estimated frequency of 3.5×10^{-6} for isoniazid and 3.1×10^{-8} for rifampin. Because the chromosomal loci responsible for resistance to various drugs are not linked, the risk of a double spontaneous mutation is extremely small: 9×10^{-14} for both isoniazid and rifampin[1]. Multidrug-resistant tuberculosis (MDR-TB), defined by the World Health Organization (WHO) as resistance to, at least, isoniazid and rifampin, will thus occur mainly in circumstances where sequential drug resistance follows sustained treatment failure.

Reports of nosocomial outbreaks of MDR-TB in institutions such as hospitals[2-5] and prisons[6] in the United States, Europe[7] and developing countries[8] have focused attention on MDR-TB as a major health issue. More than 80 % of these patients were seropositive for the human immunodeficiency virus (HIV). Infection and a few cases of active MDR-TB in health care workers following exposure to patients with MDR-TB[9-11] and limited spread of a nosocomial outbreak into a community[12,13] have also been reported. But even in these settings, most patients with active MDR-TB were immunocompromised.

Restriction fragment length polymorphism (RFLP) analysis of *M. tuberculosis* strains was used to study the disease dynamics of most MDR-TB outbreaks.[3,4,6-8,10,12,13] This technique not only confirmed transmission between patients with suspected epidemiological connection but also suggested transmission between patients not suspected to have epidemiologic links.

The most extensive MDR-TB outbreak reported to date consists of 267 cases infected with an isolate of *M. tuberculosis* with an RFLP pattern identical or closely related to strain W . Eighty-six percent of these patients were HIV positive. Seventy

percent of these patients could be epidemiologically linked, and the locations of transmission were a hospital (96 %), correctional system (2%) and community (3%).[14]

An opportunity for these outbreaks seemed to be created by the cohabitation of infectious MDR-TB patients with highly susceptible immunocompromised patients, in settings with inadequate infection control procedures. HIV is postulated as the major factor that amplified, accelerated and characterized MDR-TB outbreaks.[1]

This report documents the genotypic characterization and spread of a "strain W-like" multidrug-resistant *M. tuberculosis* strain in an urban South African community among noninstitutionalized, HIV-seronegative individuals and challenges the view that MDR-TB outbreaks only occur in very specific population groups.

MATERIALS AND METHODS

The patients described in this paper were given their diagnose between 1993 and 1997 and resided in 2 neighboring communities of metropolitan Cape Town, South Africa. This 2.5-km^2 area has a population of ~34000 people, a reported TB notification rate >1000/100,000/year[15], and ~150 *M. tuberculosis* culture-positive patients/year. Although the socio-economic living conditions are poor, there is no homelessness and no evidence of intravenous drug use. During the study period, there was a low prevalence of HIV infection in the Western Cape Province (ranging from 0.25% in 1992[16] to 3 % in 1996[17]).

Sputum samples were collected at the primary health care clinics of the 2 communities studied and were sent to the routine laboratory for staining and culture. Drug susceptibility testing was done by the indirect proportion method on Löwenstein-Jensen medium containing critical concentrations of 0.2 µg/mL isoniazid, 30 µg/mL rifampin, 2 µg/mL ethambutol, 5µg/mL streptomycin and 20µg/mL ethionamide. Kanamycin and thiacetazone were tested on Middlebrook 7H10 agar containing critical concentrations of, respectively, 5 and 2 µg/mL. Resistance was defined as 1% bacterial growth in comparison with a control by use of international criteria. Cultures resistant to isoniazid or rifampin were subsequently tested for resistance to other antituberculous drugs. Resistance to pyrazinamide was not tested.

Since late 1992, as part of a prospective study, positive cultures for *M. tuberculosis* from patients residing in these communities were genotyped by RFLP analysis (IS*6110*) according to the standardized method[18] and analyzed using Gel Compar 4.0 software (Applied Maths BVBA, Kortrijk, Belgium). This study has had a recovery rate of ~70% of positive cultures and resulted in an RFLP database consisting of 650 patients. Cluster analysis (using the Dice coefficient and an unweighted pair group method [UPGMA] clustering formula)[19] was applied to identify possible clusters of transmission. Since late 1992, 107 clusters of drug-sensitive isolates (average cluster size, 4.7; range, 2-47) and 6 clusters of drug-resistant isolates (cluster size ranging from 2 to 16) have been identified. These data were then combined with a standardized set of clinical data (date of birth, sex, history of previous TB treatment and drug sensitivity).

A cluster of identical isolates (19 IS*6110* insertion elements) was subjected to further genotyping as follows: genomic DNA was restricted with P*vu*II and *Hin*fI, electrophoretically fractionated and transferred to Hybond-N (Amersham, UK). The P*vu*II digests were sequentially hybridized with enhanced chemoluminescence-labeled IS*6110*-3' probe (complementary to the IS*6110* domain between nucleotides 631 and 875), IS*6110*-5' probe (complementary to the 5' IS*6110* domain between nucleotides 77 and 462), ^{32}P-labelled DRr probe and ISL*540* (GenBank accession number U60566). The H*inf*I digests were hybridized with ^{32}P-labelled MTB484(1) probe[20]. The respective RFLP patterns were visualized by autoradiography.

This cluster of isolates was analyzed for the following mutations: *katG*315 conferring isoniazid resistance and the silent mutation *katG*463; *rpoB*526 and *rpoB*531 conferring rifampin resistance; *rpsL*43, *rpsL*88, *rrs*513 and *rrs*491 conferring streptomycin resistance and *embB*306 conferring ethambutol resistance. Mutational analysis was done by polymerase chain reaction amplification of extracted DNA with specific primers and dot-blot hybridization for the different loci with a radiolabelled probe specific for each of the mutations[21].

Extensive clinical histories of patients infected with a strain with 19 IS*6110* copies were reviewed and detailed data concerning medical history, HIV status, previous TB treatment, type of TB, chest radiography, sputum staining and cultures, sensitivity and treatment were collected. Patients were interviewed in detail by a

medical anthropologist for completion of data regarding place of work, school, recreation, focussing on drinking places, church attendance and treatment supervisor. To maintain patients' confidentiality, other patients' names were never mentioned. To further enhance the elicitation of epidemiologic links, patients were asked for the names of their social contacts in certain streets.

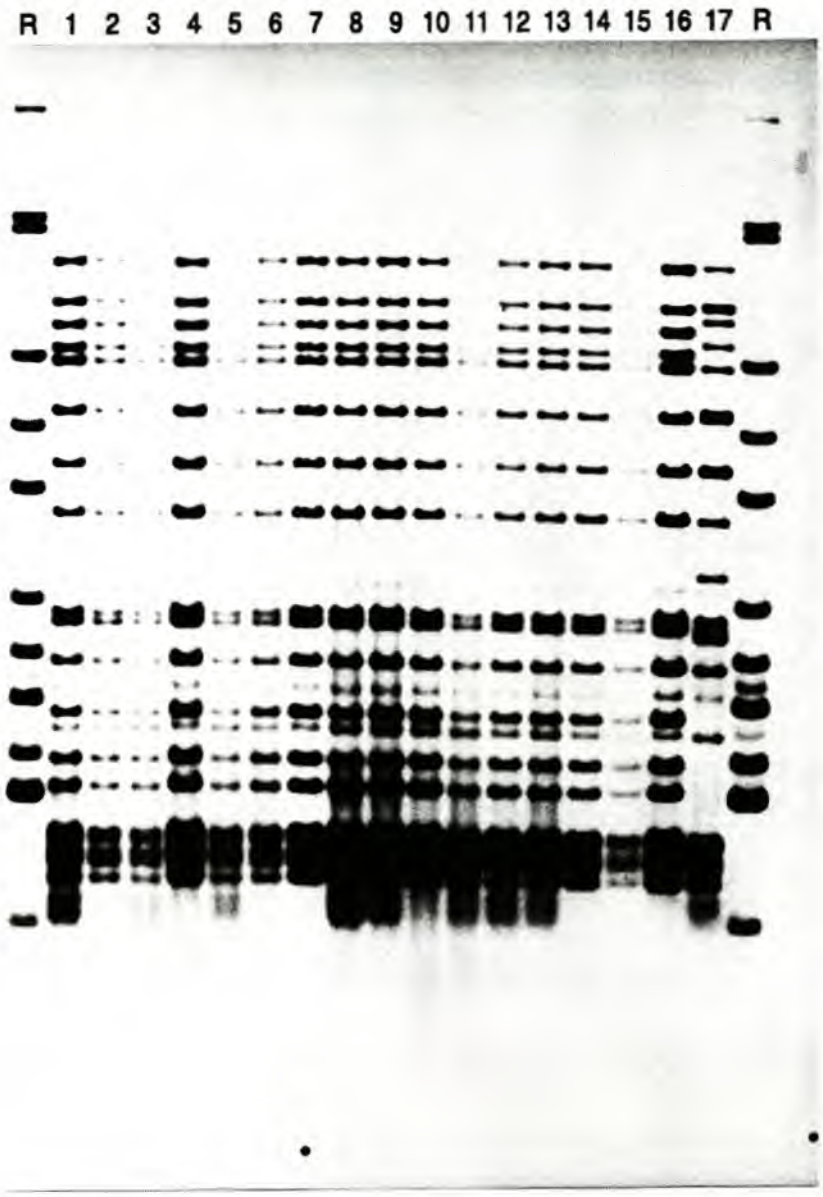

Figure 1.Autoradiograph of RFLP patterns (IS*6110* 3') of *Mycobacterium tuberculosis* isolated from case patients.

Lane R, MTB 14323 (reference strain)[18]; Lanes 1-16, patients infected with *M. tuberculosis* strain U; Lane 17, patient infected with a *M. tuberculosis* strain closely related to strain U.

RESULTS

From the strains collected between January 1993 and March 1997, 21 patients were identified with isolates resistant to at least 4 front-line drugs (isoniazid, rifampin, streptomycin, ethambutol). In 16 of these patients, genotype identity of the infecting *M. tuberculosis* strain was shown by standardized IS*6110* typing (fig 1). These isolates had 19 copies of IS*6110* and were arbitrarily called strain U. For 12 patients, >1 isolate with a U-strain pattern on RFLP analysis was available. All 4 remaining patients had clinical evidence of active TB and multiple positive cultures with similar resistance patterns. There was thus no evidence of laboratory cross-contamination[21]. One additional patient was infected with a *M. tuberculosis* strain closely related to strain U (similarity index of 79% [IS*6110*-3'] by use of the Dice coefficient and UPGMA clustering formula; figure 1). The other 4 patients with isolates resistant to the 4 front-line drugs, were infected with an isolate characterized by 5, 7, 10 and 12 copies, respectively, of IS*6110*. During the entire prospective study period (late 1992-mid 1998) no samples with strain U were recovered from 585 patients with drug-sensitive tuberculosis.

All 16 patients infected with strain U had pulmonary MDR-TB, 94% had smear-positive TB and 80% had cavities on chest radiograph at the time of diagnosis. Nine patients were female, 7 were male. The average age at diagnosis of MDR-TB was 28 years (range, 11-50; figure 2). All 16 patients were seronegative for HIV and none had a malignancy or received steroid therapy, which could lead to immunosuppression. None of the patients had been hospitalized for TB treatment or other respiratory diseases prior to the diagnosis of MDR-TB. All patients were treated at the primary health care clinics of the 2 communities; 13 patients also recieved treatment as inpatients for a median duration of 107 days at a hospital that specialized in TB treatment. All but 1 patient were treated with 4 drugs to which the organism was susceptible (i.e., appropriate treatment). On average, the median time between diagnosis and initiation of appropriate treatment was 94 days (range, 0-555 days). Ten of the 16 patients were compliant (>80% of prescribed dosages). Only 13 patients achieved bacterial conversion. The median time patients were sputum positive was 238 days (range, 35-1102 days). Patients who achieved bacterial conversion were treated for a median duration of 380 days (range, 121-637 days) after conversion.

Patient	Sex	Age	Diagnosis DR-TB	Epidemiological link	Resistance pattern INH	RIF	SM	EMB	THIA	ETH
1*	m	23	Jan-93	housemate of "index case"						
2	m	21	Mar-93	brother of patient 1						
3	m	42	Aug-93	friend of sister of patient 1						
4*	f	15	Aug-93	sister of patient 5 and 6						
5*	m	28	Sep-93	brother of patient 4 and 6						
6	f	18	Sep-93	sister of patient 4 and 5						
7	m	29	Oct-93	no interview (died)						
8*	f	29	Nov-93	sister of patient 1						
9	f	11	Mar-94	daughter of patient 8						
10*	m	24	Apr-94	friend of patient 1, 2 and 8						
11*	f	28	Sep-94	cousin of patient 4, 5 and 6						
12*	f	32	Nov-94	no interview (moved)						
13	f	39	Jun-95	no epidemiological link						
14*	m	50	Feb-96	no epidemiological link						
15*	f	37	Mar-97	cousin of "index case"						
16	f	23	Mar-97	friend of patient 8						

* initial isolate available for mutation analysis

resistance detected by conventional susceptibility testing on initial isolate

additional resistance detected by mutation analysis only on initial isolate

additional resistance detected by conventional susceptibility testing on follow-up isolate

additional resistance only detected by mutation analysis only on follow-up isolate

Figure 2 Patient characteristics and resistance pattern of strain U *M. tuberculosis* isolates. m = male; f = female; DR-TB = drug resistant tuberculosis, INH = isoniazid; RIF = rifampin; SM = streptomycin; EMB = ethambutol; THIA = thiacetazone; ETH = ethionamide

RFLP analysis by use of the IS*6110*-3' probe (figure 1) and IS*6110*-5' probe (data not shown) showed that all 16 samples representing the infecting strain of each patient had an identical pattern of 19 IS elements. In addition to IS*6110*, 2 additional *M. tuberculosis* subtyping probes, DRr and MTB484(1), confirmed that the genotypes were identical for all 16 isolates (data not shown). Comparison of the IS*6110*-3' and IS*6110*-5' RFLP analysis of strain W^{22} and strain U suggested that strain U may be genetically related to strain W (figure 3). The similarity index calculated by using the Dice coefficient and UPGMA clustering formula was 81.1% (IS*6110*-3') and 78.9% (IS*6110*-5'). To test further for similarity, a genomic sequence (ISL*540*.3) present in H37Rv and most clinical isolates but absent in all strain U isolates, was hybridized against DNA of strain W (961-0874). No hybridization was detected suggesting a common ancestry (data not shown).

DNA from the initial isolate of the MDR-TB episode was available for mutational analysis in 9 cases. In all of these isolates, the following mutations could be demonstrated: *katG*315, *katG*463, *rpoB*531, *rrs*513 and *embB*306, conferring for resistance to, respectively, isoniazid, rifampin, streptomycin and ethambutol. No mutations were identified in the *rpo*B526, *rpsL43*, *rps*L88 loci or in *rrs*491position. In 6 of the 7 patients from whom no initial isolate was available, analysis of a follow-up isolate revealed identical mutations (figure 2). This result confirms the identical genotype of the isolates as suggested by RFLP analysis. Mutation analysis of the strain closely related to strain U revealed identical mutations responsible for resistance to isoniazid and ethambutol but different mutations for rifampin (*rpoB*526) and streptomycin (*rpsL*43).

Initially, the outbreak propagated rapidly with 8 cases in 1 year. Thereafter, the spread continued at a slower rate, with 8 new cases over the following 3 years (figure 4). Figure 4 might suggest a single chain of transmission; however, conventional contact tracing refutes this. Of the 14 patients who could be interviewed, there were only 2 patients who had no apparent epidemiologic link with any of the other patients. Among the 12 patients with social links, there was 1 group consisting of 8 people who were either relatives or friends and another group of 4 people who were close or distant family. No relation between these 2 groups could be found (figure 2);

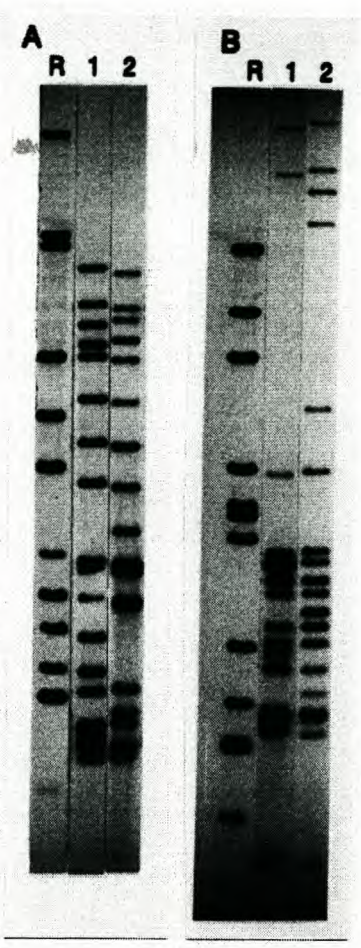

Figure 3. Southern blot comparison of *M. tuberculosis* hybridized with A, IS6110-3' (IS6110 nucleotides 631-875) and B, IS6110-5' (IS6110 nucleotides 77-462). Lane R, MTB 14323; lane 1, strain W (961-0874); lane2, strain U

Social anthropology data suggest that patient 1 was most likely infected by a close friend, an HIV-seronegative MDR-TB patient resistant to isoniazid, rifampin and ethambutol who was culture-positive >1 year prior to his death in May 1993. As this person lived outside the communities studied, his *M. tuberculosis* isolates were not available for RFLP or mutational analysis. Because this person was infectious for such a prolonged period of time, it is possible that ha was the source case for >1 of the 8 related patients. As this study was performed in 2 communities with a high population density (14,500 people/km^2), casual or unrecognized contact is a likely explanation for the patients with no apparent epidemiologic link. Moreover, all residents attend the primary health care clinic as a first line for all health problems, which makes the clinic itself another possible contact place.

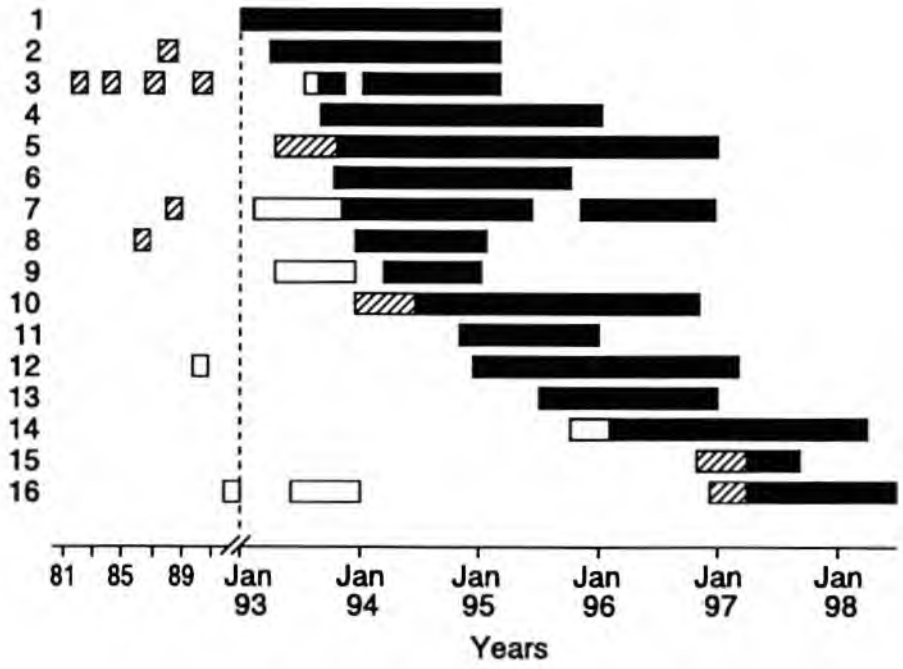

Figure 4 Schematic representation of a multidrug-resistant tuberculosis outbreak, caused by strain u, in 16 patients residing in a south african community.
Horizontal bars denote duration of treatment for: solid box, multidrug-resistant tuberculosis; open box, drug susceptible tuberculosis; diagonal shading, culture positive tuberculosis, drug susceptibility not tested.

The majority of patients (11/16) received antituberculous treatment prior to the diagnosis of MDR-TB. Four patients received an average of 5 months of treatment before drug-sensitivity tests were requested. Seven patients received a prior course of treatment for an episode of TB, predating the MDR-TB episode by an average of 3 years. In 2 cases, DNA was available from a specimen obtained prior to the diagnosis of MDR-TB. Patient 3 received treatment for culture-proven TB three months before diagnosis of MDR-TB. These bacilli were sensitive to all drugs and had an RFLP pattern of 15 IS*6110* elements not related to the strain responsible for the MDR-TB episode (similarity index of 33% as calculated by use of the Dice coefficient and UPGMA clustering formula). Patient 16 received a full treatment course for drug-sensitive TB 3 years before diagnosis of MDR-TB, the isolate was then characterized by 9 IS*6110* elements on RFLP analysis, again not related to the strain responsible for the MDR-TB episode (similarity index of 27% [data not shown]). Both patients were compliant during the treatment for drug-sensitive TB as judged on the basis of attendance of directly observed treatment (taking >80% of prescribed dosages).

DISCUSSION

As far as could be ascertained, this is the first report of an MDR-TB outbreak occurring in a community without the involvement of a single HIV-seropositive or institutionalized patient and where transmission was proved by modern molecular biology techniques, such as RFLP and mutation analysis.

In the pre-AIDS era, outbreaks of MDR-TB were occasionally reported.[23, 24] Such outbreaks were characterized by a slow spread over a number of years and a limited number of patients. Only 1 of these outbreaks reported before 1990 involved cases resistant to both isoniazid and rifampin, the current WHO definition of MDR-TB[25]. Suspected epidemiological links could only be confirmed by phage typing [26,27], a far less precise technique than RFLP analysis. The rare occurrence of outbreaks of drug-resistant TB was ascribed to the conviction that drug-resistant *M. tuberculosis* strains are less virulent.[28] However, recent studies demonstrated that drug-resistant strains do not differ from drug-sensitive strains in their ability to create infection or disease.[29,30]

This report of a cluster of 16 MDR-TB patients confirms that drug-resistant strains of *M. tuberculosis* can be transmitted, and not only to the immunocompromised host.

In 1990, the first outbreak of MDR-TB involving 10 epidemiologically related, noninstitutionalized, HIV-seronegative cases was reported by the Centres for Disease Control and Prevention of the United States.[31] However, drug resistance was proven in only 8 of the 10 cases, the initial resistance pattern was the same in only 2 of the cases and no further analysis (phage typing or genotyping) was performed to establish whether all cases were part of a chain of transmission caused by 1 specific strain.

The results of our study indicate that a difference in resistance pattern on conventional susceptibility testing of the initial isolate does not exclude recent transmission. The resistance pattern of initial isolates showed resistance to isoniazid, rifampin, streptomycin and ethambutol in only 6 of the 16 patients. In 3 of these and 6 other patients, the initial isolate was available and mutational analysis showed the presence of the same 5 mutations conferring resistance to all 4 drugs, even if conventional methods reported the isolates as susceptible to 1 of those 4 drugs. This problem, and the fact that susceptibility testing is not routinely performed, are factors that lead to suboptimal treatment of patients with primary MDR-TB. This can in turn cause prolonged infectivity and further transmission within the community. Efficient molecular procedures such as described in this study could therefore be valuable in the quick diagnosis and prevention of spread of MDR *M. tuberculosis* strains.

Our study also indicates that history of previous treatment is another clinical parameter that by itself cannot be the sole factor to reject the possibility of recent transmission of MDR-TB. It was believed that exogeneous reinfection was rare because of the possibility of protective immunity acquired after a first infection. A person with drug-resistant TB and a history of a previous TB episode was therefore classified as having acquired drug-resistant TB. There have been a few reports of exogenous reinfection in immunocompromized patients[13,32,33] and 1 report of exogenous reinfection in an immunocompetent patient.[34] Eleven of the 16 immunocompetent patients reported in our study had a history of previous treatment. In 2 patients, RFLP analysis of both the drug-sensitive and drug-resistant isolate could be performed. This showed that in both these patients the 2 TB episodes were caused by two different, unrelated strains, thereby

proving exogenous reinfection during treatment (patient 3) or after completion of a previous treatment episode (patient 16). The presence of 5 identical mutations in genes conferring resistance to antituberculous drugs (*kat*G315, *kat*G463, *rpo*B531, *rrs*513 and *emB*306) in all patients also supports transmission rather than acquisition as the mechanism responsible for drug-resistant TB in these patients.

MDR-TB outbreaks became an established phenomenon as hundreds of cases were reported world-wide and transmission between cases was proven by molecular epidemiologic techniques.[2-7] These outbreaks were characterized by rapid spread, accelerated progression from infection to disease and high mortality rates. The overwhelming majority of the patients were HIV seropositive and/or institutionalized (hospitals, prisons). The characteristics of the outbreak described in this report are very different from the above. This outbreak propagated over a number of years, the mortality rate was low (12%), patients were HIV seronegative, and transmission occurred in the community. However, strain W (New York) and the "strain W-like" strain U (Cape Town) were responsible for >80% of cases resistant to at least 4 drugs, respectively, in New York City and the 2 Cape Town communities. Because these 2 strains are not identical, as shown by a related but non-identical RFLP pattern and a difference in the mutation conferring resistance to streptomycin (*rpsL*43 in strain W and *rrs*513 in strain U), strain U cannot be the result of a recent transcontinental spread of strain W. These 2 genomically-related strains thus independently arose in 2 different geographic locations and in 2 very different patient groups suggesting that this *M. tuberculosis* strain family is prone to the development of mutations conferring resistance.

Based on the finding that >95 % of 267 cases of MDR-TB caused by outbreaks of the strain W in the United States over a 3-year period were likely to have been nosocomial infections, it was suggested that transmission of MDR-TB was almost exclusively confined to institutional settings, with no significant spread beyond the walls of institutions.[14] This would imply that attention can be focused on hospitals serving at-risk populations as a unique locus of transmission of MDR-TB[35]. The outbreak described in this report challenges this idea. A community can be the location of rapid spread of MDR-TB even if there is a low prevalence of HIV and no

homelessness or intravenous drug use. A factor that could have contributed to the extent of this outbreak is the prolonged period of infectiousness in these patients. This study describes an outbreak in an area with a high incidence of TB, where the priority is the application of direct observed therapy short course (DOTS) to treat all smear-positive (drug-sensitive) cases effectively to reduce the infectious pool and prevent the acquisition of drug resistance. The DOTS program for low-income countries does not require sensitivity testing on a routine basis and gives no priority to effective personalized treatment for patients with MDR-TB. This results in delayed diagnosis and a delay in initiation of effective MDR-TB treatment, leaving patients infectious for a prolonged period. Transmission of MDR-TB to nonimmunocompromised individuals creates a reservoir of persons infected with an MDR-TB strain, an infection for which there is currently no efficient disease-prevention strategy. To prevent an outbreak of MDR-TB, as well as the creation of a pool of persons infected with MDR-TB strains within a community, prevention of transmission of multidrug-resistant bacilli should be targeted by adequate case investigation (including contact investigation), rapid and correct diagnosis of drug resistance and adequate treatment of patients with MDR-TB.

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CHAPTER 8

The use of a Geographic Information System to study the epidemiology of drug-resistant tuberculosis

The use of a geographic information system (GIS) to study the epidemiology of drug-resistant tuberculosis

Background: A GIS has been shown to be a powerful tool in epidemiologic and public health studies, especially of infectious diseases. The aim of this study was to use a GIS in combination with molecular epidemiological data to study the socio-economic risk factors for occurrence and transmission of drug-resistant tuberculosis. Setting: Fifty-nine patients with drug-resistant TB residing in two South African communities, characterized by a high TB notification rate, were studied. Results: According to molecular epidemiological analysis, 29 patients were classified as cases of transmitted drug-resistant TB. Cases infected with an identical drug-resistant *Mycobacterium tuberculosis* isolate (cluster) were spatially aggregated within the community. The geographic distribution of occurrence and transmission drug-resistant TB was correlated with the geographic distribution of TB incidence. There was also a correlation between the occurrence and transmission of drug-resistant TB and poor socio-economic conditions. Conclusion: Drug-resistant TB and its transmission occur in geographically distinct areas of poor socio-economic conditions and high TB incidence.

INTRODUCTION

The historical roots of epidemiology lie with Hippocrates who, in 400 BC, introduced the term "epidemeion" as "diseases that visit the community". Modern epidemiology is the quantitative study of the distribution and determinants of disease in human populations. John Snow's study in the 1850's of a cholera outbreak in London is the first good example of an observational study integrating geography into medical research. By referring to maps of two water-providing companies and to the 1851 census figures, Snow was able to examine the relationship between the number of cholera deaths and the number of people living in specific areas[1]. The power of these observations led to the hypothesis of the water-borne mode of cholera transmission.

Geographic Information Systems (GIS) emerged in the 1980s as an innovative component in the field of epidemiology and public health. GIS allows a spatial perspective on disease by analysing associations between location, environment and disease on international, national and local level[2]. GIS has been used in the identification of locations of high disease prevalence (onchocerciasis[3]), the analysis of disease transmission (malaria[4]), the study of environmental risk factors (Lyme disease[5]), in disease surveillance (malaria[6]), the identification of space-time (leukemia[7]), the visualisation of temporal patterns of disease spread (AIDS[8]) and in health policy planning (drancunculiasis[9]). These examples indicate that GIS can be used to identify problems, produce hypotheses and develop strategies for control of infectious diseases.

GIS has been also used in studies of tuberculosis. A study in South Africa investigated spatial implications of the DOTS strategy and demonstrated an important impact of community health workers on access to treatment[10]. Another South African study, by the TB research group of the University of Stellenbosch, evaluated the distribution of tuberculosis within one high incidence community over a 10-year period and demonstrated that cases were unevenly distributed[11]. A study in Baltimore, USA, combined a GIS with traditional and molecular epidemiology to study the patterns of tuberculosis transmission. Results demonstrated that recently transmitted TB cases occurred in geographically distinct areas of low socio-economic status and high drug abuse[14].

A GIS was used by myself and others in studies on risk factors for childhood tuberculosis which could show a relation between crowding, poor socio-economic factors and the incidence of TB in children[12,13].

In this study, we made use of a the combination of a GIS with molecular epidemiological data to study the role of socio-economic risk factors in the occurrence and the transmission of drug-resistant TB in an area characterized by a high TB incidence.

MATERIALS AND METHODS

Setting

The study area consists of two communities of Cape Town, South Africa with a total surface area of 2.42 km^2 and a population of 34.294 (1991 census). During the apartheid era in South Africa, this area was created as a residential area for Coloured people. In 1991, 99.7% of the residents were of the coloured ethnic group. The population increased explosively from the 1960s to 1980s and stabilised thereafter. It is an area with characteristics of rapid urbanisation in a developing country. The population pyramid has a broad base (birth rate of 29.3/1000 population) and a narrow top. Housing varies from freestanding brick houses to one-room flats and rickety backyard shacks. Living conditions have improved over the last years. Sewage, electricity and running water is now available for all residents. Two primary health care clinics and an adjacent tertiary hospital serve the area. During the study period, the national TB program required the notification of all new cases of active TB and treatment under supervision at the local health authority clinic. The TB case notification rate in these two suburbs is reported as more than 1000/100 000[11]. Within the area, socio-economic conditions are related to the TB notification rates. The area with the highest case notification rate in children aged 0 to 5 years of age (14062/100 000/year) had a high level of crowding (1.5 adults per room), a low mean annual household income (4450 Rand) and a low level of parental education (6 years of schooling)[12].

Geographic information system

Different sets of data were integrated to study the disease dynamics of drug-resistant TB. The TB notification rate for the adult population was calculated for each enumerator subdistrict (ESD) by linking the 1991 population census data (most recent available census data), 1991 notification data and geographically mapped home addresses of all patients notified as cases of active TB. The 1991 population census data divide the two suburbs into 39 ESD's with an average population of 900 people (minimum 627 and maximum 1 267). The notification data provided the name, address, age and type of TB of each notified case. All duplicate notifications and previously treated patients were eliminated to avoid inaccuracies. The home addresses of patients with drug-resistant tuberculosis identified between 1 April 1992 and 31 March 1997 were subsequently geographically mapped using the computer based GIS (ARC-INFO®). The distribution of adult TB notification rates of the ESD where patients with drug-resistant TB reside could then be evaluated. The correlation between the frequency of different types of drug-resistant TB as determined by Restriction Fragment Length Polymorphism (RFLP) analysis and the adult tuberculosis notification rate in the two communities was also studied.

The 1991 census report provides for each ESD demographic variables such as age and sex, social variables such as number of households, education, type of housing and economic variables such as employment and annual income per capita. From this data, we calculated one value for each ESD for crowding, economic status and education level. Crowding was calculated as the average number of adults (>15 years of age) per room (sleeping and living rooms). Economic status was calculated as the average annual household income. Education level was calculated as the percentage of adults (>15 years of age) who had completed primary school education. The distribution of crowding, schooling and economic status among patients with drug-resistant tuberculosis and different subgroups of drug-resistant tuberculosis were then evaluated.

RFLP analysis

Mycobacterium tuberculosis isolates from patients with drug-resistant tuberculosis were genotyped by Restriction Fragment Length Polymorphism[15]. Cluster

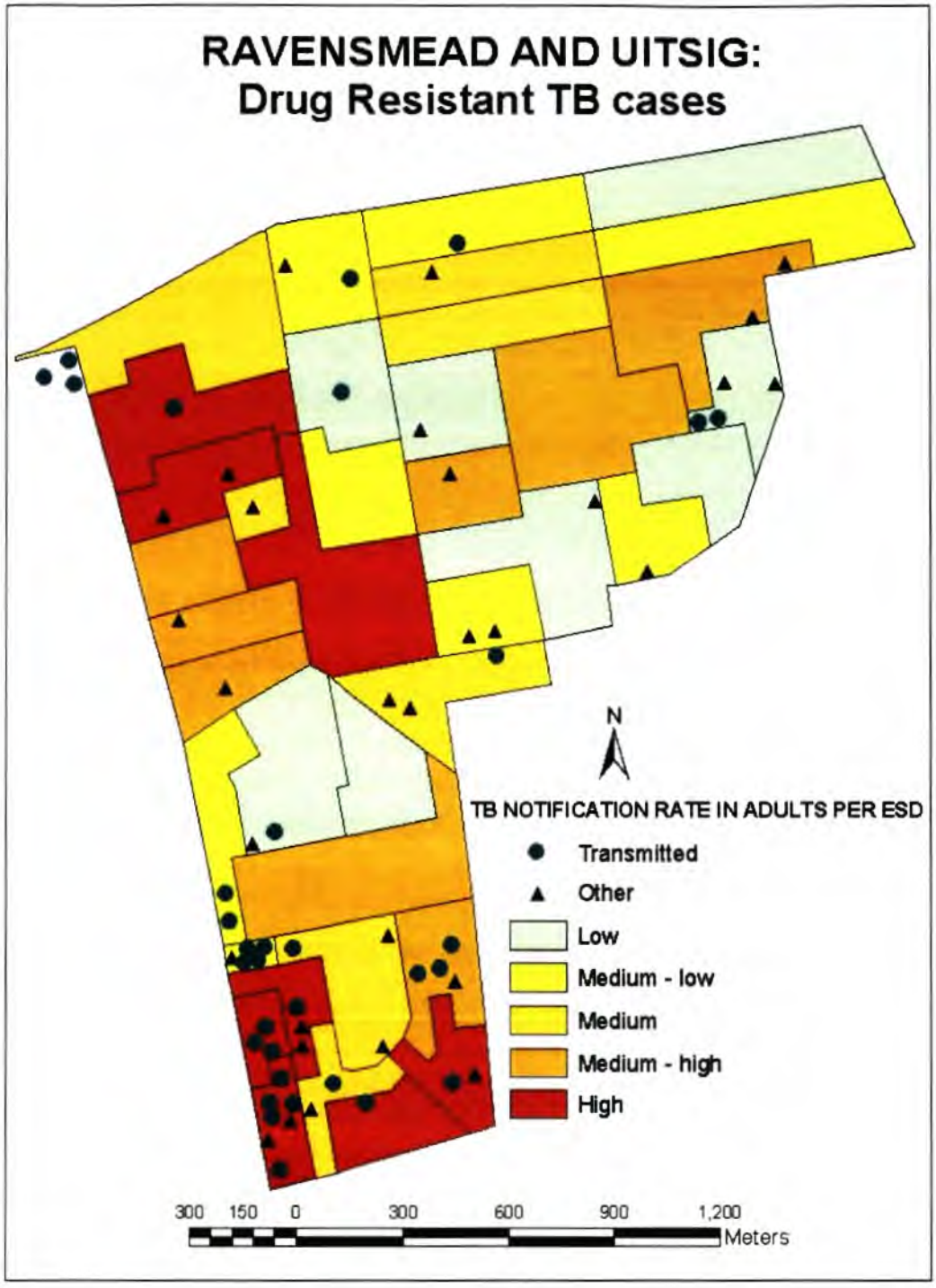

Figure 1. Geographic distribution of cases with drug-resistant tuberculosis. Low TB notification rate represents areas with a TB notification rate < 600/100 000/year, High TB notification rate represents areas wait a TB notification rate > 2000/100 000/year.

analysis was done by using the Dice coefficient and UPGMA clustering formula. To enhance the accuracy, clusters of identical drug-resistant isolates identified by IS6110-3' were subjected to further genotyping with secondary probes (IS6110-5', MTB484(1) and DRr)[16]. Isolates were classified as clustered or unique, depending on respectively the presence or absence of isolates with identical drug-resistant *M. tuberculosis* strains retrieved from patients in the studied communities. According to the formula suggested by Small et al, all clustered cases except for the index case of each cluster were classified as cases of transmitted TB[17].

Statistics

Analysis was performed using the non-parametric Mann-Whitney U test.

RESULTS

During the study period (1 April 1992 to 31 March 1997), 70 patients were treated for drug-resistant TB at the primary health care clinics of the two studied communities. In 63 of them, DNA of the infecting *M. tuberculosis* was available for RFLP analysis. Of these, 4 patients resided in the study communities but outside the area covered by the 1991 census report of the communities (figure 1). The remaining 59 patients constitute the cases of drug-resistant TB used for the geographical distribution analysis. The control group consists of the 1991 population divided into 39 enumerator subdistricts.

According to RFLP analysis, 23 cases were infected with a unique strain while 36 cases were infected with an isolate belonging to one of 6 clusters of drug-resistant *M. tuberculosis* strains. Thirty cases were classified as *transmitted* drug-resistant TB, 29 cases were classified as *other* drug-resistant TB (23 infected with a unique strain and 6 index cases of a cluster).

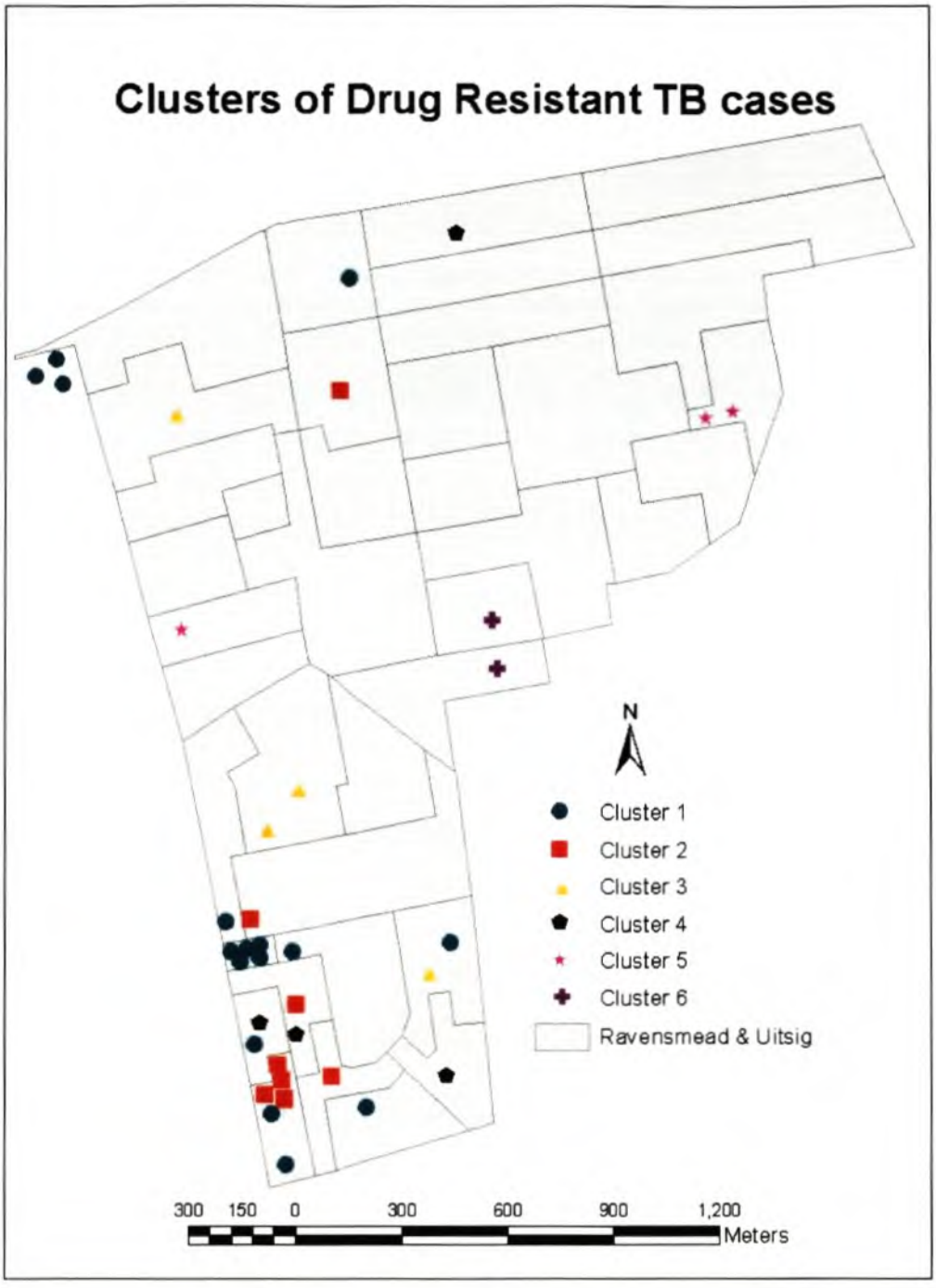

Figure 2. Geographic distribution of patients infected with clustered strains

Geographic distribution of cases of drug-resistant tuberculosis.

All cases of drug-resistant tuberculosis were geographically mapped (figure 1). At least one case occurred in 31 of the 39 ESDs. There was a trend of a higher frequency of drug-resistant TB cases with an increasing TB incidence of the ESD. The number of drug-resistant TB cases per ESD ranged from 1.12 cases per ESD for ESDs with a "low" TB notification rate to 2.28 cases per ESD for ESDs with a high TB notification rate. The distribution of the TB notification rate among the ESDs in which cases (patients with drug-resistant TB) and controls (population divided into 39 ESDs) resided was compared (table 1). The average TB notification rate per ESD was higher for the case group (1339/100 000/year) than for the control group (1085/100 000/year) but this was not significant (p = 0.24) (table 1).

Table 1: Average value (and standard deviation) of adult TB notification rate, social and economic risk factors of ESDs with-drug-resistant TB cases and the population of the communities

	ESDs with drug-resistant TB cases	**Total population**	**p value**
TB notification rate (per 100 000 per year)	1339 (887)	1085 (764)	0.24
Schooling (% completion of primary schooling)	71.5 (11.7)	76.2 (10.7)	0.042
Crowding (adults per room)	1.45 (0.29)	1.32 (0.3)	0.02
Economic status (Annual household income in Rand)	16396 (8017)	19831 (7435)	0.057

Cases of drug-resistant tuberculosis were classified and geographically mapped according to the RFLP category of the infecting isolate (figure 1). The majority of transmitted cases were grouped in the southern part of the area, where the TB notification rate is the highest (figure 1). The average TB notification rate of the ESDs where cases with transmitted drug-resistant TB reside was 1525/100 000/year. This was substantially higher but not significantly different (p = 0.285) from the average TB notification rate for the ESDs where other cases of drug-resistant TB reside (1147/100000/year). (table 2).

All patients infected with a *M. tuberculosis* bacillus that belonged to a cluster of drug-resistant isolates were also mapped with a specific code for each cluster (figure 2). On average, 80 % (range 67 – 100 %) of cases within each cluster resided within 600 meters of each other (figure 2).

Table 2: Average value (and standard deviation) of adult TB notification rate, social and economic risk factors of ESDs with cases of transmitted and other types of drug-resistant TB

	ESDs with Transmitted drug–resistant TB cases	**ESDs with Other drug-resistant TB cases**	**p value**
TB notification rate (per 100 000 per year)	1525 (1015)	1147 (689)	0.285
Schooling (% completion of primary schooling)	69.5 (12.7)	73.6 (10.3)	0.22
Crowding (adults per room)	1.50 (0.3)	1.40 (0.27)	0.18
Economic status (Annual household income in Rand)	13732 (7757)	19152 (7439)	0.013

Socio-economic risk factors for drug-resistant tuberculosis

Socio-economic risk factors for TB, such as crowding, level of schooling and economic status, were compared between the ESDs in which cases resided and the 39 ESDs in which the population of the two communities resides. The average socio-economic status was poorer for the ESDs in which the cases reside as compared to the average socio-economic status of the population (table 1). The difference was significant for schooling ($p = 0.042$) and crowding ($p = 0.02$) but not for economic status ($p = 0.057$).

Socio-economic risk factors for transmission of drug-resistant tuberculosis

Socio-economic risk factors were evaluated to determine factors influencing the transmission of drug-resistant TB. The distribution of crowding, schooling and economic status (annual household income) of the ESDs in which cases of transmitted drug-resistant TB reside were compared with the ESDs in which other cases of drug-resistant TB reside. There was a trend towards lower socio-economic conditions in the

group of transmitted drug-resistant TB cases compared with the other cases (table 2). The average percentage of adults completing primary schooling was 69.5% for ESDs where cases with transmitted drug-resistant TB reside and 73.6% for the ESDs where other cases of drug-resistant TB reside. There was only a minor difference in crowding for both groups: 1.76 and 1.74 adults per room for the transmitted and other group respectively. The difference in annual household income between the two groups was striking: 13732 Rand in the transmitted group compared to 19152 Rand in the other group. This trend of lower socio-economic status among cases with transmitted drug-resistant TB was significant only for economic status ($p = 0.013$).

DISCUSSION

Tuberculosis is not uniformly distributed throughout the world, within one country or between different population groups. Even within one community, there can be vast differences in disease incidence[11]. It is necessary to understand trends of disease frequency, demographic characteristics and geographic distribution of drug-resistant TB in a defined area, in order to design effective programs for disease control through measures assuring that acquisition and transmission of drug-resistant TB is stopped. The goal of the evaluation of the disease dynamics of drug-resistant TB by the use of a GIS was to determine the risk factors for the occurrence and transmission of drug-resistant TB.

In this study, we found that 80% of cases within each of the 6 clusters of identical drug-resistant strains resided within 600 m of another case of the same cluster. Furthermore, clusters occurred in geographically distinct areas within the community. This supports the generally accepted assumption that isolates with identical RFLP fingerprints are epidemiologically linked and belong to a chain of transmission. Drug-resistant TB cases, especially cases of transmitted drug-resistant TB, were concentrated in the areas with the highest TB incidences. The occurrence and frequency of transmission of drug-resistant TB thus correlated with the overall TB incidence. This is not surprising, as the chance of more cases developing drug resistance will be higher in areas where there is more TB. Once drug-resistant TB is present in a specific area, transmission occurs and even more cases of drug-resistant TB will be produced.

Another finding was the association between drug-resistant TB and lower socio-economic living conditions such as housing, schooling and income. The observed trend was more pronounced for the occurrence than for the transmission of drug-resistant TB. The association between poverty and health is well documented. Specifically for TB, socio-economic status appears to be closely related to disease rates. Areas with a high proportion of people who are poorly educated, poorly paid or poorly housed have high TB rates[18]. It was surprising that crowding was not associated with transmission of drug-resistant tuberculosis in our study. This may have been the result of low case numbers resulting in insufficient statistical power.

Because TB has a long incubation period and only approximately 10% of infected individuals progress to active disease, the disease dynamics are best understood by looking at the pathogenesis in the two stages of infection and active disease. The relationship between low socio-economic living conditions and risk of infection is clear, but little is known about the relationship between socio-economic conditions and risk of disease among infected individuals. What leads to a high probability of infection may have little to do with the probability that disease will develop later. This study, based on DNA fingerprinting results, could only investigate the individuals that progress to disease but missed all the infected individuals that remain disease-free. This might be another reason why it is difficult to obtain a significant relation between socio-economic factors and risk of transmission of drug-resistant TB.

A further problem with the study is a problem common to all ecological studies: the ecological fallacy. Even if the ESDs are relatively small and demographically rather homogenous, as in this study (average of 900 people per ESD), not all individuals have demographic characteristics equal to the mean value of the ESD in which they reside. Group (ESD) based mean values of demographic factors may thus erroneously have been assigned to individuals within the group.

Even though this study has intrinsic problems and can therefore not lead to certainties but only to probabilities and trends, it is the first study we are aware of which uses a GIS to examine the relationship between socio-economic factors and the occurrence and transmission of drug-resistant tuberculosis. We found that both the occurrence and transmission of drug-resistant tuberculosis show a trend of increased

frequency with lower socio-economic living conditions and with increases in overall TB incidence. A prospective study, collecting data on individual socio-economic conditions of cases with drug-resistant TB will be important to confirm or reject the trends observed by this study.

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CHAPTER 9

Outcome of multidrug-resistant tuberculosis in a South African community

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submitted

Outcome of multidrug-resistant tuberculosis in a South African community

Multidrug resistant tuberculosis (MDR-TB) is characterized by a low cure rate and high mortality rate, which can be improved by appropriate treatment. Data on outcome of MDR-TB in low and middle-income countries is scarce. We present a population-based study of 42 MDR-TB patients residing in two South African communities. All patients tested (95%) were HIV seronegative. Forty-one patients had cavitary TB and 88% were smear positive. *M. tuberculosis* was resistant to a mean of 4.2 drugs. Virtually all (98%) patients received appropriate treatment with ≥ 3 drugs, however after a long delay (2.8 months) after diagnosis. Thirty (71%) patients were hospitalized in a specialized MDR-TB treatment unit. Patients were followed-up for a median of 4.2 years after diagnosis. By the end of treatment, 20% of patients had died, 42% were cured and 38% had defaulted. The overall rate of bacterial conversion was 74%, 2-year survival rate was 90% and 5-year survival rate 71% (Kaplan-Meier analysis). Bacterial conversion was significantly associated with survival (p=0.00001). Compliance with therapy was associated with survival and bacteriological conversion (p=0.011 and p= 0.0016). The results suggest that an individualised approach and the availability of a specialized MDR-TB treatment unit can improve the outcome of MDR-TB.

INTRODUCTION

Multidrug-resistant tuberculosis (MDR-TB), defined as resistance to at least isoniazid and rifampin, is a worldwide problem posing a threat to global TB control. Previous studies have found that patients with TB resistant to rifampin and especially patients with MDR-TB are less likely to benefit from treatment than those infected with drug susceptible organisms[1-3]. Patients infected with MDR strains are not only difficult to cure and more costly to treat[4], but are also more likely to remain sources of infection for a longer period of time resulting in exposure to other persons and outbreaks of MDR-TB[5].

Investigations of outbreaks of human immunodeficiency virus (HIV)-related MDR-TB have found a strikingly high case fatality rate[3,6,7]. The outcome of HIV seronegative patients with MDR-TB is substantially better when optimal therapy is provided, but still poor compared to patients with drug susceptible TB[8-10]. Most studies on the outcome of MDR-TB are investigations performed in high-income countries and are hospital based[8-11]. Data on the outcome of MDR-TB in middle and low-income countries, where most MDR-TB patients reside, is scarce 12,13.

We present a population-based study involving two neighbouring communities of a middle-income country where, during a 5-year period, 42 patients (of whom 40 were HIV seronegative) received treatment for MDR-TB. Demographic, clinical and treatment characteristics are presented, long term clinical and bacteriological outcome are evaluated and factors influencing the outcome are analyzed.

MATERIALS AND METHODS

Setting and study population

The area studied consists of two neighbouring Cape Town Metropolitan communities (Ravensmead and Uitsig) with a total rsidential surface area of 2.42 km^2 and a population of 34,294 of the Coloured ethnic group living in poor socio-economic conditions. The incidence of new (never previously treated) bacteriologically positive

cases in the area is 225/100000/year[14]. The notification rate of all bacteriologically positive cases (new and retreatment cases) in the area is 363/100000/year. A survey of drug resistant tuberculosis in the Western Cape province conducted in 1992-1993 found a rate of 8.6% acquired and 3.2% initial drug resistance in the West Cape region[15]. The reported prevalence of HIV infection in the province ranged from 0.25% in 1992 to 3 % in 1996 (data from the National HIV surveys on women attending antenatal clinics, conducted by the Department of Health and Population Development, South Africa).

All TB patients were treated at a primary health care clinic under direct supervision. During the time of the study, there were no official National Tuberculosis Program (NTP) guidelines for the treatment of drug-resistant TB. However, in the Western Cape Province, centres specialised in MDR-TB treatment have been developed. These centres provide in-patient treatment as well as treatment advice and follow-up for outpatients with MDR-TB. Patients in the communities studied had the option to be treated at the primary health care clinic or to receive inpatient care at a centre specialised in MDR-TB treatment. The drug regimen prescribed was determined by the susceptibility pattern of the individual isolates in combination with the availability of drugs. No standardized regimens for MDR-TB treatment were followed.

Laboratory procedures

Sputum samples were collected at the health care clinics of the communities and sent to the routine laboratory for microscopy, culture and drug susceptibility testing if requested by the managing health professional. Drug susceptibility testing was performed by the economic version of the indirect proportion method[16] on Löwenstein-Jensen medium containing critical concentrations of 0.2 μg/ml isoniazid, 30 μg/ml rifampin, 2 μg/ml ethambutol, 5 μg/ml streptomycin and 20 μg/ml ethionamide. Kanamycin and thiacetazone resistance was tested on Middlebrook 7H10 agar containing critical concentrations of respectively 5 and 2 μg/ml. Resistance was defined as 1% or more bacterial growth in comparison with a control using international criteria. Initially, cultures were tested for resistance to isoniazid and rifampin. If resistance to these drugs was detected, cultures were subsequently tested for resistance to the other antituberculous drugs as mentioned above. Resistance to pyrazinamide was not tested.

Patient data and definitions

All patients residing in the studied area with a positive culture for *Mycobacterium tuberculosis* resistant to at least one antituberculous drug between April 1,1992 and March 31,1997 were included. Information on age, sex, medical history, HIV status, previous tuberculosis treatment, results of sputum microscopy and cultures, susceptibility testing, treatment and survival were obtained by review of medical files and compliance charts from the clinic and the specialized MDR-TB inpatient units. If a patient had been transferred during treatment or had moved after completion of treatment, the nursing staff of the relevant clinic was asked to do a home visit to ensure survival of the patient and to collect a sputum sample. Cases were followed until May 1999.

The date of diagnosis of MDR-TB was defined as the date of the first clinical isolate positive for *M. tuberculosis* resistant to at least isoniazid and rifampin.

A drug was considered appropriate for treatment if *M. tuberculosis* was not proven to be resistant to this drug *in vitro*.

Treatment was considered completed if a patient received 12 months of therapy (with 3 appropriate drugs) after sputum conversion or 18 months of appropriate treatment. Treatment compliance was determined as the intake of at least 80% of prescribed dosages as determined by clinic attendance for the course of therapy. A patient was considered cured if he/she completed therapy and remained culture negative for at least 6 months after ending therapy. A patient was classified as defaulted if treatment was interrupted before completion was reached. The outcome was determined as bacteriological and clinical success. Clinical success was defined as; bacteriological success was defined as bacteriological conversion. The date of bacterial conversion was defined as the first date of three consecutive bacteriologically negative isolates.

Data management and statistical analysis

Data was analyzed using Epi-Info 6.1 and STATISTICA software programs. Analysis focused on factors suspected to be associated with bacterial conversion and with survival. Candidate independent variables were treated as two-level dichotomous

factors dividing, if possible, the study population into two equally sized groups by using the mean or median of the independent variable. Factors investigated were age, sex, history of prior TB, number of drugs to which the isolate was resistant, and bacterial conversion. Characteristics of the treatment for MDR-TB were considered, such as: number of episodes, compliance with therapy, hospitalisation, administration of 3 appropriate drugs within 96 days of MDR-TB diagnosis and for an uninterrupted period of 14 months, administration of 4 appropriate drugs within 87 days of diagnosis and for an uninterrupted period of 11 months and administration of 5 appropriate therapy drugs. The outcome (dependant) variables were also dichotomous and were either "bacterial conversion" or "no bacterial conversion" and "survival" or "death". Relative risk ratios and their corresponding 95% confidence intervals (95% CI) were computed from 2 x 2 tables of dichotomous independent and dependent variables with the use of the Chi-square test or Fisher's exact two-tailed test (depending on whether the expected cell frequency was > or < 5). Survival analysis and identification of prognostic factors for bacterial conversion and survival were carried out using the Kaplan-Meier method. Patients were censored at the time follow-up ended, when they refused further follow-up (n=3) or when they became lost to follow-up (n=0). Patients who died of causes other than tuberculosis and had no evidence of tuberculosis at death were censored at their date of death. Determination of statistical significance of median time to bacterial conversion and median survival time between groups was calculated using the Log-rank test for bacterial conversion analysis (emphasis on differences occurring early in the time frame investigated) and the Peto & Peto Wilcoxon test for survival analysis (emphasis on differences occurring late in the time frame investigated). The results did not differ between the 2 methods (2 x 2 table and Kaplan-Meier analysis). For clarity of the text, the authors have chosen to show only the data of the Kaplan Meier analysis as this analysis gives not just a crude analysis of survival/death and bacterial conversion yes/no, but also takes a (clinically important) time factor into consideration.

RESULTS

Demographic and clinical characteristics

Half (52%) of the 42 patients included were female. The mean age was 33 years. Forty (95%) patients were tested for HIV, all tested negative. One patient refused HIV testing and is still alive 4.3 years after diagnosis of MDR-TB; the other patient was never tested and died 7 years after diagnosis of MDR-TB, at the age of 59 years. All patients had pulmonary tuberculosis and 37 (88%) were sputum smear positive for acid-fast bacilli. On chest radiograph, 41 (98%) patients had cavitary disease. Of the 42 patients, 30 (71%) had a history of antituberculous treatment prior to the diagnosis of MDR-TB ('acquired MDR-TB'). The median time period between the initial diagnosis of TB and the first diagnosis of MDR-TB was 60 months (range 5 months to 14 years). Nineteen (63%) of these patients had received one episode of TB treatment prior to the diagnosis of MDR-TB, 5 (17%) had received two episodes of TB treatment and 6 (20%) patients had received 3 or more episodes of treatment.

M. tuberculosis was resistant *in vitro* to a mean of 4.1 drugs. Only 3 (7%) patients had isolates resistant only to isoniazid and rifampin. Fifteen (36%) patients' isolates were resistant to 3 drugs, 6 (14%) were resistant to 4 drugs, 10 (24%) were resistant to 5 drugs, 7 (17%) were resistant to 6 drugs, 1 (2%) was resistant to 7 drugs and 1 (2%) patients' isolate was resistant to all 8 drugs tested *in vitro*. Drug susceptibility patterns of the 42 MDR-TB isolates are presented in table 1.

Treatment characteristics

One patient received only first-line antituberculous drugs; all other patients received a combination of first line and second-line drugs. The median time to initiation of second-line drugs was 90 days (range 0 days to 1.6 years) from the time of MDR-TB diagnosis. During treatment for MDR-TB, patients received a median of 4 first-line drugs (range 2 to 5) and 4 second-line drugs (range 1 to 6) (table 1).

Table 1: Drug susceptibility pattern and drugs used for treatment in 42 MDR-TB patients

Drug	Resistance pattern (%)	Drug used in treatment (%)
Isoniazid	100	93
Rifampin	100	57
Pyrazinamide	Not tested	83
Ethambutol	45	81
Streptomycin	79	50
Ethionamide	14	88
Kanamycin	14	66
Thiacetazone	48	73
Therizidine	Not tested	61
Ofloxacin	Not tested	54
Clofazimine	Not tested	20
Cycloserin	17	2

In most patients, the treatment regimen used changed during their treatment. Forty-one patients (98%) received at least 3 appropriate drugs, 38 (90%) patients received at least 4 appropriate drugs and 26 (62%) patients received 5 or more appropriate drugs. The median time to initiation of 3 appropriate drugs was 2.8 months (range 0 to 12 months). The longest uninterrupted period of treatment with 3 appropriate drugs was a mean of 14.2 months (range 1 to 39 months). The median time to initiation of 4 appropriate drugs was 3.4 months (range 0 to 19 months). The longest uninterrupted period of treatment with 4 appropriate drugs was a mean of 12.3 months (range 1 to 34 months). Patients were treated for MDR-TB for a mean duration of 20 months (range 4 to 55 months). Patients who achieved bacterial conversion were treated for a median duration of 1 year after bacterial conversion (range 1 to 38 months). Twenty-three (55%) patients were compliant during treatment, 19 (45%) were non-compliant. Thirty (71%) patients were hospitalized for a median duration of 3.2 months (range 6 days to 19 months) after a median interval of 2.9 months since MDR-TB diagnosis (range 0 – 31.9 months). In only 3 patients (7%) was lung surgery undertaken.

Outcome characteristics

Patients were followed up for a median duration of 4.2 years after diagnosis of MDR-TB. The median duration of follow-up in the group of patients who survived was 4.5 years. Of the 40 patients who stopped treatment before May 1999, 8 (20%) had died, 17 (42.5%) were cured and 15 (37.5%) had defaulted. By end of the study (May 1999),

3 additional patients had died, 23 patients were bacteriologically negative, two patients remained bacteriologically positive but refused treatment, one patient had relapsed and restarted treatment, 2 other patients were still on treatment. The remaining 3 patients refused follow-up in 1999. In only one case was death not related to tuberculosis. The average time of follow-up after the end of treatment of 29 patients who had finished treatment by May 1999 and survived, was 2.2 years (range 2.5 months to 5.9 years).

The 5 year survival rate (estimated by Kaplan Meier analysis) was 71 % (SE 8%), the 2 year survival rate was 90% (SE 4%) (figure 1). No patient died within one year of diagnosis of MDR-TB. The median time interval between diagnosis and death was 29 months (range 12 to 57 months).

Figure 1. Kaplan-Meier curves for survival after diagnosis of 42 MDR-TB patients residing in two South African communities.

The cumulative percentage of patients who reached bacterial conversion, estimated by Kaplan Meier analysis, was 79% (figure 2). The time by which 50% of the 42 patients reached bacterial conversion was 9.4 months. Of the patients who converted, the median time for bacterial conversion was 7.2 months (range 1 to 33.9 months) after initiation of treatment and 3.2 months (range 0 to 32.1 months) after initiation of therapy with at least 3 appropriate drugs. Bacteriological conversion took place up to 34 months after diagnosis. However only 3 more patients converted after 15 months of treatment.

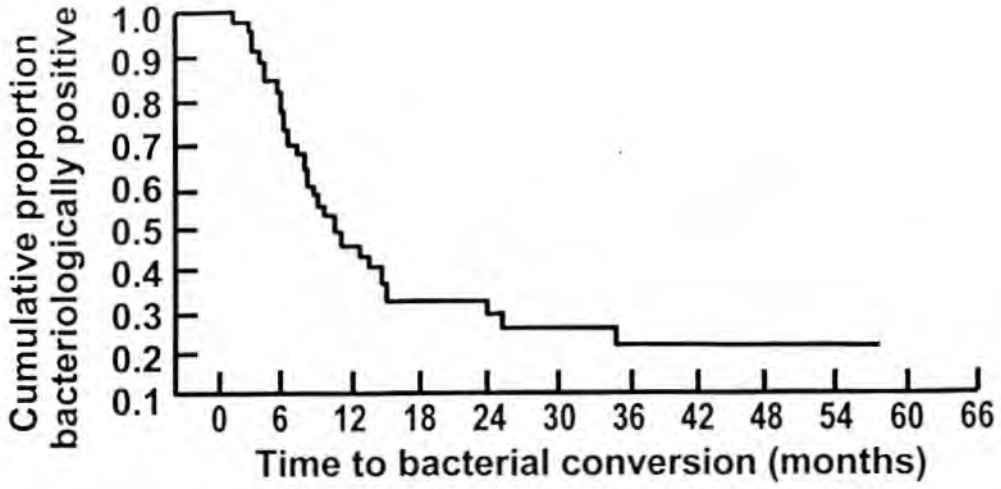

Figure 2. Kaplan-Meier life-table analysis indicating time to bacterial conversion after initiation of treatment in 42 MDR-TB patients.

Five patients became bacteriologically positive again after achieving initial bacterial conversion. In only one patient, the only one that did receive 3 appropriate drugs, did this occur after the end of treatment. The other 4 patients became positive again during their treatment, in two patients most likely due to non-compliance. Three of these patients reconverted to a bacteriologically negative status: 2 after a change in therapeutic regimen, one after lung surgery. The other patient remained non-compliant and bacteriologically positive. The overall rate of bacterial conversion was thus 74%.

Determinants of treatment outcome

Factors investigated for their associations with the outcome of MDR-TB treatment are listed in table 2. Patients who achieved bacterial conversion early were more likely to be younger than 30 years ($p=0.046$), compliant during the MDR-TB treatment ($p=0.0016$), not have interrupted their treatment ($p=0.0002$), treated with at least 4 appropriate drugs for an uninterrupted period extending 11 months ($p=0.05$) and treated with 5 or more appropriate drugs ($p=0.029$). Hospitalisation did not influence the outcome. However, among the hospitalized patients, those who were admitted at an

earlier stage of disease (within 96 days of diagnosis) converted earlier compared to those admitted after a longer delay (p=0.005).

TABLE 2: Characteristics associated with outcome of MDR-TB in 42 patients

Risk factor	p value* for median time to bacterial conversion	p value* for 5 year survival
age (> or < 30 year)	0.046	0.02
Sex	0.078	0.36
prior TB treatment (acquired resistance)	0.035	0.12
TB diagnosis > 60 months before MDR-TB	0.42	0.034
1 or more episodes of treatment	0.66	0.001
Isolate resistant to more then 4 drugs	0.41	0.69
MDR -TB treatment		
Without interuption	0.0002	0.018
Compliant with treatment	0.0016	0.011
Hospitalisation for treatment	0.75	0.54
within 96 d of diagnosis	0.005	0.41
3 appropriate drugs within 87d	0.088	0.86
3 appropriate drugs for >14m	0.06	0.06
4 appropriate drugs within 104d	0.065	0.11
4 appropriate drugs for >11m	0.05	0.05
5 appropriate drugs	0.029	0.04
bacterial conversion		0.00001

* p values are calculated by Kaplan Meier survival analysis

Patients who survived were more likely to be younger (p=0.02), compliant during treatment for MDR-TB (p=0.011), not have interrupted their treatment (p=0.018), treated with at least 4 appropriate drugs for an uninterrupted period extending 11 months (p=0.05) and treated with 5 or more appropriate drugs (p=0.04). The factor most strongly associated with survival was the achievement of bacterial conversion (p=0.00001) (figure 3).

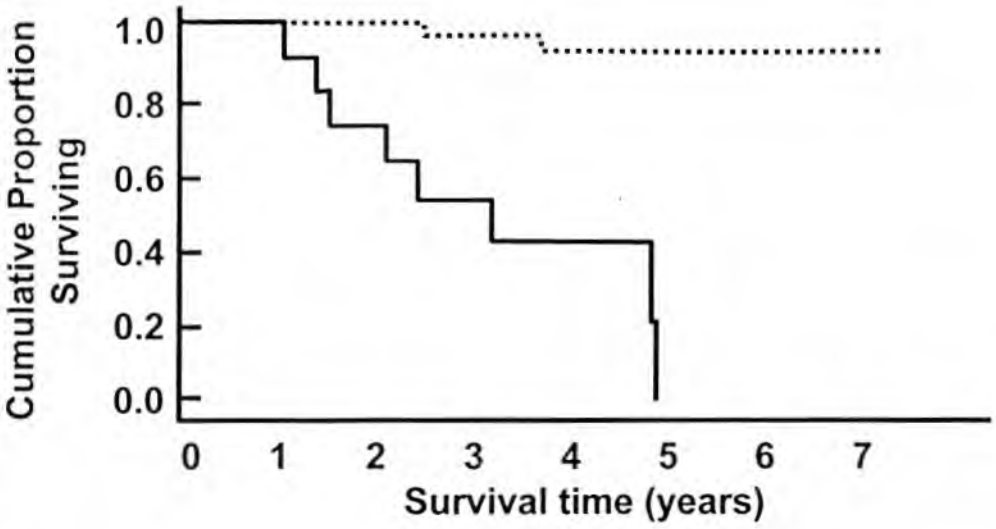

Figure 3. Kaplan-Meier curves for survival after diagnosis of 42 MDR-TB cases comparing cases without bacterial conversion (full line) to cases where bacterial conversion was successful (dotted line).

A history of prior antituberculous treatment in itself was not associated with survival. However, among the patients with a history of TB treatment, patients with chronic tuberculosis (history of multiple episodes of tuberculosis treatment) responded less well to chemotherapy (p=0.001). A long interval (>60 months) between the first diagnosis of TB and the diagnosis of MDR-TB (p=0.034) was also associated with poorer survival.

The number of drugs the isolate was resistant to did not influence bacteriological or clinical success.

DISCUSSION

The optimal treatment for drug-susceptible TB is well established. Modern chemotherapy, appropriately prescribed and administered as in the WHO advised DOTS regimens, can cure over 85% of previously untreated pulmonary tuberculosis cases[17]. No data are available from controlled trials regarding the treatment of patients with

MDR-TB. The current WHO guideline for treatment of MDR-TB cases consists of at least 3 drugs to which the isolate is sensitive for a period of 18 months after bacteriological conversion[18]. In low and middle-income countries, treatment regimens for MDR-TB are not included in National Tuberculosis Program guidelines.

TABLE 3: Literature review of studies on MDR-TB outcome in HIV negative patients

Author, reference and location	Study period	Patient selection	HIV neg. Patients analyzed	Mean duration of follow up (months)	% bacterial conversion	% death total /TB-related
Goble (11) (USA)	73-83	hospital	134*	51	56	34 / 20
Park (9) (USA)	83-93	hospital	41	NA (range 0-120)	NA	24 / 17
Schaaf (13) (RSA)	87-93	population	226*	60 (survivors)	87 (survivors)	48 / NA
Telzak (8) (USA)	91-94	hospital	25	20	65	4 / 4
Turret (10) (USA)	91-93	hospital	4	NA (range 0-34)	100	0 / 0
Flament (2) (France)	94	population	43	NA (range 24 –36)	41	NA / 46
Park (12) (Korea)	96	hospital	63	17	82.5	NA / 0

*Patients were not tested for HIV status, however, considering the HIV incidence in the studied area at the time, one can assume that, at least the majority of these patients, would have been HIV negative.

Several studies have analyzed the outcome of MDR-TB patients[2,8-13]. Results indicate that HIV seropositive cases clearly have a poorer outcome compared to immunocompetent patients. It is however difficult to compare results of these studies, even among studies on outcome of MDR-TB in HIV seronegative patients. This is due to differences in definitions of outcome (bacteriological or clinical success), duration of follow-up and patient characteristics (table 3). In addition, analysis is carried out in various ways, rarely using survival analysis and frequently not displaying results of the whole cohort due to lack of follow-up data on all patients. The figures on 'favourable outcome' among HIV seronegative patients varied from 41% to 100%[2] for bacteriological conversion and from 52%[13] to 100%[10,12] for survival. However, the most optimistic figures are reported by two studies, one with only 4 patients[10] and one which was biased by the exclusion of 41% of patients from the analysis because of surgery (21%), loss to follow-up (19%) and adverse drug reactions (1%)[12].

In the present study, where virtually all patients were proven to be HIV seronegative, 74% of patients achieved bacterial conversion. Not surprisingly, compliance with treatment was the most important factor influencing bacteriological success. The 2-year survival was 90% and decreased to a 5-year survival of 71%. This underlines the necessity of a long-term follow-up of HIV seronegative MDR-TB patients. As described by Goble et al.[11], the most predictive factor for survival was bacteriological success. Younger age at diagnosis, compliance with treatment and treatment with 4 or more appropriate drugs for a period extending 11 months were other factors associated with improved bacteriological success and a greater chance of survival. Telzak et al.[8] noted, as we also found, that multiple previous episodes of treatment were associated with a less advantageous outcome. This may be due to extensive lung damage.

The lack of association of outcome to the number of drugs to which the isolate was susceptible is in agreement with some reports[8,10] but in contrast to others[2,11,12].

As virtually all of our patients received appropriate treatment, we could not analyse the finding that institution of appropriate treatment is the factor most strongly associated with a favourable outcome[9]. We did however find that the strength of association with favourable bacteriological and clinical outcome increased with the number of appropriate drugs used, reaching significance at the level of 5 appropriate drugs. The time to initiation of appropriate treatment did influence the outcome. This is clinically very important, not only on an individual level but also on a community level as unsuccessfully treated patients can transmit their disease to other persons in the community and thus constitute a public health problem[19,20].

In the same province (Western Cape, South Africa), a study determining the outcome of MDR-TB had been previously undertaken[13]. The 5-year survival rate was then determined to be 46%. At the time of that study, patients with MDR-TB were treated without the benefit of a centralised specialist facility, by directly observed therapy, in the same primary health care clinic system as patients with drug-susceptible TB. There were no second line drugs included into the treatment regimen of MDR-TB patients. This is still the scenario in most low and middle-income countries, but also in

some high-income countries[2]. The survival of MDR-TB cases in that study was not markedly different from that of TB patients before chemotherapy was available[21].

In our series, as in most other series[2,8-13], the majority of patients with MDR-TB had isolates resistant not only to INH and RIF but also to a mean of 2 other first-line agents. Empirical therapy recommended for patients who are not multidrug resistant would therefore not include the administration of 3 appropriate drugs, possibly resulting in monotherapy and may thus aggravate the problem ("amplifier effect")[22]. With the introduction of a specialist clinic in the study area for the evaluation and management of patients with MDR-TB, second line drugs became consistently available for MDR-TB treatment. Subsequently, the 5-year survival of MDR-TB patients in the area has increased from 46 to 71%.

The results of this study suggest that a more individualised approach to the management of MDR-TB could be the most important factor contributing to improvement in the outcome of MDR-TB patients. Even though virtually all patients in this study received treatment with 3 appropriate drugs, the median time between diagnosis and initiation of appropriate therapy was unnecessarily long, and longer than that reported elsewhere[8]. The fact that MDR-TB patients included in this study were not systematically sent to a specialist MDR-TB clinic might be an explanation for the long delay in initiation of appropriate treatment and the long time to bacterial conversion. Similarly to the only other population-based study on outcome of MDR-TB[2], hospitalisation alone had no influence on outcome, although hospitalisation early in the course of the disease may have been beneficial. Attention to these factors might further improve the outcome of MDR-TB patients.

The present study indicates that the availability of a specialized centre for the management of MDR-TB, the only institution through which second line drugs can be obtained for treatment, can improve the outcome of MDR-TB treatment. The results therefore support the development of these centralised, specialised MDR–TB treatment units as part of a rational "DOTS plus" strategy for management of MDR-TB patients[18,22,23]. Even with these improvements, however, further progress is needed in development of the treatment of such cases. In the meantime, every effort must be made to avoid the promotion of drug resistance through appropriate treatment of the initial

drug sensitive TB episode because, in the end, clinically significant drug resistance is virtually always to result of a failure of medical practice[24].

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CHAPTER 10

General Conclusion

General Conclusion

A. Dogmas on tuberculosis and drug-resistant tuberculosis

Tuberculosis is a chronic infectious disease with a complex natural history. Transmission occurs by droplet infection and its occurrence is directly associated with the bacillary load in the sputum, cough frequency and environmental factors such as closeness of contact and duration of exposure. In only 5-15% of cases will active TB follow infection after an asymptomatic incubation period ranging from weeks to decades[1]. Disease in a previously infected individual may be caused by reactivation of dormant bacilli (endogenous reactivation) or it may be the result of a new challenge caused by reinfection with a different strain of *Mycobacterium tuberculosis* (exogenous reinfection). The proportion of tuberculous disease attributable to endogenous reactivation and exogenous reinfection has been the subject of a debate for decades[2]. Currently, it is generally accepted that the majority of cases of TB are a consequence of endogenous reactivation[3]. However, there is also evidence to support that the relative contribution of exogenous reinfection increases in parallel with the TB incidence[4,5].

Tuberculosis is a disease that may have killed more individuals than any other single pathogen. The enthusiasm was great when the first antituberculous drugs became available. But this enthusiasm was soon tempered as drug resistance arose after the introduction of antituberculous drugs. Drug resistance is the result of selection of spontaneous mutants that occur even prior to contact with antituberculous drugs[6]. Selection results from treatment errors, such as prescription of inappropriate regimens and patient non-compliance[7]. Multiple treatment errors lead to sequential acquisition of drug resistance[8]. Most cases of drug-resistant TB arise in chronic patients and result from poor treatment programmes[7]. Grzysbowski et al. estimated that the outcome of patients will be chronic in only 3% of patients under a good TB control programme while 25% of patients will become chronic in a poor TB control programme[9]. Drug-resistant TB is thus a man-made problem with an incidence that is related to the quality of the National Tuberculosis control Programme. However, it is also possible that patients are infected by transmission of a drug-resistant *M. tuberculosis* strain (primary drug resistance). Until recently, it was generally believed that this occurs with a

relatively low frequency, presumably because the metabolic compromises made by the bacillus to enable drug resistance makes the bacillus modestly less virulent[10]. Even a small reduction in pathogenic capacity would make transmission of drug-resistant disease quite uncommon in the normal host, whose immune system has a 90% chance of containing the infection for a lifetime. Only in specific population groups, such as institutionalised patients co-infected with HIV and TB, is it believed that there is a high risk for outbreaks of drug-resistant TB[10].

B. Re-evaluation of these dogmas

The aim of this study was to re-evaluate these dogmas in an area with one of the highest reported TB incidences in the world. This was possible through the use of new techniques such as a computerised geographic information system, and new molecular genetic techniques such as Restriction Fragment Length Polymorphism (RFLP) and mutation detection analysis.

RFLP results demonstrated that community outbreaks of drug-resistant TB occur, even without the involvement of a single immunocompromised (HIV seropositive) patient. Not only do outbreaks occur within the community, but the majority of patients with drug-resistant TB were infected with a strain belonging to a cluster of drug-resistant isolates, indicating transmission from one infectious patient to another person. The assumption that patients infected with identical strains are epidemiologically linked and therefore indicate transmission from one person to another was supported by additional data. First, results of mutation analysis by dot-blot hybridisation analysis demonstrate that the initial isolate of all patients infected with an identical drug-resistant *M. tuberculosis* strain contained the exact same combination of mutations conferring resistance. This was true for all isolates except for 3 cases infected with a strain characterised by an exceptionally high genetic stability. This makes the possibility of transmission of a drug sensitive strain with subsequent acquisition of drug resistance in individual patients highly unlikely. Second, results from the investigation of an outbreak of drug-resistant TB by combining molecular and traditional epidemiologic data showed epidemiologic links among 12 of the16 patients involved in the outbreak. Third, an analysis combining a Geographic Information System (GIS) and

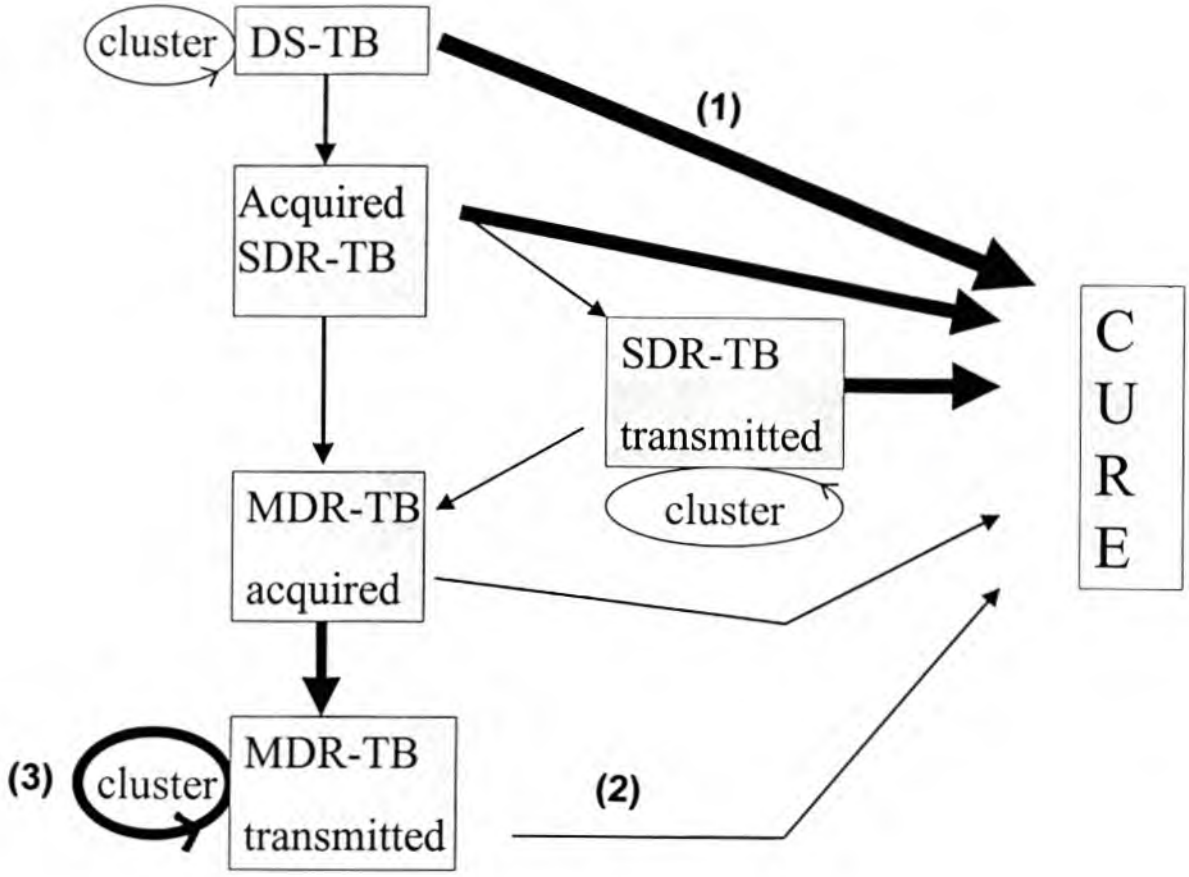

Figure 1. Influence of the presence of DOTS and the absence of DOTS-plus on the outcome of cases with drug-sensitive and drug-resistant tuberculosis.

Dark (thick) arrows indicate the outcome of the majority of cases. Light (Thin) arrows indicate the outcome of a minority of patients. SDR-TB stands for single-drug-resistant tuberculosis.
(1) under DOTS regimen, the majority patients with drug-sensitive TB will be cured
(2) in the absence of an effective MDR-TB programme, few patients with MDR-TB will be cured
(3) the majority of MDR-TB patients will remain infectious for prolonged periods of time in the absence of effective treatment which can lead to transmission of MDR-TB strains and the creation of clusters.

molecular epidemiologic data and showed that 80% of cases within each cluster of drug-resistant TB resided within 600 meter of another case and this in geographically distinct areas for each cluster.

There was a difference in the relative contribution of transmission among different types of drug-resistant TB: transmission was the most important mechanism for multidrug-resistant TB (MDR-TB) while acquisition was the predominant mechanism for single drug-resistant TB. I would like to hypothesise that these results are the consequence of the history of TB control strategies in South Africa, representative for many middle income counties (Fig 1). The key factors leading to this situation are (i) an area endemic for TB where people are in repeated contact with infectious TB patients (ii) a suboptimal TB control programme in the past leading to errors in drug prescription and patient non-compliance, (iii) a current DOTS program leading to high case holding rates, high cure rates and the protection of front line drugs and (iv) the lack of an effective MDR-TB programme. Under such conditions, the overwhelming majority of new patients with drug-sensitive TB will be cured by the DOTS programme and only a very small percentage will acquire drug resistance. However, a pool of chronic TB patients with a relatively high percentage of drug resistance, especially to the "older" TB drugs such as isoniazid and/or streptomycin, will have been inherited from the past. A DOTS regimen can be effective in the treatment of single-drug-resistant TB, as the regimen will still provide drugs to which the isolate is sensitive. Most single-drug-resistant TB patients will thus be cured and only a few of these patients will evolve to MDR-TB cases by acquisition of additional resistance. Patients with single-drug-resistant TB will thus not remain infectious for prolonged periods of time and the transmission rate of single drug-resistant TB will be comparable to that of drug sensitive TB. Another inheritance from a poor program in the past is a pool of MDR-TB patients. In the absence of a specialised MDR-TB programme with access to first and second line drugs, these patients cannot be effectively treated. Few MDR-TB patients will be cured and the majority will remain infectious for prolonged periods of time. They can infect other people leading to cases of primary MDR-TB. Cases of primary drug resistance will remain undiagnosed for months, as the DOTS programme does not require routine culture and drug-susceptibility tests at diagnosis. This allows transmission with further enlargement of the pool of people infected with drug-resistant strains of *M. tuberculosis*.

Another hypothesis that could explain the difference in therelative importance of transmission of different types of drug-resistant tuberculosis could be the transmissability of the organism. Of all unique single-drug-resistant isolates, the mutation conferring resistance to isoniazid was detected in only 27% of cases. This is in sharp contrast to the MDR-TB isolates in which a *katG*315 mutation was detected in 89% of isolates. It is possible that the mutation(s), as yet unidentified, do not only confer resistance to isoniazid but also reduce transmissability. This would confirm the historical studies in which it was shown that isoniazid resistant bacilli were reduced in virulence[11].

Surveillance of drug-resistant TB cases is important for evaluation of TB control programmes. It has been suggested that classification of patients according to pathogenic pathway of drug resistance is essential for better insight[7]. The standard classification of patients into acquired (previously treated patients) and primary (patients without a history of prior treatment) drug-resistant TB reflects the general belief that the majority of drug-resistant TB occurs as a consequence of acquisition of drug resistance in the individual patients due to treatment errors[7]. When comparing the classification of patients according to the clinical classification (acquired or primary) and the RFLP classification (unique or cluster) it was clear that the group of patients with acquired drug-resistant TB does not only include patients who truly acquired drug resistance during treatment but also includes patients initially infected with a drug-resistant strain as well as patients re-infected with a drug-resistant strain. Surveys based on the standard classification may therefore result in misleading interpretations and incorrect evaluations of the quality of TB control programmes.

The group of patients re-infected with a drug-resistant *M. tuberculosis* strain was a numerically important group: 25 of the 45 (56%) patients with a prior history of antituberculous treatment. If re-infection is an important mechanism in the occurrence of drug-resistant TB after prior treatment, re-infection should also be an important mechanism in the occurrence of relapse after prior treatment for drug-sensitive TB. We evaluated this hypothesis by RFLP analysis of the infecting strains in patients presenting with active TB after prior cure for drug-sensitive TB. For 12 of the 16 patients, the RFLP pattern for the isolate obtained after relapse was different from the

RFLP pattern for the isolate from the initial TB episode. These findings indicate the importance of re-infection as the cause of recurrence of TB after curative treatment for drug-sensitive TB.

Treatment of drug sensitive TB is highly efficacious, but the treatment options for drug-resistant TB and especially MDR-TB are limited. A standard DOTS programme with first-line drugs regimen can not only manage drug sensitive TB but can also decrease the prevalence of single-drug-resistant TB[12]. In Korea, after the introduction of a good quality TB control programme, the prevalence of INH resistance dropped from 25% in 1980 to 4.9 % in 1995[13]. Similarly, in Algeria, the prevalence of acquired INH resistance decreased from 34% (1975-1980) to 10% (1981-1985)[14]. The options for treatment of patients with MDR-TB are (i) standard DOTS regimen as for patients with drug sensitive TB, (ii) standard regimens including second line antituberculous drugs, selected on the basis of the most common drug resistance profiles in the community/country or (iii) individualised drug regimens, adjusted to the drug resistance profile of the infecting bacillus. Because of the extremely high cost of second line drugs, the WHO has opted for the use of standard DOTS regimens and discouraged the use of routine drug resistance testing in the diagnosis of new TB cases in low and middle income countries. When the WHO launched their new TB control strategy, it was hoped for that DOTS would also control MDR-TB and that MDR-TB would simply disappear if DOTS were established worldwide. The experience gained since the introduction of DOTS shows that MDR-TB persists[15-18]. In Korea, the rate of acquired MDR-TB increased from 14.5 to 19.8% between 1985 and 1995[13]. In Algeria, prevalence of acquired MDR-TB increased from 1.7% to 11%[14]. The implementation of a DOTS regimen in the prisons in the former Soviet Union will not lead to a reduction in MDR-TB, seen that the overall success rate was only 27% for patients with confirmed MDR-TB[19]. Outcome data from patients in the Western Cape treated for MDR-TB by a supervised standard first line drug regimen, indicate that the outcome is comparable to that obtained in patients before the era of antituberculous drugs: a 5 year survival rate of 46% of patients[20].

Clinical data of the patients with drug-resistant TB were used to study the outcome of MDR-TB in an area endemic for TB after the introduction of a specialised MDR-TB

treatment centre in the Western Cape Province. The majority of patients (98%) were now treated with at least 3 drugs to which the isolate was sensitive. This individualised approach to the treatment of MDR-TB was not yet optimal: appropriate treatment was only started after an average delay of 2.8 months and the longest uninterrupted period of treatment with 3 appropriate drugs was only a mean of 14.2 months instead of the proposed 18 to 24 months. Despite these shortcomings, the 5-year survival rate of patients with MDR-TB in the area increased from 46% to 71%. These results suggest that an individualised approach and the availability of a specialised MDR-TB unit can, even in non-industrialised countries, improve the outcome of MDR-TB.

C. Public health implications of the results

Results obtained in this study have enabled the re-evaluation of general beliefs concerning the disease dynamics of TB and more specifically the disease dynamics of drug-resistant TB. The ultimate aim is to go one step further and use the new insight to improve TB control. That this is possible has been shown, for example, by the population-based molecular epidemiological work performed in San Francisco. Control measures directed towards reducing transmission of *M. tuberculosis* in high-risk groups were implemented on the basis of the finding of a high proportion of clustered cases. This resulted in a decrease of the rate of clustered cases as well as a decrease of the annual TB case rate[21].

The results of this study indicate that, if exogenous reinfection is common, the use of antituberculous chemoprophylaxis in people recently exposed to infectious TB might be prudent regardless of the prior history of TB infection or disease. With a high degree of exogenous reinfection one could also expect a greater impact of sound TB control strategies than when endogenous reactivation is the predominant mechanism[22].

The results of this study might be helpful in answering questions concerning the format of future DOTS-plus programmes for MDR-TB. In agreement with Portaels et al[18], I would like to state that the central elements of a standard DOTS strategy might need to be modified.

1. Diagnosis based on microscopy or routine drug susceptibility tests?

The DOTS strategy currently employs standardised treatment regimens depending on the patients' past treatment history. This approach is based on results of trials, which failed to show a significant difference in outcome among patients with initial drug-resistant TB whose treatment was or was not adjusted on the basis of initial drug susceptibility results[23-25]. However, these studies were performed when resistance to rifampin was rare. The limited data available on the outcome of MDR-TB under DOTS regimen are disappointing[13-20].

The high failure and death rates are not the only problem involved in the management of MDR-TB by standard DOTS program. There is evidence that MDR-TB patients may develop additional resistance to SM, EMB or PZA under standard DOTS regimens (amplifier effect)[15,18,26]. Furthermore, MDR-TB patients will remain infectious and transmit drug-resistant bacilli into their environment. A question to be answered is whether the cost of treating all future secondary cases infected by MDR-TB patients exceeds the cost of routine culture and drug-susceptibility tests at diagnosis.

Heifets et al. have argued that the lack of proper laboratory support for TB control programmes in countries such as South Africa, is often not a pure matter of affordability, but rather an underestimation of its importance and the consequence of the implementation of a DOTS strategy requiring that diagnosis is based on acid-fast bacilli smear examination only[16].

If a decision for routine drug susceptibility tests at diagnosis is made, a further question raised by the work of this study is whether the standard culture-based drug-susceptibility tests will be the screening test of choice in a DOTS-plus strategy. The success of prevention of transmission of MDR-TB depends on the time a patient remains infectious. Phenotypic drug resistance testing is technically very demanding and requires a minimum of 3 to 6 weeks for a positive culture and a further minimum of 2 to 3 weeks for susceptibility testing[27]. The use of high quality national and supranational laboratories increases the reliability of results but also increases the already long turn-around time of laboratory reports. Results of genotypic drug resistance testing based on mutation analysis can be available within 24 hours after positive

culture and has even been successful directly on sputum. In this study, the cost of genotypic testing could be reduced as the evaluation of 3 selected codons (rpoB531, rpoB526 and katG315) allowed identification of 90% of MDR-TB cases. Furthermore, we have been able to show that, for MDR-TB diagnosis, the performance of genotypic drug resistance testing was superior to phenotypic drug resistance testing under routine conditions. Further research should be directed at the possibility of the development of a commercial kit testing a limited number of mutations conferring resistance.

2. Passive or active case detection?

Following the DOTS guidelines, case detection is passive. Contact tracing is considered ineffective, inadequate and therefore not cost-effective. However, the efficiency of contact tracing can be improved as shown by a study in San Francisco where investigators were trained to improve communication with specific populations at risk. The median number of contacts per new case increased and the percentage of cases with no contacts identified decreased from 25% in 1990 to 3% in 1997[21].

For this study, epidemiological data on a community outbreak of MDR-TB was collected by a medical anthropologist and showed that 12 of the 16 patients involved in the outbreak had social connections with at least one other person also involved in the outbreak. Only 4 of these patients were detected by contact tracing through the clinic system. Because of the importance of early diagnosis of MDR-TB, selective active tracing for patients in contact with MDR-TB cases could be a cost-effective procedure.

3. Treatment with a standardized DOTS-plus regimen or an individually adjusted DOTS-plus regimen?

Data on the outcome of MDR-TB under standard DOTS regimen show disappointing results[13-20]. Adjusted MDR-TB programmes have had better success rates, especially in HIV seronegative patients. Standardised regimens, consisting of a combination of first and second line drugs selected on the basis of susceptibility profiles of MDR-TB strains in the community have been successful in Rwanda[28]. Individualised regimens, based on the drug susceptibility pattern of the patients' isolate, have been successful in the USA[29,30], Peru[31] and Korea[32]. The majority of MDR-TB patients

included in this study were treated by individualised treatment regimens: 98, 90 and 62 % of patients received respectively at least 3, 4 and 5 appropriate drugs according to the results of the drug susceptibility tests. This increased the 5-year survival of MDR-TB patients in the area from 46% (treatment by standard first line regimen) to 71% (treatment by individualised MDR regimen).

Future research should be directed at multicentre clinical trials evaluating the outcome and cost-effectiveness of standardised versus individualised MDR-TB treatment regimens.

D. Final comment

MDR-TB is clearly a problem confronting a DOTS programme in its success in TB control. Mathematical modelling suggests that MDR-TB needs to be aggressively managed to prevent future epidemics[33]. Data indicating that MDR-TB is not uncontrollable exist. In response to large nosocomial outbreaks of HIV related MDR-TB, resources (>1000 million US$) were provided to re-establish the decayed TB control programme of New York City. An effective DOTS programme was introduced, contact tracing was intensified, the use of isoniazid preventative therapy was increased and individualised treatment of MDR-TB patients was initiated. Subsequently, the number of cases of MDR-TB in New York City decreased by 44% from 1992 through 1994. Epidemiological patterns suggest that the decrease resulted from interruption in the ongoing spread of MDR-TB[34].

It has been shown that a sound DOTS programme can stop acquisition of drug resistance. The work in this study showed that, even in poorer countries, treatment of MDR-TB patients could be made much more effective by the institution of specialised MDR-TB care. Because of the relatively high proportion of MDR-TB caused by transmission, one can expect effective treatment to have an important impact on the prevalence of MDR-TB, as effective treatment will reduce the high rate of ongoing transmission of MDR-TB. It should therefore be possible to turn the tide, not only in rich industrialised countries but also in poorer countries.

Until recently, treatment of MDR-TB in resource poor countries had been deemed by many to be too difficult and too costly to even attempt; others declared that MDR-TB and transmission of MDR-TB was simply not a problem[16,35]. It is now clear that MDR-TB in the developing world has become a problem that can no longer be ignored[31,35,36]. Worldwide, all TB patients, including MDR-TB patients, should have a right to effective treatment. Otherwise, we will create enormous problems for the future and will again be faced with a situation in which medical advances cannot be used in the poorer population most in need. However, it has been shown that a poor TB control programme can be more harmful than no programme[9]. The same might be true for a DOTS-plus programme for MDR-TB. Therefore, excellent operational studies will be necessary to decide on the optimal format for DOTS-plus programmes. It is in this context that the WHO established the WHO Working Group on DOTS-plus for MDR-TB in 1999. International political commitment and financial support will be necessary for the implementation of a DOTS-plus programme in selected areas with median and high levels of MDR-TB. But the most crucial element in the decision for implementation of a DOTS-plus programme, directed at stopping the spread of MDR-TB, will be the existence of a sound DOTS programme to stop further acquisition of MDR-TB.

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