Tuberculosis in Ndola Urban District, Copperbelt Province, Zambia: Molecular epidemiology, drug resistance, access to quality of care

Faculty of Pharmaceutical, Biomedical and Veterinary Sciences

Dissertation for the degree of doctor in biomedical sciences at the University of Antwerp to be defended by

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Tuberculose in het Ndola Stedelijk District, Copperbelt Provincie, Zambia: Moleculaire epidemiologie, antibiotic resistentie en toegang tot kwaliteitsvolle zorg.

Tuberculosis in Ndola Urban District, Copperbelt Province, Zambia: Molecular epidemiology, drug resistance, access to quality of care

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To our God the Father, You brought me to it, and you have taken me through it!

Contents

Abbreviations

AhpC	alkyl hydroperoxide reductase
ACP	acyl carrier protein
AIDS	acquired immune deficiency syndrome
B.C.	before Christ
BCG	bacilli Calmette-Guerin
CAS	Central Asian spoligotype
CFP-10	culture filtrate protein 10
CT	computed tomography
CRI	colorimetric redox indicator
DHMT	district health management team
DR	direct repeat
DNA	deoxyribonucleic acid
DOTS	directly observed therapy short course
DST	drug-susceptibility testing
E	ethambutol
EAI	East African Indian spoligotype
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot
EmbB	an arabinosyl transferase inhibited by ethambutol
ESAT-6	early secretory antigenic target 6
ETH	ethionamide
FDC	fixed dose combinations
FDA	Federal Drug Agency
FQ	fluoroquinolone
GC	guanine-cytosine
H	isoniazid
HIV	human immunodeficiency virus
IFN-γ	interferon gamma
IGRA	interferon gamma release assay
IS	insertion sequence
KAN	kanamycin
LAM	Latin American Mediterranean spoligotype
LAMP	loop-mediated isothermal amplification
LED	light-emitting diode
LJ	Löwenstein-Jensen
LPA	line probe assay
MDGs	Millennium Development Goals
MDR-TB	multidrug-resistant TB
MGIT	mycobacteria growth indicator tube
MIRU	mycobacterial interspersed repetitive units
MIRU-VNTR	mycobacterial interspersed repetitive units- variable number of tandem repeats
MODS	microscopic observation drug-susceptibility

MOH	Ministry of Health
MPTR	major polymorphic tandem repeat
MTB	*M. tuberculosis*
MTT	3-(4,5-dimethylthiazol-2-5-Diphenyl) tetrazolium bromide
NAAT	nucleic acid amplification test
NRA	nitrate reductase assay
NTLP	National Tuberculosis and Leprosy Programme
NTM	non-tuberculous mycobacteria
NTP	National Tuberculosis Programme
OFL	oflaxicin
PCR	polymerase chain reaction
PGRS	polymorphic GC-rich sequence
POA	pyrazinoic acid
PPD	purified protein derivatives
QFT-G	QuantiFERON-Gold
R	rifampicin
REMA	resazurin microtitre assay
RFLP	restriction fragment length polymorphisms
RNA	ribonucleic acid
rRNA	ribosomal RNA
RT-PCR	real-time PCR
S	streptomycin
SAF1	Southern Africa Family 1
SCC	short course chemotherapy
SNP	single nucleotide polymorphisms
ST	shared types
STD	sexually transmitted diseases
SNP	single nucleotide polymorphism
TLA	thin layer agar
TST	tuberculin skin test
TB	tuberculosis
VNTR	variable number tandem repeats
WHO	World Health Organization
XDR	extensively drug-resistant
Z	pyrazinamide
ZN	Ziehl–Neelsen

Summary

Despite worldwide tuberculosis (TB) control efforts, 1.3 million people died from TB worldwide in 2009. Africa accounts for approximately 30% of global TB cases and deaths. Similarly, the TB burden in Zambia has continued to be high, despite years of directly observed treatment strategy (DOTS) implementation.

In this thesis, we investigated the management of TB in Ndola Urban District by combining molecular and conventional tools to study the epidemiology of TB and access to TB care.

Firstly, we determined the levels of TB drug resistance. Sputum samples were collected from all consecutive smear-positive, new and previously-treated patients, from four diagnostic centres. Drug-susceptibility testing was performed using the proportion method against four first- and two second-line TB drugs. Results revealed relatively low levels of any resistance to first-line drugs (8.8%), 1 case of MDR, 1.3% and 3.3% rifampicin mono-resistance (new and previously-treated, respectively) and absence of resistance to ofloxacin and kanamycin,.

Secondly, we determined the types of circulating *Mycobacterium tuberculosis*-complex strains in Ndola and possible modes of transmission using spoligotyping and 15-locus MIRU-VNTR typing. The results showed that majority (87.7%) of isolates belonged to SAF1 spoligotype family with a high clustering rate of 37.7%, suggesting significant recent transmission levels.

Lastly, we investigated TB service delivery and aspects of health seeking behaviours of TB patients. In a retrospective descriptive study of 105 previously treated sputum-smear positive pulmonary TB subjects, structured questionnaire administration identified that patients delayed to seek treatment (68.0%) even when, knowledge of TB symptoms was high (78.1%) or patients suspected TB (73.3%). Patient adherence to taking medication was high (77.1%) but low submission of follow-up sputum (47.6%) was observed.

We conclude that Ndola has managed to maintain low levels of TB drug resistance most likely attributed to long-standing DOTS implementation. Further, only a small number of genotypes account for TB cases in Ndola indicating recent transmission plays an appreciable role in TB transmission. Many aspects of TB service are well established in Ndola Urban District as per national TB guidelines. However, sensitisation on care-seeking and

closer patient follow-up monitoring systems is recommended to strengthen TB control program.

Samenvatting

Ondanks de inspanningen om tuberculose (tbc) te bestrijden, stierven er in 2009 wereldwijd 1,3 miljoen mensen aan tbc. Afrika is goed voor ongeveer 30% van de tbc-gevallen en –sterfte. Ook in Zambia blijft de ziektelast hoog ondanks de jarenlange implementatie van de DOTS (directly observed treatment strategy) strategie.

In dit proefschrift onderzochten we de epidemiologie van tbc en de toegang tot tbc-zorg in het Stedelijk District van Ndola.

Ten eerste hebben we de mate van resistentie aan vier eerstelijns en twee tweedelijns anti-tbc middelen bepaald. Sputummonsters werden verzameld van opeenvolgende, nieuwe en eerder behandelde patiënten in vier diagnostische centra. De resultaten toonden een relatief laag niveau van resistentie tegen eender welk eerstelijns middel (8,8%), één geval van multi-resistente tbc, 1.3% en 3.3% van mono-resistentie aan rifampicine (nieuwe en eerder behandelde, respectievelijk) en afwezigheid van resistentie tegen ofloxacine en kanamycine.

Ten tweede hebben we de aard van de circulerende Mycobacterium tuberculosis-complex stammen in Ndola gedocumenteerd met behulp DNA-fingerinting technieken. De resultaten toonden aan dat de meerderheid (87.7%) van de isolaten behoorden tot de SAF1 spoligo familie met een clusteringgraad van 37.7%.

Tot slot onderzochten we de tbc-dienstverlening in de gezondheidszorg en het gedragspatroon om hulpverlening te zoeken bij tbc patiënten. In een retrospectieve, beschrijvende studie bij 105 eerder behandelde pulmonaire tbc patiënten, gaf een gestructureerde vragenlijst aan dat de patiënten de stap naar hulpverlening uitstelden (68.0%), zelfs wanneer de kennis van tbc-symptomen goed was (78.1%) of patiënten vermoedden dat ze tbc hadden (73.3%). De therapietrouw was volgens de patiënten hoog (77.1%), maar de naleving voor het indienen van opvolg sputum was laag (47.6%).

We concluderen dat Ndola erin geslaagd is om lage niveaus van resistente tbc te behouden, waarschijnlijk toe te schrijven aan langdurige DOTS implementatie. Verder blijkt slechts een klein aantal genotypen verantwoordelijk voor de tbc-gevallen in Ndola wat aangeeft dat recente transmissie hier een aanzienlijke rol speelt in de tbc epidemiologie. Howel, veel aspecten van de tbc dienstverlening hier goed zijn ingeburgerd, blijft sensibilisering om de stap naar hulpverlening niet uit te stellen en het

nauwer naleven van patiëntopvolging zijn aangeraden om het tbc-bestrijdingsprogramma te versterken.

Chapter 1: Introduction

1.1 *Mycobacterium tuberculosis*

1.1.1 The genus *Mycobacterium*

Bacterial species within the genus *Mycobacterium* are slender, slightly curved or straight rods with a width of 0.2-0.6µm and length of 1.0-10µm. They are non-motile, non-spore forming, acid-alcohol fast, gram-positive aerobes. Their genomic makeup consists of high guanine-cytosine (GC) content (61% -71%). Mycobacteria are also characterized by a very unusual, complex cell wall structure in which up to 60% is composed of lipids consisting mainly of uncommonly long-chain a-branched ß-hydroxylated fatty acids with 60-90 carbons called mycolic acids. The complex cell wall confers this genus with resistance to dehydration, acids and alkalis, and prevents decolouration by acid-alcohol (1-3). Phylogenetically, *Mycobacterium* is the only genus of the family Mycobacteriaceae within the order Actinomycetales of the class Actinomycetes. Figure 1 shows the different groups within the genus *Mycobacterium* (4).

With respect to their clinical significance, *Mycobacterium* species can be classified into three groups; obligate parasites that are pathogenic to vertebrates, potential (opportunistic) pathogens, which can be found in clinical samples as well as water, soil and other environmental sources, and saprophytes that rarely cause disease and are found in the environment.

1.1.2. *Mycobacterium tuberculosis* complex

M. tuberculosis, the organism responsible for the human infectious disease tuberculosis (TB) is one of several closely related bacterial species known as tubercle bacilli or *M. tuberculosis* (MTB) complex, which belong to the genus *Mycobacterium*. This group includes *M. tuberculosis*, *M. africanum*, *M. microti*, *M. bovis*, *M. bovis BCG*, *M. canettii*, *M. pinippedii* and the recently described *M. mungi* (5).

Whilst other mycobacterial species are widespread in the environment, members of the MTB complex are obligate pathogens. With a doubling time in the order of 18 to 24 hours for *M. tuberculosis* and *M. bovis*, MTB complex species are very slow growers both *in vitro* and *in vivo*. The slow doubling time can be attributed to the complex synthesis of the cell wall which involves multiple genes, additionally, the large waxy cell wall restricts intake of nutrients through the pores slowing down bacterial growth. Consequently, growth of identifiable colonies will only appear after 4 to 6 weeks on solid media inoculated with clinical material, whilst infection is an insidious chronic

process, which may take several weeks or months before manifesting disease. The MTB complex exhibits very little phenotypic and genetic variation among its members, complicating differentiation between species in routine diagnosis (6-7).

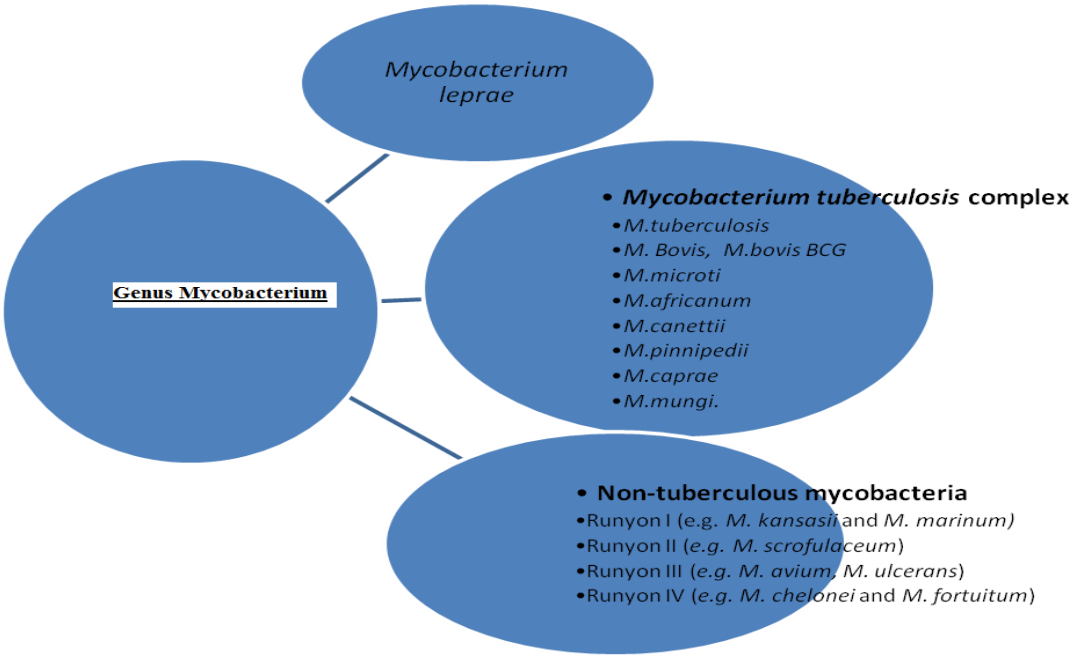
The host range of MTB complex species does however present some variations. Whereas, *M. tuberculosis* is known to be the principal cause of human TB (8-9) and *M. africanum* has been reported to be a significant contributor to human TB infection in West Africa (10-11), *M. bovis* has a wider host range. Although *M. bovis* preferentially affects bovinidae and cervidae, it is also the main cause of TB in domestic and wild animals (12-13) and known to affect humans. Unpasteurised milk and milk products are regarded as the main route of transmission of zoonotic TB caused by *M. bovis* in countries where there are no effective eradication programmes for bovine TB in cattle. Other members of the MTB complex such as *M. canettii* have been reported to occasionally cause human infections (14-16), while some species are associated with particular animals such as voles (*M. microti*), goats (*M. caprae*) or seals *(M. pinnipedii*), with few reports of human infection (17-22), and banded mongooses (*M. mungi*) with no reported cases of human infection.

Another species in the genus *Mycobacterium*, M. *leprae*, is a pathogenic mycobacterium, which causes leprosy in humans, and has also been reported in wild nine-banded armadillos and in three species of non-human primates (chimpanzees, sooty mangabey monkeys and cynomolgus macaques (23-25). The disease affects peripheral nerves, skin, and mucous membranes. Skin lesions, areas of anaesthesia, and enlarged nerves are the principal signs of leprosy.

Other members of the genus *Mycobacterium* are collectively referred to as non-tuberculosus mycobacteria (NTM), "atypical", 'anonymous', 'environmental', 'saprophytic', 'opportunistic' mycobacteria or 'mycobacteria other than *M. tuberculosis* (MOTTs)'. They comprise several species, which may cause a wide range of clinical conditions involving several organ systems. NTM comprise over 100 species (http://www.bacterio.cict.fr/m/mycobacterium.html), which were originally classified into four groups on the basis of growth rates, colony morphology, and pigmentation in the presence and absence of light (Runyon I – IV). Although now outdated, the Runyon classification proved useful in identification of the individual species of NTM in microbiology laboratories. Of note under NTMs is *M. ulcerans*, the causative agent of Buruli ulcer, a pathogenic NTM endemic in many tropical and sub-tropical countries, especially in West Africa. Buruli ulcer ranks third among the important mycobacterial diseases world-wide in humans, after TB and leprosy. *M.*

ulcerans has now been detected in water detritus and aquatic organisms in swampy areas, and transmission to man occurs presumably through minor skin traumas.

Figure 1: Classification of mycobacteria



1.1.3. The *Mycobacterium tuberculosis* genome

The complete genome sequencing of H37Rv, the best characterized *M. tuberculosis* laboratory strain, was a major milestone in TB research in 1998 (8), which opened avenues for new scientific opportunities in both basic science and clinical research on TB. Since then, genome sequences have been made for other *M. tuberculosis* strains including the CD1551, Haarlem, F11, Beijing C and H37Ra, and for over 20 other mycobacteria strains including *M. africanum*, *M. bovis*, *M. avium*, *M. leprae* and *M. smegmatis* (26).

The genome of the H37Rv *M. tuberculosis* strain is a chromosome with 4,411,529 base pairs with a high GC content of 65.6%. Although the H37Rv genome is smaller than that of *Escherichia coli* - 4.6 million base pairs (27) - it is very versatile, coding for most of the usual bacterial anabolic and catabolic pathways and amino acid synthesis/degradation. Another feature that distinguishes *M. tuberculosis* from other bacteria is the presence of 3,924 protein encoding genes, mainly coding for enzymes involved in lipolysis (for bacterial survival inside its host) and lipogenesis (for cellular envelope synthesis). However, only 40% of these protein-encoding genes can be said to be functional, based on their resemblance to other known

proteins. In addition, 10% of the coding capacity of the mycobacterium genome is devoted to two large unrelated families of acidic, glycine-rich proteins, the Proline-Glutamate (PE) and Proline-Proline-Glutamate (PPE) families. These proteins appear to be related to antigenic variation thereby interfering in the host's immunological response, and consequently, ensuring a greater survival probability to the bacteria (8, 28).

Notable too, is the presence of around 250 genes involved in fatty acid metabolism, essential for the complex cell wall structure these organisms possess. Unlike most bacteria, which contain one system for fatty acid synthesis (fatty acid synthase II), mycobacteria are known to harbour two entire systems: fatty acid synthase I (mainly found in eukaryotes and plants) and fatty acid synthase II. These multiple fatty acid synthase systems are associated with anti-mycobacterial drug targets.

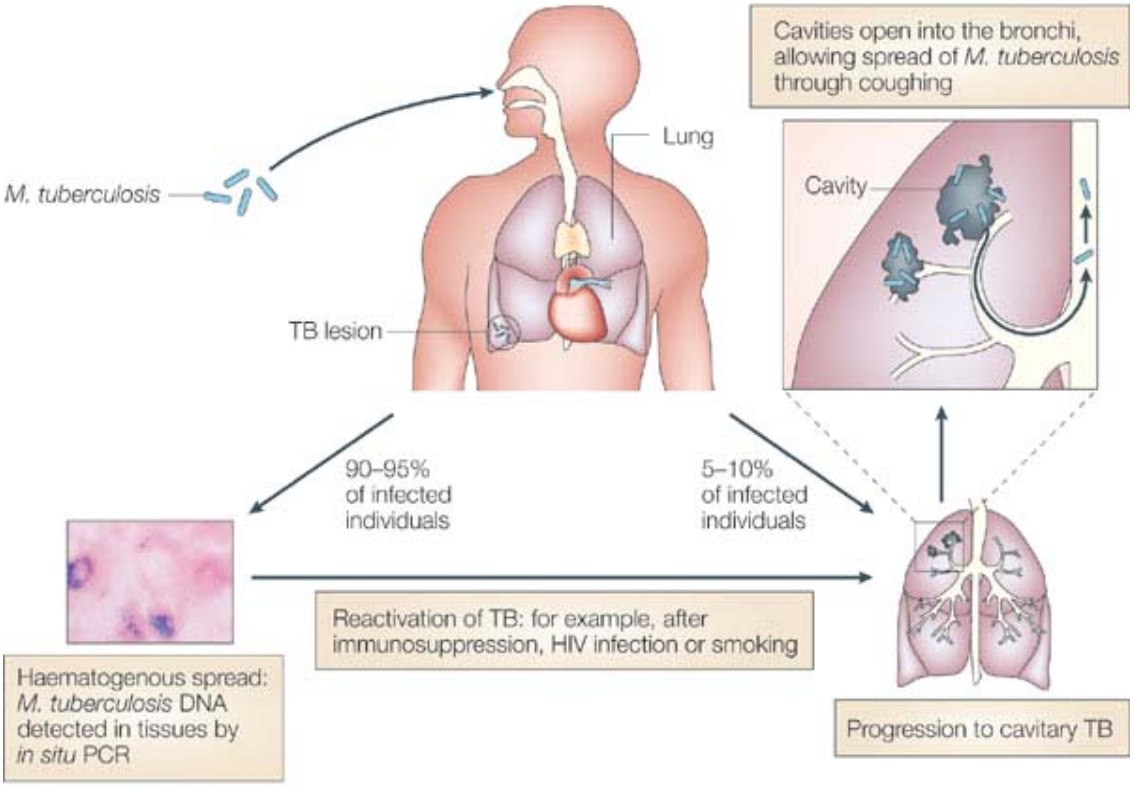
The *M. tuberculosis* genome is also rich in repetitive DNA, especially insertion sequences (IS), inserted in intergenic or non-coding regions. In particular, the H37Rv strain contains 16 copies of IS*6110* and eight copies of IS*1081*, both useful in the identification and characterization of *M. tuberculosis* strains. A further 32 insertion elements of different IS families are contained in this genome (8, 29-30).

1.1.4 The pathogenesis of TB

Tuberculosis infection occurs by airborne transmission of droplet nuclei containing a few (no more than 3) viable, virulent bacilli produced by a sputum-positive (infectious) individual, usually through coughing. These droplet nuclei are very small (approximately 1-5 μm) and coughing may generate about 3000 droplet nuclei. These infectious droplet nuclei can remain suspended in the air for hours, rendering TB a highly contagious disease. Upon inhalation, bacilli are deposited in the terminal alveoli of the lungs (Ghon focus), where they are engulfed by alveolar macrophages. Ingested bacilli can either be eliminated immediately or grow in the intracellular environment in localized lesions called tubercles. Thereafter, establishment of cellular immunity occurs, 2 to 6 weeks post infection, resulting in the elimination of most of the bacilli. This stage - usually symptom-free - is referred to as primary infection. In most cases, however, some of the engulfed bacilli may remain within its human host as a quiescent or dormant form of infection for long periods of time – referred to as latent TB, often reactivating in situations of low immunity such as in old age, HIV infection and malnutrition. People harbouring latent infection have a 10% risk of developing active TB during their life-time, while this risk is increased in HIV-infected individuals (Fig 2).

Following primary infection, characteristic foci with solid caseous necrosis (granuloma or Gohn complex) are formed from an accumulation of immune cells as a result of an inflammatory response. If the immune system mounts a successful response, the granulomas shrink and calcify, causing residual damage to the lungs. If on the other hand, the immune system fails to contain the infection, bacilli in the granuloma will proliferate causing the granuloma to increase in size and cellularity and ultimately necrosis will follow. Expansion of this necrotic reaction results in cavity formation in the lungs through which massive bacterial dissemination into the air through coughing can occur. The pathological and inflammatory processes produce typical TB symptoms such as weakness, fever, weight loss, night sweat, chest pain, respiratory insufficiency, and cough; advanced pathology may also cause blood vessel disruption, which leads to hemoptysis.

Figure 2: Pathogenesis of TB



In about 15% of the patients, early in infection, bacilli invade the blood stream, thereby disseminating to various parts of the body, such as the pleura, lymph nodes, liver, spleen, bones and joints, heart, brain, genito-

urinary system (frequently kidney or bladder), meninges, peritoneum, and skin, resulting in extra-pulmonary TB (28, 31-33).

1.2 Epidemiology

1.2.1 History of the disease

Although TB is still ravaging the human race, the disease is not new to mankind. Modern day scientific methods like DNA-based methods and indeed microscopy, have been able to demonstrate existence of TB in humans (Egyptian mummies) as far back as 2,400 before Christ (B.C.). The first actual documented description of a disease called 'pthisis', meaning 'wasting away' in Greek, was written as early as 400 B.C. by Hippocrates in his 'Of Epidemics'. One begins to see consistent documentation of the disease by the 17th century when death from TB had reached high levels in Europe, and by the end of the 19th century and the beginning of the 20th century, TB was the major cause of death in Europe. TB became well established in all the social levels at this time affecting even royalty, much unlike today, where TB is known as the disease of the poor. Famous personalities that fell victim to TB include Vivian Leigh, Eleanor Roosevelt, George Orwell, John Keats, Charlotte Bronte and St Francis of Assisi. Although more is written about TB in Europe (the white plague), it appears that TB existed in the Americas before Europe (28, 34).

Throughout history, people have referred to TB by different names depicting its devastating effects, including consumption, King's Evil, lupus vulgaris, phthisis, the white plague, 'captain of all these men of death' and many more. Initial efforts toward the discovery of its causative agent can be attributed to the work performed in 1680 by the French man Franciscus Sylvius. In his anatomic-pathologic studies in TB patients, he proposed the pulmonary nodules, which he named 'tubercula', as part of the disease pathogenesis. Later in 1722, the British doctor Benjamin Marten, proposed that TB could be transmitted by the 'breath' of a sick person. In 1865, TB transmission was demonstrated in experiments using rabbits, by the French military surgeon Jean-Antoine Villemin. The actual identification of *M. tuberculosis* occurred in 1882 by Robert Koch, when he isolated and cultured the bacilli from crushed tubercles.

Following the identification of the bacilli, further work by Koch soon led to the development of a "TB therapeutic drug", tuberculin, which turned out to be useful in the detection of TB infection; sick animals inoculated with this drug developed an intensified reaction (1890). Subsequently, tuberculins like purified protein derivatives (PPD), PPD_S and PPD RT23 have been produced. Robert Koch further went on to develop staining methods for identification of TB bacillus. These staining techniques were first improved by

Paul Ehrlich in 1885 and finally by Ziehl and Neelsen (28, 35-36). TB diagnosis using the Ziehl-Neelsen staining technique still forms the cornerstone for diagnosis in the developing world, which bears the largest burden of TB today.

Treatment at the time was based on fresh air, rest and good nutrition, often offered in sanatoria. Other progress included the development of the live attenuated vaccine in 1921, now known as BCG (bacilli Calmette-Guerin), and introduction of antibiotics for TB treatment, such as streptomycin (S) in 1947, isoniazid (I) and *p*-aminosalicylic acid (1962), while rifampicin (R) and ethambutol (E) were developed later.

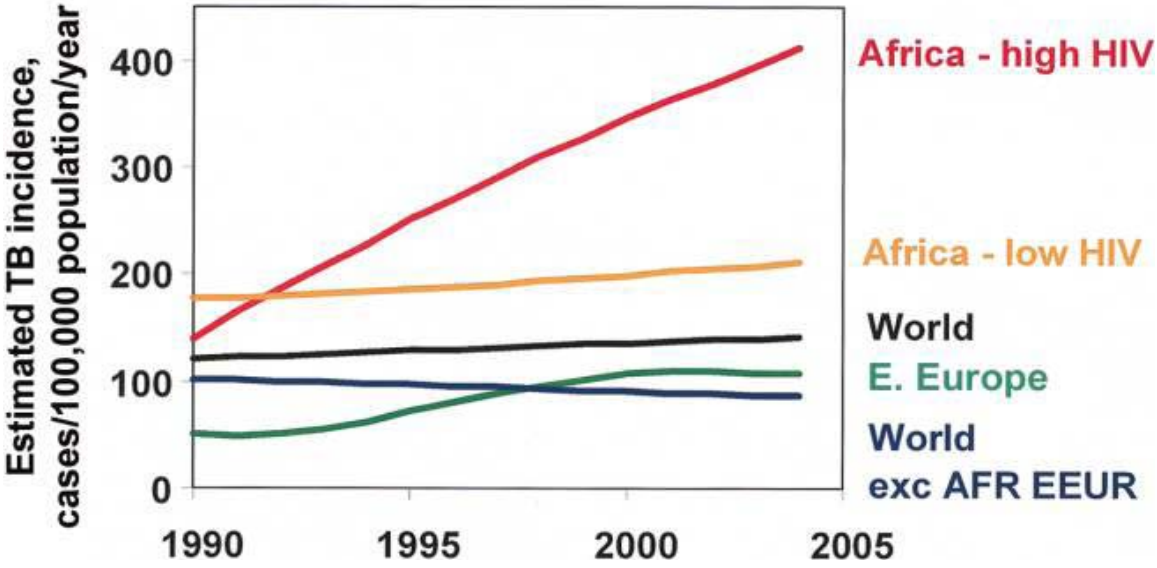
1.2.2 Current situation of the disease

1.2.2.1 The Global TB epidemic

TB remains a major challenge to global public health in the 21st century, constituting one of the leading causes of morbidity and mortality worldwide, and the most common cause of adult death from a curable infectious disease. It is estimated that 2 of the 6 billion people on the face of the earth is latently infected by *M. tuberculosis* (37).

Following tremendous declines in global TB cases at the beginning of the century, the world experienced a global resurgence in the 1990s resulting in the declaration of TB as a global emergency in 1993 by the World Health Organization (WHO). The re-emergence was mainly driven by the human immunodeficiency virus (HIV) pandemic, as evidenced by the similarity in global trend patterns between TB incidence and HIV prevalence, especially in Africa (Figure 3).

Figure 3: TB/HIV Trends: Incidence estimates of all forms of tuberculosis (TB), per 100,000 general population, by year, 1990–2004.



"Africa–low HIV" denotes all sub-Saharan African countries where HIV infection prevalence in the adult (15–49 years of age) population is <4%. "Africa–high HIV" denotes all sub-Saharan countries where HIV infection prevalence in the adult population is >4%. "World exc AFR EEUR" denotes the world with the exception of Africa and Eastern Europe[38]

Despite worldwide TB control efforts, current WHO estimates state that in 2008, there were still 11.1 million prevalent cases of TB worldwide, an equivalent of 164 cases per 100 000 population. An estimated 9.4 million new cases of TB occurred (an equivalent to 139 cases per 100 000 population), an increase from the 9.3 million TB cases estimated to have occurred the year before. Asia and Africa accounted for 85% of these cases, with 80% occurring in the WHO 22 high burdened countries, the majority of which are in the sub-Saharan region. In the same period, an estimated 1.3 million deaths occurred (an equivalent to 20 deaths per 100 000 population) due to TB.

Nonetheless, declines in TB incidence appear to have occurred in most regions of the world since 2004, according to the WHO. The rates of decline vary from less than 1% per year in the South-East Asia Region to around 4% per year in Latin America (37).

1.2.2.2 The TB epidemic in Africa

Despite an apparent stabilization and decline in overall TB globally, Africa continues to be one of the hardest hit regions. Estimated TB prevalence, incidence and death rates all continued to increase along the HIV epidemic. Notification rates in the WHO African Region rose from 82 per 100,000 inhabitants in 1990 to 160 in 2006. To address the increasing trend in the TB epidemic, the 55th session of the Regional Committee of Ministers of

Health of the WHO African Region declared TB an emergency in 2005 and called for urgent and extraordinary actions to rapidly bring the TB epidemic under control (39).

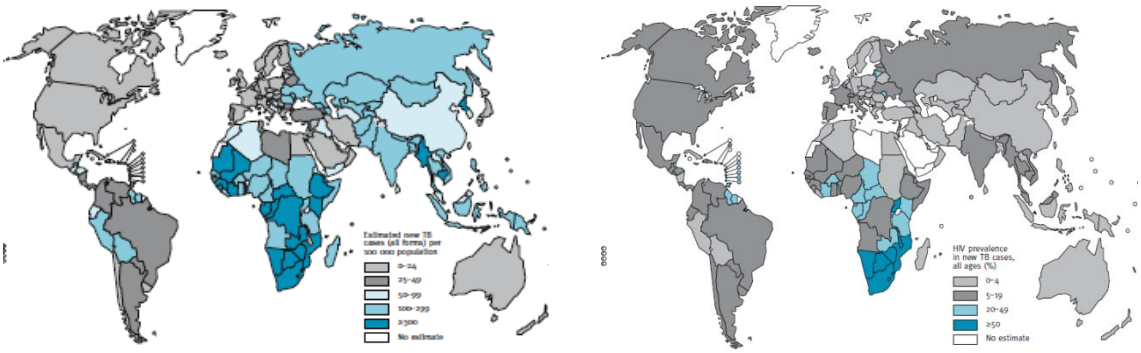
In 2009, there were an estimated 3.9 million prevalent TB cases with an estimated 430,000 TB deaths in the African region. Furthermore, with an incidence rate of 2.8 million, Africa accounted for 30% of global TB cases. Of the 15 countries with the highest estimated TB incidence rates in the world, 13 are in Africa, perhaps not surprising considering the high rates of HIV co-infection. Even so, Africa has seen a reversal in the TB epidemic since 2004 in countries with high HIV-prevalence rates, probably due to the levelling off of the HIV epidemic in sub-Saharan in the 1990s (40), but this decline has not occurred in those African countries with low prevalence of HIV. Africa is also the only WHO region worldwide where TB incidence continues to rise (37).

1.2.3 TB and HIV- co-infection

Tuberculosis and HIV are the two leading causes of infectious disease–associated mortality worldwide. Furthermore, HIV-associated TB is a major global public health challenge. WHO estimated that in 2009, 1.1 million of the 9.4 million incident TB cases (12% of all TB cases) that occurred worldwide were HIV-associated. The African region accounted for the majority (80%) of these HIV-positive TB cases. In some sub-Saharan Africa countries, HIV/TB co-infection is well over 50% of TB cases. During the same year, approximately 0.4 million people died from HIV-associated TB worldwide (37).

The detrimental synergistic association between TB and HIV is well known; these two diseases affect each other in many aspects from pathogenesis and the epidemiologic profile, to clinical presentation, treatment, and prevention, to larger issues of social, economic, and political consequence. Currently, HIV infection represents the major risk for progression of a latent TB infection into active disease. Patients co-infected with HIV and MTB have a greatly increased risk of developing active TB (annual risk 10% compared with a lifetime risk of only 8–10% in those solely infected with MTB (32). Furthermore, TB is the most important disease associated to HIV and AIDS-defining disease, TB accelerates progression to AIDS in HIV-infected patients and is the principal cause of mortality among HIV patients in hospitals. Epidemiologically, TB incidence increase coincides within period, region and population with the appearance of HIV (Figure 4).

Figure 4: Estimated HIV prevalence among new TB cases (left) and TB incidence rates (right) in 2009[41]



Clinically, HIV has made TB diagnosis more difficult to perform. For example, high rates of false-negative tuberculin skin tests and modest sensitivity of Interferon-gamma release assays (IGRAs) to detect active TB disease among HIV-positive patients has been reported (42-43). Also, HIV-infected pulmonary TB patients present with atypical chest X-ray findings and/or with sputum smears that are negative for acid fast bacilli (44-46). Besides, this population is more likely to suffer from extra-pulmonary TB (47) and opportunistic infections, caused by NTMs such as *M. avium* complex, *M. kansasii*, *M. fortuitum*, and *M. chelonae* especially in low TB-prevalence countries (3). HIV has also complicated TB therapy in that adverse drug reactions to anti-TB medications are more common in HIV-infected TB patients, for instance, the use of thiocetazone in HIV-infected individuals is discouraged because of the risk of severe toxicity.

Another complication of the HIV-TB scourge is the emergence of drug-resistant MTB strains. Multi-drug resistant TB (MDR-TB), which is defined as simultaneous resistance to R and H (the most powerful drugs against MTB) with or without resistance to other drugs, is currently known as the most malignant opportunistic infection yet associated with HIV infection. Although there does not appear to be a direct epidemiologic association between HIV infection and MDR-TB, the high mortality rates among HIV-positive patients infected with MDR-TB, frequently exceeding 80%, and the rapid progression to death (between 4 to 16 weeks) (48-49), are a worrying fact. Additionally, the recently described extensively drug-resistant TB (XDR-TB), defined as MDR TB resistant to any fluoroquinolone (FQ) and at least one of three injectable second-line drugs (i.e., amikacin, kanamycin (KAN), or capreomycin), associated with a very high mortality rate among HIV-infected individuals (50-51), has further undermined TB control efforts in some settings.

1.3 Global TB control

1.3.1 Control strategies

Following years of neglect, global efforts to control TB were reinvigorated in the early 1990s when the world realized that TB was fast becoming a major global public health problem. In 1993, the WHO declared TB a global emergency. The following year, the internationally recommended TB control strategy, later named DOTS, was launched. This strategy relied on the implementation of five elements:

1. Political commitment;
2. Case detection utilizing smear microscopy;
3. Standardized short course chemotherapy;
4. A regular uninterrupted supply of all essential anti-TB drugs; and
5. Programme supervision and evaluation.

To further augment efforts towards TB control, the WHO launched The Stop TB Partnership in 1998 with the ambitious goal of eliminating TB as a public health problem by 2050.

The UN declaration of the Millennium Development Goals (MDGs) in 2000, which committed nations to a new global partnership to reduce extreme poverty by setting out a series of time-bound targets (with a deadline of 2015), also advanced the cause for TB control. TB-control related goals within the MDGs pertain to goal 6 "Combat HIV/AIDS, malaria and other diseases" and target 6c "Halt and begin to reverse the incidence of malaria and other diseases". More specifically, indicator 6.9 and 6.10 refer to "Incidence, prevalence and death rates associated with TB" and "Proportion of TB cases detected and cured under DOTS" respectively.

The DOTS strategy was originally developed as a public health approach to control TB in a cost-effective manner in resource-limited situations, prioritizing smear-positive patients (the most infectious group). With time, however, major public health challenges such as the TB/HIV co-epidemic and the emergence of MDR-TB could not be ignored in TB control efforts. Additionally a change in the global environment towards a more human approach to public health has led to disease control efforts becoming increasingly patient-centred and directed towards universal access to care.

To address the aforementioned issues and enhance their response in achieving targets for TB control of the Stop TB Partnership and MDGs, the WHO launched the Stop TB Strategy in 2006. Its main goal is to reduce substantially the global burden of TB by 2015 in line with the MDG and Stop TB Partnership targets, and to achieve major progress in the research and

development of the tools needed for TB elimination. The 2015 targets are 'to halt and began to reverse the incidence' of TB and to reduce by 50% prevalence and mortality rates relative to 1990 levels.
A fundamental change between the 1990s DOTS strategy and the 2006 Stop TB Strategy is the enhancement of the concept of patient-centred care for all individuals with TB. The Stop TB Strategy has six components:

1. Pursue high-quality DOTS expansion and enhancement;
2. Address TB/HIV and MDR-TB and other special challenges;
3. Contribute to health system strengthening;
4. Engage all care providers;
5. Empower people with TB and communities;
6. Enable and promote research.

1.3.2 Vaccines

Globally, BCG remains the only vaccine against TB in humans. BCG is a live attenuated vaccine derived from *M. bovis*. Advantages of the BCG vaccine include: it has a long established safety profile, it is of easy inoculation and can also be administered as an oral vaccine, it requires a single immunization and can confer immunity for a long period, it is a very efficient adjuvant for immunity induction, and it has a low cost of production. Early infant BCG vaccination can protect infants from severe forms of the disease such as miliary TB and TB meningitis. However, reports on its efficacy to prevent pulmonary TB have been variable and in some instances conflicting. The protection conferred by the vaccine against TB disease arising from a new infection, even when it is correctly administered to newborns, is incomplete and extremely variable in different populations. It does not protect against reactivation disease of the already infected and its efficacy wanes with age (52-53). Consequently, the role of BCG as a TB control tool is limited.

The need for new and improved vaccines against TB cannot be overemphasized. Current vaccine development is complicated by strain-specific epidemiologic differences in prevalence, transmissibility, and disease severity and the notion that some *M. tuberculosis* strains elicit distinct host immune responses. Furthermore, the extent of immunity against *M. tuberculosis*, either from early exposure or prior disease, is not well understood. HIV disease also presents another challenge in that HIV infected individuals may not be able to mount an effective T-cell response (54-59). Notwithstanding, currently, there are numerous candidate vaccines utilizing various design strategies in the pipeline. In total, 11 vaccine candidates against TB have entered clinical trials within the last several years. The mechanism of most of these current candidate vaccines is reliant on T cell immunity via stimulation of Th1 cells, which activate antimycobacterial

capacities in macrophages. The majority of these vaccines are preventive pre-exposure vaccines, i.e., given prior to MTB infection. They mainly reduce initial bacterial burden and contain the MTB. This way, infection, primary disease, latent infection and reactivation of latent disease can be averted. Future TB vaccine development should target vaccines that can achieve sterile eradication of MTB or prevent stable infection of MTB altogether (54, 60).

1.4 Diagnosis of TB

Nowadays, there are a myriad of tools that have been developed for the diagnosis of TB. In general, TB diagnosis can be categorized in two groups; clinical diagnosis, which includes medical history, clinical and radiological examination, and laboratory diagnosis, which includes microbiological, immunological and molecular-biological investigations.

1.4.1 Clinical diagnosis

Although non-specific, in general clinical manifestations of illness can be considered the first clue to the diagnosis of TB. In fact, in countries with a high prevalence of TB, it is essential that health staff maintain a high index of suspicion in patients that present with signs and symptoms suggestive of TB, which include, fever and night sweats, chest pain, shortness of breath (dyspnoea), loss of body weight, cough which may or may not be productive of sputum for more than 3 weeks and haemoptysis. Other non-specific symptoms include loss of appetite, general malaise and weakness. Patient history, for example, known history of TB contact among children can also guide diagnosis. A thorough examination of the chest and respiratory system that reveals crepitations, bronchial breathing, reduced air entry, dullness or stony dullness or hyperesonance, enlarged lymph nodes and pleural effusions is also suggestive, albeit neither sensitive nor specific. However, when it comes to extra-pulmonary TB, clinical manifestations depend on the organs or systems involved. Therefore, TB is usually considered in the differential diagnosis of any localized inflammatory process that does not respond well to general antibiotic or surgical treatment.

Other tools used in the clinical diagnosis of TB include radiography of the chest and other newer high resolution imaging techniques, such as chest computed tomography (CT), are employed predominantly in the developed world. Although highly suggestive, images from these techniques are not absolutely specific to TB. Classical chest radiographic abnormalities include features of ‘primary’ TB, for example, unilateral hilar lymph node enlargement, parenchymal airspace consolidation and/or pleural effusion, or features of ‘reactivation’ TB, for example, focal or patchy heterogeneous

consolidation involving the apical and posterior segments of the upper lobes and the superior segments of the lower lobes, poorly defined nodules, linear opacities and cavitations (61-63).

An addition to the clinical tools for TB diagnosis is the tuberculin skin test (TST) usually used as a screening method for the identification of persons with a positive immune response against MTB complex. The TST utilizes a standard preparation of PPD, an extract of the sterile supernatant of *M. tuberculosis*-cultured filtrate, which is administered intradermally and results in a delayed type hypersensitivity reaction represented by a local skin induration. The diameter of the induration is then measured 48–72 h post antigen injection, using the 'ballpoint technique'. A positive TST however does not distinguish latent from active TB infection (64-65).

1.4.2 Laboratory-based diagnosis

Sputum smear microscopy has proved invaluable in TB diagnosis and remains the cornerstone of TB diagnosis in most high-burden, low-income countries, because it is a rapid, cheap (light microscopy) and simple method to use. Smear microscopy relies on the retention of stain following the application of acid and thereby enabling visualization under a microscope. The conventional methods use carbol-fuschin [Ziehl–Neelsen (ZN)] or Kinyoun stain followed by light microscopy examination, whereas the more recently introduced fluorescence microscopy uses auramine-based stains (auramine O or auramine–rhodamine). The latter method has become acceptable as a more sensitive method. However, the cost attached to fluorescence microscopy, mainly due to the high cost of their bulbs, has slowed down uptake in low-income countries. More recently, the development of fluorescent microscopes using light-emitting diode (LED) technology, not only has comparable sensitivity to the conventional fluorescent microscope, but is also more affordable.

Nonetheless, sensitivity of microscopy remains a concern, as it is only able to detect bacilli if present at a concentration of at least 5,000 –10,000 bacilli/ml in sputum specimens. The sensitivity of sputum smear microscopy is at best 70–80% in HIV-uninfected adults, and decreases in the settings of high HIV/TB co-infection, in paediatrics and in extrapulmonary TB (44, 46, 66-67). Various physical and chemical processing methods, which include centrifugation, sedimentation and pre-treatment with bleach, have been shown to increase sensitivity, when used prior to microscopy (68).

To date, culture methods remain the gold standard for diagnosing TB. Traditionally, culture methods have mainly used egg-based [Lowenstein Jensen (LJ) or Ogawa] or agar-based (Middlebrook 7H10 and 7H11) solid media. However, with a doubling time of 20 – 22 hours of mycobacteria, this

method takes time to obtain results (minimum of 3 weeks). Identification of mycobacteria using these conventional culture methods mainly depends on growth characteristics, such as, growth rate, colony morphology and pigmentation, and also biochemical reactions that include susceptibility to p-nitrobenzoic acid, thiophene-2-carboxylic acid, hydroxylamine, niacin production, nitrate reductase, catalase and urease testing (69-70). These characteristics also afford this method the ability to differentiate between different mycobacterial species. The prolonged incubation time and the laboratory infrastructure required are major limitations, especially in resource-limited countries. Furthermore, bacterial culture and isolation of *M. tuberculosis* are still required for drug-susceptibility testing (DST), and these procedures require several weeks to yield results.

The recent past has seen the development and improvement of several automated liquid-based bacterial systems, such as the mycobacteria growth indicator tube (MGIT) system (Becton Dickinson). These systems depend on detection of metabolic activity and have greatly reduced the time to detection of a positive culture by 50–60%, compared with culture on LJ slopes, shortening the time to detection to about 10–14 days instead of weeks. Other liquid-based systems include the BACTEC systems (460, 9000, Becton Dickinson, Sparks, MD) and the Versa TREK formerly known as the ESP culture system II (Trek Diagnostic systems, West Lake, OH) (71-73). However, wide use of these systems is limited in resource-limited countries because of the high initial capital investment required, and the need for proprietary consumables with limited shelf lives. Other cheaper alternatives being proposed include the non-commercial microscopic observation drug-susceptibility (MODS) assay with the benefit of simultaneous susceptibility testing. The assay entails inoculating a sputum sample into wells of a tissue culture plate containing Middlebrook 7H9 broth with some wells containing anti-TB drugs Time to detection of culture for this assay is approximately 8 days. Performance for this assay has been shown to be at least as good as, or better than, automated broth-based methods (74-76). The MODS assay however, still requires substantial laboratory infrastructure, well trained staffing and is labour-intensive.

The development of serological tests as alternative methods in TB diagnosis has been ongoing for a long time. These tests employ the principle of antibody-antigen reactions to determine exposure to disease. With the current global need for fast, at-point-of-care diagnosis, and cheap methods for TB diagnosis, serological tests are an obvious alternative, as has been seen in their use in other diseases. Added to this is the urgent need to strengthen early diagnosis and diagnosis in paucibacillary cases, such as, pulmonary TB with negative sputum smears in adults, extrapulmonary TB, childhood TB and TB patients with HIV co-infection.

Currently, there exist numerous systems that have been developed as serological diagnostics. Each system employs different antigens including heat shock proteins, proteins, lipids and polysaccharides and uses different laboratory techniques such as, Enzyme-linked immunosorbent assay, immunochromatography, immunodot rapid test and kaolin agglutination test. Sadly, despite the numerous assays developed in this category, none have produced impressive results with regards to their sensitivity and specificity, and are therefore deemed not ready to replace microscopy yet (77-79).

In addition to antibody-antigen reactions, developments in TB diagnosis include those in cellular immunodiagnostics, which rely on the concept that T cells of individuals sensitized with *M. tuberculosis* antigens will release interferon gamma (IFN-γ) when they re-encounter mycobacterial antigens. So far, the IGRA is regarded by many as the most important development in the diagnosis of *M. tuberculosis* infection over the last decade. IGRA targets two proteins, namely, the early secretory antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10), which are produced by *M. tuberculosis,* but not by all strains of BCG and the majority of environmental Mycobacteria (except *M. Kansasii*, *M. szulgai*, *M. marinum*, *M. flavescens* and *M. gastri*). This makes IGRA relatively specific to *M. tuberculosis (80-81).* Unfortunately, IGRA performed on peripheral blood alone cannot distinguish between individuals with latent TB infection, active TB or past TB (44, 82-84).

At present, there are two IGRA commercialized systems that have been developed; QuantiFERON-Gold (QFT-G) (Cellestis Ltd, Carnegie, Australia) measures IFN-γ in IU/mL using an enzyme-linked immunosorbent assay (ELISA), and T-SPOT.TB (Oxford Immunotec Ltd, Abingdon, UK) counts the cells releasing IFN-γ visualized as spots with the enzyme-linked immunospot (ELISPOT) technique. During the last several years, these systems have been approved in various countries and the findings of their diagnostic performance have been accumulated and characterized. When compared to TST, numerous studies have shown the specificity of IGRAs to be consistently higher and obviously superior, whereas sensitivity is rather variable between studies. This difference may be due to various patient characteristics such as TB disease condition, age, extent of immunosuppression due to underlying illnesses, and so on (83, 85-86).

Another assay showing some promise as a future test in this category is the ELISA-based assay detecting lipoarabinomannan in unprocessed urine (Chemogen, USA). It is a cell wall lipopolyscaccharide specific for the *Mycobacterium* genus, but not to MTB complex alone. Although still under

evaluation, the test has shown to be more sensitive than smear microscopy for diagnosis of active disease. Although the sensitivity of the assay is not high enough, its use in smear-negative disease has been suggested due to its higher sensitivity compared to microscopy. Urinary LAM may thus find a place in future clinical algorithms, but at present cannot be used to reliably exclude a diagnosis of TB (46, 87-90).

Due to their slow turnaround time (culture), low sensitivities (smear microscopy) and specificities (X-ray), the traditional methods of diagnosing TB are proving inadequate in TB control. The many challenges HIV/TB co-infection poses to conventional methods of TB diagnosis include rapid progression to TB disease, higher levels of smear-negative (pauci-bacillary) in pulmonary TB and in extra-pulmonary TB and infection with NTM. The concurrent increase of MDR- and XDR-TB globally further emphasises the demand for methods that will rapidly diagnose TB, be sensitive enough to detect TB in samples with low bacterial loads, specific enough to differentiate MTB complex from NTMs, and rapidly detect presence of drug resistance. Methods that can directly detect TB from clinical samples, for example sputum, will be an added advantage.

Several molecular methods have been introduced and evaluated to address the above challenges. These methods include nucleic acid amplification tests (NAATs) which mostly utilise the PCR technique to amplify short sequences of DNA or RNA specific for the MTB complex. The most commonly used target for identification of MTB complex is the insertion sequence IS*6110*. Several 'in-house' NAATs have been described, with overall reported sensitivity and specificity ranging between 84–100% and 83–100%, respectively, for respiratory specimens. Lower sensitivity and specificity have been reported for non-respiratory specimens (91-93).

So far, there are two commercially available FDA-approved NAATs. Firstly, the Amplicor series, which includes the Mycobacterium Tuberculosis Test (Amplicor) (Roche Diagnostic Systems Inc., NJ), the improved automated versions - COBAS Amplicor MTB, COBAS Amplicor analyzer (Roche Diagnostics, Switzerland), and COBAS TaqMan MTB. These assays are based on the amplification of the 16S rRNA gene using genus-specific primers, which, after hybridization to oligonucleotide probes, is detected in a colorimetric reaction in a microwell plate format (94) or in real time in the tube. The second FDA-approved NAAT is the Amplified Mycobacterium Tuberculosis (MTB) Direct Test (MTD) (Gen-Probe Inc., San Diego, CA) based on isothermal amplification of 16S ribosomal transcripts which are detected in a hybridization protection assay with an acridinium ester-labeled MTB complex-specific DNA probe (95).

Although not FDA-approved for the direct detection of *M. tuberculosis* in clinical samples, the BD ProbeTec MTB Test (Becton Dickinson, Sparks, MD) and its improved version, the BDProbe Tec ET, are also commercially available NAATs. These systems are based on the strand-displacement amplification technique that uses enzymatic replication of target sequences in IS*6110* and the 16S rRNA gene. The amplified products are then detected with a luminometer.

A promising addition to the list of NAATs is the loop-mediated isothermal amplification (LAMP) assay (96). The method is based on auto-cycling strand displacement DNA synthesis using the large fragment of Bst DNA polymerase, enabling the detection of trace amounts of DNA under isothermal conditions. LAMP is a simple, rapid and cost-effective nucleic acid method, and the resulting amplicons can be detected by confirming the presence of generated white precipitate (magnesium pyrophosphate).

The performance and usefulness of these NAAT assays in the clinical diagnosis have been widely evaluated and due to the significant heterogeneity of sensitivity and specificity results (Table 1), it does not appear that they will be replacing conventional tests for diagnosing pulmonary TB as yet.

Table1: Sensitivity and Specificity of NAA tests in Clinical specimens[97]

	Smear-positive pulmonary		Smear-negative pulmonary		Extrapulmonary	
	Sensitvity	Specificity	Sensitvity	Specificity	Sensitvity	Specificity
Amplicor	97	>95	40-73	>95	27-98	>95
AMTD	92-100	>95	40-93	>95	93	>95
BDProbe Tec	90-100	92	33-100	83-97	76	>90
Real-time PCR	78	100	78	100	80	100
LAMP	97.7	99	48.8	99	ND	ND

Although traditional methods for species identification of mycobacteria are simple to perform and require minimal equipment, they tend to be labour-intensive and cumbersome and ultimately take time to obtain results. Hence molecular methods provide a rapid alternative. Here too, in-house and commercially available methods exist. The array of in-house methods are based on sequencing of RNA and DNA fragments such as the 16S rRNA gene (also used as the reference standard), the 23S rRNA gene, the 65-kDa heat shock protein genes, *rpo*B, *sod*A , *rec*A, *hsp65* gene and *gyr*B.

The AccuProbe (Gen-Probe Inc.) is one of the first commercially available systems. It is based on species-specific DNA probes that hybridize to rRNA for the identification of several important mycobacteria, including the MTB complex, *M. avium*, *M. intracellulare*, the *M. avium* complex, *M. kansasii* and

M. gordonae. More recent systems based on line probe assay methods are able to identify many more species, these include the INNO-LiPA MYCOBACTERIA v2 (Innogenetics NV, Ghent, Belgium), and the GenoType MTB Complex and GenoType Mycobacterium (Hain Lifesciences, Nehren, Germany), both to be applied on positive cultures. INNO-LiPA MYCOBACTERIA v2, based on nucleotide differences in the 16S–23S rRNA gene spacers, simultaneously detects and identifies the genus *Mycobacterium* and 16 different mycobacterial species and can be performed on liquid or solid cultures. The GenoType MTB Complex is based on a 23S rRNA gene fragment specific for the MTB complex, together with *gyr*B sequence polymorphisms, and the RD1 deletion for identification of *M. bovis* BCG, whereas, the GenoType Mycobacterium is based on regions of the 23S rRNA gene for the identification of 35 species of mycobacteria. Extensive evaluation of these systems has been performed and they have shown relatively high sensitivity and specificity (>90%).

More recently, a fully automated molecular test for the detection of MTB and R resistance, the Xpert®MTB/RIF (Cepheid, Canada), has been developed. It uses a heminested real-time (RT) PCR assay to amplify an MTB-specific sequence of the *rpoB* gene, which is probed with molecular beacons for mutations within an 81-bp core region of the *rpoB* gene, referred to as the rifampicin-resistance determining region (RRDR). Testing is carried out on the MTB/RIF test platform (GeneXpert, Cepheid, Canada) which integrates sample processing and PCR in a disposable plastic cartridge containing all reagents required for bacterial lysis, nucleic acid extraction, amplification, and amplicon detection. The only manual step is the addition of a bactericidal buffer to sputum before transferring a defined volume to the cartridge. The MTB/RIF cartridge is then inserted into the GeneXpert device, which provides results within 2 hours. Evaluation of this system in a multi-centre study reported that it was able to identify 98% of culture-confirmed TB cases, including 72% of those with smear-negative disease, with specificity of 99%. Performance for case detection and discrimination of R-resistance was similar across diverse sites in many countries. The increased sensitivity compared to microscopy, especially among patients with smear-negative TB, puts the Xpert®MTB/RIF at an advantage as a tool for rapid TB detection. Advantages of the Xpert®MTB/RIF include that it is a simple-to-use, non-invasive, almost point of care test that can diagnose active pulmonary TB and allow a decision on treatment initiation, with same-day turnaround. However, the cost aspect, reliance on a power supply and the yearly calibration requirements, may limit the use of this technology at the peripheral level (98-99).

Other molecular methods have also been developed for the detection of drug resistance; this topic is discussed in chapter 1.4.

1.5 TB Treatment

TB is a curable disease for which effective treatment has been made globally available; the average cost to treat a patient infected with drug-sensitive TB is $22.40 (100). The aims of TB treatment are to cure patient of TB, prevent death from active TB or its late effects, prevent relapse, reduce transmission, and prevent development of drug resistance. Treatment of TB is a vital component in TB control primarily because cure of infectious cases will interrupt the chain of TB transmission in a community.

The bacillary population in a patient is not homogeneous but consists of an active subpopulation undergoing active metabolism and a semi-dormant subpopulation with spurts of metabolism. The existence of multiple bacillary subpopulations calls for a combination regimen including drugs with distinct pharmaco-dynamic properties to ensure complete cure. Actively replicating bacilli can lead to therapy failure or early death and therefore must be killed rapidly by early bactericidal drugs, whereas semi-dormant bacilli can cause relapse and require sterilising drugs. Semi-dormant bacilli are difficult to eradicate with most drugs.

Current anti-TB drugs can be considered in two categories, first-line and second-line drugs, referring to their application. First-line anti-TB drugs are used in standard treatment regimens commonly referred to as the short course chemotherapy (SCC). They are mainly bactericidal, and combine a high degree of efficacy with a relative toxicity to the patient during treatment. They include H, E, R, S, and pyrazinamide (Z). Isoniazid and R are the most powerful bactericidal drugs, active against all populations of TB bacilli, while R is also the most potent sterilizing drug available. Pyrazinamide is bactericidal, but only active in an acid (intracellular) environment. Streptomycin is bactericidal against rapidly multiplying TB bacilli, whereas E is bacteriostatic and has a synergistic action in association with more powerful drugs to prevent the emergence of resistant bacilli.

Standard SCC for drug-susceptible TB uses the first-line drugs for 6–9 months and is considered in two phases, namely the intensive and the continuation phase. The intensive phase lasts 2 -3 months and is designed to kill actively growing and semi-dormant bacilli, thereby reducing the duration of infectiousness of an individual. The continuation phase on the other hand, works to eliminate bacilli that are still multiplying and also reduces the risk of failure and relapses. This phase lasts between 4 – 6 months depending on disease site and drugs used. SCC is highly effective with a greater than 95% success rate and has been validated through randomized controlled trials.

Due to the increased risk of acquired drug-resistant TB in patients who have had previous exposure to anti-TB drugs, TB cases are defined according to treatment history (Table 2). This strategy enables the prescription of appropriate treatment regimens that prevent acquired resistance, and is also essential for epidemiological monitoring of the TB epidemic at regional and country level.

Nowadays, almost every country subscribes to a limited number of standardized TB treatment regimens, depending on availability of financial resources, efficacy, effectiveness and applicability in the current national health system network, and population distribution and mobility. Standardised treatment regimens have been designed on the basis of representative drug–resistance-survey data in well-defined patient populations. All patients in a patient group or category receive the same treatment regimen.

Fixed dose combinations (FDCs) tablets with different drug combinations, for example, HR, HE, HRZ and HERZ, have been introduced on the market. FDC tablets provide the following advantages:

- Prevention of drug resistance [when given under Directly Observed Therapy Short course (DOTS)]
- Simplification of treatment
- Simplification of management
- Reduction of misuse of the drugs for treatment of conditions other than TB

The main disadvantage with FDC tablets is in treatment management, especially in handling side effects. For this reason, programmes should always have a limited stock of single dose formulations (101).

WHO treatment guidelines historically placed great emphasis on the treatment of the most infectious patients i.e. sputum smear-positive pulmonary TB cases. The categories I–IV prioritized sputum smear-positive patients for treatment whilst relegating smear-negative TB patients third priority, and MDR-TB patients as fourth priority. More recent WHO guidelines now categorise patients (and standard regimens recommended for each group) according to the likelihood of having drug resistance. Drug resistance is a critical determinant of treatment success, and prior TB treatment confers an increased risk. Therefore, patients are grouped similar to regis-tration groups used for recording and reporting, by differentiating new patients from those with prior treatment, and specifying reasons for retreatment, that is failure, relapse, or default (102).

Table 2: WHO Patient Categories according to Treatment history

New	A patient who has never had treatment for TB or who has taken anti-tuberculosis drugs for less than 1 month
Relapse	A patient previously treated for TB who has been declared cured or treatment completed, and is diagnosed with bacteriologically positive (smear or culture) TB
Treatment after failure	A patient who is started on a re-treatment regimen after having failed previous treatment
Treatment after default	A patient who returns to treatment, positive bacteriologically, following interruption of treatment for 2 months or more
Transfer in	A patient who has been transferred from another TB register to continue treatment
Other	All cases that do not fit the above definitions. This group includes a chronic case, a patient who is sputum-positive at the end of a re-treatment regimen.

Adapted from the WHO Treatment of tuberculosis: Guidelines for national programmes – 3rd edition[101]

In addition, the WHO has recently issued new recommendations with regards to treatment of TB in persons living with HIV. The recommendations state that TB patients with known positive HIV status and all TB patients living in HIV-prevalent settings should receive daily TB treatment at least during the intensive phase and if possible for the continuation phase (102). There are also recommendations by some experts to prolong TB treatment in persons living with HIV to 8 or more months of R-containing regimens (102-103).

Failure of patients to adhere to medications as prescribed or failure of physicians to prescribe an adequate regimen can result in drug resistance. MDR-TB is associated with high death rates of 50% to 80%, with a relatively short duration (4 to 16 weeks) from diagnosis to death (48-49, 104).

1.6 Drug resistance

1.6.1 The mechanism of drug-resistant TB

Mycobacteria and related pathogens possess an inherent resistance to most common antibiotics which limits chemotherapeutic options of disease. This resistance, referred to as intrinsic drug resistance, is attributed to the synergistic mechanisms of the selective permeability of the cell wall and a

repertoire of internal defense systems that are induced by antibiotics (105). In addition to the above, drug resistance also results from spontaneous genetic mutations that occur naturally in individual mycobacteria. Although, this rate is low in mycobacteria (eg 3.5×10^{-6} for H and 3.1×10^{-8} for R), prolonged exposure to a single drug or suboptimal therapy may lead to the selection and expansion of resistant MTB strains. Further, because the chromosomal loci responsible for resistance to various drugs are not linked, the risk of a double spontaneous mutation is extremely low (9×10^{-14} for both H and R). This therefore implies that resistance to more than one drug will occur mainly in circumstances where sequential drug resistance follows sustained treatment failure (49, 104, 106). Genetic and molecular analysis of drug resistance in MTB suggests that resistance is usually acquired by the bacilli either by alteration of the drug target through mutation (107) or by titration of the drug through overproduction of the target (108). This type of drug resistance resulting from drug pressure/selection is referred to as acquired drug resistance. An individual can also be infected with a resistant MTB strain, commonly referred to as primary drug resistance.

An individual can harbour bacilli that are resistant to one or more anti-TB drugs known as mono or poly-drug resistance, respectively. Within poly-resistance is the important subgroup of MDR-TB. Inadequate treatment of MDR-TB may result in XDR-TB (refer Chapter 1.2.3 for definitions of MDR and XDR). Further types of poly resistance have been described, as pre–XDR-TB, defined as MDR-TB resistant to either any fluoroquinolones or at least one second-line injectable drug, but not to both (109-110). Another type referred to as Total Drug Resistant (TDR) TB, defined as M tuberculosis isolates that are resistant to all first - second-line drugs (111) or TB for which no effective treatment are available (112).

Resistance to first-line anti-TB drugs has been linked to mutations in at least 10 genes; *katG*, *inhA*, *ahpC*, *kasA* and *ndh* for H resistance; *rpoB* for R resistance, *embB* for E resistance, *pncA* for Z resistance, and *rpsL* and *rrs* for S resistance (106, 113-114).

Isoniazid inhibits the biosynthesis of cell wall mycolic acids, resulting in loss of cell wall integrity and ultimately bacteria death. Activation of H pro-drug requires the enzyme catalase-peroxidase which is coded by the *katG* gene. However, not all mutations in this gene result in resistance, for example the *katG* 463 (Arg-Leu) substitution is the most common polymorphism found in the *katG* gene, but it is not associated with H resistance. Often associated with *katG* gene mutations are mutations in the alkyl hydroperoxide reductase (AhpC) gene which result in over expression of the AhpC protein. The AhpC protein is able to detoxify the effects of organic peroxides within the cell wall and thereby protect bacteria against oxidative damage but does

not provide protection against H. One of the targets for activated H is the protein encoded by the *inhA* locus. InhA is an enoyl–acyl carrier protein (ACP) reductase which is proposed to be the primary target for resistance to H and ethionamide (ETH). Six point mutations associated with H resistance within the structural *inhA* gene have been identified (Ile16Thr, Ile21Thr, Ile21Val, Ile47Thr, Val78Ala and Ile95Pro). To date, approximately 70% to 80% of H -resistance in clinical isolates is associated with mutations in the *katG* and *inhA* genes. Mutations in the *kasA* gene have also been described to confer low levels of H-resistance. This gene encodes a β-ketoacyl-ACP synthase involved in the synthesis of mycolic acids (106, 113, 115-116).

Rifampicin works by binding specifically to the β-subunit of the RNA polymerase and thereby hindering transcription, resulting in death of the bacteria. Mutations in the *rpoB* gene cause conformational changes that result in defective binding of the drug. Most mutations are dominated by single nucleotide changes, and restricted to the RRDR of the *rpoB* gene. The most frequently seen mutations in R-resistant isolates are those of changes in the codons Ser531 and His526 (more than 70%) (49, 106, 113, 117).

Pyrazinamide targets an enzyme involved in fatty acid synthesis and is responsible for killing persistent (semi-dormant) bacilli. Z is converted to its active form, pyrazinoic acid (POA) by pyrazinamidase (PZase) encoded by *pncA*. Accumulation of POA in the cytoplasm results in the lowering of intracellular pH to a level that inactivates a vital fatty acid synthase. The activity of Z is highly specific for *M. tuberculosis. M. bovis* is naturally resistant to Z due to a unique C-G point mutation in codon 169 of the *pncA* gene. Various *pncA* mutations have been identified in more than 70% of Z-resistant clinical isolates scattered throughout the gene (106, 118-119).

Ethambutol inhibits an arabinosyl transferase (EmbB) involved in cell wall biosynthesis. Mode of action involves interaction of E with the EmbCAB proteins encoded by the *emb*C, *emb*A, and *emb*B genes, leading to inactivation of arabinogalactan synthesis. Mutations in the *emb*B locus cause alterations in EmbB, possibly leading to an altered target for E. Alternatively, hyperexpression of the EmbCAB proteins could lead to E-resistance. Up to 70%-90% of all E-resistant isolates are associated with five identified mutations in codon 306. Action of E is specific for mycobacterial species (120-121).

By interacting with the 16S rRNA and S12 ribosomal protein S induces ribosomal changes, which cause misreading of the mRNA and ultimately results in inhibition of protein synthesis and bacterial death. Mutations in the *rrs* and *rpsL* genes encoding 16S rRNA and S12 ribosomal protein account for 65-75% of S resistance. Additionally, it has been suggested that low

levels of S resistance are also associated with altered cell permeability or rare mutations which lie outside of the *rrs* and *rpsL* genes (106, 113).

Fluoroquinolone (ofloxacin, moxifloxacin, gatifloxacin) resistance is associated with mutations in the *gyrA* and *gyrB* genes which encode for DNA gyrase. The FQs target and inactivate DNA gyrase, a type II DNA topoisomerase. 75–94 % of FQ-resistant strains have mutations in the *gyrA* gene. However, there exists a polymorphism at gyrA codon 95 that is not associated with FQ resistance. As for aminoglycosides, resistance to these drugs is associated with mutations in the *rrs* gene encoding for 16s rRNA, although in a different region (1400bp) as S. Aminoglycosides inhibit protein synthesis by binding to bacterial ribosomes and thereby disturbing the elongation of the peptide chain in the bacteria. Similarly, the peptides viomycin and capreomycin also inhibit protein synthesis and mutations in the *rrs* gene have been associated with resistance (51, 122).

In addition to mutations in the *inhA* gene conferring resistance to ethionamide, mutations in the *ethR* gene, encoding a repressor for the expression of EthA. EthA is necessary for the activation of ETH and therefore reduced EthA activity will result in ETH resistance (49, 106, 114, 123).

Given the wealth of data that has accumulated on mutations associated with resistance to specific anti-TB drugs, a new database devoted to drug resistance mutations in TB, was established in 2008. This database called the TB Drug Resistance Mutation Database (TBDReaMDB), is an interactive database, publicly available on the Website http://www.tbdreamdb.com/, giving a comprehensive listing of mutations associated with TB drug resistance and the frequency of the most common mutations associated with resistance to specific drugs. This database will serve as a resource for: the development of new diagnostic tests that can rapidly and accurately diagnose drug resistant TB, new technologies in drug discovery, sequencing projects and geographical distribution and surveillance of mutations (124).

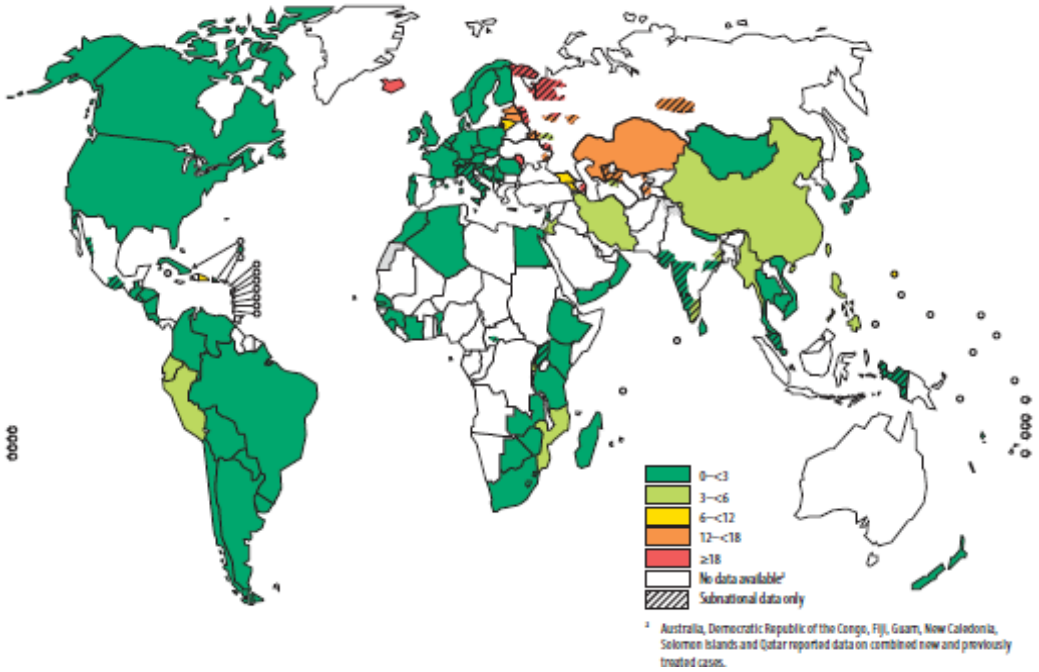
1.6.2 The prevalence of drug-resistant TB

In resource-limited countries, drug shortages, interruptions in drug supplies and poor quality drugs may contribute to the development of drug resistance. Tuberculosis drug resistance occurs worldwide and its prevalence shows great variations in different regions. The 2008 WHO Global Project on Anti-Tuberculosis Drug Resistance Surveillance reports that the global resistance to at least one anti-TB drug (any resistance) was 20%. Any resistance was higher in previously-treated TB patients (0% to 86%) compared to new cases (0% to 56%). Global MDR prevalence among all TB cases was estimated at 5.3% or approximately 500 000 cases in 2007. Global distribution of MDR is uneven (Fig 5), with the former Soviet Union,

India and China accounting for approximately 60%. MDR-TB caused an estimated 150 000 deaths in 2008. Further, approximately 6% or 40, 000 of the MDR cases were estimated to be XDR (125).

Data on drug-resistant TB from Africa are limited mainly due to insufficient laboratory capacity for DST, poor surveillance mechanisms and reporting systems, outdated databases and suboptimal coverage of the infrequent surveys. This has probably resulted in gross underestimation of the drug-resistance problem in Africa. According to the WHO, of the 22 African countries that provided data on drug-resistant TB, MDR was relatively low, ranging from 0.5% to 3.9% among new TB cases and 0.0% to 16.7% among previously-treated cases. The 2010 drug-resistance report estimated that 69, 000 MDR-TB cases emerged in Africa in 2008. Only three countries, Rwanda, the United Republic of Tanzania and South Africa (not national survey) reported data on XDR-TB cases. Both Rwanda and the Tanzania reported absence of XDR in their surveys, whilst, South Africa reported a 5.7% XDR among MDR-TB cases from a review of the country's laboratory database (126-127).

Figure 5: Global distribution of drug resistant *M. tuberculosis*[126]



1.6.3 Detection of drug resistance

1.6.3 .1 Culture-based methods

Phenotypic DST relies on detection of the effect of the drugs on bacterial multiplication or metabolism, compared to controls not exposed to the drug. DST can be done on solid or liquid culture media. The conventional DST

assays done on solid media detect the growth of *M. tuberculosis* in the presence of anti-TB drugs by one of three methods; proportion, resistance ratio or absolute concentration method. However, these methods require a minimum of 4 weeks to give results. Broth-based culture methods which rely on early detection of growth via detection of oxygen consumption, radio-active CO_2 production, *M. tuberculosis* specific enzymatic activity, or atmospheric pressure changes in culture vials, have greatly reduced time of obtaining results to 10 – 12 days. These methods include commercial systems like the semi-automated BACTEC 460 TB system (Becton Dickinson) and the fully automated BACTEC MGIT 960 TB system (Becton Dickinson). These systems have high requirements, cost- and skill-wise, for low-income countries.

Other cost-effective media-based methods more suited to resource-poor settings that have been developed include the thin layer agar (TLA) assay (71, 128-129), MODS test (75, 130-131), colorimetric redox indicator (CRI) methods (132-133), nitrate reductase assay (NRA) (134-135) and mycobacteriophage-based (136-137) methods. The MODS and TLA tests are both based on the characteristic chord formation of MTB when growing in medium. To detect drug resistance, drug free and drug-containing media are inoculated with specimens from patients, and cultures are microscopically examined for early growth or microcolonies. Systematic review and meta-analysis studies revealed the TLA assay sensitivity and specificity to detect R and H resistance was 100%, while the MODs test showed sensitivity and specificity for R resistance at 98% and 99.4% respectively, and 97.7% and 95.8% respectively for H resistance (0.1 µg/mL) (138).

The CRI methods are based on reduction of redox indicators that are added to culture medium during *in vitro* growth of *M. tuberculosis*. Examples include the tetrazolium salt-based assay utilizing 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) which is yellow in color in its oxidized form but is reduced to blue/ purple colored compound during growth of micro-organisms, and the resazurin microtitre assay (REMA) which is based on oxidation of resazurin by a growing culture. The nitrate reductase assay depends on ability of viable *M. tuberculosis* to reduce nitrate to nitrite (71, 114, 128, 138-139).

The phage-based assays use mycobacteriophages to infect live *M. tuberculosis* in the absence and presence of anti-TB drugs and detect the bacilli using either the phage amplification assay or production of light. A commercial phage-based assay available is the FAST Plaque TB-Response assay (Biotec Laboratories Ltd.) which detects drug resistance of *M. tuberculosis* directly in sputum specimens (136, 140). The method provides rapid (within 2 days) and accurate results when compared with the

proportion method and the BACTEC radiometric method, but has relatively low sensitivity and specificity. The other non-commercial methods described above are able to provide results within 8 – 14 days.

1.6.3.2 Molecular methods

Delayed detection of drug resistance means delayed adjustment of treatment with an effective drug treatment regimen and poses the danger of MDR-TB outbreaks. Several molecular tests to detect drug resistance more rapidly have been developed. These methods are based on the detection of specific gene mutations known to be associated with resistance to a particular drug. However, not all mechanism for drug resistance for all drugs are fully understood yet and the fact that drug resistance to a particular drug is not associated to a single mutation in most cases further complicates the situation.

The line probe assay (LPA) technique has proved useful in the rapid detection of drug resistance and has been recommended for DST by WHO because of their combination of speed and high accuracy, relatively low technical requirements and ease of sample transport.

There are currently three commercially available solid phase reverse hybridization assays for the rapid detection of drug resistance in MTB complex: INNO-LiPA Rif TB Assay (Innogenetics; Belgium) for detecting resistance to R, the GenoType® MTBDR*plus* (Hain Lifesciences, Germany) for the simultaneous detection of R and H resistance, and the GenoType® MTBDR*sl* for the detection of E and the main second-line TB drugs. Analysis of evaluations of these kits indicate high sensitivity and specificity for INNO-LiPA (>95%) for detecting TB and correctly identifying R resistant and sensitive strains, whereas a recent review of the GenoType® MTBDRplus found over 95% sensitivity and 100% specificity in most studies on isolates. A comparison of the GenoType® MTBDR*sl* with DNA sequencing revealed 100% correlation, with sensitivity compared to phenotypic DST reaching 89% for OFL, 75% for amikacin and 87% for capreomycin, but only 38% for E resistance. KAN- and latest generation FQ-resistance have not been evaluated, so that the clinical usefulness of this test is not yet entirely clear (139).

The Xpert®MTB/RIF earlier described (section 1.4.2), has also shown impressive performance in the rapid detection of drug resistance to rifampicin. Evaluation of this system in a multi-centre study reported that R-sensitivity results agreed with phenotypic DST in more than 97% of patients. Performance for discrimination of R-resistance was similar across diverse sites in many countries (98-99, 141).

The successful implementation of this assay will allow for more rapid and decentralized detection of R resistance. As encouraging as these findings may be, more studies are needed to resolve a number of issues involving performance of the MTB/RIF assay. These issues include, its limitations in; a real appreciation of implementation in peripheral labs, testing only for R resistance - a platform that detects a relatively small number of mutations, inability to indicate which patients are "sputum smear–positive" for reporting purposes, infection-control intervention, and treatment monitoring, impact of NTMs and performance in children (142-148).

Other technologies that have shown potential for use in the rapid detection of drug resistance include microarray technologies and high performance liquid chromatography techniques. However, from the few evaluation studies done, sensitivity of these techniques is still an issue. Coupled with the fact that they require expensive reagents and equipment, skilled technical personnel, these techniques are still a while before use in routine labs.

With the emergence of MDR and XDR-TB, the need for rapid DST is urgent even in low-income countries. But given the limitations of the promising technologies described above, for management of MDR/XDR in these low-income countries, surveillance targeting continuous monitoring of drug resistance among first re-treatment cases using the slow but probably more accurate and less costly conventional methods are being recommended (139).

1.6.4 MDR- and XDR-TB treatment

In situations where first-line drugs prove ineffective i.e. in MDR-TB, second-line drugs are employed. They are mainly bacteriostatic, and in general, less efficacious than first-line drugs and are more expensive, toxic and require long periods of administration. There are several second-line drugs available; these include FQs such as ofloxacin, moxifloxacin and gatifloxacin, aminoglycosides such as KAN and amikacin, cyclic polypeptides such as capreomycin, and others such as para-aminosalicyclic acid, cycloserine, linezolid and ethionamide. Treatment for MDR-TB is longer (at least 18–24 months) and success rates are substantially lower than for drug-susceptible TB (around 75%), even worse in HIV-infected patients. FQ use has been associated with improved MDR treatment outcomes. MDR-TB treatment regimens should include at least 4 drugs with presumed susceptibility, including an injectable agent and a FQ in the initial phase, and at least 3 of the most active and best-tolerated drugs in the continuation phase. An initial phase of at least 6 months should be followed by a continuation phase of 12-18 months (102).

Treatment of XDR-TB is similar to principles used in MDR-TB treatment, in that the regimen should consist of drugs to which the patient's MTB isolate is supposedly susceptible. Treatment is complex and should be done in consultation with an expert. The regimen should consist of 4 – 6 drugs selected from, first-line and second-line drugs or from the third-line category of drugs with uncertain anti-TB activity, which include clofazamin, linezolid, amoxicillin/clavulanate, imipenem or macrolides (51, 149) (Table 3).

Drug(s)	Chemical description	Route	Class	Mode of action	Biological process inhibited	Gene target for resistance	Most common resistance-conferring mutations
I	Nicotinic adic hydrazide	Oral	1st-line	Bactericidal	Mycolic acid synthesis	*katG* *inhA* *aphC*	S315T, S315N, S315R -15C/T, -8T/A, -8T/C -46G/A, -39C/T
R	Rifamycin derivative	Oral	1st-line	Bactericidal	Protein synthesis	*rpoB*	H526T, H526/D, S331L, L533P
Z	Nicotinamide derivative	Oral	1st-line	Bactericidal	Unknown	*pncA*	G162D, R140S, V128G
E	Ethylene dimino-di-1-butanol	Oral	1st-line	Bacteriostatic	Lipid/cell wall synthesis	*embB* *iniA*	M306V, M306I S501W, Gly308R
RBU	Rifamycin derivative	Oral	2nd-line	Bactericidal	Protein synthesis		
S	Aminoglycoside	Injectable	2nd-line	Bactericidal	Protein synthesis	*rrs* *rpsL*	513A/T, 491C/T L43R, L88Q, L88R
KAN/AMI	Aminoglycoside	Injectable	2nd-line	Bactericidal	Protein synthesis	*rrs*	1401A/G, 1484 G/T
CAP/VIO	Cyclic peptide	Injectable	2nd-line	Bactericidal	Protein synthesis	*rrs*	1401A/G, 1484G/T
CIP/OFL	Fluorquinolone	Oral	2nd-line	Bacteriostatic	DNA replication	*gyrA* *gyrB*	A90V, D94N, D94G N510D
LFX	Fluorquinolone	Oral	2nd-line	Little bactericidal	DNA replication	*gyrA* *gyrB*	A90V, D94N, D94G N510D
MFX/GFX	Newer fluorquinolone	Oral	2nd-line	Bactericidal	DNA replication	*gyrA* *gyrB*	A90V, D94N, D94G N510D
ETH/PTH	Isonicotinic acid derivative	Oral	2nd-line	Bacteriostatic	Mycolic acid synthesis	*inhA*	-15C/T
PAS	Para-amino-salicylic acid	Oral	2nd-line	Bacteriostatic	Unknown		
CS	D-Cycloserine	Oral	2nd-line	Bacteriostatic	Cell wall synthesis		
TAC	Thiacetazone	Oral	2nd-line	Bacteriostatic	Mycolic acid synthesis		
CLR	Erythromycine derivative	Oral	3rd-line	Bactericidal	Protein synthesis		
AMX/CLA	β-lactam with β-lactamase inhibitor	Oral	3rd-line	Bactericidal	Cell wall synthesis		
CFZ	Iminopherazine derivative	Oral	3rd-line	Bacteriostatic	Cell membrane function		
LZD	Oxazolidinone derivative	Oral	3rd-line	Bactericidal	Protein synthesis		

Table 3: I, isoniazid; R, rifampicin; Z, pyrazinamide; E, ethambutol; RBU, rifabutine; S, streptomycin; KAN, kanamycin, AMI, amikacin; CAP, capreomycin; VIO, viomycin; CIP, ciprofloxacin; OFL, ofloxacin; LFX, levofloxacin; MFX, moxifloxacin; GFX, gatifloxacin; PAS, para-amino salicylic acid; CS, cycloserine; TAC, thiacetazone; CLR, clarithromycin; AMC, amoxicillin β-lactam antibiotic with clavulanate β-lactamase inhibitor; CFZ, clofazimine; LZD, linezolid.
Mutations are expressed as amino-acid change AxxxB (with A and B representing the amino-acid code and xxx the codon) or nucleotide changes xxA/B (with xx representing the nucleotide position and A and B the nucleotide). Adapted from (114) and (124)

1.7 Role of social factors in TB control, prevention and care.

Many lives have been saved in the past two decades by the increased global efforts in TB control, focused on improving diagnosis and treatment through the DOTS and Stop TB strategies. However, the epidemiological impact has been less impressive. Although NTPs continue to report decreases in prevalence of- and mortality due to TB, incidence estimations have not yet decreased to the same degree. This has driven the need to revisit the control strategies. It has become apparent that the current approaches have laid emphasis on diagnosis and treatment rather than on prevention. While biomedical approaches to prevention, such as the development of more effective tools for early diagnosis, effective treatment and vaccine development, can greatly enhance TB control, addition of strategies that address social, economical and health system factors, will likely provide greater epidemiological impact.

It has been long known that TB is a social disease, affecting mainly the lower socio-economic strata of society. Several of the associated determinants of TB disease, e.g., nutritional status, crowding, migration, smoking, alcohol, poor economic status and low education, are poverty related. Successful TB control will depend on accessible and effective public health systems which address the local social determinants. Tuberculosis control cannot reach its proposed global targets without adequate investment in an adequate network of accessible, effective and comprehensive health services (150). Further, accessibility and effectiveness of service delivery not only depend on the service provider, but also on the patient. Therefore an understanding of barriers to access to healthcare and the consequent implementation of interventions that address these barriers will likely improve service delivery. Understanding how poverty, stigma, exclusion, low awareness, low education level and other competing priorities in a community contribute to people's access to quality diagnosis, treatment and care, can help to fill in the gaps in service delivery (151-152). Fortunately, health systems improvement is already firmly on the WHO Stop TB control community's agenda (153-154).

1.8 Molecular typing of the MTB-complex

In general, disease-related molecular epidemiology attempts to utilize a multidisciplinary approach to identify factors that determine disease causation, propagation/dissemination, and distribution (in time and space). This is primarily achieved by associating demographic/epidemiologic characteristics with the biologic properties of clinical isolates recovered from infected individuals. The conventional methods used in TB species typing,

such as identifying variations in colony morphology, comparing growth rates, determining susceptibility to select antibiotics, and phage typing, do not provide sufficient discrimination, limiting their utility in TB epidemiology. The advent of (PCR-based) MTB-specific genotyping methods and the unravelling of the complete MTB genome provided for significant improvement in this aspect.

1.8.1 DNA-typing methods

While the MTB complex genome is highly conserved in relation to other bacterial pathogens, it still possesses sufficient polymorphic genomic regions, which allow for discrimination between individual strains. The first genotyping technique for typing MTB complex was reported in 1991 (155) and standardised in 1993 (156). This was a non-radioactive restriction fragment length polymorphisms (RFLP) based technique in which high-molecular-weight fragments from digested genomic DNA was visualized with digoxigenin-labeled *M. tuberculosis* DNA following Southern blotting. Thereafter, several other alternative genotyping technologies with varying practicalities and efficiencies have been developed, which have proved helpful in studying transmission dynamics, outbreaks, nosocomial infections, phylogenetics, distinguishing exogenous re-infection from endogenous reactivation, and laboratory contamination. The most common genotyping methods currently used are described below.

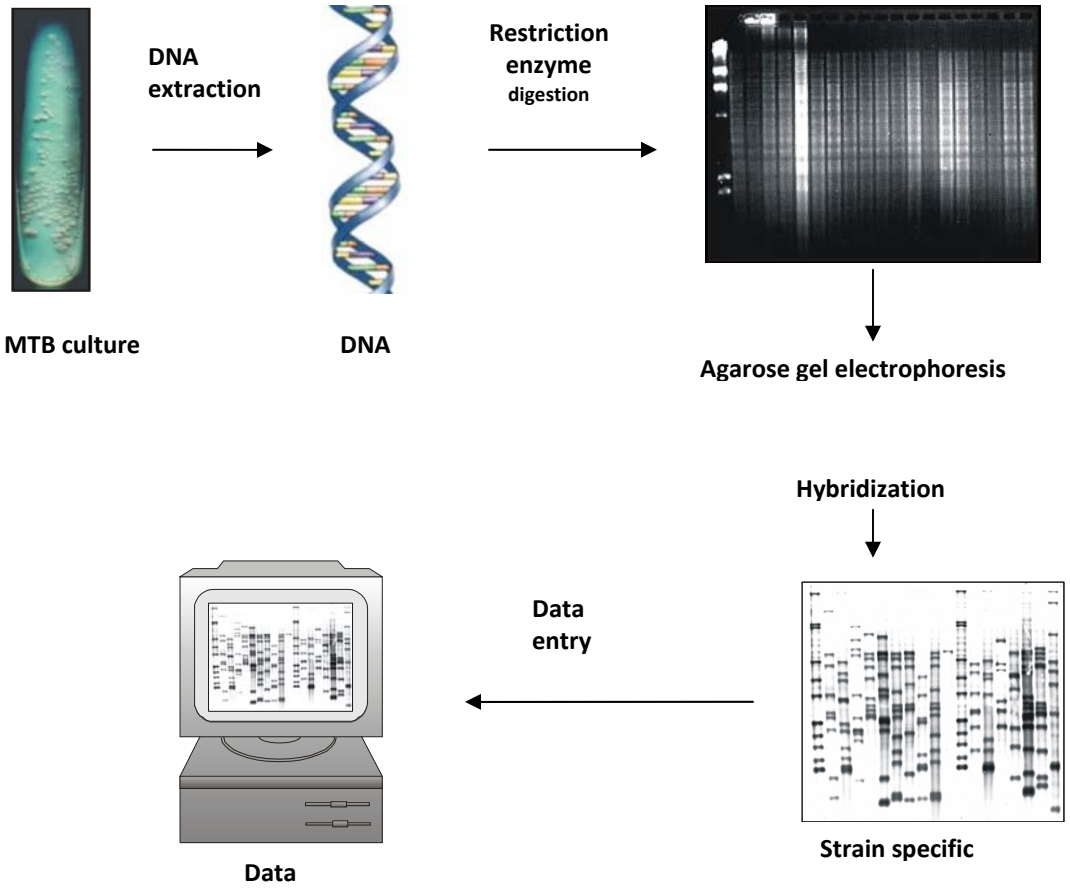
1.8.1.1 IS6110 RFLP

This is the gold standard for typing MTB complex isolates and is based on differences in number and arrangement of the insertion sequence (IS) *6110* fragments in a chromosome. The number of copies of IS*6110* elements in MTB strains ranges from 0 to 25. The technique involves digestion of extracted high molecular weight mycobacterial DNA with *Pvu*II, which cleaves the DNA at specific sequences within and flanking IS*6110* elements. The DNA fragments are then separated on agarose gel, transferred to a nylon membrane by Southern blotting and then hybridized with a radioactive or non-radioactive probe which can be visualised using an enzyme-based chemiluminescence or radioactivity on a light-sensitive film. Discrimination of isolates is based on differences in number and arrangement of the IS*6110* fragments in a chromosome, reflected as differences in number and position of bands on the film (Fig 6). Other targets used for RFLP in MTB include IS*1081,* the polymorphic GC-rich sequence (PGRS), the Direct Repeat (DR) sequence, mpt40 (a fragment from the phospholipase C gene, specific for *M. tuberculosis*) and a major polymorphic tandem repeat sequence of *M. tuberculosis* (MPTR).

IS*6110*-based RFLP is highly reproducible, and in isolates with more than five IS*6110* elements, highly discriminatory. However, this method is

technically demanding, labour intensive, requires large amounts of high quality DNA and expensive computer software for analysis. The latter requirement makes intra- and inter-laboratory comparisons and sharing of data difficult (59, 157-159).

Figure 6: Diagrammatic representation of the IS*6110*-RFLP method



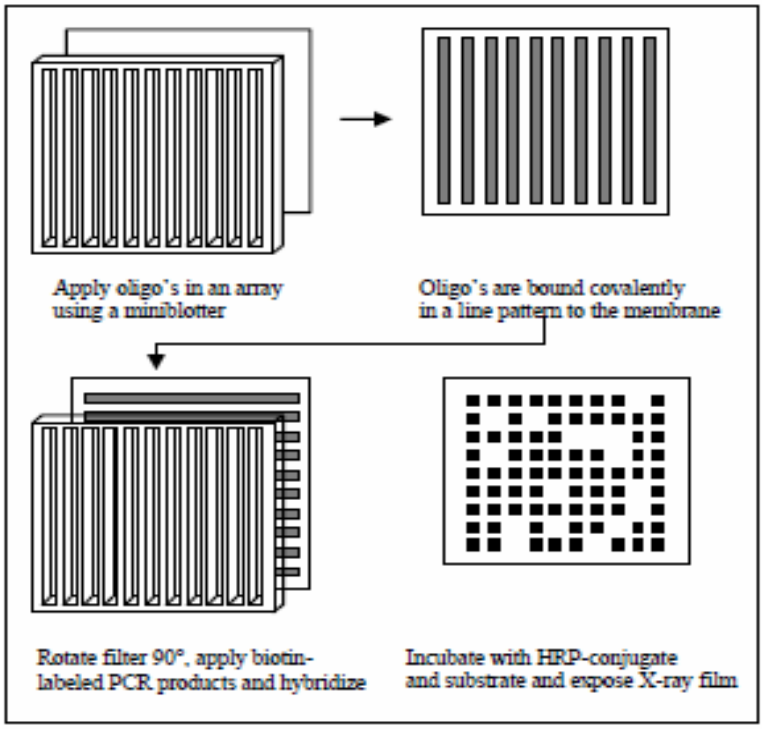
1.8.1. 2 Spoligotying

Spacer oligotyping (Spoligotyping) is based on DNA polymorphism of non-repetitive spacers sequences of 35-41 bp long that are interspersed among identical direct repeats present at one particular chromosomal locus, the Direct Repeat (DR) locus. The DR is MTB complex specific. The DRs are 36 bp long and are present in 30-50 copies in the DR locus. The number of repeats and spacer sequences varies in different strains and is the basis of differentiating MTB complex isolates.

Identification of different MTB strains using spoligotyping involves the detection of the presence or absence in the DR region of 43 spacers of known sequence. Membranes spotted with 43 immobilized synthetic

oligonucleotides, representing each of the unique spacer sequences are hybridized with biotin labeled PCR-amplified DR locus of the test strains (reversed hybridisation). After addition of a conjugate and substrate, this reaction results in a pattern that can be detected by chemiluminescence (160) (Fig 7). The results are highly reproducible, and the binary (present/absent) data generated can be easily interpreted and computerized, making it easier for inter-laboratory comparison (161-162). Although, still labour intensive, minimal DNA material is required for this method and non-viable material can be used. The main drawback to this method is its low discriminatory power.

Figure 7: Diagrammatic representation of the spoligtyping method[160]



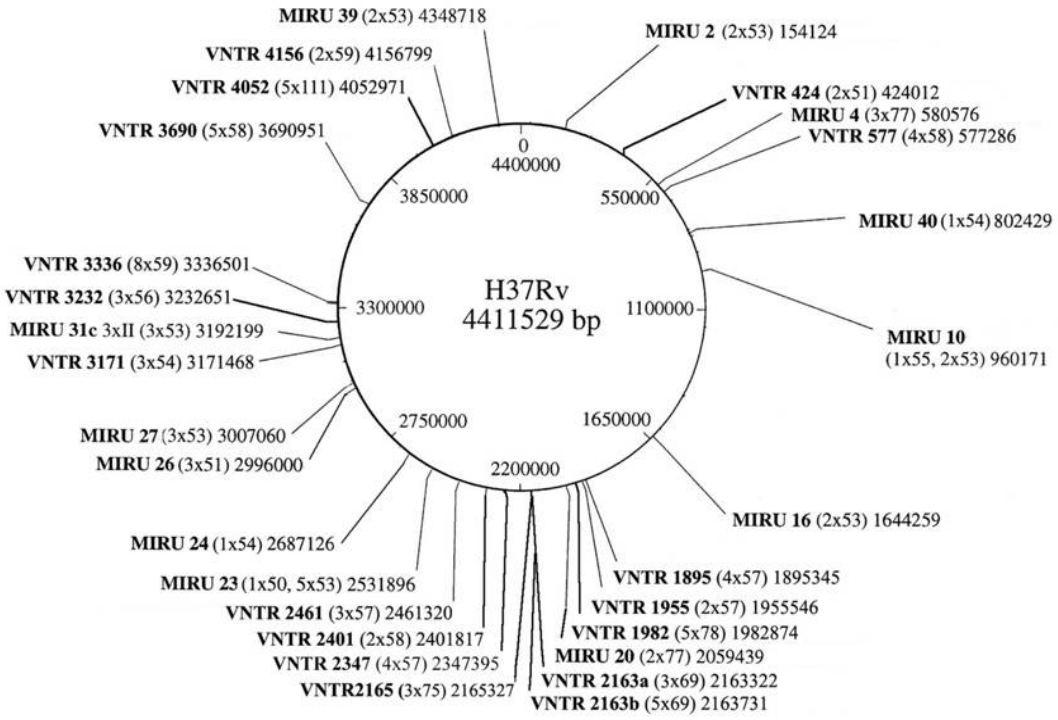
The level of differentiation by spoligotyping is less compared to IS*6110*-RFLP for strains having five or more IS*6110* copies, but higher for strains with less than five copies. Thus spoligotyping is a preferred method to type *M. bovis* strains, which usually contain only one or two IS*6110* copies (158, 163).

1.8.1. 3 Mycobacterial Interspersed Repetitive Units- Variable Number of Tandem Repeats (MIRU-VNTR) typing

Variable number tandem repeats (VNTR) are short tandemly repeated DNA sequences that vary in length and number in different loci. In MTB complex, VNTRs with tandem repeats of 40 to 100 bp (minisatellites) dispersed

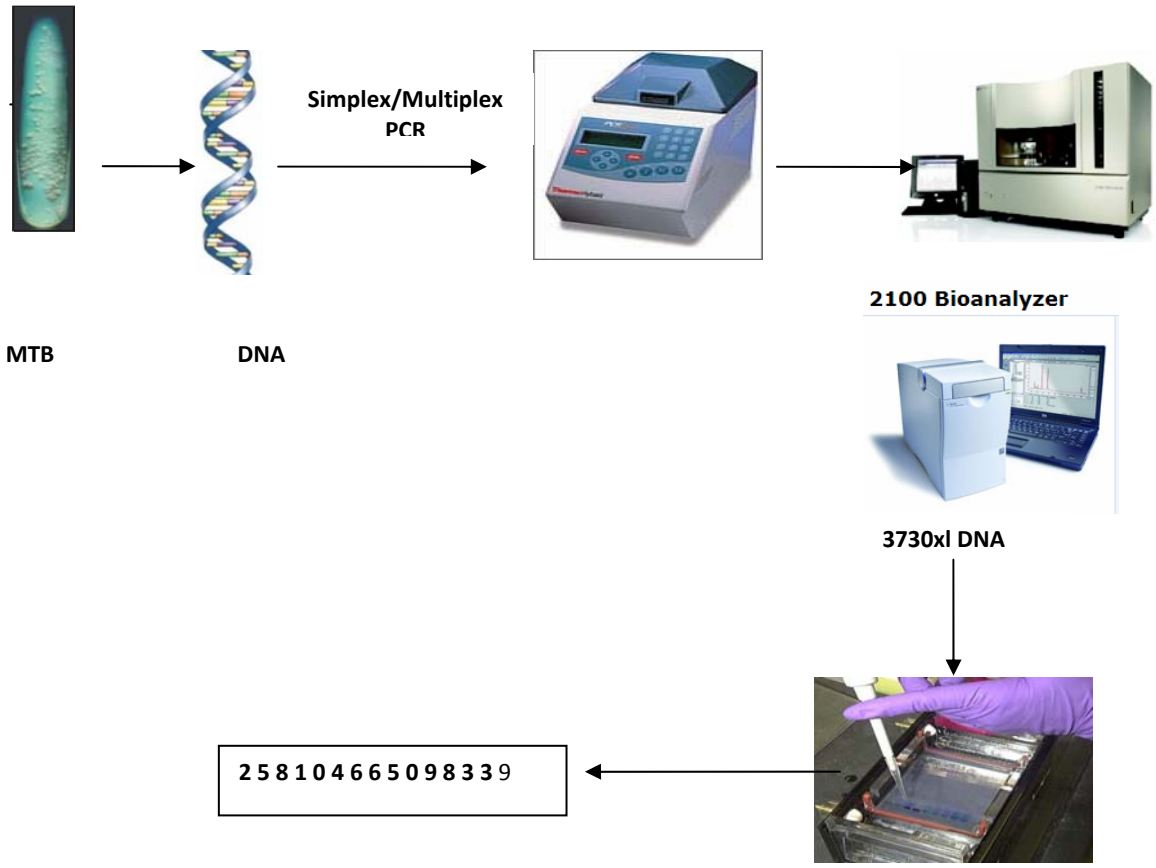
throughout the genome have been identified and are referred to as mycobacterial interspersed repetitive units (MIRU) (164-166) (Fig 8).

Figure 8: H37Rv genome showing minisatellites[166]



The MIRU-VNTR genotyping technique relies on PCR amplification of DNA sequences in various loci using primers targeting flanking regions of respective VNTRs and on the determination of the sizes of the resulting amplicons, after electrophoretic separation. As the length of the repeat units is known, these sizes reflect the numbers of VNTR copies present in that specific locus. The final result is a numerical code corresponding to the repeat number in each VNTR locus (Fig 10).

Figure 9: Diagrammatic representation of the MIRU-VNTR typing method



Initial VNTR typing systems for MTB complex strains made use of very limited sets of loci, which were not sufficiently discriminatory. More extensive sets of VNTR loci have been described subsequently, including a system based on 12, 15 and 24 loci, which has been shown to be applicable for reliable genotyping and molecular epidemiology studies of MTB (167-169). Further, a MIRU-VNTR-based high throughput genotyping system has been developed, which combines the analysis of multiplex PCRs for the target loci on a fluorescence-based DNA analyzer with computerized automation of the genotyping. Both this system and the simpler system using electrophoresis with agarose gels are highly reproducible at intra- and inter-laboratory levels. The discriminatory power of MIRU-VNTR is similar to that of IS*6110* RFLP and better for low-copy IS*6110* strains. In addition, this method can be applied directly to clinical specimens with a high bacillary load. However, the manual method of VNTR can be labour intensive (170-172).

1.8.1. 4 Deletion oligotyping (deligotyping)

Deletion oligotyping (deligotyping) is based on detection of known genomic deletions as genetic markers. Genomic deletions of large sequences are irreversible and often unique events. Consequently, once a deletion occurs in the progenitor strain, the specific deletion can serve as a genetic marker for the genotyping progenies of this strain (59).

The method assesses the presence or absence of 43 genomic regions using multiplex PCR, amplicons from test strains followed by reverse hybridisation on a membrane containing the target sequences of the 43 loci. Deligotyping has been shown to be highly sensitive and specific (99.9% and 98.0%). However, high-throughput deligotyping needs to be evaluated against different panels of clinical strains and in different epidemiologic and geographic settings (173-175).

1.8.1. 5 Single Nucleotide Polymorphisms (SNPs)

Another typing method that has proved useful in species differentiation of MTB strains and phylogenetics is the use of synonymous single-nucleotide polymorphisms (SNPs). Synonymous SNPs are single nucleotide changes that do not result in amino acid changes (neutral). Due to their neutral nature, these SNPS are ideal for population-genetic studies examining phylogenetic relationships among bacterial strains. The technique has high resolution and since the technique can be automated, it can be used for large-scale genotyping. However, a disadvantage to this technique is that it requires extensive genomic sequencing of multiple chromosome targets. Once lineage-specific SNPs are determined they can be used in more simple PCR-based assays (176-178).

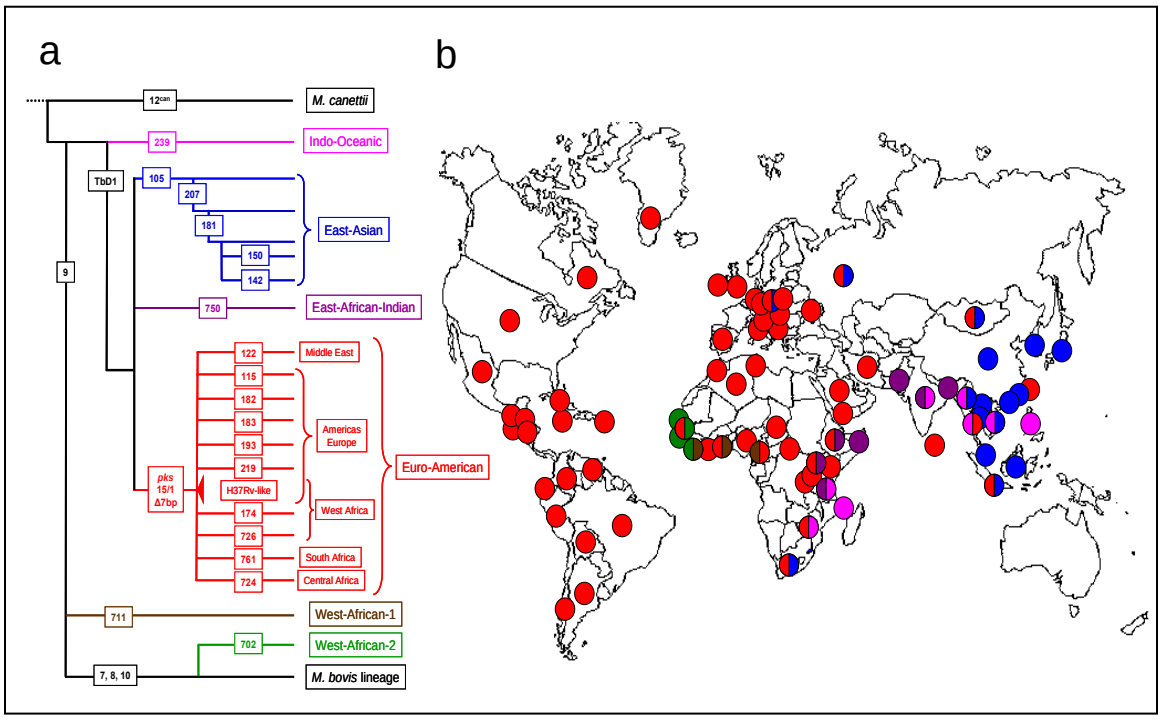
1.8.2 TB evolutionary lineages

In an attempt to analyze MTB population structure and to assess the complexity of the global epidemiology and evolutionary genetics of tubercle bacilli, a genetic diversity database consisting of spoligotype patterns of isolates from various regions of the world was first constructed in 1999 (179). Over the years, this publicly available international database has continued to be updated. The genotype patterns are grouped according to their similarity and phylogenetic signatures and classified in different genotypic families/clades/lineages. Isolates with identical spoligotypes patterns are referred to as shared types (ST). By systematically analyzing published spoligotypes, the latest, SpolDB4 now contains a total of 39,295 strains containing 62 families with 1,939 different shared types, representing 141 countries (161).

SpolDB4 has defined 10 main lineages, namely, the Latin American Mediterranean (LAM), W-Beijing, Central Asian (CAS), East African Indian (EAI), Haarlem, MANU, Beijing and Beijing-like, X, T and S. Global distribution of these lineages shows varied predominance of the different lineages for different regions. Haarlem, LAM, and T lineages predominate in Africa, Central America, Europe and South America whereas, the Beijing, Central Asian (CAS) and East African Indian (EAI) are predominant in Asia and the Oceania. The Beijing genotype which may have been endemic in China for a long time is emerging in some parts of the world, especially in countries of the former Soviet Union, and to a lesser extent in the Western world (180).

Another free online reference database available for standard genotyping of MTB complex is the MIRU-VNTRplus (181). Alternative lineages based on SNPs have been proposed (Fig 10). Because lineage-specific SNPs are mutually exclusive in the MTB complex, they provide better definition in phylogenetic relationships in contrast to Spoligotyping and MIRU-VNTR, which have a propensity for convergent evolution and homoplasie (182-183).

Figure 10: Global distribution of major TB lineages: SNP analyses [184]



1.8.3 Application of molecular epidemiological tools

The desirable characteristics of a good typing method include the ability to be applied to all organisms within a species, the ability to cluster epidemiologically related organisms and to differentiate epidemiologically unrelated organisms. The method should also be highly reproducible, technically less demanding, reasonably priced with an acceptable turnaround time. Given the number of molecular tools now available, it is important to choose (an) appropriate method(s) to address a particular study question, e.g., transmission dynamics, outbreaks, or phylogenetics.

The field of molecular epidemiology has made possible several epidemiological investigations, such as suspected outbreaks in various settings (185-187), confirmation of laboratory error/cross contamination (188-190), understanding spatiotemporal transmission and evolutionary dynamics (175, 191), determination of the fraction of cases attributable to recent transmission or reactivation (192-194), distinguishing between endogenous reactivation and exogenous re-infection (195-197), investigation of properties and patterns of drug resistance with specific populations or groups of strains (198-200), and better understanding of transmission dynamics within specific populations (201-204). Molecular methods can also be used to evaluate host- and strain-specific risk factors and possible genotypic-specific differences in phenotypes such as virulence, organ tropism, and transmissibility (205-208). It should be borne in mind that to obtain a holistic picture, both molecular and conventional epidemiologic data sources should be combined.

1.9 TB in Zambia

Figure 11: Map of Zambia[209]

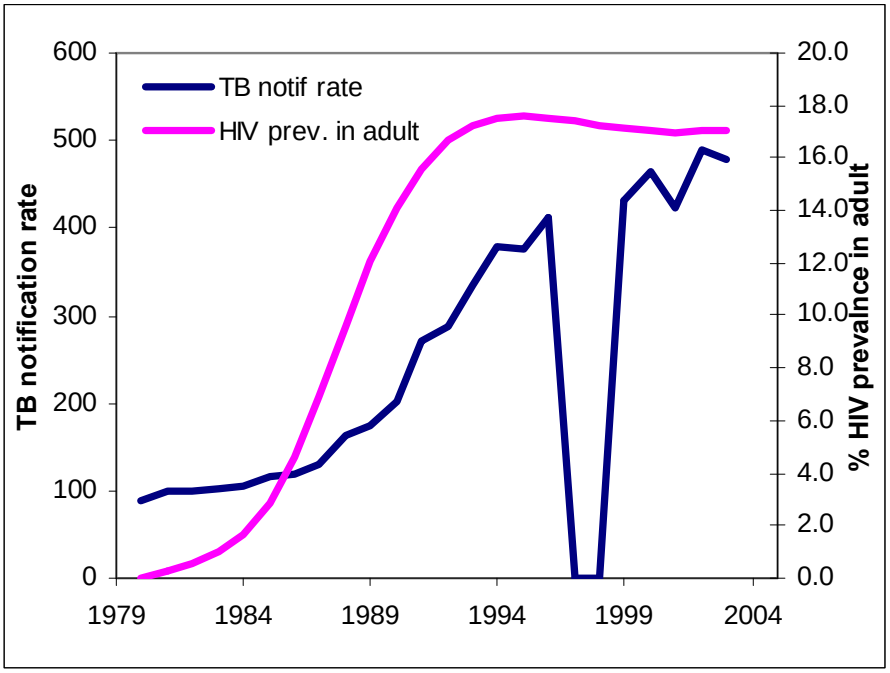
Zambia (Fig 11) is a relatively large country, 752,610 Km^2, with a population estimated at 12.525 million people (210). It is a landlocked country in sub-Sahara Africa sharing borders with Angola, the Democratic Republic of Congo, Malawi, Mozambique, Namibia, Tanzania, Zimbabwe and Botswana. Administratively, the country is divided into nine provinces and 72 districts. Of the nine provinces, two are predominantly urban, namely Lusaka and Copperbelt provinces. The remaining provinces - Central, Eastern, Northern, Luapula, North-Western, Western, and Southern - are predominantly rural provinces.

1.9.1 Prevalence and incidence

Tuberculosis remains a major public health problem and ranks sixth in causes of death in Zambia (211). Just like the rest of the African region, Zambia has seen the concurrent rise in TB cases in concert with the rise in HIV (Figure 12). Even though significant achievements have been made in the fight against TB, Zambia still continues to battle with a high TB burden

made worse by the HIV scourge. 2007 WHO TB estimates show that incidence for all forms of TB cases in Zambia was 553/100,000, while that for sputum smear-positive was 228/100,000, corresponding to around 67,800 and 28,000 cases, respectively (41).

Figure 12: Trends of TB and HIV in Zambia: *No data available for 1998-2000 following integration of vertical programme, the NTLP almost collapsed due to lack of focus on TB control.



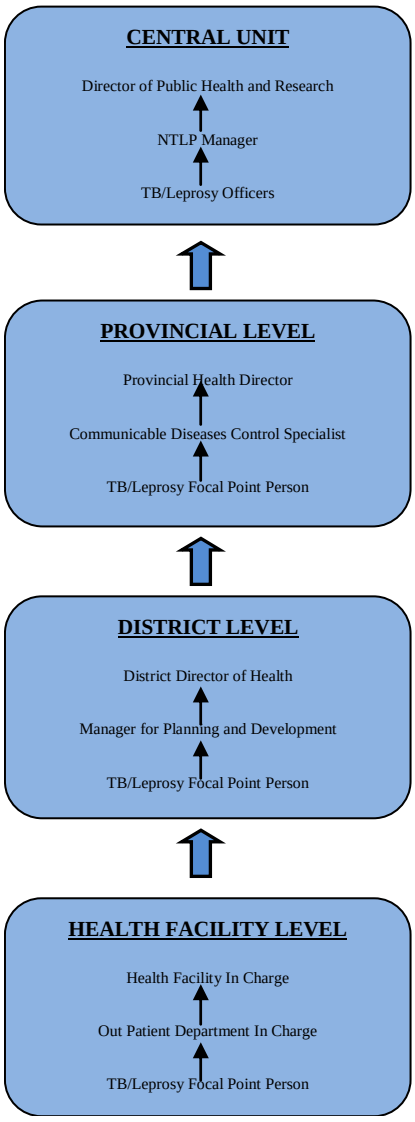
Compilation of TB data from a national TB technical review meeting in March 2008 showed total TB notifications for 2007 at 50,415 (approximately 500/100,000 population). Cohort analysis of the 2006 TB cases showed that the proportion of new smear-positive cases detected out of the estimated total TB cases, was 52% and treatment success rate stood at 85.1%. Other major outcomes reported included death rate (6.6%), treatment failure rate (0.6%), transfer out rate (5.1%) and default rate (2.8%) (210). Drug resistance appears to be relatively low in Zambia. However, this data is old, the last available national drug-resistance survey data is from 1999, and this survey indicated MDR at 1.8% and 2.3% in new and previously-treated cases, respectively.

TB/HIV data was available in full for the first time in 2007 following introduction of new recording and reporting tools in mid 2006. This data showed a 68.5% prevalence of co-infection in the 23,356 TB patients tested for HIV. Currently, all the facilities are testing TB patients for HIV co-

infection. However, WHO notes that although uptake from patients has been good, documentation systems require strengthening and supervision in order to obtain better estimates of the burden of HIV in TB patients. Further, the integration of TB and HIV&AIDS services is better at district and health centre levels, but remains a challenge at big urban centres and at hospitals (210).

1.9.2 The National Tuberculosis Programme

Figure 13: Organogram of the Zambian NTLP[212]



The National TB Programme was established in 1964 and operated as a vertical programme. In 1992, there was a Health Sector reform according to the National Health Policies and Strategies. The main feature of the

organizational and institutional restructuring implemented under the health sector reform programme was the decentralization of health service delivery, through devolution of key management responsibilities and resources from central to district level. In 1993, the TB Programme was combined with the AIDS and Sexually Transmitted Diseases (STD) Programmes to form the National AIDS/STD/TB/Leprosy Programmes.

In line with the Health Sector Reforms, by 1997 full integration of the vertical TB Programme had occurred and specific TB posts at provincial and district levels had been abolished. However, following decentralization, the TB and Leprosy Control Programme (NTLP) almost collapsed in the late nineties due to lack of focus on TB control at all levels as a result of loss of structure, staff trained and guidance in TB control. Intensified efforts from government and stakeholders revived and strengthened the programme later. Current TB control in Zambia is well integrated in the primary health care services with a well defined structure and functions. The NTLP structure consists of a TB Focal Person (TBFP) at each level of the health system, i.e. at National, Provincial, District and Health Facility Level (Fig 13).

The Zambian NTLP TB control strategies which include, case detection based on sputum smear microscopy, short-course supervised treatment and BCG vaccination in infants, stem from WHO guidelines whose main focus is to interrupt transmission through early case detection and effective treatment. Introduction of DOTS to some urban areas was as early as 1993, and full geographic coverage was reported in 2006. Further, given the high sero-prevalence of HIV in Zambia (14.3%), and co-infection with TB of approximately 68.5%, Zambia has adopted the new Stop TB strategy.

Like many low-income countries, smear microscopy is the main diagnostic tool for TB. In this regard, the NTLP has been working to improve access to laboratory diagnosis for every TB suspect, by putting in place systems that network centres with microscopy facilities (diagnostic centres) and those without (treatment centres). Zambia introduced the 8-month SCC for sputum-smear positive patients as early as 1986. In 2009, the 6-month treatment regimen was introduced for new smear-positive, smear-negative and extra-pulmonary TB. Fixed-dose combinations containing 4 drugs (HERZ) are now available in health facilities.

NTLP manual also stresses the importance of, and offers guidelines on patient compliance through DOTs, patient education and patient monitoring and follow-up. Following the New Stop TB Strategy, integration of TB and HIV care has been intensified at all levels of health care service provision. Other guidelines are also provided for management of TB in children, special

situations such as pregnancy and MDR (although not diagnosed routinely, management of MDR is included in the Guidelines).

1.9.3 Ndola Urban District

The Copperbelt province is predominantly a copper mining region. It is the most industrialized and urbanized province in Zambia, located approximately 300 Kms north of the capital, Lusaka. The province has well developed road and railway networks with an estimated population of 1.911 million people, the province has 10 districts (7 urban and 3 rural). HIV prevalence for the province was estimated at 17.0% in the 2007 Demographic Health Survey (209). Further, the province accounted for 27.6% of the country's TB cases in 2004 (213)).

Ndola is the capital city of the Copperbelt province, with an area of 1,103 Kms^2 and has an estimated population of 374,757 persons (census). Ndola DHMT provides health care services through 26 health centres. With regards to TB services, whereas all 26 health centres offer treatment and care services, only 8 offer diagnostic (smear microscopy) services. However, a network has been put in place in which treatment centres are able to access microscopy services for their clients. In the year 2009 Ndola DHMT recorded 2,827 TB cases (all types) of TB of which 770 cases were new (Data from Ndola DHMT office).

Organisation of TB care service delivery in Ndola is centred on the TBFP at each health centre, in agreement with the National TB guidelines. The TBFP can be a nurse or a clinical officer at the health centre who would have been trained in the fundamentals of TB care and treatment. Accountable for all TB activities at the health centre, the TBFP is responsible for ordering medicine and supplies from central stocks at DHMT, follow up of patients and submitting reports to the NTP. However, the TBFP is not a full-time engagement and the nurse or clinician in this position also performs other duties at the health centre. This no doubt puts tremendous pressure on the TBFP to execute his/her duties efficiently. Urban health centres have more staff, yet also have a larger population to care for. Given these staffing constraints, the treatment supporters and community health workers play a major role in ensuring patient follow-up and treatment. The former are usually relatives of the patient who get trained on administration of directly observed treatment, whereas, the latter are volunteers from the community, sometimes former TB patients, who help to follow up patients and pick up medication and sputum samples for the very sick.

Patient flow at the clinic is as follows: from the consultation room, if a clinician suspects TB, the patient is sent to the 'TB corner', where the TBF registers the patient and sends him/her to the laboratory for smear

microscopy. An '(on the) spot' sputum sample is collected immediately and the patient is given a container with instructions for the collection of a morning sample. The patient will return the following day to the lab with the sample and another 'spot' sample is collected. Depending on whether the health centre is a diagnostic centre or a treatment centre, the patient will be asked to return the next day for smear microscopy results (in the case of a diagnostic centre), or given a return date for results after samples have been sent to a diagnostic centre (in the case of a treatment centre). Availability of results at the treatment centre depends on the collection and delivery system of samples and results, by motorcycle, popularly called the 'Honda man', which is once or twice a week.

Following positive smear microscopy results, a patient is given information on TB basics and treatment at the 'TB corner' and given the first dose of medication. Health centres differ in how many times they require their patients to pick up doses, weekly or bi-weekly for the intensive phase, and monthly for the continuation phase. However, defaulters and retreatment cases are required to pick up medications daily. Patients are given treatment cards on which they indicate daily drug intake; it is the duty of the TBFP to follow-up a patient who does not show up to pick up his medication. The TBFP also schedules follow-up sputum submissions to coincide with drug pick up at month 2, 4 and at the end of treatment. Apart from a few centres with non-governmental support, no food supplements are given to patients on TB treatment. Further, the frequent visits to the health centre can be costly to the patient, with regards to transport, in areas that are distant from the health centres. There is no provision of reimbursement for transport from the healthcare system.

Chapter 2: Rationale and Purpose of the Study

Zambia continues to grapple with the TB epidemic. With an incidence of 553/100,000 and a TB/HIV co-infection rate of nearly 70%, the need for investigations into the dynamics of the Zambian TB epidemic cannot be over-emphasised. It is worrying that even after years of DOTS implementation; the TB burden continues to be high. No doubt, formulation of any strategy to combat the epidemic should be based on a thorough appreciation of the problem, especially in high TB incidence settings like ours, where determination of distinct transmission patterns is often complex.

The low levels of basic epidemiological data, especially the biological baseline data, to feed policy formulation in Africa may contribute to the slower-than-desired decline of the TB burden in the region. Until recently, very little work has been published on TB-related work in Zambia compared to most of its neighbours. For example, it will be very useful, especially following the decentralization of health delivery systems, to look at accessibility of quality TB services and the impediments to health seeking in the communities, as well as determining the levels of drug resistance and the types of strains circulating in Zambia. Synthesis of biological and sociological factors that contribute to the epidemic through use of molecular and conventional epidemiological tools may greatly enhance the accuracy and resolution of the epidemiological picture in Zambia.

General objective

- To asses at the management and control of TB on the Copperbelt province of Zambia.

Specific Objectives

- To determine the level of TB drug resistance to first- and second-line TB drugs in Ndola Urban District
- To determine the types of circulating MTB strains and transmission modes of TB in Ndola
- To investigate TB service delivery and study health seeking behaviours of TB patients in Ndola

Chapter 3: RESULTS

3.1. Low Occurrence of Tuberculosis Drug Resistance among Pulmonary Tuberculosis Patients from an Urban Setting, with a Long-Running DOTS Program in Zambia

Preamble

Monitoring trends of drug resistance levels is an important aspect of TB control. It is extremely useful as the ultimate measure of the effect of TB control programs and makes it possible to select the most appropriate drug regimens for a population. The limited number of effective drugs against TB entails that development of drug resistance be avoided at all costs, especially in the low-income/high-burden countries that already struggle to treat pan-susceptible TB. In general, data on drug resistance in Africa are inadequate and outdated, while data on drug resistance to second-line drugs is practically non-existent. Zambia is no exception, up until now, the only national drug-resistance data available is from 2001 and did not investigate resistance to second line drugs.

Author's contribution: CM was involved in the conceptualizing and organization of the study. She took the lead in the setting up of the cultures and subsequent drug susceptibility testing, and finally in the data analysis of the results and manuscript preparation.

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Research Article

Low Occurrence of Tuberculosis Drug Resistance among Pulmonary Tuberculosis Patients from an Urban Setting, with a Long-Running DOTS Program in Zambia

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We set out to determine the levels of *Mycobacterium tuberculosis* resistance to first- and second-line TB drugs in an urban population in Zambia. Sputum samples were collected consecutively from all smear-positive, new and previously treated patients, from four diagnostic centres in Ndola between January and July 2006. Drug susceptibility testing was performed using the proportion method against four first- and two second-line TB drugs. *Results*. Among 156 new cases, any resistance was observed to be 7.7%, monoresistance to isoniazid and rifampicin was 4.5% and 1.3%, respectively. Of 31 retreatment cases, any resistance was observed to be 16.1%, monoresistance to isoniazid and rifampicin was 3.3% for each drug, and one case of resistance to both isoniazid and rifampicin (multidrug resistance) was detected. No resistance to kanamycin or ofloxacin was detected. *Conclusion*. Although not representative of the country, these results show low levels of drug resistance in a community with a long-standing DOTS experience. Resource constrained countries may reduce TB drug resistance by implementing community-based strategies that enhance treatment completion.

1. Introduction

Despite a long-running National Tuberculosis and Leprosy Program (NTLP), Zambia has seen a rapid increase in TB cases, especially after 1983, synchronous with the beginning of the HIV era in Zambia. The World Health Organization (WHO) estimates the prevalence of all forms of TB in Zambia at 707/100,000 and ranks Zambia as ninth in the world for TB incidence rate with an incidence of smear-positive cases at 280/100,000 [1].

Decentralization of the health sector in the late 1990s almost led to the collapse of the program, but its revival and recovery was realised through government's renewed focus and reorganization. Zambia adopted the Directly Observed Therapy Short Course (DOTS) strategy since 1993 and achieved 100% geographical DOTS coverage by 2004. According to the NTLP, in 2005, the Copperbelt and Lusaka provinces were responsible for 60% of the nation's notified cases and also showed some of the highest HIV prevalence rates at 17% and 20.8%, respectively [2]. Furthermore, a recent study in selected District Health clinics in Lusaka showed TB/HIV coinfection at 59% [3].

The prevalence of multidrug-resistant (MDR) TB in Zambia was determined to be relatively low in 2001; 1.8% and 2.3% in new and previously-treated cases, respectively [4]. Other reported data on TB drug resistance in Zambia

stem from a survey in Zambian prisons in 2000–2001, which reported combined MDR among inmates at 9.5% [5]. Notwithstanding, because access to drug susceptibility testing (DST) is limited and not performed routinely, the picture of drug resistance in Zambia may be imprecise. This study was set out to document the prevailing drug resistance levels to four first-line drugs and two second-line drugs from an urban setting, where implementation of the DOTS strategy has been ongoing since the early 1990s.

2. Materials and Methods

2.1. Study Design. This is a part of a prospective cohort study in subjects with sputum smear-positive pulmonary TB (PTB).

2.2. Study Setting and Population. The study was conducted in health facilities in Ndola district under the Ndola District Health Management Team (DHMT). The Ndola DHMT has a catchment population of 374,757 persons [6] and 26 health centres, most of which are able to deliver TB treatment and care (treatment centres), but only six are able to perform smear microscopy (diagnostic centres). TB patients were treated according to the national TB guidelines [7], in line with the WHO treatment guidelines [8]. All new patients received an eight-month daily Category I regimen consisting of INH, RMP, pyrazinamide (PZA), and ethambutol (EMB) for two months followed by six months of INH and EMB. Patients who had previously taken TB drugs for more than one month received Category II treatment; INH, RMP, streptomycin (SM), EMB and PZA for two months daily followed by INH, RMP, EMB, and PZA for one month daily and INH, RMP, and EMB for five months administered daily. For this study, all consecutive previously-untreated and previously-treated smear-positive cases were enrolled from 4 of the 6 TB diagnostic centres. Data from the National Reference Laboratory for proficiency testing in smear microscopy show that, in 2006, 3 of the 4 participating diagnostic centres took part in the national program, with an average performance of 80% (75%, 80%, and 85%). The two clinics not included in the study were left out mainly because of their comparatively low population catchment areas at the time. All sputum smear-positive TB patients aged 15 years and above were included, whereas children below the age of 15 years were excluded. Taking into account WHO guidelines for resource-limited settings [9], sputum smear-negative TB patients were excluded as well.

2.3. Sample Size. Taking into consideration the 1.8% reporting national MDR prevalence among new TB cases and further assuming 15% losses that could arise from failed or contaminated cultures, a sample size of 344 would allow us to estimate the level of RMP resistance with a precision of 0.5% and 95% confidence interval.

2.4. Laboratory Methods. Sputum samples were stored in cetylpyridinium chloride (CPC) transport medium at ambient temperatures until the weekly collection to the Tropical Diseases Research Centre (TDRC). Samples were later transported to the reference laboratory in Lusaka and the Chests Diseases Laboratory (CDL) for culture on Löwenstein-Jensen (LJ) medium following decontamination using the Petroff method [10]. Cultures were incubated at 37°C and read weekly for growth for at least eight weeks. Successfully grown cultures were transported back to TDRC for storage and onward transportation of isolates to the Institute of Tropical Medicine (ITM, Antwerp, Belgium) for drug susceptibility testing (DST).

Isolates were identified as mycobacteria by smear microscopy and as *M. tuberculosis* by growth rate and temperature, colony morphology, and susceptibility to p-nitrobenzoic (PNB) acid [11]. Further identification was performed at TDRC using the Gen-Probe Accuprobe System for identification of *M. tuberculosis* complex (MTBC) and *M. avium-M. intracellular* complex (MAC) (Gen-Probe, San Diego, Calif.).

Drug susceptibility testing was performed using the proportion method on LJ medium against four first-line drugs, that is, INH (0.2 and 1.0 μg/ml), RMP (40 μg/ml), SM (4 μg/ml) and EMB (2 μg/ml), and two second-line drugs,that is, ofloxacin (OFL; 2 μg/ml) and kanamycin (KAN; 30 μg/ml) [12]. The latter were chosen to detect possible extensively drug-resistant (XDR) TB, and pre-existing resistance to these drugs in the general population. Due to practical reasons, DST for PZA was not done. Detected RMP and INH resistance was further confirmed using the GenotypeMDRTB/*plus*/(Hain LifeScience, Nehren, Germany) following the manufacturers's instructions.

2.5. Genotyping of Failed Cultures for Drug Susceptibility. In addition, we were able to test a randomly selected subset of heat-inactivated bacterial suspensions that failed on subculture and subsequent DST at ITM and were kept at −20°C for DNA fingerprinting purposes. We performed sequencing of the rpoB gene according to Rigouts et al. [13] on 41 isolates. Further sequencing of the katG gene, according to Telenti et al. [14], was performed on isolates that revealed rpoB mutations.

2.6. Data Collection Methods. The study was conducted under routine TB care. Pulmonary TB patients were registered onto the TB program following sputum smear-positive microscopy using Ziehl Neelsen staining results. In the laboratory, all smear-positive samples were preserved in 1% CPC and periodically transported to the central laboratory for further processing. At the end of each day, data for all smear-positive samples was abstracted from the TB clinic register, into a register provided specifically for the study. Data routinely collected at diagnosis by the TB focal person included sociodemographic (name, sex, age, residence) and clinical (case type and microscopy result) data. Other data included treatment regimen, follow-up microscopy results and treatment outcome at the end of treatment. The study registers were checked against the clinic TB registers at the end of the study period to complete any missing data and for verification.

2.7. Ethical Consideration. Before commencement of the study, approval for the study protocol was obtained from the Ethics Committee at TDRC.

Table 1: Phenotypic drug resistance patterns to first-line and second-line antituberculosis drugs in 193 *M. tuberculosis* isolates from treated and previously treated subjects.

Resistance pattern	New cases *n* (%)	Previously treated cases *n* (%)	Missing information *n* (%)	Total *n* (%)
Total	156 (80.8)	31 (16.1)	6 (3.1)	193 (100)
Pan-susceptible	144 (92.3)	26 (83.9)	6 (100)	176 (91.2)
Any resistance	12 (7.7)	5 (16.1)	0	17 (8.8)
INH	8 (5.1)	3 (9.7)	0	11 (5.7)
RMP	2 (1.3)	3 (9.7)	0	5 (2.6)
SM	3 (1.9)	3 (9.7)	0	6 (3.1)
EMB	0	1 (3.2)	0	1 (0.5)
Mono-resistance				
INH	7 (4.5)	1 (3.2)	0	8 (4.1)
RMP	2 (1.3)	1 (3.2)	0	3 (1.6)
SM	2 (1.3)	0	0	2 (1.0)
EMB	0	0	0	0
Polyresistance				
MDR (INH+RMP+SM+EMB)	0	1 (3.2)	0	1 (0.5)
Non-MDR				
INH+SM	1 (0.6)	1 (3.2)	0	2 (1.0)
RMP+SM	0	1 (3.2)	0	1 (0.5)
OFLO	0	0	0	0
KAN	0	0	0	0

INH: isoniazid; RMP: rifampicin; SM: streptomycin; EMB: ethambutol; Oflo: ofloxacin; Kan: kanamycin, MDR, multidrug resistance.

2.8. Statistical Methods. The data were double entered in Epi Info (Version 3.2.2, Centers for Disease Control and Prevention, Atlanta, GA, USA). All the electronic records were manually counterchecked against the source records for completeness and consistency. We performed data analysis using SAS (version 9.1.2, SAS Institute, Inc, Cary, NC, USA). The two-sided Pearson's asymptotic and exact chi square tests were appropriately used for comparisons to assess associations of sex, age, and treatment history using SAS 9.2 (SAS Institute Inc., Cary, NC, USA.) and StatXact 4.0.1 (Cytel Software Corp., Cambridge, MA, USA.). A *P*-value less than .05 was considered statistically significant.

3. Results

A total of 361 sputum smear-positive PTB subjects from the four selected diagnostic centres in Ndola from January to July 2006, were enrolled into the study. This represented 72% (361/499) of all the smear-positive PTB patients recorded in Ndola district during the same period. However, only 276 subjects yielded valid cultures and were identified as *M. tuberculosis* complex. Samples for the remaining 85 subjects, yielded either contaminated ($n = 10$) or negative ($n = 75$) cultures. A further 82 isolates had lost viability upon subculture of isolates for DST, and as a result, only 194 isolates were finally available for phenotypic DST. Additionally, we had to disqualify a result from the analysis because the patient was less than 15 years.

Of these 193 subjects, 132 were males and 61 were females, giving a male to female ratio of 2 : 1. The median age of these subjects was 31 (range: 15–79). Among these 193 subjects, 156 (80.8%) had never received TB treatment, while 31 (16.1%) were retreatment cases, and six (3.1%) had missing case type data. Comparison, for subjects recruited on the study, between the group for whom we obtained DST and those we could not obtain DST showed no statistical difference with regards to age ($P = .999$), sex ($P = .467$), and treatment history distribution ($P = .999$).

As shown in Table 1, overall DST patterns showed that of the 193 subjects investigated, 17 (8.8%) were resistant to at least one of the four first-line drugs tested, and only one MDR case was detected. Resistance to INH was observed in 11 (5.7%), SM resistance in six (3.1%), RMP resistance in five (2.6%), and only one (0.5%) case showed resistance to EMB. Further, overall monoresistance was observed as follows: against INH in eight (4.1%), against RMP in three (1.6%), and against SM in two (1.0%) subjects.

3.1. Drug Resistance among New Cases. Among the 156 new cases, any resistance was observed in isolates from 12 (7.7%) subjects, of which monoresistance to INH was detected in seven (4.5%) subjects and monoresistance to RMP was detected in two (1.3%) subjects. One patient exhibited non-MDR polyresistance to SM and INH. There was no resistance observed against the two second-line drugs tested, OFL and KAN.

3.2. Drug Resistance among Previously Treated Cases. Among the 31 retreatment cases studied, 28 were relapse cases, one

treatment failure and two defaulters. Of these, only five (16.1%) subjects showed any form of resistance to the four drugs, all being relapse cases. Mono-resistance was observed in one patient (3.3%) for INH and in another for RMP. Two subjects exhibited non-MDR polyresistance to SM and INH and to SM and RMP, while MDR was observed in another patient, who exhibited resistance to all four first-line drugs tested. Again, there was no resistance observed against OFL and KAN (Table 1).

3.3. Genotypic Drug Resistance. All isolates found to be RMP- and INH-resistant by phenotypic DST were confirmed to harbor mutations in the *rpo*B- and *kat*G genes, respectively. In addition, of the 41 cases that underwent sequencing of *rpo*B and *kat*G genes, two failed PCR or were too weak to yield valid sequencing results, whilst 39 yielded successful results. Of these 39 cases, 27 were new cases, 10 were previously treated cases and 2 had missing case-type data. Two (5.1%, 2/39) isolates were found RMP-resistant (1 Leu456 and 1 Glu438 mutation according to the *M. tuberculosis* nomenclature) of which one was found to be additionally INH resistant (Thr315 mutation). The former—likely RMP-monoresistant—subject was a 15-year-old new case, whereas the MDR subject was a 19-year-old relapse case.

4. Discussion

Our study revealed relatively low levels of any resistance to first-line drugs (8.8%) in Ndola, and for the first time systematically investigated and documented absence of resistance to second-line drugs (OFL and KAN). These levels of resistance to any of the first-line drugs are at the lower end of the spectrum when compared to the other 22 African countries reported in the WHO global project on anti-TB drug-resistance 2008 report, whose range is between 3.8%–39%. Further, compared to the only available countrywide drug-resistance data, for Zambia, the 2000 DR survey [4], MDR levels of 1.8% and 2.3% in new and previously treated cases, respectively, were reported, and we found MDR to be rare in this population. We cannot directly compare the results of the two; admittedly, the relatively low sample size of our study may have reduced the probability of picking up MDR cases and additionally, the two are different in coverage, one being nationwide and the other localized. But we cannot also exclude the fact that some of the isolates that did not grow in subcultures were MDR isolates, as it has been shown that some MDR isolates have reduced fitness [15]. However, among the 39 isolates that failed in phenotypic DST in our study, only one turned out to be MDRTB, suggesting that the proportion of MDR among the lost isolates was not significantly higher as compared to those with successful phenotypic DST (P-value = .241). It is possible that Ndola District, itself, may have low levels of resistance, being one of the earliest Districts to have implemented DOTS in Zambia. Through this long-standing experience, the Ndola District has benefited from the use of community volunteers and treatment supporters in their TB programme to assist in ensuring patients complete treatment, as evidenced by their relatively high treatment success rates of over 80% in 2008 (Provincial District Health Office). The WHO TB country profile data show treatment success rates for Zambia in 2006 to be 85% [16]. Data for Ndola District for that year were not available.

A successful national TB program will strive to avoid the emergence of drug resistance, particularly to the two most important anti-TB drugs, RMP and INH, to avoid development of MDR and eventually XDR *M. tuberculosis* strains. Unlike the earlier DR survey, which did not detect any RMP mono-resistance, this study, albeit with a relatively small sample size, detected RMP mono-resistance at 1.3% and 3.3% among new and previously-treated cases, respectively. Considering that over 96% of RMP-resistant cases can be detected by molecular tools [13], the rate might even be at 1.6% among new cases and drop to 2.4% among previously-treated cases if we include the subjects with only molecular analyses. Admittedly, we can not firmly conclude that this case was indeed RMP-mono-resistant as we did not test genes conferring resistance to SM and EMB, and as *kat*G mutations represent only between 50% and 95% of INH resistance [17]. Nevertheless, an unusually high rate of RMP mono-resistance of 8.9% was also observed in the prisons study mentioned earlier [5], and although this data was not confirmed by molecular techniques, these high levels of RMP-monoresistance might be attributed to high levels of HIV infection in prisons, reported in Zambia, at 26.7% [18]. These results may still imply an emerging undetected problem in the population or may indicate high transmission of RMP-mono-resistant strains among prisoners.

Scrutiny of the available drug-resistance data in the African region, suggests that RMP-mono-resistance continues to be low. Of the 22 African countries reported in the WHO global project on anti-TB drug-resistance, only nine reported RMP mono-resistance in their population. Our results for RMP mono-resistance in new cases fall within the range of those reported by the WHO report for the 22 African countries (range 0.8% – 1.8%), but appears to be well above the figures reported by the 4 countries that reported RMP mono-resistance in retreatment cases (range: 0.8% – 1.3%) [4]. Our results were also higher than those reported in another noncountrywide survey in Bujumbura, 0.6% and 1.4% RMP mono-resistance in new and previously-treated subjects, respectively, with a combined resistance at 0.7% [19]. Another noncountrywide survey in Benin showed 2.2% of combined RMP mono-resistance [20]. Low RMP mono-resistance (1.0%) in retreatment cases was also reported in the Western Area and Kanema districts of Sierra Leone [21].

Until recently, RMP mono-resistance was rarely encountered worldwide. Knowledge of the mechanisms by which this resistance is developed remains vague. Multiple risk factors associated with mono-resistance to RMP have been suggested, including irregular drug intake, inadequate treatment of prior TB episode, prior history of TB, and prior use of rifamycins and rifabutin in treatment of TB and other bacterial infections [22–24]. Further, some studies have suggested that this type of resistance is rarely a result of transmission [25–27], while others show resistance in new cases as in our study, suggesting possible primary acquisition

[19, 23]. Molecular typing results of this population are to be presented in another paper, but spoligotyping analysis did not indicate transmission of a single strain in samples exhibiting RMP mono-resistance. HIV disease has also been associated with the development of RMP mono-resistance due to malabsorption of anti-TB drugs [26, 28, 29]. Due to both nonavailability of routine VCT for TB patients at the time and the logistical limitations of the study, we were not able to determine the TB/HIV coinfection for the study population. Ndola's HIV prevalence in 2006 was estimated at 22.5% [30]. Additionally, DHMT data for Ndola district for 2008 cohort analysis indicate a 60.4% TB/HIV co-infection in smear-positive cases. RMP mono-resistance in immunocompromised populations may have detrimental consequences in terms of transmission and accumulation of polydrug resistance and MDR in a population [31].

On the other hand, INH mono-resistance seems to be more common globally, as was the case in our study. Our combined prevalence of 4.2% (4.5% and 3.3% in new and previously-treated cases) is within the range obtained in most of Africa. We observe a higher rate in new cases, contrary to what has been observed in most countries. Again, as mentioned earlier, HIV disease may play a role in this type of drug resistance [28].

Our study also detected two MDR cases of which one had full phenotypic DST results; this patient was resistant to all four first-line drugs. This case was a registered relapse case. However, after category II treatment, this case was declared cured for the second time, but died the following year. The cause of death could not be confirmed.

No resistance against OFL and KAN was observed in this population, even in the MDR patient. This may be indicative of low use/access to these drugs in Zambia. So far, resistance to second-line drugs has been reported to be low in most African countries [32] except in some provinces of South Africa [4]. However, we must be wary that very few countries in Africa have tested these drugs [32], and the non-availability of data does not necessarily mean absence of resistance, and as such, mechanisms to monitor this trend should be encouraged.

We acknowledge that the relatively high proportion of subjects for whom we could not obtain DST results due to negative culture or contamination could have introduced some bias. However, comparison of demographic characteristics of the two groups (those that had DST results and those without) did not show any statistical difference, and molecular analyses for part of the isolates with DST results showed only one additional MDR case and one mono-RMP case. Further, misclassification of patient history by clinic staff is possible, even though verification from subjects was obtained whenever possible. We were unable to obtain subjects' HIV test results for reasons mentioned earlier. Consequently, we are unable to link HIV status with drug resistance patterns observed in this population. Another limitation to our study is that due to logistical constraints smear-negative patients were excluded from the study. We acknowledge that in a high-HIV prevalence country like Zambia, smear-negative patients may contribute to the notifiable TB case load. However, there is no strong evidence to indicate that the proportion of cases that have DR varies substantially according to whether the TB case is smear-positive or smear-negative [9].

5. Conclusions and Recommendations

Although, the study may not be representative of the whole country, and the results are not necessarily comparable to previous data, our findings suggest that Ndola has maintained low levels of anti-TB drug resistance. These findings lend support to the notion that it is possible to keep TB drug resistance levels low even in resource constrained countries by implementing strategies that reduce treatment interruption. However, the appearance of mono-resistance to RMP, not previously reported in the general population, coupled with the sustained levels of INH resistance, may require further investigation.

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3.2 Diversity of Mycobacterium tuberculosis genotypes circulating in Ndola, Zambia

Preamble

The epidemiology of TB in Zambia still remains largely unknown, and consequently, epidemiological data cannot help focus TB control strategies. Efforts in TB control have relied on generic recommendations mainly derived from studies done in a low-incidence setting context. It is clear that there is a great deal of diversity within the TB epidemic world-wide and therefore for policies/strategies to be successful, they should be made in context. Evidently, the need to understand more fully disease dynamics in specific areas will help identify additional strategies that would improve TB control.

Efforts should be made to understand the fine detail of the TB epidemic in Zambia, a high-incidence setting. Although, molecular tools have been shown to provide new insights into the dynamics of the disease, it is also recognized that as a result of their inherent characteristics, molecular tools may be differentially applicable, not only to specific questions, but also to particular locale. For example, in high-incidence settings with numerous, often closely related *M. tuberculosis* strains, a molecular marker with a much higher discriminatory power, maybe more appropriate.

Author's contribution: CM was involved in the conceptualizing and organization of the study. She performed most of the spoligotyping, and the manual MIRU-VNTR typing. She took the lead in data interpretation of the result, subsequent data analysis and manuscript preparation.

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Diversity of *Mycobacterium tuberculosis* genotypes circulating in Ndola, Zambia

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Abstract

Background: Tuberculosis (TB) is one of the major public health problems in Zambia. However, information about lineages of *M. tuberculosis* complex (MTBC) isolates useful for epidemiology investigations is unknown. In this study, we investigated the diversity of MTBC isolates from Ndola, a typical Zambian urbanized city with a documented high HIV prevalence.

Methods: This was part of a prospective cohort study in subjects with sputum smear-positive pulmonary TB. Spoligotyping was used to genotype the MTBC isolates and establish the circulating lineages. The 15-locus Mycobacterial Interspersed Repetitive Units - Variable Number Tandem Repeats (MIRU-VNTR) typing was used to study recent transmission.

Results: A total of 98 different spoligotypes were identified among 273 MTBC isolates. The majority (64.8%) of the isolates belonged to 9 known families, while 96 (35.2%) of the isolates were orphans. While LAM (41.8%) was the largest spoligotype family observed, most of the isolates (87.7%) belonging to the SAF1 family, with a significant portion coming from the T (13.6%), and X (5.9%) families. A few isolates (3.6%) belonged to the CAS, EAI, H, S, X1-LAM9 or U families. MIRU-VNTR typing was highly discriminatory (h = 0.988) among the 156 isolates tested in our sample, and increased the discrimination among 82 SAF1 isolates from 6 to 46 distinct patterns. In addition, 3.2% (5/156) of cases with available MIRU-VNTR results harbored more than one MTBC strain.

Conclusions: Our findings show a limited diversity of MTBC in Ndola with a high clustering rate (37.7%), which indicates that recent transmission plays an appreciable role in the dynamics of TB disease in this setting. This conclusion emphasizes the importance of early diagnosis and timely treatment. The results also confirm that MIRU-VNTR typing is suitable for studying the molecular epidemiology of TB in Ndola.

Background

Zambia is ranked among the world's top 10 high TB incidence countries with an incidence rate of 280 smear-positive tuberculosis (TB) cases per 100,000 inhabitants [1]. The World Health Organization (WHO) estimates the prevalence of all forms of TB in Zambia at 707/100,000 [1]. According to the National Tuberculosis Leprosy Program (NTLP), in 2004, the Copperbelt Province was responsible for nearly a third (27.6%) of the nation's notified TB cases. It was also one of the provinces with the highest Human Immunodeficiency Virus (HIV) prevalence (17%) in Zambia [2]. While efforts have been made to identify drivers of the HIV pandemic in Zambia, similar efforts for the TB epidemic lag far behind. A number of surveillance activities, both biological and socio indicator - studies, which are useful in identifying the risk factors that play a role in driving the HIV epidemic in Zambia, have been implemented. However, efforts in TB have mainly relied on generic recommendations to prevent TB. The epidemiology of TB in Zambia still remains largely unknown, and consequently, epidemiological data cannot help focus TB control strategies. In high TB incidence settings, determination of distinct transmission patterns is often indefinable, but may be greatly enhanced by the use of both molecular and conventional epidemiological tools.

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Use of molecular markers for strain-specific differentiation of *Mycobacterium tuberculosis*-complex (MTBC) isolates in epidemiological studies became available in the last decades. Some of the more popular MTBC typing methods being used include IS*6110*-based restriction fragment length polymorphism (RFLP) [3] and PCR-based methods like spoligotyping [4], mycobacterial interspersed repetitive units - variable number of tandem repeats (MIRU-VNTR) [5-8], single-nucleotide polymorphisms [9,10] and large-sequence polymorphism analysis [11-14]. Furthermore, apart from differentiating MTBC strains, MIRU-VNTR typing can easily identify mixed infections in patient isolates. Even so, the choice of typing methods used in studies should be considered carefully to provide meaningful analysis because their ability to discriminate MTBC in different settings varies widely. In addition, knowledge on the (over-) representation of specific genotype families in a community can be important, especially if these families have been implicated in disease complications like drug resistance, severe disease, or increased transmissibility.

In this study, we set out to investigate the circulating MTBC isolates, and use spoligotyping and 15-locus MIRU-VNTR typing to distinguish MTBC isolates from Ndola, an urban city on the Copperbelt Province of Zambia.

Methods

Study setting and population

This was part of a prospective cohort study in subjects with sputum smear-positive pulmonary TB, conducted in the Ndola urban district on the Copperbelt Province of Zambia. The Ndola District Health Management Team (DHMT) is responsible for health care service delivery in the district and has a catchment population of about 374,750 persons [15]. The district has 21 health centres, 3 military clinics, and 2 tertiary care hospitals. Most of these health centres are able to deliver TB treatment and care and are referred to as treatment centres, but only 6 are able to provide laboratory services for smear microscopy and are referred to as diagnostic centres.

For this study, sputum samples were collected from all consecutive sputum smear-positive pulmonary TB subjects at 4 of the 6 existing TB diagnostic centres between January and July 2006 as per routine. Both previously-untreated (new) and previously-treated (retreatment) cases were enrolled. The two clinics not included in the study were left out mainly because of their comparatively low population catchment areas at the time and because of logistical problems. All TB patients were treated according to the national TB guidelines [16] in line with the WHO treatment guidelines [17]. Smear-negative subjects were excluded because of logistical limitations such as budgetary and manpower constraints.

Laboratory methods

After routine microscopy, sputum smear-positive samples were stored in cetylpyridinium chloride (CPC) transport medium and kept at ambient temperature until they were taken to the Tropical Diseases Research Centre (TDRC) on a weekly basis. The samples were later transported from TDRC to the Chest Diseases Laboratory (CDL), a reference laboratory in Lusaka, for culture on Löwenstein-Jensen (LJ) medium following decontamination using the Petroff method [18]. Culture tubes were incubated at 37°C and were read weekly for growth for at least eight weeks. Successfully grown cultures were transported back to TDRC for storage and onward transportation of isolates to the Institute of Tropical Medicine (ITM, Antwerp, Belgium) for further analysis.

Data collection methods

The clinics were provided with a register dedicated for the study to record study-subject information, which included socio-demographics (name, sex, age, residence) and clinical data (case type, smear-microscopy results for three time points during treatment treatment regimen followed, and treatment outcome). HIV data could not be collected at that time, because routine counseling and testing (CT) for TB patients had not yet been implemented by 2006, and it was not logistically possible to capture this data in the study. As quality control, the study register was checked against the clinic TB registers at the end of the collection period.

DNA extraction

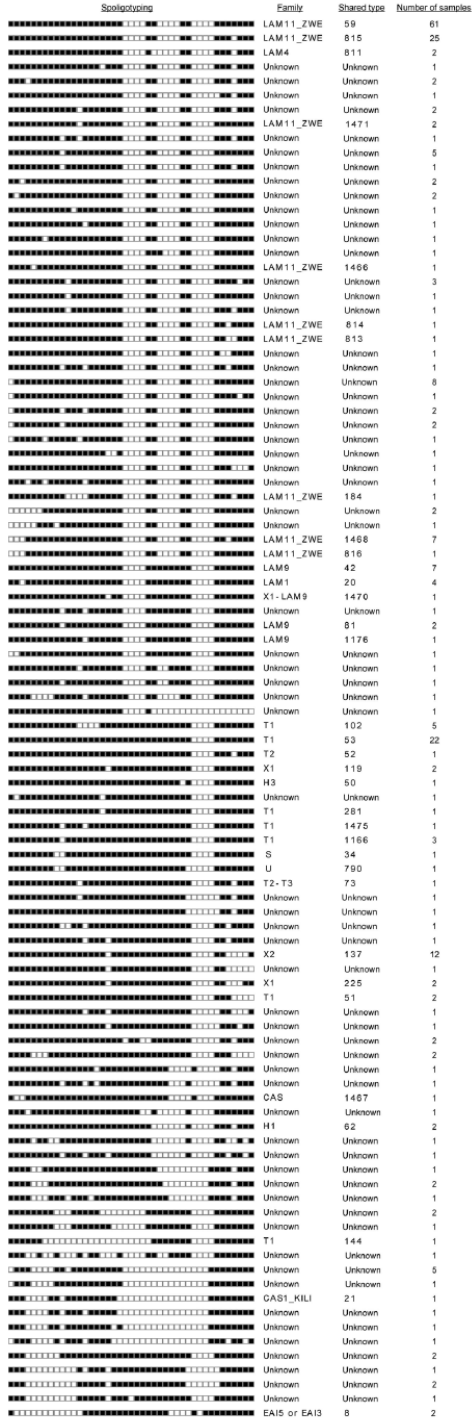
To obtain genomic DNA for spoligotyping and MIRU-VNTR typing, mycobacterial colonies grown on LJ medium were resuspended in 200 μl 1 × Tris EDTA buffer (10 Mm Tris-HCl, 1 Mm Ethylenediaminetetracetic acid disodium [pH8.0]) and then boiled for 10 min. The suspension was centrifuged at 15 000 g for 1 min to pellet cell debris. The supernatant containing DNA was harvested and used in PCR reactions.

DNA fingerprinting

Spoligotyping was performed using a commercial kit (Isogen Bioscience B.V., Maarssen, The Netherlands) according to Kamerbeek *et al.* [4]. Standardized MIRU-VNTR typing based on 15 loci was performed using the manual method [19] or by the automated method at Genoscreen in Lille, France.

Investigation of laboratory cross contamination

The investigation of possible laboratory cross contamination or error was performed by reviewing the DNA-fingerprint patterns of clustered isolates from samples that were processed on the same day from respective laboratories.



Spoligotyping	Family	Shared type	Number of samples
	LAM11_ZWE	59	61
	LAM11_ZWE	815	25
	LAM4	811	2
	Unknown	Unknown	1
	Unknown	Unknown	2
	Unknown	Unknown	1
	Unknown	Unknown	2
	LAM11_ZWE	1471	2
	Unknown	Unknown	1
	Unknown	Unknown	5
	Unknown	Unknown	1
	Unknown	Unknown	2
	Unknown	Unknown	2
	Unknown	Unknown	1
	Unknown	Unknown	1
	Unknown	Unknown	1
	Unknown	Unknown	1
	LAM11_ZWE	1466	1
	Unknown	Unknown	3
	Unknown	Unknown	1
	Unknown	Unknown	1
	LAM11_ZWE	814	1
	LAM11_ZWE	813	1
	Unknown	Unknown	1
	Unknown	Unknown	1
	Unknown	Unknown	8
	Unknown	Unknown	1
	Unknown	Unknown	2
	Unknown	Unknown	2
	Unknown	Unknown	1
	Unknown	Unknown	1
	Unknown	Unknown	1
	Unknown	Unknown	1
	LAM11_ZWE	184	1
	Unknown	Unknown	2
	Unknown	Unknown	1
	LAM11_ZWE	1468	7
	LAM11_ZWE	816	1
	LAM9	42	7
	LAM1	20	4
	X1 - LAM9	1470	1
	Unknown	Unknown	1
	LAM9	81	2
	LAM9	1176	1
	Unknown	Unknown	1
	Unknown	Unknown	1
	Unknown	Unknown	1
	Unknown	Unknown	1
	Unknown	Unknown	1
	T1	102	5
	T1	53	22
	T2	52	1
	X1	119	2
	H3	50	1
	Unknown	Unknown	1
	T1	281	1
	T1	1475	1
	T1	1166	3
	S	34	1
	U	790	1
	T2 - T3	73	1
	Unknown	Unknown	1
	Unknown	Unknown	1
	Unknown	Unknown	1
	Unknown	Unknown	1
	X2	137	12
	Unknown	Unknown	1
	X1	225	2
	T1	51	2
	Unknown	Unknown	1
	Unknown	Unknown	1
	Unknown	Unknown	2
	Unknown	Unknown	2
	Unknown	Unknown	1
	Unknown	Unknown	1
	CAS	1467	1
	Unknown	Unknown	1
	H1	62	2
	Unknown	Unknown	1
	Unknown	Unknown	1
	Unknown	Unknown	1
	Unknown	Unknown	2
	Unknown	Unknown	1
	Unknown	Unknown	2
	Unknown	Unknown	1
	T1	144	1
	Unknown	Unknown	1
	Unknown	Unknown	5
	Unknown	Unknown	1
	CAS1_KILI	21	1
	Unknown	Unknown	1
	Unknown	Unknown	1
	Unknown	Unknown	1
	Unknown	Unknown	2
	Unknown	Unknown	1
	Unknown	Unknown	2
	Unknown	Unknown	1
	EAI5 or EAI3	8	2

Figure 1 Distinct Spoligotypes of *M. tuberculosis* isolates from Ndola. The dendogram was generated using the dice coefficient and the unweighted pair group method with arithmetic averages (UPGMA) using the MIRU-VNTR*plus* program [20].

Fingerprint analysis

Spoligotyping and MIRU-VNTR patterns were compared to the international Spol DB4.0 database using MIRU-VNTR*plus*, a freely available web-based program [20]. This allowed assignment of shared international spoligotype numbers (ST) to known profiles. Spoligotypes that were not present in the Spol DB4.0 are referred to as 'orphan' types. MIRU-VNTR profiles with double alleles at a single locus were considered to be clonal variants of the same strain, whereas those with double alleles at 2 or more loci were considered to be mixed infections [21,22]. Identical spoligotypes and MIRU-VNTR patterns were considered to be in a cluster. Dendograms were generated using the dice coefficient and the unweighted pair group method with arithmetic averages (UPGMA). The clustering rate was defined as $(n_c - c)/n$, where n_c is the total number of clustered cases, c is the number of clusters, and n is the total number of cases in the sample. A cluster was defined as two or more patterns with identical DNA genotypes. The discriminatory power of DNA fingerprinting methods was calculated using the method described by Hunter and Gaston [23].

Ethical Consideration

Before beginning the study, approval for the study protocol was obtained from the Ethics Committee at TDRC. In addition, approval and support was also obtained from the Director of the Ndola DHMT. The protocol was implemented in such a way as to have minimum interference with routine work at the clinics. The study did not require any additional (invasive) sampling, data collected was anonymized, there was no direct contact with the patients, and the outcome of the research data would not have an influence on patient management. As a result, we did not ask informed consent from the subjects.

Statistical methods

Epidemiological and laboratory analysis data were double entered and descriptive analyses done in Epi Info™ (Version 3.2.2, Centers for Disease Control and Prevention, Atlanta, GA, USA). All the electronic records were manually counterchecked against the source records for completeness and consistency.

The two sided Pearson's asymptotic and exact chi square tests were appropriately used to assess associations of sex, age, geographic origin and drug-resistance profiles with spoligotyping families or MIRU-VNTR clusters using SAS® 9.2 (SAS Institute Inc., Cary, NC, USA.) and StatXact® 4.0.1 (Cytel Software Corp., Cambridge, MA, USA.). A P value less than 0.05 was considered statistically significant.

Results

Subjects and isolates

A total of 361 sputum smear-positive PTB subjects from the four selected diagnostic centres in Ndola were enrolled into the study from January to July 2006. Isolates were successfully obtained for 273 subjects, representing 54.7% (273/499) of all smear-positive PTB patients recorded in Ndola district during this period. Samples for the remaining 88 subjects included in the study, yielded either contaminated (n = 10) or negative (n = 78) cultures.

Of the cultures from 273 different subjects available for DNA fingerprinting, 85 (31.1%) were female and 188 (68.9%) were male with an age range between 14 and 79 years and a median age of 31 years. All the isolates were confirmed to be *M. tuberculosis* with an overall low drug resistance level (unpublished data).

Characterization of *M. tuberculosis* lineages

We used spoligotyping to determine lineages of circulating *M. tuberculosis* strains in Ndola. A total of 98 different spoligotypes were obtained among the 273 isolates analyzed. Patterns from 177 isolates belonged to nine families in the Spol DB4.0, whereas 96 (35.2%) isolates could not be matched to any lineage, and are thus referred to as 'orphan'.

The largest spoligotype family was the Latin American Mediterranean (LAM) that accounted for 41.8% (114 isolates) of the total isolates, most (100 isolates) of which belonged to the LAM11_ZWE sub-family designated Southern Africa Family 1 (SAF1) [24]. The next most common family was the T family with 37 isolates (13.6%), followed by the X family at 5.9% (16 isolates). A few isolates (3.6%) belonged to the CAS, EAI, H, S, X1-LAM9 or U families (Figure 1). Although our 'orphan' isolates were not described in the Spol DB4.0, six of them showed spoligotypes identical to previously reported orphan isolates in Zambia (n = 2), Zimbabwe (n = 2) and Cape Town (n = 1) [24]. There was a uniform distribution of spoligotypes from the various study centers (data not shown). Further, no significant statistical differences were observed in the distribution with regards to age (p = 0.5073), sex (p = 0.0896) and treatment history (p = 0.1824) between the group for whom we were able to perform spoligotyping and the group for which we could not.

Transmission analysis

To gain insight into the transmission rate of *M. tuberculosis* in Ndola, 156 (57.1%) out of the 273 samples with spoligotyping results were randomly selected and typed by 15-locus MIRU-VNTR.

MIRU-VNTR analysis revealed five isolates with clonal subpopulations, i.e. the presence of double alleles at a single locus suggestive of possible ongoing evolution within a strain, and five mixed infection cases (3.2%) i.e. isolates with double alleles at 2 to 5 MIRU-VNTR loci among the 156 isolates with MIRU-VNTR results. The mixed infection cases were removed from the analysis whereas the isolates with clonal subpopulations were included in the analysis with the double alleles at a single locus treated as missing data. Thus, further analysis was performed on the 151 isolates (Figure 2).

Not surprisingly, spoligotyping alone had the lowest ability to differentiate the isolates in our sample (clustering rate of 74.2%) and a discriminatory power of 0.840. MIRU-VNTR alone yielded a clustering rate of 39.1% and a discriminatory power of 0.988. The highest discrimination was achieved when spoligotyping and MIRU-VNTR were used together (h = 0.989; clustering rate of 37.7%), which was marginally better than that of MIRU-VNTR alone (Table 1 and Figure 2).

MIRU-VNTR typing of SAF1 isolates

We also assessed the genotypic similarity of isolates belonging to the major spoligotype family SAF1 among the above 151 isolates by 15-locus MIRU-VNTR. Of the 82 SAF1 isolates evaluated, MIRU-VNTR split the family into 46 different patterns i.e. 13 clusters comprising 49 isolates and 33 unique patterns (Figure 3). All isolates that were different by spoligotyping were also different by MIRU-VNTR. The differentiation of SAF1 isolates in clusters was mostly limited to one or two loci.

We did not observe any significant differences between the group of patients for whom we performed MIRU-VNTR and those we did not with regards to age (p = 0.8884), sex (p = 0.7350) and treatment history (p = 0.1536).

Discussion

This study reports the first utilization of spoligotyping and MIRU-VNTR typing to study the diversity of *M. tuberculosis* isolates in Ndola using a large number of samples. Ndola is an urbanized city with a high prevalence of HIV (17%) and is representative of many urban towns along the line of rail in Zambia. Our results demonstrate that the SAF1 family, and to a lesser extent the T family are the main circulating TB genotypes in Ndola, causing half (50.2%) of the TB cases in the city. The predominance of ST59 and ST53 of the SAF1 has been shown to be ubiquitous in the southern African region [24,25]. The small group of genotypes accounting for most TB cases in Ndola may imply their long-standing presence in the area. The clonal variations we saw for the SAF1 family appear to support this notion.

The 15-locus MIRU-VNTR in our sample performed very well. Although the comparison with IS*6110*-RFLP was not available, the high discriminatory value achieved by MIRU-VNTR in this study suggests that the technique

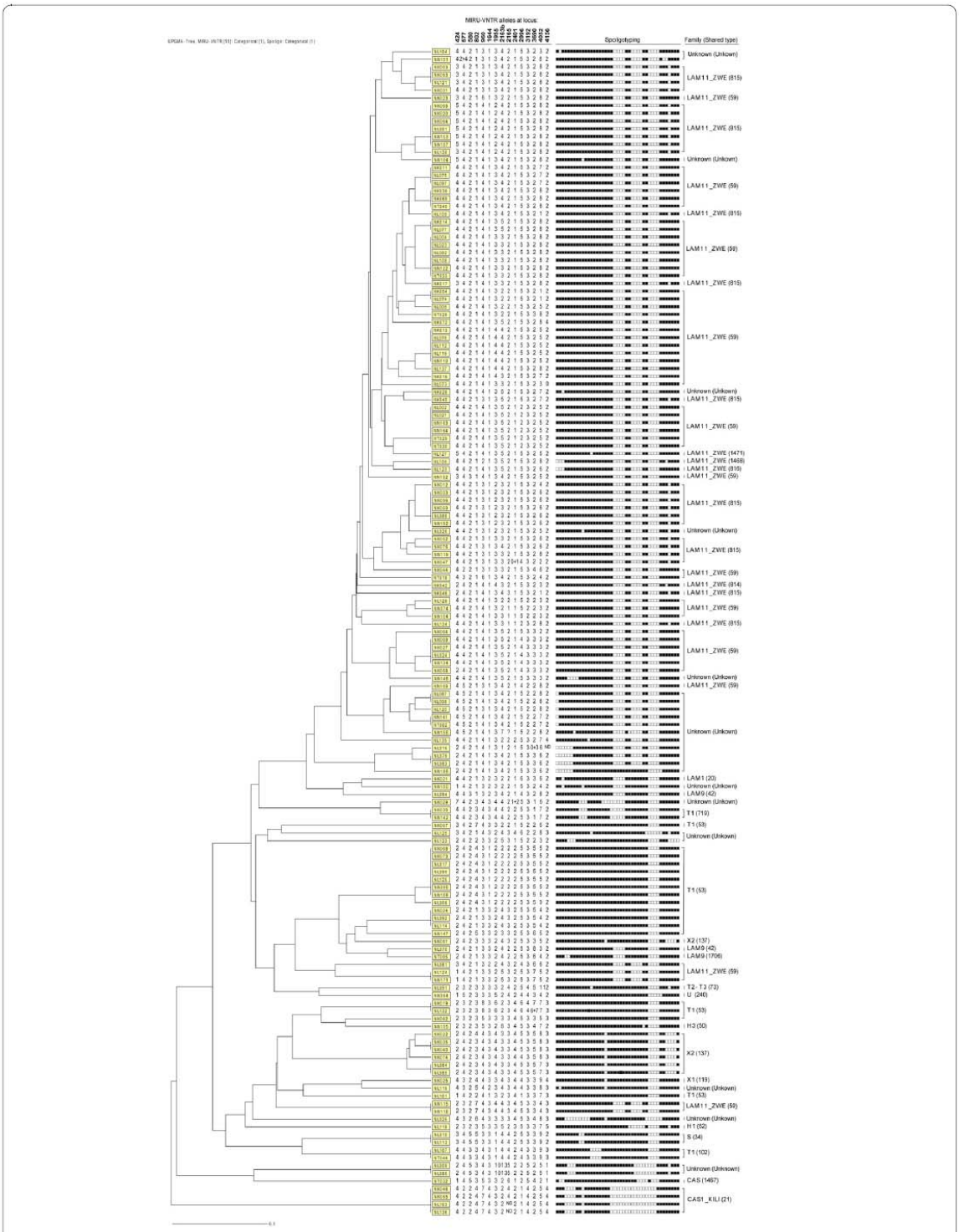


Figure 2 Spoligotyping and MIRU-VNTR clustering of representative *M. tuberculosis* isolates from Ndola. The dendogram generated using the dice coefficient and the unweighted pair group method with arithmetic averages (UPGMA) using the MIRU-VNTR*plus* program [20]. ND: Not determemined.

Table 1: Discriminatory power of spoligotyping and 15-locus MIRU-VNTR among 151 *M. tuberculosis* isolates from Ndola, Zambia

Genotyping method	Number of different profiles	Number of isolates with unique profile	Number of clusters	Number of isolates in clusters	Clustering rate (%)	h index
Spoligotyping	39	27	12	124	74.2	0.840
MIRU-VNTR	92	65	27	86	39.1	0.988
MIRU-VNTR + spoligotyping	94	68	26	83	37.7	0.989

is suitable for studying the molecular epidemiology of TB in Ndola. The high clustering rate (37.7%) exhibited in this study suggests likely high transmission in the community that may have occurred both before and during our study period since the study included both new and retreatment cases. We did not find any evidence of laboratory cross-contamination as a possible explanation for the high cluster rate. Also, we did not observe any significant differences between age, sex and treatment history among analyzed and non-analyzed cases by either of the typing techniques. The potential association of specific genotypes or clusters with the HIV status of the patients could not be investigated because HIV data was not routinely captured by the clinics format during the time of study. Conventional epidemiological investigations of contacts could not be performed due to budgetary constraints.

Our relatively short time window of sampling (7 months) is probably still acceptable to interpret the observed clustering as resulting from 'recent transmission' [26] and might not be surprising in a high TB and HIV incidence setting. Studies on clustering rates for other African countries are rare and diverse both in methodology and results; only a few of them have used highly discriminatory typing techniques. In Botswana, 25% of investigated isolates from four communities (18 months sampling) clustered by IS*6110*-RFLP [27] whereas a population-based nationwide study with a 21 months sampling period showed a 38% cluster rate [28]. Clustering was not associated with HIV or demographic characteristics in both studies except for prior imprisonment in the first study. In Ethiopia, 41.2% of 121 isolates (12 months sampling) clustered by IS*6110* and a clear association with HIV infection and female sex was observed [29]. In Benin, 34% of isolates (15 months sampling) clustered by 12-loci MIRU-VNTR and spoligotyping, with no parameters linked to clustering [30]. These and our cluster rates are higher than the estimated 9-13% of TB cases due to recent transmission in Malawi, where nearly half of the cases acquired TB from an HIV-positive subject [31].

Given the relatively short time window of our study compared to other African studies, our clustering rate should be considered high, and probably reflects a high recent transmission rate emphasizing the importance of early diagnosis and timely treatment. Further investigation on the link with HIV infection is required. We acknowledge that the interpretation of transmission dynamics data in this study may be limited because we did not include smear negative subjects, who are known to contribute to TB transmission [32-34].

On the one hand, these findings lend support to the premise that *M. tuberculosis* in endemic areas with predominant family strains can still possess sufficient genetic diversity when the appropriate molecular method is applied, enabling more detailed epidemiologic investigations. On the other hand, since differentiation of SAF1 isolates by 15-locus MIRU-VNTR was mostly limited to one or two loci, application of the standardized 24-locus MIRU-VNTR [35] - not used in this study due to financial constraints - may increase the ability to discriminate more MTBC among the clustered SAF1 strains.

This study also detected mixed infections in five subjects. Except for 1 of these 5 subjects, who exhibited INH resistance, isolates from the other 4 subjects, were pan-susceptible to the anti-TB drugs tested. The rate of mixed infections detected in this study (at least 3.1% observed among 156 isolates with MIRU-VNTR results) is comparable to previous reports from high-incidence populations [21,22,36-38]. Although this observation does not necessarily pose a serious threat for patient management owing to the relatively low level of drug resistance in this setting, it is potentially an important factor to consider particularly for treatment of compliant subjects with unexplained changes in drug resistance patterns during the course of chemotherapy.

Conclusion

This study has shown that the majority of MTBC isolates in Ndola belongs to the SAF1 family with a high clustering rate and that the 15-locus MIRU-VNTR typing is suitable for studying the molecular epidemiology of TB in

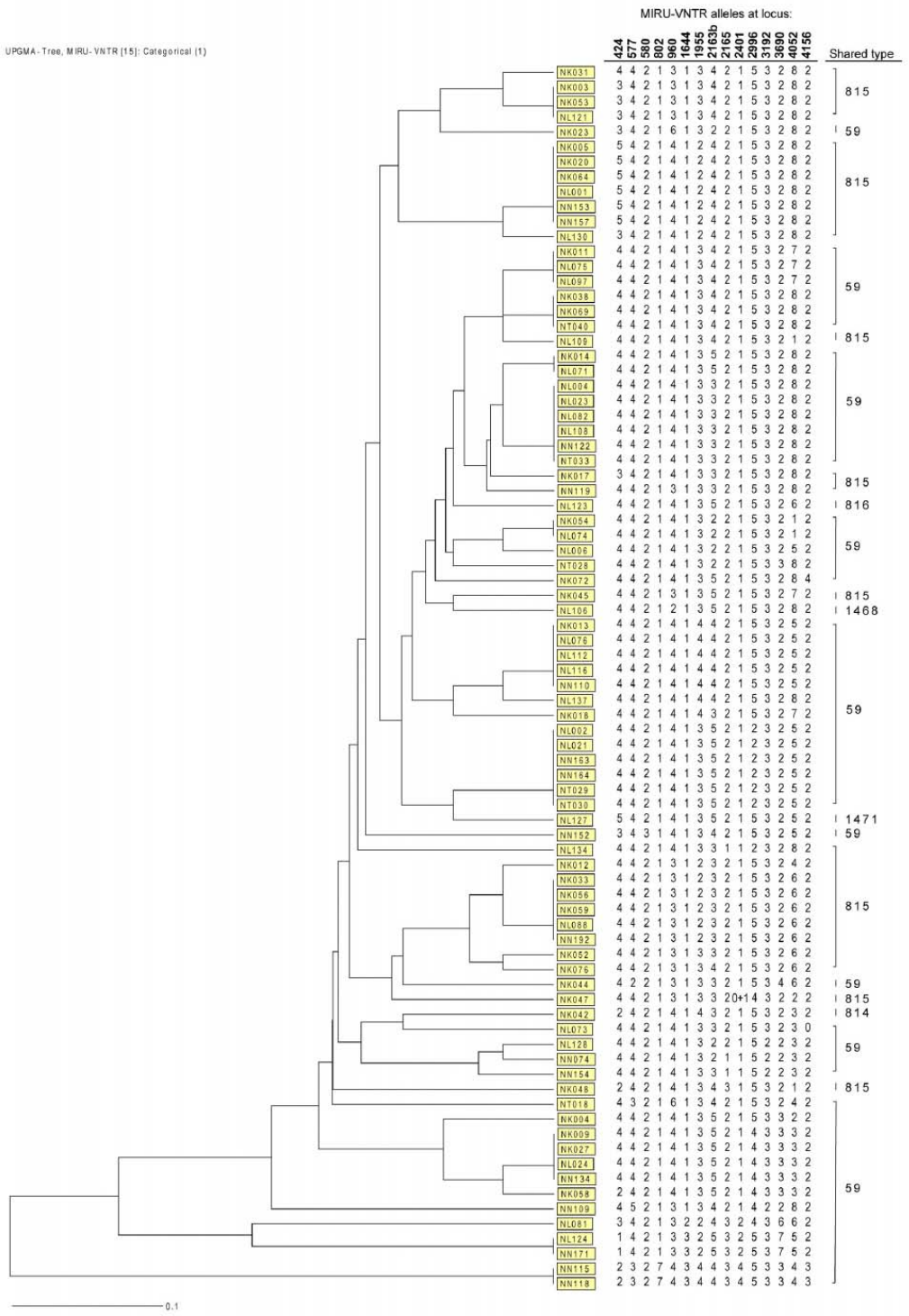


Figure 3 MIRU-VNTR clustering of *M. tuberculosis* isolates belonging to the SAF1 family. The dendogram was generated using the dice coefficient and the unweighted pair group method with arithmetic averages (UPGMA) using the MIRU-VNTR*plus* program [20].

Ndola. Finally, the probable high recent transmission rate underlines the importance of early diagnosis and timely treatment.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
CM was involved in the design, implementation of the study, and drafted the manuscript. ICS conceived and designed the study and critically revised the manuscript. FP critically revised the manuscript and LR was involved in the implementation and critically revised the manuscript. DK performed statistical analysis and critically revised the manuscript. NK critically revised original study design and the manuscript. All the authors read and approved the final manuscript.

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3.3. Management of pulmonary tuberculosis patients in an urban setting in Zambia: a patient's perspective

Preamble

The continued high incidence and prevalence of TB in Africa despite a reported DOTS coverage of over 90% (WHO African region annual report), is a cause for concern. Current global TB control strategies focus on case detection and treatment with a view to interrupt transmission. However, even though these control strategies have been effective in curing patients and saving lives, the epidemiological impact has so far been less than desired.

The success of a national TB program is multi-faceted and complex supported by findings that have shown TB trends are strongly associated with biological, social and economic factors (150-151). As alluded to earlier, a combination of conventional and molecular tools will provide a wholesome picture. Understanding the factors that affect or influence care actions in different settings, are an important component in TB control.

Author's contribution: CM was involved in the conceptualizing and organization of the study. She developed the questionnaire and was involved in the piloting of the questionnaire. The data analysis was performed by a biostatistician, but CM took the lead in the data interpretation of the analysis results and prepared the manuscript.

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RESEARCH ARTICLE Open Access

Management of pulmonary tuberculosis patients in an urban setting in Zambia: a patient's perspective

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Abstract

Background: Zambia continues to grapple with a high tuberculosis (TB) burden despite a long running Directly Observed Treatment Short course programme. Understanding issues that affect patient adherence to treatment programme is an important component in implementation of a successful TB control programme. We set out to investigate pulmonary TB patient's attitudes to seek health care, assess the care received from government health care centres based on TB patients' reports, and to seek associations with patient adherence to TB treatment programme.

Methods: This was a cross-sectional study of 105 respondents who had been registered as pulmonary TB patients (new and retreatment cases) in Ndola District between January 2006 and July 2007. We administered a structured questionnaire, bearing questions to obtain individual data on socio-demographics, health seeking behaviour, knowledge on TB, reported adherence to TB treatment, and health centre care received during treatment to consenting respondents.

Results: We identified that respondents delayed to seek treatment (68%) even when knowledge of TB symptoms was high (78%) or when they suspected that they had TB (73%). Respondent adherence to taking medication was high (77%) but low adherence to submitting follow-up sputum (47%) was observed in this group. Similarly, caregivers educate their patients more often on the treatment of the disease (98%) and drug taking (100%), than on submitting sputum during treatment (53%) and its importance (54%). Respondent adherence to treatment was significantly associated with respondent's knowledge about the disease and its treatment ($p < 0.0001$), and with caregiver's adherence to treatment guidelines ($p = 0.0027$).

Conclusions: There is a need to emphasise the importance of submitting follow-up sputum during patient education and counselling in order to enhance patient adherence and ultimately treatment outcome.

Background

Tuberculosis (TB) continues to be a major health problem in Zambia, despite a long running National Tuberculosis and Leprosy Programme (NTLP). In 2007, the World Health Organization (WHO) estimated the TB burden in Zambia to be at 60,337 cases (all forms of TB) [1]. The TB control efforts have been hampered by the high level of human immunodeficiency virus (HIV) infection, especially in urban settings where prevalence is estimated to be 19.7% [2]. As a result the number of TB and HIV cases threatens to overwhelm the capacity of the general health systems. HIV-TB co-infection rates in Zambia have been estimated at 70% [1].

Zambia adopted the WHO recommended Directly Observed Treatment Short course (DOTS) strategy as its primary approach in TB control in 1993 and has officially reported 100% DOTS coverage in all nine provinces since 2003 [3]. A good functioning primary health care system is crucial in the implementation of DOTS. In Zambia, the NTLP activities have been

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integrated into the primary health care services. The decentralisation of TB treatment services has provided for more responsibility at the lower levels of the health care system and in the face of an overwhelming TB case-load, this move has proved to be beneficial to the practical implementation of the programme. Despite the human resource challenges, the use of treatment supporters and community volunteers in the implementation of DOTS has contributed to the improvement in cure rates over the past decade from 67% in 2000 to the global target of 85% by 2006 [3]. The goal of the Zambian NTLP is to prevent and control TB through the provision of quality diagnostic and treatment services for TB and TB/HIV- infected individuals at all levels of the health care delivery system [4].

Assessing access to quality of healthcare service delivery is complex and multidimensional and will depend on several aspects that are both patient/community-related and/or health systems/service related. Several questions could be considered in this vein, for example, are patients seeking help when they are sick, and when they do seek healthcare, are they getting the appropriate care they require when they need it and ultimately, is this care effective when they get it? Understanding the factors that affect or influence care actions in different settings, will ultimately result in an improvement in healthcare delivery.

Although there are several reports about health seeking behaviour of TB patients and factors related to their delay in seeking health care, compliance to treatment and the role of these factors in treatment outcome, only a few studies describe patient experience in accessing TB care throughout treatment. This study describes and assesses the care received by pulmonary TB patients from government health care centres, and the association with patient adherence to TB treatment based on previous TB patients' reports. The study also alludes to patient's attitude to seek health care for TB.

Methods

Study design and population

This was a cross-sectional study of subjects who had been treated for pulmonary TB through the NTLP at government health centres in Ndola, an urbanized city on the Copperbelt Province of Zambia with an estimated population of 374,757 persons [5], representative of many urban towns along the line of rail in Zambia. At the time of the study, the Ndola District Health Management Team (NDHMT) provided health care services through 26 health centres. All the health centres provided TB treatment and care (treatment centres), but only six were able to perform Acid Fast Bacilli (AFB) smear microscopy (diagnostic centres).

Sampling and sample size

The sampling frame comprised the names of all the smear-positive TB patients, new and retreatment cases, registered in the TB microscopy laboratory registers at the six diagnostic centres between January 2006 and July 2007, as a record of all smear-positive patients undergoing treatment in the 26 treatment centres in that period. Those that had received treatment from private clinics or hospitals and children less than 18 years of age were not included. A sample of 105 respondents was randomly selected from the sampling frame. The sample size was calculated using Epi Info 3.5.1 (Centers for Disease Control and Prevention, Atlanta, GA, USA). Based on pre-test results, we expected a frequency of patient compliance and adherence to treatment of 50% ±10%, at a confidence interval of 95%, and non-response level of 10%, and therefore estimated a sample size of 105 as sufficient.

Data collection, management and analysis

Initial contact with the selected respondents was made through the TB focal persons at the health centres. Trained research assistants from the Tropical Diseases Research Centre (TDRC), interviewed consenting participants using a structured questionnaire at their homes. The questionnaire, bore questions to capture individual data on socio-demographics, knowledge on TB, health seeking behaviour, adherence to TB treatment, and reported health centre care during treatment. Most of the questions were closed ended. The questionnaire was pre-tested before use and modifications incorporated in the final version.

The collected data was entered in an MS Access database using Epi Info™3.5.1 (Centers for Disease Control and Prevention, Atlanta, GA, USA), with in-built consistency and range checks. The database was converted to SAS® 9.2 (SAS Institute Inc., Cary, NC, USA) for recoding where necessary and final analyses. Fisher's exact Chi-squared test was used to examine associations of factors. A $p \leq 0.05$ was considered significant.

National Guidelines for management of TB

The management of TB patients in Zambia has been standardised under guidelines provided by the NTLP [6]. Except for the seriously ill and identified multidrug resistant (MDR) cases, TB patients are treated on an ambulatory basis. Patients are instructed to pick up medication at TB treatment centres once or twice a week during the intensive phase and once monthly during the continuation phase. The national guidelines stipulate that treatment during the intensive phase should be under direct observation by a trained treatment supporter - usually a relative, while the continuation phase can be self-administered but with monthly supervision

from the health centre. Patient education is an important aspect of TB treatment management and is also included in the guidelines to improve cure rates and compliance. Further, as part of patient monitoring and follow up, microscopy is to be repeated at 2, 5 and 8 months. To ensure and improve compliance to sputum follow-up, it is the duty of treatment centres to (1) ensure patients make follow-up visits and submit sputum specimens as required (2) deliver sputum specimens to the nearest diagnostic centre for microscopy and (3) collect microscopy results from diagnostic centres and make available to patients for appropriate care. Patients do not visit diagnostic centres themselves.

Conceptual framework

The following concepts were used to make analysis.

Respondent treatment adherence

Respondents that reported to have completed eight months of taking medication without interruption, and submitted sputum at least twice post diagnosis - one time point being at eight months - were considered to have adhered to the treatment programme.

Care giver treatment guidelines adherence

Caregivers that were reported by the respondents to have enquired about patient's TB history, provided patient information (on TB disease and its treatment, how to take medication, the requirement to submit follow-up sputum during treatment and the importance of submitting follow-up sputum), and gave the patient an opportunity to ask questions, were considered to have adhered to the TB treatment guidelines.

Respondent knowledge

Respondents that were able to name the correct mode of TB transmission, at least two correct symptoms of TB and knew the importance of treatment completion and sputum submission were considered to be knowledgeable about the disease and its treatment.

Health centre systems access

Health centre delivery systems were considered to be adequate if respondents reported that: the distance to the health centre was less than 30 minutes walk from their home, he/she was commenced on TB treatment not more than 5 days post laboratory diagnosis, and he/she used the same clinic for follow-up treatment and follow-up sputum submission.

Ethical consideration

Approval for the study protocol was obtained from the Ethics Committee at TDRC. Approval and support were also obtained from the Director of the NDHMT. Consenting respondents were asked to sign an informed consent following an explanation of the study. Interviewers were not part of the health care system. Respondents were assured of anonymity and confidentiality.

Table 1 Socio-demographic characteristics of the respondents (N = 105)

	n	%
Sex		
Female	50	48
Male	55	52
Age (years)		
15 - 24	13	12
25 - 34	33	31
35 - 44	29	28
45 - 54	16	15
55 - 64	7	7
> 65	7	7
Marital Status		
Married/Cohabiting	58	55
Single	23	22
Divorced/Separated	11	11
Widowed	13	12
Education		
None	8	8
Primary	44	42
Secondary	50	48
Tertiary	3	3
Employment		
Formal	18	17
Informal	44	42
Housewife	13	12
Dependent	15	14
Unemployed	15	14
Distance to clinic		
5-10 minutes	41	39
20-30 minutes	43	41
45 minutes	9	9
1 hour	10	10
Too far to walk, need to get bus	2	2
Previous episode of TB		
Yes	23	22
No	82	78

Results

Respondent characteristics and health seeking attitudes

Basic Respondent socio-demographic characteristics are shown in Table 1. Other results showed that 68% of respondents waited for one month or more since the onset of symptoms before going to the health centre. When asked why they waited that long, most of the respondents (76%) thought the symptoms will go

away. The most common response for how they coped with symptoms prior to visiting the health centre was self-treatment (64%). Most of the respondents (98%) only presented at the health centre when they were feeling very sick. When asked if they suspected that they had TB, 30 respondents (29%) responded in the affirmative. However, 73% of these respondents still waited for at least one month before going to the health centre.

Respondent treatment adherence

When respondents were asked if they had stopped taking their medication at some point during treatment, 22% said yes, and the most common reason for stopping was that the respondent felt better (55%). Among respondents that were asked the number of times they submitted sputum after initiation of treatment, 32% reported submitting sputum at three time points, 25% at two time points, whilst 43% submitted sputum only once post treatment initiation. Two thirds (67%) of the respondents reported submitting sputum at the end of treatment, (eight months). Adherence to treatment of respondents is shown in Table 2 (A).

Care giver treatment guidelines adherence

The majority of respondents (84%) confirmed that they were asked if they had suffered from TB previously before commencement of TB treatment. To the questions enquiring whether the health-worker explained how to take the medication and whether the instructions were clear, nearly all responded favourably. When asked if the health worker informed them at the initiation of treatment that they would have to submit more sputum samples during treatment, 53% said yes; all of whom reported that the health centre staff explained to them the importance of submitting follow-up sputum specimens. Forty-nine (47%) respondents reported that they were given an opportunity to ask questions for clarifications. Table 2 (B) shows performance of caregivers' adherence to treatment guidelines.

Respondent knowledge and awareness of TB

When asked to name some symptoms of TB, a significant proportion of the respondents (78%) was able to mention at least two symptoms, with cough being the most identified symptom (89%). A considerable number (69%) of the respondents correctly knew the mode of

Table 2 Distribution of respondent and caregiver adherence and health systems access in Ndola, Zambia (N = 105)

	n	%
A. Respondent adherence to treatment programme		
Respondents that complied and adhered to treatment programme	45	43
1. Respondents that completed medication without stopping at any point	81	77
2. Respondents that submitted sputum as required	50	48
B. Caregiver adherence to treatment guideline		
Respondents whose caregivers adhered to treatment guidelines	26	25
1. Respondents whose caregivers enquired about their TB history	88	84
2. Respondents whose caregivers educated them on:		
the disease and its treatment	103	98
how to take medication	105	100
requirement of submitting follow-up sputum	56	53
the importance of follow-up sputum submission	57	54
3. Respondents who were given an opportunity to ask questions	49	47
C. Respondents' knowledge on the disease		
Respondents that demonstrated knowledge on the disease and its treatment	30	29
1. Respondents that gave the correct mode of TB transmission	73	70
2. Respondents that gave at least two correct symptoms of TB	82	78
3. Respondents that knew the importance of treatment completion	94	90
4. Respondents that knew the importance of follow-up sputum submission	57	54
D. Health centre systems access		
Respondents that reported adequate healthcare systems access	84	80
1. Respondents who reported the distance to the health centre as being too far	84	80
2. Respondents who reported commencing treatment within a week of diagnosis	105	100
3. Respondents who reported using the same clinic for treatment and sputum submission	77	73

transmission of TB, however, 13% incorrectly cited using the same utensils. Knowledge of the importance of completing medication for eight months was high (89%) but knowledge for the importance of submitting follow-up sputum was lower (55%). Ninety-one percent of the respondents reported that they knew they were cured of their last TB episode, but when asked how they knew they were cured, reasons ranged from feeling better (80%), the fact that they took medication for eight months (15%), to that laboratory results were negative (4%). Table 2 (C) shows performance of respondents with regards to knowledge and awareness of TB. Most respondents (71%) did not suspect that they had TB despite the large number (85%) naming cough as one of the symptoms they experienced.

Healthcare systems access

TB treatment centres appeared relatively close to the respondents' homes: 80% lived within 30 minutes walk, 18% lived within an hour's walk and 2% said it was too far to walk and needed to take a bus. Respondents were also asked how long after being diagnosed with TB it took before starting medication; all the respondents reported that they were started on treatment within one week of diagnosis, with 86% starting within two days.

When respondents were asked if they had used the same clinic for their follow-up visits and drug collection throughout treatment, affirmative responses were 87%. Respondents were further asked if they had submitted their follow-up sputum samples to the same clinic they went for reviews and collected drugs from, and 73% said yes. Table 2 (D) shows performance of health centres with regards to access as reported by the respondents.

Factors significantly associated with respondent adherence

The results showed that, using our conceptual framework, respondents' adherence to treatment was not only significantly associated with respondent's knowledge about the disease and its treatment ($p < 0.0001$), but also with reported caregivers' adherence to treatment guidelines ($p = 0.0027$) and reported adequate healthcare systems access ($p < 0.0001$) (Table 3).

Further analyses showed that caregivers explaining the importance and schedule of follow-up sputum submission was significantly associated with respondents' adherence to sputum submission as required ($p < 0.0001$), but not with respondents' completing medication for eight months ($p = 0.0562$).

Discussion

The success of a national TB program is multi-faceted and complex. Community awareness; patients' adherence to treatment; patient access to quality of care through competent healthcare staff who are able to provide quality of care through prompt diagnosis and referral, prescription of correct treatment regimens and treatment follow-up; and accessible TB services, are important components of a successful TB program.

Consequently, it is important that people in communities are aware and able to suspect TB in persons who show signs and symptoms suggestive of the disease, such as prolonged cough, persistent fevers, and weight loss. Maybe not surprising, as previous TB patients our respondents showed a good level of knowledge on the symptoms and modes of transmission of TB, attributable to caregiver education during treatment. However, our study revealed vast differences in knowledge regarding the importance of treatment completion compared to knowledge of the importance of follow-up sputum submission; whereas, nearly 90% knew the importance of treatment completion, only 57% knew the importance of the latter, reflective of the low importance given to the relevance of education on this issue. Similarly, other studies have shown that most TB patients know the importance of treatment completion [7-9]. According to our conceptual framework, overall knowledge of the disease was low, mainly due to the low knowledge gap in the role of sputum microscopy in TB treatment by the respondents.

Despite the high knowledge levels of TB symptoms shown in our study, most respondents not only, reported not to have suspected they had TB, but also reported that they delayed seeking care (even when they suspected they had TB). Whereas it is possible that respondents were truly unaware of TB symptoms prior to TB treatment, several other studies have shown that there are various reasons why patients delay seeking care at a health centres. Loss of income, health centre systems or staff attitudes, stigma of the HIV association, severity of disease, lifestyle, for example, alcohol abuse, are among the many explanations [9-13]. The most common reasons in our study, 'I was thinking the symptoms will go away' or 'I did not think it was serious' also appear to be common in different settings [8,12]. This may be reflective of the commonly practiced self-treatment, which may ameliorate initial symptoms thus temporarily masking the severity of disease and consequently 'buy them time' to continue with their daily income generating endeavours. Only 17% of our study population were in formal employment suggesting that for most respondents an income was dependent on their daily efforts and therefore may not afford the time at the health centre. Further, the period of the study, were the early days of scaling up of free antiretroviral therapy in Zambia and so people may still have been feeling helpless against HIV infection.

Our results showed that only 47% of respondents reported to have submitted follow-up sputum at least twice post diagnosis and that 67% reported submitting follow-up sputum at the end of treatment. These results

Table 3 Respondent adherence associations to Caregiver adherence, Respondent knowledge and Health system accessibility (N = 105)

	Respondent adherence to treatment programme		
Characteristics	Adhered	Did not adhere	*P value
A. Caregiver adherence to treatment guidelines			
Did not adhere to guidelines	27	52	
Adhered to guidelines	18	8	0.0027
B. Respondents' knowledge on TB			
Not knowledgeable	20	55	
Knowledgeable	25	5	< 0.0001
C. Health centre systems access			
Not good/not efficient	0	21	
Good/efficient	45	39	< 0.0001

*P values are based on Fisher's exact chi square test.

may be cause for concern because sputum re-examination at the end of the patient's treatment is a much stronger indicator of treatment success than 'treatment completion. Further, data in one of our studies in this population, has shown that among subjects who experienced another episode of TB within one year of completing treatment, there were more who harboured the same *M. tuberculosis* strain as that of the previous episode (relapses/treatment failures) than those that had a different strain (re-infection) (unpublished data). Furthermore, our study showed a high proportion of respondents taking of drugs for the complete period of treatment (89%) with a notable proportion (22%) reporting stopping medication at some point during treatment. Over half (55%) cited that they stopped because they were feeling better, similar to many other studies [14,8,15].

The role of the health worker on patient compliance has been described many times [16-18]. Patient counselling and good communication [19,20] can improve patient compliance. Our study showed high levels of patient satisfaction when it came to health provider explanation regarding medication. However, we did not see the same positive response with regards to health provider explanation on the role of follow-up sputum submission. Only about half of the respondents reported that they were informed about the requirement (53%) and importance (54%) of submitting follow-up sputum. In fact, these two parameters were shown to be significantly associated with respondent adherence ($p < 0.0001$ for both). A study in Egypt demonstrated that adherence to recommended sputum smear microscopy schedule was significantly associated with treatment success [21]. Our study also showed that respondent adherence to treatment was significantly associated with respondent's knowledge about the disease and its treatment ($p < 0.0001$) in contrast to other studies [22,8].

Moreover, caregivers' communication skills fell short on account of dialogue, giving the patient a chance to ask questions, an important aspect in patient management that ensures patient understanding of disease and treatment. The effects of non-dialogue counselling were demonstrated in a study in Madagascar where reported lack of opportunity to ask questions by patient was significantly associated with non-adherence [16].

Other features of the health system, like distance, convenience of TB services (microscopy, antiretroviral treatment services), how long it takes to see the clinician, prompt diagnosis and referral of TB patients presenting with TB-related symptoms at primary health care facilities, may have an effect on patient access to healthcare. Distance to the health centre for this population was not an issue. Delays in the commencement of treatment have been documented in some settings [23], our study, however, showed that all the respondents were given medication within one week of diagnosis, with 84% commencing treatment within two days post laboratory diagnosis. The NTLP in Zambia has given full responsibility of sputum transportation plus obtaining and communicating results for each patient, to the treatment centres. This not only reduces on the number of patients, who remain undiagnosed following initial health centre visit, but also removes the inconvenience and added travel costs from the patients. The majority of our respondents reported that they used the same treatment centre for sputum submission. Our results indicate that facility-service related factors may not be the main issue in patients' access to TB care in Ndola, unlike the study from KwaZulu-Natal where systems failure was reported as contributing to the ineffectiveness of the National Tuberculosis Program [24].

Admittedly, because this study asked questions about past events, participants' recall may have biased our

results. In addition, since the interview was anonymous to ensure complete confidentiality, we were not able to go back to the patient's data files to verify the self-reported data. Nevertheless, the implied cure rate for this sample population is comparable to the average cure rate data for the same period from Ndola. Another limitation for this study is that we did not establish from the respondents how long it took for laboratory results to be available for diagnosis, a factor that could well contribute to delay in TB patient care. However, enquiries from TB focal persons indicated a turnaround time for lab results ranging from the same day to a week. Further, our study did not include all components of TB treatment and care in the National Guidelines and consequently, other components that contribute to this package have not been discussed. Lastly, it is well known that respondents usually consider the interviewer to represent authority or the healthcare system and therefore tend to bias their answers in the way they expect they should to please the interviewer. Consequently, although the study made efforts to use researchers from outside the respondents' healthcare system, it is difficult to completely remove this perception in communities.

Conclusions

In conclusion, TB treatment systems appear to be well in place in NDHMT. However, taken together, these results suggest that closer monitoring systems on guidelines adherence at health centres may need strengthening and more patient counselling on treatment of disease and importance of sputum submission may improve cure rates.

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Authors' contributions

CM was involved in the design and implementation of the study, and drafted the manuscript.
ICS conceived and designed the study and critically revised the manuscript. HM, DK and KV performed statistical analysis and critically revised the manuscript. SK and NK critically revised original study design and the manuscript. LR supervised the implementation and critically revised the manuscript. All the authors have read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Chapter 4: Discussions and Perspectives

The first priority for national TB programs (NTPs) is to cure patients with active disease so as to reduce suffering, avert mortality, cut transmission, and avoid development of drug resistance. This thesis attempted to investigate the management of TB in Ndola Urban District on the Copperbelt Province of Zambia by combining molecular and conventional tools to study the epidemiology of TB and access to TB care. As development of drug resistance is a big concern of any NTP, the study examined the levels of drug resistance to both first- and second-line drugs in Ndola Urban District using conventional DST methods supplemented by molecular analyses. The study also used molecular methods to investigate transmission dynamics including strain diversity in the study community. In addition, the quality of TB service delivery to this community was investigated, from the patients' perspective.

The study reports in Chapter 3.1 low occurrence of drug resistance in Ndola to first-line drugs and no resistance to second-line drugs. These findings are in concert with reports in many settings in sub-Saharan Africa outside of South Africa and can in part be attributed to the lack of wide-spread R use prior to the last two decades, history of well-functioning control program, and a long running DOTS programme in the study area. It is also plausible that reported low levels in many African settings might be due to underreporting of drug-resistant TB because of the limited capacity for laboratory DST and reporting systems (214)).

Treatment of TB via DOTS has repeatedly been proven to decrease relapse rates, reduce rates of both primary and acquired drug resistance, reduce treatment failure, increase cure rates and decrease death. Data from some countries have shown rapid declines in TB during periods that coincided with the implementation of TB programmes having the basic DOTS elements in place (151). In the study site, the treatment component under DOTS is well established, using community treatment supporters to achieve directly observed treatment to all patients. This has enhanced treatment completion rates. Over 80% of participants in the study reported completing treatment (Chapter 3.3), thereby contributing to the lowering of development of MDR that could arise through poor adherence. This completion rate is comparable to current DHMT treatment completion data from cohort analysis of 79.5% for smear positives. Of the less than 20% (n=18) of patients that reported not to have completed treatment, over half stopped because they felt better, a common reason given for treatment incompletion. Three patients reported running out of medicine and five reported that they went to the village, common practice when someone becomes very ill, emphasising the importance of patient education, improved follow-up and referral systems.

A second reason for low reported drug-resistance in Africa could be systematic underreporting due to limited capacity for laboratory-based DST. Indeed, low- and middle-resource countries today are limited to periodic surveys due to, not only limited laboratory capacity but also to the logistical complexities of organising culture-based DST. Ultimately these surveys are either rarely performed or performed at irregular intervals. For example, Zambia has only conducted two drug-resistance surveys thus far: one in 2001 and another in 2007; results for the latter are still not available. Limited capacity to perform DST is evident in Zambia where only 3 public sector laboratories are currently performing DST. Furthermore, the referral system for sputum specimens requiring culture/DST in Zambia is still very weak, and still not included in the TB Manual. Given the distances of the countrywide-spread health centres, the NTP referral system is still struggling with the logistics of transporting samples from health centres to the referral laboratories (located in Ndola and Lusaka only) and reporting results back to the health centres on time. In addition, conventional culture-based DST suffers from technical and operational difficulties like high contamination rates. Our study also faced problems of contamination and lack of growth, especially when strains had to be stored and later transported between laboratories. The aforementioned problems limit routine collection of data on drug resistance in Ndola, like most of Africa. Given the limited laboratory capacity for DST in Africa, the expense involved in conducting drug-resistance surveys and the track record of producing results from these surveys, it is probably more sustainable and practical to advocate strengthening implementation of routine drug resistance surveillance (125). With the advent of molecular tools to detect resistance to R, H, FQs and injectable second-line drugs, drug-resistance surveys or even continuous surveillance might become feasible, on condition that the prices (both of equipment and reagents) lower and transfer of technology takes place (215-216).

Despite the observed overall low drug-resistance levels in this setting, the study also revealed existence of R mono-resistance (1.3% combined resistance) in the study population. This type of R resistance was not reported in the earlier countrywide survey of 2001. Some studies have suggested that it is rarely a result of recent transmission (200, 217-218), while others show R mono-resistance in new cases as in the study, suggesting possible primary acquisition (219-220). However, Spoligotyping analysis (Chapter 3.2) in the population did not indicate clonal distribution of R mono-resistant strains, albeit the final sample size might have limited documentation of all recent transmissions. Other risk factors associated with mono-resistance to R include irregular drug intake, inadequate treatment of prior TB episode, prior history of TB, and prior use of rifamycins in treatment

of TB and other bacterial infections (220-223). Although, late introduction of R in Zambia may as well contribute to the reported low levels of resistance, one also notes that its introduction in Zambia coincided with the onset of the HIV epidemic and therefore it is possible that the development of R resistance could have been accelerated by HIV infection in the population. The detrimental synergistic association between TB and HIV has been mentioned earlier (section 1.2.3), but additionally, HIV has been implicated in development of R-resistance due to malabsorption of anti-TB drugs (217-218, 221, 224-225).

The study also attempted to detect possible XDR-TB, and any preexisting resistance to the major second-line drug classes in the general population, by testing OFL and KAN. Our results revealed absence of this type of resistance. This may not be surprising considering the low use of these drugs even in treatment of other diseases in Zambia. Obviously, results from the national DR survey may paint a better picture. While the DR situation may not appear serious in Zambia at the moment, the possibility of an XDR outbreak is a very daunting reality that would have disastrous consequences owing to the weak monitoring/surveillance systems, inadequate capacity to diagnose XDR and poor infection control strategies, transmission of an XDR strain in a community in Zambia would have disastrous consequences, especially with such a high prevalence of HIV, where mortality rates are known to be extremely high (50). The need for new diagnostic tests which can rapidly identify MDR and XDR from clinical samples is all too clear. In Zambia, currently, use of rapid culture-based DST (MGIT) is limited to the three Reference Laboratories only and a few research centres.

Introduction of the new GeneXpert assay in health care centres in Zambia may fare well in urban settings like Ndola, compared to remote rural health centres. There has been an effort to improve infrastructure and logistical issues in urban areas, but apart from limited infrastructure in some remote rural health centres, unreliable electricity access may limit use of the machine and storage of reagents. Further, urban health centres tend to be better staffed, with usually more than one laboratory technologist. Training and standardized guidelines for the application of the assay and interpretation of results will be cardinal in delivering quality care.

This work , demonstrated that more than half (50.2%) of the TB cases in Ndola are caused by only two *M. tuberculosis* families, namely the SAF1 family and to a lesser extent the T family (Chapter 3.2) as determined by spoligotyping. However, the study could not demonstrate a link between prevailing genotypes and drug resistance, age or sex in the study population. Unfortunately HIV data were not available to investigate possible links between HIV-infection and specific MTB lineages. A high clustering rate

in Ndola was also seen within a relatively short time frame, raising the possibility of a high rate of recent transmission occurring (Chapter 3.2) which can contribute to continued high incidence rates. Similarly, other country-level investigations on the impact of DOTS programmes have indicated that, after several years of apparent successful implementation (as measured by high case detection and treatment success), incidence is not falling as rapidly as was expected (226). In fact, in the countries where a decline in incidence was observed, it has been difficult to separate out the effects of DOTS from those of social and economic development (151, 227). A number of countries have attributed this high incidence to the increase among young people presumably due to their higher exposure to various risk factors for TB infection (228). Other possible sources of transmission of TB in Ndola and many urban towns in Zambia include social interactions at bars (which are often badly ventilated) and wakes, where mourners are expected to spend at least one night at the funeral house, with many women sharing a single room (also usually badly ventilated). Reactivation of old disease may also play a part in transmission in this community considering the high prevalence of HIV.

Upon applying MIRU-VNTR, more strain diversity in these families was seen, confirming that these two methods if used in concert, can be discriminatory enough to be used in typing MTB in Zambia.

Many authors have suggested that to gain more insights into the dynamics of TB transmission in high-incidence countries, studies need to be conducted in-county. For this reason, low resource countries not only need to develop skill in molecular typing methods but also go a step further and identify which molecular techniques are applicable to their local situation, i.e. which methods are feasible and which methods will give sufficient information to answer pertinent questions. This begs for enough political will to invest in building capacity for more 'specialised' laboratories to be able to perform some of these techniques so that they are able to inform policy by conducting comprehensive studies.

Zambia, like many sub-Saharan countries, has reported successes in expansion to access for many health programmes like anti-retroviral treatment, immunization and TB treatment through DOTS. However, one of the challenges they face is the delivery of quality of care in these programmes to ensure good treatment outcomes (229). Admittedly, there are many aspects to good TB service delivery: accessible TB services; access to quality care through competent healthcare staff able to provide prompt diagnosis and referral, prescription of correct treatment regimens and documented treatment follow-up; good patient information; and patients' adherence to treatment, all play a part. TB control has greatly benefited

from well standardized treatment strategies applicable at all levels of health systems care and therefore, diligent monitoring of the implementation of these strategies will result in well functioning primary health care systems, crucial in the genuine successful implementation of DOTS.

Our study and others (230) have shown relatively high levels of knowledge of TB symptoms in the study population. However, of concern is the continued delay to seek treatment. Most of the respondents (98%) only presented at the health centre when they were feeling very sick. Despite the high knowledge levels of TB symptoms shown in the study, most respondents not only reported not to have suspected they had TB, but also reported that they delayed seeking care, even when they suspected they had TB in some instances. This negative care seeking behavior no doubt compounds transmission in a community. In Zambia, TB awareness campaigns, by way of posters and community theatre groups, disseminating TB messages in communities have been going on for over a decade. Perhaps these modes should also be used to place emphasis on the importance of seeking early treatment, i.e., on the risk of continued TB transmission in the community.

A major concern from the study was the much lower levels of smear examination of follow-up sputum, much more so at the end of treatment. Microscopy results at the end of treatment are a better indicator of cure than treatment completion. Access to microscopy centres did not appear to be the problem in this setting. Rather, importance placed on the role of microscopy in determining cure, appeared to be a possible barrier. Whereas, the importance placed on treatment completion was evident even from the patients themselves, importance of microscopy in TB treatment follow-up was not so evident in this setting as nearly 90% of the subjects knew the importance of treatment completion but only 57% knew the importance of submitting follow-up sputa. The role of microscopy in patient management cannot be over-emphasized in the improvement of NTP performance.

In general, many of the aspects of TB service are well established in Ndola urban District as per national TB guidelines provided by NTLP: (1) Access to health centres does not appear to be a problem as most participants reportedly reside near health centres, (2) there did not seem to be major problems at the health centres in terms of delays to commence patients treatment, (3) providers seem to be prescribing the appropriate medication, stemming from the fact that patients reported that the providers enquired about their TB history, and (4) providers seem to be providing education about the disease and its treatment to the patients. The major drawback observed in the study was the limited use of dialogue by the providers (Chapter 3.3). Allowing patients to ask questions and seeking clarification is

an important way to assure full comprehension by patients about their disease and its treatment. Recently, the NTLP has made further efforts towards improving TB service delivery by producing four TB training modules for health centre staff, adapted from WHO modules, namely: (1) Detect Cases of TB, (2) Treat TB Patients, (3) Inform Patient About TB, and (4) Identify and Supervise Community TB Treatment Supporters. These modules give step by step guidance to primary health care givers on complete TB care, and are currently being field-tested.

The notion of using program data for surveillance as opposed to periodical surveys, as mentioned earlier, is being advocated in other infections like HIV (231-233). To facilitate continuous DR surveillance, will require efficiently run laboratory systems that are able to perform DST. Initially, this continuous surveillance should focus on retreatment cases. However, in low income countries, another hurdle so far reported has been the quality of data systems in these programs, which needs to be improved (196).

Overall, this work provides pertinent data for Ndola Urban District, even though application of the findings to the general population in Ndola Urban District maybe limited due to the relatively small sample size, mainly arising from technical problems from growing TB cultures. Further, Ndola Urban District maybe representative of most of the urban towns but not of rural areas, and its longstanding DOTS programme may influence results of the TB programme. Therefore, it would be advisable to perform similar more in-depth studies using the more representative population-based sample sizes and taking into account the urban and rural divide in order to make inference from the findings.

Chapter 5: Conclusions

The following conclusions were made from the studies:

- Ndola Urban District has maintained low levels of anti-TB drug resistance presumably due to the longs standing DOTS programme.
- Spoligotyping revealed a limited diversity of MTB complex in Ndola Urban District, dominated by the members of the SAF1 family.
- The high clustering rate in Ndola Urban District is suggestive of probable high recent transmission which underlines the importance of early care seeking, early diagnosis and timely treatment.
- The 15-locus MIRU-VNTR typing is suitable for studying the molecular epidemiology of TB in Ndola Urban District.
- Patients in Ndola Urban District continue to delay to seek treatment.
- TB patients need to be educated about the importance of submitting follow-up sputum samples for microscopy during treatment.
- In general, TB treatment systems appear to be well in place in Ndola Urban District.

Perspectives

Drug Resistance surveillance:

- Although drug-resistance data can be obtained from the national drug-resistance surveys, lack of data in Zambia points to the need for strengthening of DST in the country. Zambia needs to develop systems to monitor drug resistance. For example, the appearance of mono-resistance to R, not previously reported in the general population, coupled with the sustained levels of H resistance in Ndola Urban District, may require further investigation to determine whether this is limited to Ndola Urban District. Zambia should consider drug-resistance surveillance through sentinel sites, and using rapid tests for drug resistance that require little infra-structure. Obviously, establishment of this surveillance should place emphasis on failures and relapses after treatment, and put in place good quality assurance systems.

Epidemiology and Transmission

- Affirmation of the suitability of the use of spoligotyping and MIRU-VNTR to differentiate MTB complex in Ndola Urban District implies its probable use for epidemiological studies in Zambia. To obtain more accurate data, detailed studies using larger and more representative sample sizes, combining conventional and molecular epidemiology, should be conducted to investigate transmission patterns in Zambia such as rural versus urban

settings and those with longstanding DOTS versus recent introduction of DOTS.

Access and Quality of TB Care

- Although the study appears to suggest that TB treatment systems in Ndola Urban District are functional, the findings should be interpreted cautiously as information from all components of TB treatment and care stipulated in the National TB Guidelines was not captured. More comprehensive studies here too, will better inform policymakers.

The aspect of access and quality of care is an important issue that should be given priority. NTPs need to develop systems that will monitor quality of care being delivered in their programmes, not forgetting the importance of data quality. Information on the baseline and future trends in burden of diseases and related risk factors is important for planning and monitoring local and national primary-care responses.

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Professional Career of Chanda Mulenga

Chanda Mulenga obtained her first degree from the University of Sussex, England, in 1993. Thereafter she returned to Zambia in 1994 with a conviction to work in medical research. These were harsh economic times in Zambia with an employment freeze in government institutions. Hence she requested the then Director of the Tropical Diseases Research Centre (TDRC) for an attachment to get introduced into research. After about 8 months, an opening became available in the Nutrition Department for which she applied. Chanda was finally employed in August 1996 as a Staff Development Fellow at the TDRC and has worked there since. In 1999, Chanda obtained a scholarship to study at Karolinska Institutet where she carried out research on interactions of the trypanosome with the Blood Brain Barrier, working with animal models. She obtained a Licentiate degree from the Neuroscience Department in 2001. She was promoted to Head of Immunology Unit, Biomedical Department soon after her return from Sweden and has held that position to date. In 2005, Chanda began her PhD as a sandwich student at the Institute of Tropical Medicine, Antwerp, Belgium.

In her capacity as Head of Unit, Chanda has spearheaded a number of national surveys and researches. She has been pivotal in the provision of national HIV and STI data for Zambia by providing leadership for a number of national surveys, for example, the bi-annual ANC National Sentinel Surveillance for HIV and STIs, Zambia Demographic Health Survey and Behavioural and Biological Surveillance Surveys. In her work Chanda has gained a lot of experience in the development and implementation, including coordination of field activities, for both research and Public Health Surveys. Her experience has also resulted in provision of technical assistance in the African region for the implementation of Demographic Health Surveys, namely, Lesotho and Ethiopia. At the national level, Chanda has coordinated National trainings for laboratory technicians and technologist in HIV/ STI diagnosis and AFB smear microscopy.

Chanda's involvement in research is listed below:

- Study on Tuberculosis Drug Resistance and Treatment Outcome On The Copperbelt Province of Zambia, 2005 – 2010. **PI (PhD project)**

- Study on Institutionalisation of HIV/AIDS Workplace Program in Lusaka Water and Sewerage Company: **Consultant** for HIV prevalence component. 2010 -2011
- Zambia Demographic and Health Survey 2004, 2006-2007, 2012 – **Coordinator for Biomarker component**
- National Sentinel Surveillance for HIV/Syphilis 2004, 2006, 2008, 2011 – **Co-PI**
- Validating the BED IgG Capture Enzyme Immunosorbent Assay to Estimate HIV Incidence in a Generalised Epidemic, 2006 – to date – **Co-investigator**
- A Sero-Prevalence and Behavioral Survey of the HIV/AIDS Situation in Zambian Prisons, 2009 – **Co-investigator**
- Biology and Clinical Staging of Trypanosome Neuroinvasion in Sleeping Sickness, NEUROTRYPS INCO project. 2007 – 2010 **– Country Partner**
- Studies of HIV Viral Diversity and Neutralizing Antibody Characterization In Zambia, 2006 - 2010 – **Laboratory Coordinator**
- HIV prevalence and incidence among Migrant and No-migrant Farm workers in Mazabuka, Zambia. 2006. **Laboratory Coordinator**
- Sexually Transmitted and Blood-borne Infection Prevalence Assessment in High-risk Populations: The First (baseline) Behavioral and Biologic Surveillance Survey for HIV/STI Risk Related Behaviors and Sexually Transmitted Infection Prevalence Assessment in Female Sex Workers in, Ndola, Zambia (BBSS), 2005 – **Co-Investigator**
- Epidemiology of Trichomonas Vaginalis: Prevalence of Trichomonas Sp., and Sexual and Hygienic Risk Factors Associated with Genital Trichomoniasis, 2004 - **Project coordinator**
- Evaluation of the Syndromic Management Algorithms for treating Sexually Transmitted Infections in Primary Health Clinics in Zambia, 2003 – 2005 - **Project coordinator**
- HIV Vaccine Trial Site Development For Zambia (NIH, CIPRA grant) – 2002 **Laboratory Scientist/assistant coordinator**
- Evaluating the Role of Molecular Technologies in the Detection of Drug Resistant Tuberculosis, 2002 – 2005 - **Project Officer.**
- Sexually Transmitted and Blood-borne Infection Prevalence Assessment in High-risk Populations: The Second Behavioral and Biologic Surveillance Survey for HIV/STI Risk Related Behaviors and Sexually Transmitted Infection Prevalence Assessment in Female Sex Workers in Zambia (BBSS), 2003 – **Co-Investigator**

Other peer reviewed publications include:

- Heffron R, Chao A, Mwinga A, Sinyangwe S, Sinyama A, Ginwalla R, Shields JM, Kafwembe E, Kaetano L, **Mulenga C**, Kasongo W, Mukonka V, Bulterys M. High prevalent and incident HIV-1 and herpes simplex virus 2 infection among male migrant and non-migrant sugar farm workers in Zambia. Sexually Transmitted Infections. Sex Transm Infect. 2011:10**.**
- Crucitti T, Jespers V, **Mulenga C**, Khondowe S, Vandepitte J, Buvé A. Non-sexual transmission of Trichomonas vaginalis in adolescent girls attending school in Ndola, Zambia. PLoS One. 2011;6(1):e16310.
- Kirchherr JL, Hamilton J, Lu X, Gnanakaran S, Muldoon M, Daniels M, Kasongo W, Chalwe V, **Mulenga C**, Mwananyanda L, Musonda RM, Yuan X, Montefiori DC, Korber BT, Haynes BF, Gao F. Identification of amino acid substitutions associated with neutralization phenotype in the human immunodeficiency virus type-1 subtype C gp120. Virology. 2011;409(2):163-74.
- Crucitti T, Jespers V, **Mulenga C**, Khondowe S, Vandepitte J, Buvé A. Trichomonas vaginalis is Highly Prevalent in Adolescent Girls, Pregnant Women, and Commercial Sex Workers in Ndola, Zambia. Sex Transm Dis. 2009: 24
- **Chanda Mulenga**, Jama Mhlanga, Krister Kristensson and Brita Robertson. *Trypanosoma brucei brucei* crosses the blood-brain barrier while tight junction proteins are preserved in a rat chronic disease model. Neuropathology and Applied Neurobiology. 2001: 27: 77-85