UNIVERSITY OF CAPE COAST

MOLECULAR CHARACTERISATION OF *MYCOBACTERIUM TUBERCULOSIS*-COMPLEX IN SELECTED PRISONS AND HOSPITALS IN GHANA

BY

OTI KWASI GYAMFI

Thesis submitted to the Department of Molecular Biology and Biotechnology of the School of Biological Sciences, College of Agriculture and Natural Sciences, University of Cape Coast, in partial fulfillment of the requirements for the award of Doctor of Philosophy degree in Molecular Biology and Biotechnology.

JULY 2017

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DECLARATION

Candidate's Declaration

I hereby declare that this thesis is the result of my own original research and that no part of it has been presented for another degree in this university or elsewhere.

Candidate's Signature:..... Date: 12-09-2018

Name: Oti Kwasi Gyamfi

Supervisors' Declaration

We hereby declare that the preparation and presentation of the thesis were supervised in accordance with the guidelines on supervision of thesis laid down by the University of Cape Coast.

Principal Supervisor's Signature:..... Date: 12/9/2018

Name: Prof. Isaac K. A. Galyuon

Co-Supervisor's Signature: ..... Date: 12/09/2018

Name: Dr. Foster Kyei

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ABSTRACT

The population structure of *Mycobacterium tuberculosis* in Ghana was investigated by studying the molecular genetic nature of strains isolated from three very important sections of the human population *viz* the penal system, in presenting patients at hospitals and at post-mortem. Tuberculosis isolates were subjected to drug-susceptibility testing, the Capilia TB-Neo Immunochromatographic test, the HAIN reversed line blot tests, the multiplex ligation-dependant probe assay (MLPA), spoligotyping, and multilocus interspersed repetitive units-variable number tandem repeats (MIRU-VNTR) typing. Remanded prisoners were responsible for 80% of the TB prevalence within the prisons. Whilst there were some TB drug-resistant isolates in all the population groups, the presenting patient population had in addition a multi-drug resistant TB rate of 2.3% (4/170). Significant resistance-associated mutations were: the *katG* MUT1 mutation (AGC→ACC) and the *inhA* MUT1 (C→T in position 15) mutation; the *rpoB* MUT3 S531L mutation and the *rpoB* MUT2B H526D mutation. Spoligotyping revealed a preponderance of the T1 (SIT 53) and the LAM10_CAM (SIT 61) lineages. MIRU-VNTR typing of isolates from the presenting patients yielded a Hunter-Gaston Discriminatory Index (HGDI) of 0.99 for both 24-loci and 15-loci sets. When employed in tandem with Spoligotyping, the 24-loci MIRU-VNTR gave a near 1.0 HGDI value (0.996 ≈ 0.99) for the presenting patient population. The loci MIRU-40 (or (VNTR 0802), VNTR 2163b and VNTR 3690 were the most discriminatory for distinguishing individual members of strain populations in isolates from the prisons, presenting patients and decedents respectively.

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KEY WORDS

Drug Resistance

Molecular Typing

Presenting Patients

Post-mortem

Prisons

Tuberculosis

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ACKNOWLEDGMENTS

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I am highly indebted in gratitude and appreciation to Dr. Arend H. J. Kolk (Department of Analytical Chemistry & Forensic Science, Van't Hoff Institute for Molecular Sciences, University of Amsterdam, The Netherlands), Prof. Dr. Dick van Soolingen and Dr. Richard M. Anthony (both of RIVM, Bilthoven, The Netherlands), Prof. Dr. Paul Klatser (The Janssen Pharmaceutical Companies of Johnson & Johnson, Amsterdam, The Netherlands), Dr. Indra Bergval, Ms. Sjoukje Kuijper, and Ms. Anja Schuitema (all formerly of the Department of Biomedical Research, KIT, Amsterdam, The Netherlands).

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DEDICATION

To my Family and to the memory of my late parents, Dr Kwame Gyamfi and Mrs Hawawu Gyamfi (nee Yakubu).

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LIST OF ABBREVIATIONS

AG	Arabino-galactan
AGE	Agarose Gel Electrophoresis
AIDS	Acquired Immune Deficiency Syndrome
AMK	Amikacin
ATM	Atypical Mycobacteria
bp	Base pair
CAP	Capreomycin
CAS	Central Asian Spoligotype
CRISPRs	Clustered Regularly Interspaced Short Palindromic Repeats
CS	Cycloserine
D	Discriminatory Power
DR	Direct Repeat Region of the MTBC genome
DST	Drug Susceptibility Testing
DS-TB	Drug-susceptible Tuberculosis
E	Test Method Efficiency
EAI	East African Indian clade or spoligotype
EDTA	Ethylene diamine tetra-acetic acid solution
EMB	Ethambutol
EMB^R	Ethambutol-resistant isolate
EMB^S	Ethambutol-sensitive isolate
ETB	Extra Pulmonary Tuberculosis
ETR	Exact Tandem Repeat
HGDI	Hunter–Gaston Discriminatory Index

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INH	Isoniazid
INH^R	Isoniazid-resistant isolate
INH^S	Isoniazid-sensitive isolate
IS	Insertion sequence
IP	Institut Pasteur
ISO	International Standards Organisation
KATH	Komfo Anokye Teaching Hospital
kbp	Kilo base pairs
KBTH	Korle Bu Teaching Hospital
KIT-BR	The Royal Tropical Institute, Department of Biomedical Research, Amsterdam, The Netherlands
LiPA	Line probe assay
M	*Mycobacterium*
MAF	*Mycobacterium africanum*
Mbp	Mega base pairs
MIC	Minimum Inhibitory Concentration
MIRU	Multi-locus Interspersed Repetitive Unit
MLPA	Multiplex Ligation-dependent Probe Assay
MoH	Ministry of Health, Ghana.
MOTTS	Mycobacteria Other Than Tuberculosis Strains
MPTR	Major Polymorphic Tandem Repeat
MTB	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis*-complex
NAAT	Nucleic Acid Amplification Test
NALC	N-Acetyl L-Cysteine
NJ	Neighbour-Joining tree

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NPV	Negative Predictive Value (of a test method)
NTP	National Tuberculosis Programme, Ghana
PCR	Polymerase Chain Reaction
PM	Post mortem
PPV	Positive Predictive Value (of a test method)
PZA	Pyrazinamide
QRDR	Quinolone Resistance-determining Region
REA	Restriction Enzyme Analysis
RFLP	Restriction Fragment Length Polymorphism
RIF	Rifampicin
RIF^R	Rifampicin-resistant isolate
RIF^S	Rifampicin-sensitive isolate
RIVM	The Royal Institute for the Environment and Public Health, Bilthoven, The Netherlands.
SDS	Sodium dodecyl sulphate solution
SIT	Spoligotype International Type
Sn	Sensitivity (of a test method)
Sp	Specificity (of a test method)
SSPE	Sodium chloride - Sodium phosphate – EDTA solution
STR	Streptomycin
STR^R	Streptomycin-resistant isolate
STR^S	Streptomycin-sensitive isolate
Taq	*Thermus aquaticus* polymerase enzyme
TB	Tuberculosis
TTH	Tamale Teaching Hospital, Ghana

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UCC	University of Cape Coast, Ghana
UN	United Nations
UPGMA	Unweighted Pair Group Method with Arithmetic Mean tree
V-DICE	VNTR Diversity and Confidence Extractor
VNTR	Variable Number Tandem Repeats
W.H.O.	World Health Organisation (of the United Nations)
ZN	Ziehl-Neelsen

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CHAPTER ONE

INTRODUCTION

Background to the Study

The global burden of tuberculosis

Tuberculosis (TB) disease is one of the oldest and most challenging public health problems confronting the world today. That the ancient antecedent of tuberculosis, was a blight on the health of humanity, cannot be further emphasised. It is estimated that the aetiologic agent, *Mycobacterium tuberculosis*, MTB, is 15,000 years old (Kapur, Whittman & Musser, 1994). However, there also is evidence to show that this organism may have infected early humans about three million years ago in East Africa (Gutierrez et al., 2005). Moreover, evidence from Pharaonic (the Old World) and Incan (the New World) mummies indicate the existence of TB disease afflicting humans between the third and fifth millennia before the Christian Era (Saitou & Nei, 1987; Zink et al., 2003), and this indicates the pandemic nature of the disease, even from antiquity. The earliest descriptive reference to tuberculosis-like disease state was by Hippocrates, who used the term *phthisis* in his treatise *BOOK 1, OF THE EPIDEMICS* (410-400BCE) (Strazzulla, 2006). His description was of a disease of 'weakness of the lung' accompanied by fever and cough and almost always fatal (Herzog, 1998; Frith, 2014).

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approximately seven to eight million new TB infections are recorded annually (WHO, 2015). These infections result in the death of about one and half to two million in a third of those afflicted (WHO, 2015). The fact that only 10% of individuals diagnosed with TB portray a heterogeneous disease pattern, indicates that host factors and the natural history of the disease may play a role in disease exposure (Desikan & Narayanan, 2015). It is estimated that nearly one billion people will be infected by MTB by the year 2020.

Sub-Saharan Africa has the highest incidence of pulmonary tuberculosis (PTB) in the world. In 2014, 9.6 million people were estimated to have been sick with TB made up of approximately 56.3%, 33.3% and 10.4% men, women and children respectively (WHO, 2015). The incidence rate for Africa in the same period was 330/100,000 population. The emergence of the acquired immunodeficiency syndrome (AIDS), from infections by the human immunodeficiency virus (HIV), as a pandemic has exacerbated the problem. This is because TB is the single most opportunistic infection in HIV-AIDS afflicted individuals. In many African countries, the prevalence rate (unfortunately in tandem with HIV-AIDS) is more than 100/100,000 (WHO, 2015).

The WHO estimates that 1.2 million people with HIV also developed TB in 2014 (WHO, 2015). Also, in 2014 approximately 450,000 people died of TB in Africa, out of 3.2 million prevalent cases (WHO, 2015).

Another growing challenge is the emergence of drug-resistant tuberculosis (DR-TB), particularly, multi-drug-resistant tuberculosis (MDR-TB), as well as the extensively drug-resistant tuberculosis (XDR-TB). This

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dismal picture of the TB situation in Africa is bound to become more depressing because of the dual threats of HIV and MDR-TB. This requires the use of better diagnostic and typing tools for proper identification, treatment and control of the different strains.

Genotyping tools are invaluable to undertaking a genetic characterisation of circulating tuberculosis strains in a specified setting. Knowledge of genetic characteristics will shed light on the population structure of the circulating strains, particularly, in institutionalised sections of the Ghanaian population, such as the prisons, where overcrowding can lead to spread of the infection. Knowledge of the genetic characteristics will, in turn, inform the formulation of better and more efficient control and management strategies for TB.

In Ghana, tuberculosis is one of the commonest communicable diseases and it is estimated that 250,000 are infected by MTB each year. Although Ghana is not one of the WHO-declared 22 high-burden countries, TB control and management still remains daunting, largely because of insufficient financial resources. In the year 2001, Ghana (with a population of 18,800,000 then) had an adult prevalence rate of 1.6% (WHO, 2002). In 2014, Ghana (with an estimated population of 27 million) had a TB (including co-infection with HIV) incidence and TB prevalence (including co-infection with HIV) rates of 165/100,000 and 282/100,000 population, respectively. In 2014, case detection of TB stood at 33,000 with bacteriologically confirmed cases being 7,682 (WHO, 2014).

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In general, TB control and management paradigms, to date, have been influenced by the target group in the general population to which they have been adapted to be applied to (Tsiouris, Gandhi, El-Sadr, & Friedland, 2007; Imam et al., 2010; Chiang, Van Weezenbeek, Mori, & Enarson, 2013). Knowledge of the molecular genetic nature of circulating strains of the MTBC is of crucial importance in TB control and management. There is a paucity of information or knowledge about how the molecular genetic characteristics of circulating MTBC strains in the open Ghanaian population compare with those in institutionalised sections of the population, such as the penal system. Unearthing baseline information about the predominant MTBC strains (in terms of species-type or lineage, wild-type or drug-resistant) in circulation among infected inmates in selected Ghanaian prisons and how they contrast with strains in circulation among patients presenting at selected hospitals or diagnosed at post mortem would serve to provide invaluable insights into the proficiency of TB control and management strategies, particularly, in the prison environment.

In Ghana, TB control is integrated into the primary health care system. That is, diagnosis and control is the responsibility of the general health staff at the district and regional hospitals. National health policies, initiated by the Ministry of Health (MoH), through the National Tuberculosis Programme (NTP), are implemented by the Regional Health Management Teams (RHMT) and District Health Management Teams (DHMT).

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Tuberculosis in prisons

There are higher rates of TB infection in prison populations than in the general population. TB rates in prisons are often 5 to 10-fold higher (Maher Grzemska, Coning, & Reyes, 1998). The peculiar social characteristics of the prison population exacerbate TB infection within the walls of a prison. As such, prison inmates have a higher risk of being infected with TB than other groups within the general population. Inmates are usually from socio-economically disadvantaged backgrounds where poverty rates are high and substance abuse is prevalent. These risk factors thus fuel TB infection in the penal system (Long, Njoo, & Hershfield, 1999; Long, 2000). The backgrounds of inmates can also predispose them to TB infection. For instance, inmates from backgrounds that exhibit sexual promiscuity and/or use injectable drugs are predisposed to active TB disease once infected. These behaviours have led to higher rates of HIV infection amongst prison inmates than in the general population (U.S. Department of Health & Human Services, 1995). Moreover, the stifling environmental conditions in most prisons are conducive for the spread of TB (Chaves et al., 1997; MacIntyre, Kendig, Kummer, Birago, & Graham, 1997).

Thus, the danger of extensive transmission of TB within the prison is real. Indeed, both inmates and prison staff have been known to contract TB in prisons (Chaves et al., 1997; MacIntyre et al., 1997; Steenland, Levine, Sieber, Schulte, & Aziz, 1997; Banda, Gausi, Harries, & Salaniponi, 2009; Margolis, Darraji, Wickersham, Kamarulzaman, & Altice, 2013).

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Thus, the danger of extensive transmission of TB within the prison is real. Indeed, both inmates and prison staff have been known to contract TB in prisons (Chaves et al., 1997; MacIntyre et al., 1997; Steenland, Levine, Sieber, Schulte, & Aziz, 1997; Banda, Gausi, Harries, & Salaniponi, 2009; Margolis, Darraji, Wickersham, Kamarulzaman, & Altice, 2013).

Reports indicate that length of incarceration is significantly associated with the risk of TB infection and disease (Bellin, Fletcher, & Safyer, 1993; Kendig, 1998). Persons incarcerated in prisons are particularly at high risk of contracting TB (including DR-TB) because of over-crowding, poor health, poor diet, late diagnosis and incomplete treatment regimens (Chaves et al., 1997; MacIntyre et al., 1997; Margolis, Darraji, Wickersham, Kamarulzaman, & Altice, 2013; Reid et al., 2012). This study sought to employ some molecular techniques to, among other objectives, determine the nature and extent of TB infection (including DR-TB) amongst coughing inmates in selected prisons in the Ghanaian penal system and, thereby, help in combating the disease.

Tuberculosis, anaemia, hypertension, pneumonia and malaria, are diseases significantly afflicting Ghanaian prisons. For some institutionalised sections of the community, such as the prisons, tuberculosis is already a significant cause of death at post-mortem. In 2012, TB was the second leading cause of prison deaths (with cardiac attacks) at 9.9% behind anaemia, 13.1% (Ghana Prisons Service, 2012). In 2013 it was the third leading cause of death at 14.0% behind anaemia (17.1%) (Ghana Prisons Service, 2013). The current control strategy in Ghanaian prisons is to screen (direct microscopy with X-radiography and in some cases culture) and cure only symptomatic inmates. The fate of asymptomatic inmates is unknown and this could be extremely risky for the general prison population including warders and visitors.

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The Ghanaian prison system

There is no record of the existence of penal systems in traditional Ghanaian societies. The colonial authorities introduced penal systems, including the legal framework to regulate their operation. The first prisons were run from forts and castles dotted along the coastline of present-day Ghana. A notable example was the Cape Coast castle. The Prisons Ordinance was passed in 1860 and it detailed the operating procedures for the incarceration of convicted 'natives' (Roth, 2006). Prison conditions were generally harsh, with convicts engaged in road construction on a paltry diet. There was gradual expansion soon after and, by the 1900s, the British colonial officials managed the fledgling penal system. Ghanaian staff intrusions into management positions of the prisons gathered pace after World War II culminating, by 1962, in a situation where Ghanaians manned all staff positions (Roth, 2006). There was little urge by Ghana's first post-independence government to subject the inherited penal system to any reforms, either in terms of infrastructural expansion or legislation. After the demise of this government, through a *coup d'etat* in 1966, the in-coming military government instituted a civilian commission to delve into the penal system and make recommendations, which could lead to improvements. The report, issued in 1968, was seminal as it detailed a situation of systemic institutional failure; for example, out of the country's then 29 prisons, 9 were judged to be grossly unfit for human detention for any period of time and 13 were judged to be only fit for short-term detentions. However, the

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recommendations contained in the report were largely ignored and prison conditions continued to deteriorate.

The present state of affairs within prison walls is still harsh and de-humanising. There is massive over-crowding and general prison management is grossly under-financed. The 2003 Prison Service Annual Report revealed that there was a monthly average inmate population of 11,038 in prisons meant for 6,500 inmates (Ghana Prisons Service, 2004). Presently, in some instances, there is 300% over-crowding and the high prevalence of communicable diseases within the prisons has been blamed on this. By statute, the Ghana prison system is divided into 'adult' and 'juvenile' systems. While the Prisons Ordinance governs the 'adult' system, the country's Criminal Procedure Codes regulate the handling of juvenile offenders. There is a Prisons Service Board, which formulates policy for the control and management of the prisons system. Members of the Board include a representative each from the Public Services Commission (as Chairman), the Ghana Medical Association, the Attorney-General's Department, the Chief Director of the Ministry of Employment and Social Welfare and three other designated members. This Board advises the President of the Republic of Ghana on the appointment of the Director of Prisons. The Ghana Fourth Republican Constitution enjoins the Prisons Service Board to regularly (not less than two years) review prison conditions and make appropriate recommendations for general improvement.

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The Ghana prisons health system

Most prisons have either a 'Sick Bay' or an infirmary. Only the Nsawam Medium Security Prison (located in the Eastern Region) and the Ankaful Maximum Security Prison (located in the Central Region) can boast of a well-functioning infirmary with an attached medical laboratory manned by trained technicians. The infirmary at the Nsawam Medium Security Prison also includes an isolation ward for inmates afflicted with contagious diseases. Facilities at most of the other prison infirmaries are extremely poor and there is urgent need for renovation of infirmary facilities.

The Ghana Prison Service manages a Contagious Diseases Prison (CDP) situated in the town of Ankaful in the Central Region. It is a fenced, semi-intensive prison camp facility and serves as the main prison set aside by the Ghana Prisons Service to receive inmates afflicted by contagious diseases in the Ghanaian penal system. The CPD accepts inmates with only tuberculosis and inmates co-infected with TB and HIV. The CPD does not accept inmates infected with HIV only. Table 1 depicts the convicted inmate populations of selected prisons as at 7th May, 2014 (Data Unit, Ghana Prisons Service).

All health-related complaints are first presented at infirmaries (where available) before being sent to the nearest District or Regional hospital (Prisons Medical Suprintendent, personal communication, 9th May, 2014). Inmates suspected to harbour TB are captured in District/Regional TB registers and their sputum samples obtained for microscopy based on National

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Convict Populations of Prisons as of 7th May, 2014

PRISON	REGION	MALE	FEMALE	TOTAL
Nsawam Medium Security	Eastern	2736	69	2805
Koforidua	Eastern	432	-	432
Akuse	Eastern	218	10	228
Ankaful Contagious Diseases Prison	Central	39	-	39
Ankaful Main	Central	391	-	391
Ankaful Annex	Central	529	-	529
James Fort	Greater Accra	992	29	1021
James Camp	Greater Accra	452	-	452
Winneba	Central	214	-	214
Kumasi Central	Ashanti	1541	48	1589
Kumasi Manhyia	Ashanti	199	-	199
Sunyani Central	Brong-Ahafo	755	21	776
Tamale Central	Northern	291	12	303
Yendi	Northern	102	-	102
TOTAL		8891	189	9080

Tuberculosis Programme (NTB) criteria. The Ankaful Psychiatric Hospital and the Ankaful Leprosarium laboratories provide diagnostic and follow-up support services for TB management for the Contagious Disease Prison

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(CDP). All newly diagnosed TB cases are made to start the intensive phase of the DOTS regimen promptly. Arrangements are then made to transfer such cases to the CDP at Ankaful near Cape Coast in the Central Region. Treated inmates are sent back to their respective prisons to complete their sentences. The CPD is part of a Prison colony complex at Ankaful. This complex also comprises the Ankaful Maximum Security Prison, Ankaful Main Camp and the Ankaful Annex Prisons. Very sick inmates at the CDP are referred to the Cape Coast District Hospital, where a Chest Clinic exists. Drugs for DOTS are obtained from the Prisons Headquarters in Accra and the Komenda-Edina-Eguafo-Abrem (KEEA) District Health Directorate at Elmina, in the Central Region.

Statement of the Problem

There is a disturbing lack of knowledge on the molecular genetic nature (strain type, clade, lineage and/or family, drug resistance profiles) and extent (prevalence of strain type, clade, lineage and/or family, drug-resistant strains) of the disease in identified clusters of the population such as the penal system and in-patients presenting at health centres and even at post-mortem. Information is also lacking on similarities, if any, between the population structures of MTBC strains in the penal system and that of MTBC strains in patients presenting at health centres and MTBC strains isolated from tuberculosis diagnosed at post-mortem. Also, in the penal environment, the fate of asymptomatic inmates is unknown and this could lead to infections within the general prison population including prison warders and visitors.

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Such knowledge is urgently needed for concerted efforts to be made to improve the management and control of TB.

In Ghana, conventional methods, such as chest radiography and sputum microscopy are currently being employed to diagnose TB in the prisons. Recently, the GeneXpert or Xpert® MTB/RIF assay methodology (developed, produced and marketed by Cepheid, Sunnyvale, Ca., USA) has been employed in a few Regional Hospitals to provide more efficient diagnosis of TB, particularly that caused by resistant forms of the bacillus. The GeneXpert or Xpert® MTB/RIF assay platform has come in handy as it offers almost instantaneous information on most of the mutations on the MTBC genome implicated in isoniazid and rifampicin resistance with very minimal sputum sample preparation. Generally, information on drug-sensitivity patterns is lacking for TB control and management in Ghana. Drug-susceptibility testing on solid media is not routinely available but is carried out in only a few difficult-to-treat cases. Also introduced, albeit on experimental basis, are the HAIN MTBC Reversed Line Blot tests (Nehren, Germany) – providing capabilities in both resistance-testing format (on most SNPs associated with isoniazid and rifampicin resistance) and a genotyping format (speciation into MTBC lineages and atypical mycobacteria). Conventional drug susceptibility testing depends on the positive outcome of cultures and sub-cultures. Beyond the three to six weeks usually required for a positive culture outcome, an additional period of four weeks is required for conventional susceptibility testing. An improvement offered by the BACTEC mini-culture approach requires at least 15 days for susceptibility testing.

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Drawbacks to these conventional methods include slowness, problems of standardisation of the optimum inoculum size, anti-microbial agent stability in the media employed and the low specificity of results.

For a resource-stretched country, such as Ghana, the consequences of transmission of TB, particularly DR-TB, are real and could have deleterious public health implications especially in institutionalised sections of the population, such as the prisons. Therefore, not only is there the urgent need for more sensitive, rapid and reliable methods of diagnosis, but also a need for techniques which will shed light on the nature of the contagion, particularly its population structure. The application of molecular techniques (DNA Isolation, PCR-based mutational analytical techniques) would provide more sensitive, specific and rapid alternatives regarding molecular characterisation of circulating MTBC strains. These include Dot Blot Hybridisation, Immunochromatographic tests, Multiplex Ligation-dependent Probe Assay, the HAIN GenoType® MTBC test, the HAIN GenoType® MTBDR*plus* test, Spacer Oligonucleotide Typing or Spoligotyping, and Multilocus Interspersed Repetitive Units-Variable Number Tandem Repeats (or MIRU-VNTR typing). It must be stated that though the molecular-based methods offer a more rapid and sensitive alternative in yielding clearer information on strain types and resistance-associated mutations, they are more expensive. Also, the problems of obtaining the optimum inoculum size, anti-microbial agent stability and low specificity of results, which have bogged traditional culture-based resistance-testing and biochemical speciation methodology, are largely avoided by the molecular techniques employed in this study, particularly the nucleic acid

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amplification techniques (NAATs). Prompt diagnosis of TB (particularly primary DR-TB), which these proposed molecular techniques offer, is likely to reduce the spread of TB through the initiation of the appropriate chemo-therapeutic interventions. Also, a prompt and accurate diagnosis (including an accurate detection of all resistance-associated genes) of TB would allow for an optimisation of chemo-therapy and a mitigation of the spread of drug-resistant TB. This would minimise the public health threat posed by TB, especially DR-TB. In order to understand the epidemiology of TB in the selected prisons and in-patients presenting at selected health centres/hospitals, mutational methods of analysis, which can provide information on drug resistance profiles and strain types in circulation were employed in the study. It is also envisaged that the application of these molecular techniques would:

1. help the Ghana National Tuberculosis Programme (NTP) establish drug resistance patterns in the country since the quality of these patterns may be compromised if key sections of the population, such as the prisons are omitted.
2. provide essential inputs in the formulation of TB drug policy.
3. help the Ghana Prison Service combat TB, particularly DR-TB, among inmates in the penal system, patients presenting at health-care facilities and on TB diagnosed at post-mortem (the case of sub-clinical TB) by providing accurate genetic information
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5. help in the creation of an integrated disease management programme based on the outcomes obtained from the molecular epidemiology of TB in the selected prisons, inpatients presenting at health centres/hospitals and at post mortem.

To clearly shed light on the burden of TB, three thematic areas are immediately obvious viz:

(a) circulating strains infecting patients presenting at health centres/hospitals;

(b) circulating strains in prisons; and

(c) the problem of TB diagnosed at post-mortem or routine autopsies (i.e. the so-called 'missed TB' or sub-clinical TB).

Research Objectives

1. The primary objective of this study was to investigate the molecular genetic nature and prevalence of TB amongst coughing inmates (both convicted and remanded) in selected Ghanaian prisons, in-patients presenting in selected hospitals (closest to these selected prisons) and TB diagnosed at post-mortem.

The specific objectives were to:

2. determine the prevalence of TB amongst coughing inmates in the selected prisons, in patients presenting at selected health centres/hospitals.
3. determine the genetic diversity and prevalence of MTBC strain families/lineages/clades in circulation;

5. help in the creation of an integrated disease management programme based on the outcomes obtained from the molecular epidemiology of TB in the selected prisons, inpatients presenting at health centres/hospitals and at post mortem.

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2. determine the prevalence of TB amongst coughing inmates in the selected prisons, in patients presenting at selected health centres/hospitals.
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4. characterise TB strains and infections diagnosed at post-mortem in order to give a better understanding of the population structure of MTBC;
5. identify resistance-associated genes and rate of drug resistance in the MTBC isolates in circulation amongst the various population groups;
6. determine the discriminatory power of the various typing methods employed;
7. determine, the most discriminatory loci or group of loci to employ in epidemiological studies; and
8. evaluate the sensitivities, specificities, PPVs, NPVs and efficiencies of the analytical techniques employed and assess their use in TB control and management facilities.

Significance of the Study

This present study involved the application of novel molecular genetic analytical techniques to characterise MTB strains isolated from inmates in selected prisons, patients presenting in selected hospitals and routine post-mortem examinations. Such a study would be useful in enhancing our understanding of the diversity of MTBC species and strains and the dynamics of their transmission. This should then contribute to the efficient control of TB in the prisons and the Ghanaian population at large.

In order to shed light on the population structure of MTBC strains in circulation in Ghana, the study covered three main thematic areas, namely prisons, presenting patients at hospitals and in decedents at post-mortem with

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CHAPTER TWO

LITERATURE REVIEW

Taxonomy and Characteristics of the Genus *Mycobacterium*

The genus *Mycobacterium,* of which all Mycobacteria, and indeed *Mycobacterium tuberculosis* are members, belongs to the phylum Actinobacteria. It belongs to the Order *Actinomycetales*, Sub-order *Corynebacterineae* and Family *Mycobacteriaceae* (Brenner, Krieg, Staley, & Garrity, 2005). This genus is of economic importance because it includes pathogens known to cause serious diseases in both humans and animals. The most notable of the diseases are TB (*Mycobacterium tuberculosis*-Complex, MTBC), leprosy or Hansen's disease (*Mycobacterium leprae* and *Mycobacterium lepromatosis*), Buruli ulcer (*Mycobacterium ulcerans*) and "fish tank granuloma" or "fish handlers' disease" (*Mycobacterium marinum*). Members of this genus are characterised as being acid-alcohol-fast and are all immobile, except *Mycobacterium marinum,* which has been shown to be motile in microphages (Ryan & Ray, 2004). Also, upon maturity on solid media, members tend to possess a slender rod-like shape. They are only weakly Gram-positive, lack the capacity to sporulate, except *M. marinum* and probably *M. bovis* (Ghosh et al., 2009) and are aerobiotic. However, this view has been questioned (Traag et al., 2010). Other characteristics of the genus include a rich DNA G+C content of about 61-71% of the MTB DNA or RNA, and the ability to synthesise long-chain mycolic acids containing between 60 and 90 carbon atoms, which can pyrolytically be cleaved to C_{22} and C_{26} fatty

CHAPTER TWO

LITERATURE REVIEW

Taxonomy and Characteristics of the Genus *Mycobacterium*

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acid esters as suggested by Lévy-Frébault and Portaels (1992). Currently, the genus comprises 196 different species and 13 different sub-species (LPSN, 24th May, 2017).

Structurally, mycobacteria are held in a capsular shape, which forms a protective barrier between the pathogen and the macrophages thereby making the cell envelop impermeable (Daffé & Draper, 1998; Rastogi, Legrande, & Sola, 2001). Classification of individual species within the genus has been based on certain characteristic differences, such as species generation time (time used in producing progeny) and clinical characteristics triggered in the host by infecting species. The former mode led to categorising mycobacteria into fast or rapidly growing mycobacteria (or fast growers) and slow-growing mycobacteria (or slow growers). Usually, fast growers form visibly coloured colonies within the first seven days of inoculation onto solid media while the slow growers take between two to three weeks to grow. Slow growers tend to be pathogenic to humans and animals while the fast growers tend to be rare.

The slow growers have also been categorised further into three groups (Timpe & Ruyon, 1954) according to their ability to produce visible carotenoid pigments on exposure to light. Group I consists of Photochromogens. These represent species that produce yellow coloured colonies on exposure to light but produce non-pigmented colonies in the absence of light (e.g. *Mycobacterium kansasii*, *Mycobacterium marinum* and *Mycobacterium simiae*). Scrotochromogens form Group II and these produce deep yellow to orange coloured colonies in the presence of light or in the dark (e.g. *Mycobacterium gordonae*, *Mycobacterium szulgai* and *Mycobacterium*

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xenopi). The third group, Group III, are the Non-chromogens whose members do not produce any pigments on exposure to light. A sub-group of Group III is usually classified as Group IV. Groups III and IV are usually merged into one group and species therein are characterised by a pale yellow buff or tan-pigmented colonies, examples of which are MTBC, MAC, *Mycobacterium bovis*, *Mycobacterium ulcerans*, *Mycobacterium fortuitum* and *Mycobacterium chelonae*. A more mundane mode of classification is that based on clinical features sparked off in the infected host, that is, the MTBC group, the group of non-cultivable mycobacteria and the so-called 'mycobacteria other than tuberculosis' (MOTT). The MOTT group is also sometimes referred to as atypical mycobacteria (ATM) or non-tuberculous mycobacteria (NTM). While the MTBC group causes tuberculosis, the group of non-cultivable mycobacteria, generally represented by *Mycobacterium leprae* causes leprosy (Hansen's disease). Members of the MOTT group can also cause pulmonary disease not akin to tuberculosis, skin diseases, lymphadenitis and disseminated diseases, particularly, in immuno-compromised individuals.

Phenotypically, mycobacteria are closely related to members of *Nocardia*, *Rhodococcus* and *Corynebacterium*. Ecologically, mycobacteria are extremely widespread in terms of habitation and can be found usually in water and food sources. However, some mycobacteria, such as those of the MTBC and those implicated in the pathogenesis of leprosy and buruli ulcer, are obligate in nature and, therefore, require a host to proliferate in.

With advances in molecular-genetic techniques, it is now possible to classify mycobacteria more rapidly and accurately by the application of

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With advances in molecular-genetic techniques, it is now possible to classify mycobacteria more rapidly and accurately by the application of

techniques based on amplifications of nucleic acid sequences in the 16S ribosomal ribonucleic acid (16S rRNA) of the mycobacteria (Böddinghaus, Rogall, Flohr, Bloecker, & Boettger, 1990; Rogall, Wolters, Flohr, & Bottger, 1990). Ribosomal ribonucleic acid, (rRNA) contains highly conserved sequences, which can be used to characterise organisms (Woese, 1987).

Structurally, the rRNA is about 1,500 nucleotides in length, and it is part of the smaller 30S subunit of the 70S prokaryotic ribosome. Essentially, it contains highly conserved small sequences and differences in these sequences are now exploited to characterise specific oligonucleotides so that the organisms can be classified at the genus, group or even species level (Tortoli, 2003). It has been found that, generally, the slow growers harbour one copy of 16S rRNA except *Mycobacterium terrae* (Ninet et al., 1996) and *Mycobacterium celatum* (Reischl et al., 1998). Fast growers have also been found to harbour two 16S rRNA fragments except *Mycobacterium chelonae* and *Mycobacterium abscessus* (Tortoli, 2003).

The genus *Mycobacteria* is very important in public health because of its inherent zoonotic potential. This is not only seen in mycobacterial pathogens like MTBC, *Mycobacterium leprae* and *Mycobacterium ulcerans* but in others, including *Mycobacterium avium* subsp. *paratuberculosis* and *Mycobacterium avium* subsp. *homminissuis*, which have gained notoriety in diseases such as Crohn's disease (*Mycobacterium avium* subsp. *paratuberculosis*) and are responsible for opportunistic infections in HIV patients (Inderlied, Kemper, & Bermudez, 1993). Other mycobacteria, such as *M. marinum*, *M. fortuitum* and *M. chelonae*, are also important zoonotic agents

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since they are collectively implicated in the aetiology of piscine mycobacterioses ('fish tuberculosis'). Members of the MOTT group have also gained importance because of their opportunistic nature in infecting immune-compromised subjects including AIDS patients. MOTT also causes cervical lymphadenitis, cutaneous infections, chronic lung disease, post-surgical and post-traumatic infections, and pneumonitis. Thus, the need to correctly identifying the species involved for the initiation of appropriate treatment and the application of control and management measures cannot be overemphasised.

The Mycobacterial cell wall

The mycobacteria cell wall is made up of three different structures covalently linked together. These structures are peptidoglycan, arabinogalactan and mycolic acids. The nature of the outer wall, characterised by these structures, helps mycobacteria to maintain their structural integrity and survive and grow as intracellular pathogens (Daffé & Draper, 1998). Mycolic acids are extremely hydrophobic and are uniquely characteristic of the genera *Mycobacterium* and *Corynebacterium*. The outer peripheral surface of mycobacteria contains phenolic glycolipids, phthiocerol dimycocerosates, sulpholipids, and phosphatidylinositol mannosides woven with mycolic acids. The outermost crust is made up of the polysaccharides, glucan and arabinomannan. This complex lipoid layer inevitably works to the advantage of mycobacteria as seen in the high lipid content of the cell wall, which makes the pathogen robust in the hostile intracellular environment.

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The mycobacterial cell wall is based on three-compartment model composed of a plasma membrane, a cell wall core and the extractable non-covalently linked glycans, lipids and proteins (Alsteens et al., 2008). The cell wall core, which is insoluble, is composed of a matrix of cross-linked peptidoglycans linked to arabinogalactans esterified at the distal ends to mycolic acids (Baulard, Besra, & Brennan, 1999). Functionally, this complex cell wall core efficiently 'envelopes' the *Mycobacterium* and serves to control its growth and survival in the infected host and its participation in the immunologic response (Brennan, 2003; Daffé & Draper, 1998; Dinadayala et al., 2006). In pathogenic mycobacteria, the outer cellular layer consists mainly of polysaccharides while in non-pathogenic mycobacteria the outer cellular layer consists mainly of proteins (Malaga et al., 2008).

Also, the lipoid cell wall of mycobacteria imparts certain resilient characteristics to mycobacteria, for example, while they resist drying, they can be killed by ultraviolet radiation, sunlight and pasteurisation (Biberstein & Hirsch, 1999).

The *Mycobacterium tuberculosis*-Complex (MTBC)

The MTBC belongs to the genus *Mycobacteria,* as stated earlier, and comprises of a group of closely-related pathogens (Smith, Besra, & Brennan, 2006; Huard et al., 2006), which includes *Mycobacterium tuberculosis* (Koch, 1882), *Mycobacterium africanum* (Castets, Rist, & Boisvert, 1968; Castets, Boisvert, Grunbach, Brunel, & Rist, 1969), *Mycobacterium bovis* (Karlson & Lessel, 1970) and *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG)

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(Calmette & Guerin, 1927), *Mycobacterium caprae* (Aranaz, Cousins, Mateos, & Dominguez, 2003), *Mycobacterium microti* (Wells & Oxon, 1937; Reed, 1957), *Mycobacterium canetti* (van Soolingin et al., 1997), *Mycobacterium pinnipedii* (Cousins et al., 2003), *Mycobacterium suricattae* (Parsons, Drewe, van Pittius, Warren, & van Helden, 2013), *Mycbacterium orygis* (van Ingen et al., 2012) and *Mycobacterium mungi* (Alexander et al., 2010). Recent reports indicate that the MTBC evolved probably in the Horn of Africa (Blouin et al., 2012; Comas et al., 2013). Members of the MTBC show a 99.9% similarity at the nucleotide level with virtually identical 16S rRNA sequences (Böddinghaus et al., 1990; Sreevatsan et al., 1997; Huard et al., 2006). Despite this near-identical sequence homology exhibited by members of the MTBC, they are usually differentiated by long sequence polymorphisms (LSPs) (Mostowy et al., 2004). The MTBC exhibits a highly clonal population structure and there is little or no evidence in support for recombination between the strains making up the MTBC (Supply et al., 2003; Smith, Gordon, Rua-Domenech, Clifton-Hadley, & Hewinson, 2006). Epidemiological and host preference characteristics seem to support delineation of the MTBC into various species and also these species tend to fit in the scenario of host-adapted eco-type sets (Cohan, 2002). However, this notwithstanding, members of the MTBC can be distinguished from each other by cultural and biochemical properties (Collins & de Lisle, 1985; Grange, Yates, & Kantor, 1996).

Mycobacterium bovis is the primary causative agent of tuberculosis in bovines, such as cattle and buffalo, and other ungulates. The vaccine strain,

(Calmette & Guerin, 1927), *Mycobacterium caprae* (Aranaz, Cousins, Mateos, & Dominguez, 2003), *Mycobacterium microti* (Wells & Oxon, 1937; Reed, 1957), *Mycobacterium canetti* (van Soolingin et al., 1997), *Mycobacterium pinnipedii* (Cousins et al., 2003), *Mycobacterium suricattae* (Parsons, Drewe, van Pittius, Warren, & van Helden, 2013), *Mycbacterium orygis* (van Ingen et al., 2012) and *Mycobacterium mungi* (Alexander et al., 2010). Recent reports indicate that the MTBC evolved probably in the Horn of Africa (Blouin et al., 2012; Comas et al., 2013). Members of the MTBC show a 99.9% similarity at the nucleotide level with virtually identical 16S rRNA sequences (Böddinghaus et al., 1990; Sreevatsan et al., 1997; Huard et al., 2006). Despite this near-identical sequence homology exhibited by members of the MTBC, they are usually differentiated by long sequence polymorphisms (LSPs) (Mostowy et al., 2004). The MTBC exhibits a highly clonal population structure and there is little or no evidence in support for recombination between the strains making up the MTBC (Supply et al., 2003; Smith, Gordon, Rua-Domenech, Clifton-Hadley, & Hewinson, 2006). Epidemiological and host preference characteristics seem to support delineation of the MTBC into various species and also these species tend to fit in the scenario of host-adapted eco-type sets (Cohan, 2002). However, this notwithstanding, members of the MTBC can be distinguished from each other by cultural and biochemical properties (Collins & de Lisle, 1985; Grange, Yates, & Kantor, 1996).

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M. bovis BCG, derived from attenuated cultures of wild-type *M. bovis* is also included in the MTBC. *Mycobacterium africanum* is a significant cause of human tuberculosis in West Africa (Kallenius et al., 1999). *Mycobacterium microti* primarily affects field voles and other rodents but very rarely humans (van Soolingen et al., 1998; Rastogi, Legrande, & Sola, 2001). *Mycobacterium canettii* is very rare and the few known cases all occurred in the Horn of Africa (Pfyffer, Auckenthaler, van Embden, & van Soolingen, 1998; van Soolingen et al., 1997). *Mycobacterium pinnipedii* (the 'Seal bacillus') was first isolated from seals (Cousins et al., 1993; Zumarraga et al., 1999; Cousins et al., 2003), while *Mycobacterium caprae* was also first isolated from goats in Spain (Aranaz et al., 2003), *Mycobacterium mungi* (Alexander et al., 2010), *Mycobacterium scuricattae* (Parsons et al., 2013) and *Mycobacterium orygis* strains first isolated from banded mongooses (Alexander et al., 2010; Kahla, Henry, Boukadida, & Drancourt, 2011), meerkats (Parsons et al., 2013) and oryxes (van Ingen et al., 2012).

Mycobacterium tuberculosis

This strain, together with *Mycobacterium bovis, Mycobacterium africanum* and *Mycobacterium microti* are considered the classical members of the MTBC. Indeed, *Mycobacterium tuberculosis*, MTB, is the 'flagship' representative of the MTBC and principal causative organism of human tuberculosis. Humans are the known reservoir of this strain. This bacillus was first described by German physician and microbiologist, Robert Koch, in 1882 and as a result of his ground-breaking work, it is also sometimes referred to as

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the 'Koch's bacillus' (Koch, 1882). Tuberculosis is spread mainly through air droplets containing the bacillus. Inhalation of these droplets may almost invariably lead to infection; the most common infection site being the lungs making pulmonary tuberculosis (PTB) the commonest infection type. The TB bacterium, after infection may inhabit the microphages, surviving and multiplying there, albeit in a latent or 'dormant' state for several years. The TB bacterium is quite fastidious but rugged with a lipoid cell wall, and presents as rods when grown on solid media. It is considered Gram-positive (though weakly so) and acid-fast to Ziehl-Neelsen staining, requiring approximately 18 hours to complete one cycle of replication (Wayne & Kraus, 1977).

Earlier hypotheses speculated that MTB evolved from *M. bovis* through an initial specialised adaptation of an animal (probably mammalian) pathogen to a human host (Stead et al., 1995). However, whole genome sequence analysis and comparative genomics have revealed that the MTB genome contains several variable genomic regions within members of the MTBC (Cole et al., 1998; Brosch et al., 2002). It has been argued that the lineages of MTB and *M. bovis* separated from each other before the characteristic TbD1 deletion in certain strains of MTB occurred. Therefore, it is obvious that *M. bovis* cannot be taken as the progenitor of MTB. It is most probable that the *M. bovis* lineage evolved or emerged independently, or at best, descended from *M. tuberculosis* (Brosch et al., 2002; Cole, 2002; Huard et al., 2006). Based on the presence or absence of TbD1, MTB has now been broadly classified into 'ancestral/ancient' and 'modern' TB. Thus, the

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flagship member of the MTBC, *Mycobacterium tuberculosis,* lacks the TbD1 region and is considered as 'modern' TB whilst the rest of the MTBC have the TBD1 region on their genomes and, therefore, considered as 'ancestral/ancient'. The evolutionary path of MTB has also been characterised by DNA loss indicated by a 'Region of Difference' (RD) and nucleotide deletion events resulting in a lineage represented by *M. bovis*, *M. africanum* and *M. microti.* This lineage is believed to have diverged from the common ancestor of MTB before changes to TbD1 occurred. *M. canetti* and ancestral MTB strains do not exhibit any of these deletions and therefore are likely to have evolved from a common ancestor (Brosch et al., 2002).

Mycobacterium africanum

Mycobacterium africanum (MAF) is a very important member of the MTBC and it was first isolated from a Senegalese patient in 1968 (Castets et al., 1968). It is best described as a group, albeit heterogeneous in nature, and is considered a significant aetiologic agent of human tuberculosis in West Africa. Indeed, it is thought to be responsible for almost half of all TB infections in West Africa (de Jong, Koymans, Guillemont, Koul, & Andries, 2007; Gagneux & Small, 2007), occurring at various frequencies in different countries. Traditionally, it has always been defined based on biochemical test properties placing it intermediate between classical MTB and *M. bovis*. *Mycobacterium africanum* has been divided into two subtypes, *viz.*, *Mycobacterium africanum* I (Spoligotype Cluster F, West Africa) and *Mycobacterium africanum* II (Cluster G, East Africa) (Niemann et al., 2002).

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Recent evidence indicates that the latter subtype is MTB, *sensu stricto*, because it corresponds to a particular sub-lineage of MTB which can be classified as 'modern' tuberculosis (Mostowy et al., 2004; Gagneux et al., 2006) and which has now been designated the so-called 'Uganda' genotype (Niemann, 2004). On the other hand, the West African subtype or *M. africanum* I (MAF I) has been shown, through numerical analyses of biochemical characteristics, to be more closely related to *Mycobacterium bovis* (David, Jahan, Jumin, Grandry, & Lehmam, 1978). *Mycobacterium africanum* I has further been divided into two lineages - lineage 1 (usually denoted by 'WAF1') and lineage 2 (usually denoted by 'WAF2'). WAF1 lacks the RD711 deletion while lineage WAF2 lacks deletions in RD7, RD8, RD9, RD10, RD701 and RD702. This strain may have found a phylo-geographical niche in West Africa as it is rarely found outside the sub-region, though evidence is now beginning to show that numbers of MAF infections may be decreasing in countries such as Cameroun and Burkina Faso (Niobe-Eyangoh et al., 2003; Godreuil et al., 2007). Curiously, MTB and MAF DNA have been isolated from Egyptian mummies (Zink, Heym, Allen, Young, & Cole, 2003) indicating a different epidemiological picture in antiquity. The present preponderance of MAF in West Africa is clearly indicative of a lack of spread by this strain despite waves of recent human migrations and even the slave trade (both trans-Atlantic and trans-Saharan). This view may be supported by the fact that if MAF is to be found outside West Africa then it is very likely to be found in first generation West African immigrants (Desmond et al., 2004).

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Mycobacterium bovis and *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG)

The bovine tubercle bacillus, *Mycobacterium bovis* (Karlson & Lessel, 1970), is the causative agent of tuberculosis in a wide range of animals (domestic and wild) and also man. It has gained notoriety as an important zoonotic disease and therefore a public health problem. Tuberculosis disease emanating from *M. bovis* infection is indistinguishable from that caused by classical MTB. It is estimated that *M. bovis* is responsible for about 5% of all TB infections in humans (Cosivi et al., 1998). The facilitative natural host of this bacterium is the *Bovidae* species, particularly, cattle and buffalo. Owing to efficient husbandry practices and food safety practices such as pasteurisation of milk, the number of tuberculosis infections caused by *M. bovis* in the industrialised countries has greatly reduced. This situation is different for resource-poor regions, such as Africa, where high risk factors for *M. bovis* infection have been associated with poor food hygiene practices (consumption of unpasteurised milk) and proximate habitation to cattle kraals and the emergence of HIV-AIDS (Cosivi et al., 1998).

Mycobacterium bovis on culture displays dysgonic growth (exhibiting a 16-20 hour generation time) as opposed to classical MTB, which is eugenic. *M. bovis* also tolerates pyruvic acid on growth media and it is innately resistant to pyrazinamide (PZA).

The *M. bovis* BCG (Bacille Calmette-Guérin) strain has its origins in a wild virulent *M. bovis* strain. It was prepared by French bacteriologist Albert Calmette and his veterinarian assistant Camille Guérin (both working at the

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Institut Pasteur de Lille, Lille, France) who effected 230 passages or subcultures of the original wild virulent strain in a variety of media until it lost its virulence on a glycerine-potato medium (Luca & Mihaescu, 2013). This strain has now been accepted worldwide as a vaccine against tuberculosis and now part of the WHO list of Essential Medicines. It was first used medically in 1921 (WHO, 2014). The BCG vaccine has also found use in an immunotherapeutic treatment of superficial bladder cancer (Lamm et al., 1991).

Mycobacterium caprae

This species was first isolated from pathological tissue specimens obtained from goats in Spain (Aranaz et al., 1999). It is characterised by biochemical properties far removed from other members of the MTBC. For example, it was found to be susceptible to PZA (at a minimal concentration of 50µg mL^{-1}), did not reduce nitrate nor accumulate niacin and only resistant to thiophene-2-carboxylic acid hydrazide (TCH) at 1 and 2 pg mL^{-1} but susceptible at 5 or 10 pg mL^{-1}. Genetic analysis reveals that it displays a unique combination of polymorphisms in the *pncA*, *oxyR*, *katG* and *gyrA* genes. Whilst sequencing of the *pncA* gene in these isolates revealed a characteristic MTB polymorphism, sequencing of the *oxyR*, *katG* and *gyrA* genes revealed polymorphisms consistent with *M. bovis*. Subjecting DNA of these isolates to IS*6110* RFLP analysis, direct repeats and polymorphic G+C-rich sequence-associated RFLP analysis and spacer oligonucleotide typing (spoligotyping) revealed patterns which strongly suggested that these isolates

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are quite remote from other members of the MTBC (Aranaz et al., 1999). Further sequence analysis of the *gyrB* genes revealed unique nucleotide substitutions not seen with other MTBC members, which differentiates these isolates from *M. bovis* and other members of MTBC (Aranaz et al., 1999). These isolates are habitually linked to sources of caprine origin and therefore are considered a sub-species within the MTBC (Aranaz et al., 1999) with the name: *Mycobacterium tuberculosis* subsp. *caprae* subsp. *Novo* (Aranaz et al., 1999). Subsequent findings suggest that this class of MTBC may not be confined to Spanish goats alone, as strains have been realised from isolates obtained from cattle (*Bos taurus* Linn.), wild boar (*Suisridae scrofa* Linn.) and pigs (*Suisridae sp.* Linn.), with a geographical range of occurrence from France through Austria to Germany. This unusual member of the MTBC may pose public health problems as strains giving similar spoligotype patterns have been isolated from human patients associated with goat-farming (Gutierrez et al., 1997). Spoligotyping (Spacer Oligonucleotide Typing) experiments have revealed that these caprine mycobacterial strains may very well be members of a homogenous group or cluster characterised by a lack of hybridisation to direct variable repeat positions 1, 3-16, 30-33 and 39-43. These findings support the original assertion that these caprine mycobacterial strains form a separate taxon within the MTBC. As such, it has been proposed that the sub-species *Mycobacterium tuberculosis* subsp. *caprae* subsp. *novo* (Aranaz et al., 1999) be elevated to a species level with the name, *Mycobacterium caprae* comb. *novo* sp. *novo*. (Aranaz, Cousins, Mateos, & Dominguez, 2003) within the MTBC.

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Mycobacterium microti

Mycobacterium microti was first isolated from voles in 1937 by Wells (Wells & Oxon, 1937). Thus, it is commonly referred to as the 'vole bacillus'. The natural host seems to be small rodents but infection has also been seen in cats (*Felidae*) and pigs (*Suisridae*) (Huitema & Jaartsveld, 1967) and llamas (*Lama vicugna*) (Pattyn, Portaels, Kageruka, & Gigase, 1970). This taxon grows poorly on traditional egg-based solid media, such as Loewenstein-Jensen and Ogawa media, as well as in liquid-based media employing modern automated techniques. The poor growth properties can lead to under-estimation of its incidence. The slow growth properties also greatly impede primary culture and isolation of this taxon, which further complicates its biochemical analysis. The technique of spoligotyping has however been applied successfully to simultaneously detect and type *M. microti* (van Soolingen et al., 1998). Initially, *M. microti* was considered non-pathogenic to humans. However, recent reports seem to vindicate this (van Soolingen et al., 1998; Niemann, Richter, & Rüsch-Gerdes, 2000; Emmanuel et al., 2007). For example, Niemann and his group (Niemann et al., 2000) analysed isolates from two human infections of *M. microti* in Germany using spoligotyping and found that one isolate belonged to the llama-type with the presence of hybridisation to, or presence of spacers 4-7, 23, 24, 26, 37 and 38, and the other the vole-type, which was without hybridisation to all 43 spacers except spacers 37 and 38.

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Mycobacterium canetti (Mycobacterium prototuberculosis)

Mycobacterium canetti is probably the rarest member of the MTBC. This taxon was first isolated from a farmer by the French microbiologist, Georges Canetti, after whom it was named, in 1969. As a member of MTBC, *M. canetti* is exceptional in its morphology because it appears smooth and shiny on growth media. This strain was fully described in 1997 after an isolate was obtained from a two-year old Somali patient suffering from lymphadenitis (van Soolingen et al., 1997), where initial biochemical tests indicated similarities between this strain and MTB. Genetic analysis has confirmed that it has identical 16S rRNA sequences with other members of the MTBC. Genetic differences between *M. canetti* strains and other members of MTBC can be found in IS*1081* copy number and polymorphisms in certain house-keeping genes while phenotypic differences exist in lipid content of the cell wall and colony morphology (Brosch et al., 2002). Laboratory diagnosis of *M. canetti* infections, though seldom routine, is bound to present a conundrum since, on culture, the smooth and glossy appearance of its colonies irreversibly revert to a rough appearance (van Soolingen et al., 1997) so typical of classical MTB. Molecular genetic analysis has revealed that regions RD, RvD and TbD1 are highly conserved in the genome of *M. canetti* (as opposed to other members of the MTBC) and that this species may have diverted from a common ancestor of MTB. The genome of *M. canetti* carries 26 unique spacer sequences in its direct-repeat region, which cannot be found in or are no longer in the genome of other members of MTBC, thus lending credence to the hypothesis of diversion (Brosch et al., 2002). Epidemiologically, all

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known cases of *M. canetti* infections to date have been linked to the Horn of Africa (van Soolingen et al., 1997; Pfyffer, Auckenthaler, van Embden, & van Soolingen, 1998; Miltgen et al., 2002), suggesting it may be endemic in this region.

Mycobacterium pinnipedii

This taxon was first isolated from the lungs of seals (pinnipeds) in Australia in 1993 (Cousins et al., 1993; Cousins et al., 2003). Biochemical analysis revealed that these lung isolates belonged to a unique cluster within the MTBC. With time, more isolates were recovered from other seals and sea lions in South America. The specie *M. pinnipedii* prefers solid media enriched with sodium pyruvate. Further comparative studies on these isolates and other members of MTBC revealed that the protein, MBP70, usually secreted in culture broths by *M. bovis* was not present in *M. pinnipedii*. Microscopic studies, after Ziehl-Neelsen staining, have revealed loose cord formation. Anti-microbial agent susceptibility testing has shown that isolates of this taxon are susceptible to pyrazinamide (PZA). Sequencing the *gyrA* and *katG* genes confirmed the status of *M. pinnipedii* as belonging to a unique cluster within the MTBC. Squence analysis of the *pncA*, *oxyR* and MBP40 antigenic protein of *M. pinnipedii*, surprisingly, indicated that it was more closely related to both MTB and *M. africanum* than to *M. bovis*. A forensic study conducted recently by Bos and co-workers (Bos et al., 2014) revealed that a Peruvian skeleton dating to 1000CE was infected with a tuberculosis strain most closely related to *M. pinnipedii* and this has led to suggestions that, in the past, seals

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might have served as a possible reservoir for the transmission of tuberculosis from the Old to the New World. Spoligotyping of *M. pinnipedii* DNA yields a distinct spoligotype pattern when compared to patterns of other MTBC members (Cousins et al., 2003). These observations have led the name *M. pinnipedii* sp. *Novo* being proposed (Cousins et al., 2003). More discriminative molecular genetic techniques exist that can unequivocally differentiate, particularly, *M. pinnipedii* from *M. caprae* (Gormley, Corner, Costello, & Rodriguez-Campos, 2014).

Mycobacterium mungi

This species was first isolated from banded mongooses (*Mungos mungo*) in Botswana (Alexander et al., 2010) and, subsequently, named *Mycobacterium mungi* sp. *novo*. It is sometimes referred to as the 'Mongoose Bacillus'. Initial observations were that it caused high mortality rates in the banded mongooses, which lived in close proximity to human habitats, scavenging on the human waste therefrom, including faecal matter. Samples from infected mongooses were positive by PCR for the gene segment encoding for the mycobacterial protein MPB70, the IS*6110* element and the 16S ribosomal DNA, indicating that it was characteristically a member of the MTBC (Liebana et al., 1997). Applying an algorithmic PCR analysis gave distinct results which indicated that the *M. mungi* isolates were clearly different from other members of the MTBC (Alexander et al., 2010). Sequencing the *gyrB* gene (encoding for gyrase B) yielded no detectable single nucleotide polymorphisms (SNP's) and this led to the positioning of

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this taxon as being between the so-called 'dassie bacillus' (an MTB strain genotypically closest to *M. microti*) and the *M. africanum* subtype 1(a) (Alexander et al., 2010). Sequencing the *gyrB* gene provides the means to identify MTBC member-specific SNP's. Further analysis of these novel isolates within the regions of difference RD701 and RD702 and the lack of SNP's within *rpoB* gene (β-subunit of RNA polymerase) and *hsp65* gene ('heat shock protein' – a 65kDa dominant and well-preserved antigenic protein inducing a cellular and humoral immune response) unequivocally precluded this taxon from being an *M. africanum* subtype 1(a) sublineage (Mostowy et al., 2004; Huard et al., 2006). These findings set *M. mungi* apart from other members of the MTBC, and qualify it as a *bonafide* member of the MTBC.

Mycobacterium suricattae

This species was first observed as an agent of tuberculosis disease in free-ranging meerkats (*Suricata suricatta*) in the Kalahari Desert, southern Africa, in 2002 (Alexander et al., 2002; Parsons, Drewe, van Pittius, Warren, & van Helden, 2013). Subsequent investigations variously described it as *M. bovis* (Drewe et al., 2009) and a member of the 'animal-adapted lineage of the MTBC' (Drewe, Eames, Madden, & Pearce, 2011). However, after comprehensive analysis (Parsons et al., 2013), it was realised that this taxon was quite different from other members of the MTBC because it had a unique MIRU-VNTR number and also exhibited a unique $RD5^{das}$ sequence when compared with the 'dassie bacillus'. Experiments employing standard spoligotyping methodology (Kamerbeek et al., 1997) repeatedly failed to give

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amplifications in any of the usual 43-spacer arrays used and this probably pointed to the fact that the direct-repeat region might have been deleted in this taxon. Deletion of the direct-repeat region was finally confirmed by further unsuccessful experiments which involved an attempt to amplify, through PCR, selected gene sequences upstream and downstream of the direct-repeat region. Indeed, the results indicated a deletion of the direct-repeat region together with substantial chunks of its flanking regions – approximately 3,500bp upstream and 1,700bp downstream. These results, particularly spoligotyping data (Kamerbeek et al., 1997), clearly distinguish this taxon from other members of the MTBC and thus this taxon, *Mycobacterium suricattae*, is now considered a separate member of the MTBC.

Mycbacterium orygis

This taxon is also called the 'oryx bacillus' and has been isolated from members of the *Bovidae* family, such as oryxes, antelopes, waterbucks, gazelles and deer (van Soolingen et al., 1994; Smith et al., 2006; van Ingen et al., 2012). It is slow-growing and extremely rare and it is sometimes described as belonging to the 'antelope clade' of MTBC. In order to fully define the phylogeny of this taxon, van Ingen co-workers subjected available archived oryx bacillus samples to region of difference' (RD) and SNP analysis. Results indicated a consistent pattern for the isolates with the presence of RD1, RD2, RD4, RD5a (*Rv2348* gene), RD6, and RD13–RD16 and absence of regions RD3, RD5b (*plcA* gene), and RD7–RD12. The deleted region for RD12 (RD12oryx) was larger than those observed for *M. bovis* and

amplifications in any of the usual 43-spacer arrays used and this probably pointed to the fact that the direct-repeat region might have been deleted in this taxon. Deletion of the direct-repeat region was finally confirmed by further unsuccessful experiments which involved an attempt to amplify, through PCR, selected gene sequences upstream and downstream of the direct-repeat region. Indeed, the results indicated a deletion of the direct-repeat region together with substantial chunks of its flanking regions – approximately 3,500bp upstream and 1,700bp downstream. These results, particularly spoligotyping data (Kamerbeek et al., 1997), clearly distinguish this taxon from other members of the MTBC and thus this taxon, *Mycobacterium suricattae*, is now considered a separate member of the MTBC.

Mycbacterium orygis

This taxon is also called the 'oryx bacillus' and has been isolated from members of the *Bovidae* family, such as oryxes, antelopes, waterbucks, gazelles and deer (van Soolingen et al., 1994; Smith et al., 2006; van Ingen et al., 2012). It is slow-growing and extremely rare and it is sometimes described as belonging to the 'antelope clade' of MTBC. In order to fully define the phylogeny of this taxon, van Ingen co-workers subjected available archived oryx bacillus samples to region of difference' (RD) and SNP analysis. Results indicated a consistent pattern for the isolates with the presence of RD1, RD2, RD4, RD5a (*Rv2348* gene), RD6, and RD13–RD16 and absence of regions RD3, RD5b (*plcA* gene), and RD7–RD12. The deleted region for RD12 (RD12oryx) was larger than those observed for *M. bovis* and

M. caprae. These archived samples were further subjected to spoligotyping using standard methodology (Kamerbeek et al., 1997) and also to standard 24-loci MIRU-VNTR (Supply et al., 2006) analysis. While spoligotyping patterns qualified the isolates as *M. africanum* (after querying the Spol.DB4 database), MIRU-VNTR typing yielded closely related patterns. A minimum-spanning tree derived from the MIRU-VNTR results only confirmed the clonality of the oryx isolates with the dominant strain (14/21 samples) emanating from both human and animal sources (van Ingen et al., 2012). The HAIN GenoType® MTBC Assay (HAIN Lifesciences, Nehren, Germany) classified all the oryx bacillus isolates as *M. africanum*. Further molecular genetic work indicated the presence of the $gyrB^{1450}$ (G→T) mutation, which it shares with *M. africanum*, *M. microti* and *M. pinnipedii* (Huard et al., 2006), and this explains its apparent misclassification by the HAIN GenoType® MTBC Assay (HAIN Life Sciences, Nehren, Germany). These results indicate that there is the possibility that some oryx bacillus isolates in the past might have been misclassified as *M. africanum*. In addition to all these, however, further phylogenetic investigations (van Ingen et al., 2012) revealed that the oryx bacillus has an intact RD1 region, a unique RFLP pattern and a novel $Rv2042^{38}$ GGC mutation which sets it apart from other members of MTBC. These qualify the oryx bacillus to be considered as a subspecies within the MTBC with the binomial name, *Mycobacterium orygis*. Less than a decade ago, this bacillus had also been isolated from buffalos (van Pittius, van Helden, & Warren, 2012).

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The Molecular Genetic Basis of Drug Resistance in the *Mycobacterium tuberculosis*-Complex

Resistance is caused by chromosomal mutations in selected genes and this makes drugs employed in the chemotherapy of TB to be ineffective against the mutant bacilli. During poorly managed or ineffective chemotherapy, a drug resistant strain may well advance, through gradual selection, to become the predominant strain within the host. Resistance by the MTBC to antimicrobial agents is a consequence of an accumulation of these chromosomal mutations in certain genes serving as targets for the bactericidal activities of these agents. In the course of approximately 10^6 to 10^8 replications, wild-type strains of *MTB* may well undergo mutations that confer resistance to a single drug (David, 1970, as cited in Pinto and Menzies, 2011). It has been estimated that these mutations linked to resistance occur spontaneously with a frequency or rate of 2.56×10^{-8}, 2.25×10^{-10}, 1×10^{-7} 2.95×10^{-8} and 1×10^{-3} for INH, RIF, EMB, STR and PZA, respectively (David, 1970, as cited in Pinto and Menzies, 2011). Instances of a double mutation have been estimated to be lower - of the order of 9.0×10^{-14} for both INH and RIF (Dooley & Simone, 1994). Mutations in genes now known to be linked to resistance to the first-line drugs in the MTBC genome include *rpoB* gene for RIF resistance; *katG*, *inhA*, *aphC*, *kasA* and *ndh* genes for INH resistance; *rpsL* and *rrs* genes for streptomycin (SM) resistance; *embB* gene for ethambutol (EMB) and *pncA* gene for pyrazinamide (PZA) resistance.

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The First-line Anti-Tuberculosis Drugs

Isoniazid (INH)

Isoniazid, INH (or 4-Pyridinecarboxylic acid hydrazide), whose molecular structure is shown in Figure 1, is one of the key drugs in the treatment of TB and was first introduced in 1952 (Bernstein, Lott, Steinberg, & Yale, 1952). Wild-type MTB strains are highly susceptible to INH at the minimum inhibitory concentration range (MIC) of 0.02 – 0.2μg mL^{-1}. It is now believed that INH (Figure 1) transverses the cellular wall by passive diffusion (Bardou, Raynaud, Ramos, Laneelle, & Laneelle, 1998), acting only on growing bacilli. Isoniazid, INH, has been observed under anaerobic conditions to be ineffective against non-replicating bacilli. As is the case with most chemotherapeutic agents, adverse reactions have been found to result from INH therapy. These adverse events are hepatotoxicity and neurotoxicity.

The full mechanism of MTB resistance to INH has not been fully understood. Mutations in the catalase-peroxidase gene (*katG)*, alkyl hydroperoxidase gene (*aphC*), enoyl acyl reductase gene (*inhA)* and β-ketoacyl synthase gene (*kasA*) of the MTB genome are known to confer resistance to INH.

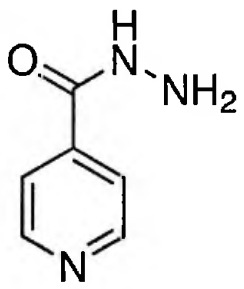

Figure 1: Molecular structure of Isoniazid (INH)

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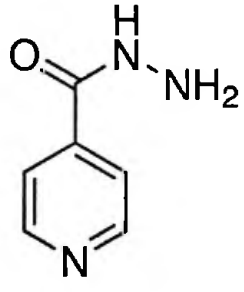

Figure 1: Molecular structure of Isoniazid (INH)

Work on 240 alleles has revealed that mutations in *katG*, *inhA* and *ahpC* genes are mostly strongly associated with INH resistance by (Hazbón et al., 2006).

Effect of katG gene mutations on Isoniazid (INH) resistance

Isoniazid, INH, permeates the bacterial cell wall as a pro-drug, being activated by a bifunctional catalase-peroxidase enzyme encoded by the *katG* gene (Zhang, Heym, Allen, Young, & Cole, 1992). Activated INH is extremely toxic, disrupting mycolic acid biosynthesis by inactivating the NADH-dependent enoyl-acyl carrier protein encoded by the *inhA* gene (Banerjee et al., 1994). Mycolic acids are long-chain α-branched β-hydroxy fatty acids, which serve as the key components of mycobacterial cell walls. Indeed, without mycolic acids, cellular integrity is compromised leading to cell death (Barry, et al., 1998). Structurally, mycolic acids are covalently attached to arabinogalactan (AG) and other non-covalently attached complex lipids and glycolipids populating the bilayer of the bacterial cell wall (Kremer, Guérardel, Sudagar, Locht, & Besra, 2002). Studies have demonstrated that the therapeutic efficacy of INH is maintained in TB strains with an efficient *katG* gene (Zhang et al., 1992). Studies have also demonstrated that strains with a 'defective' *katG* gene, usually occasioned by deletions, lead to INH resistance (Zhang, Garbe, & Young, 1993). It has been estimated that the Serine-to-Threonine substitution at codon 315 occurs in 30-60% of INH-resistant isolates (Ramaswamy & Musser, 1998; Musser et al., 1995). For the *katG* gene, the most commonly occurring mutation is found at codon 315 (Slayden and Barry, III, 2000). However, there are also other widespread

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mutations that could result in inactivation of the catalase-peroxidase system under a MIC range of 0.2 – 256 mg L^{-1}.

Effect of mutations of the ahpC gene on Isoniazid (INH) resistance

The *ahpC* gene encodes for alkyl hydroperoxide reductase protein C, which is implicated in resistance to reactive oxygen and reactive nitrogen intermediates. An increase in the concentration of the alkyl hydroperoxide reductase protein C (*ahpC*) has been observed for TB strains with diminished *katG* gene efficiency (resulting from the *ser*315*thr* amino acid substitution) in producing the catalase peroxidase enzyme required for activating INH into a toxic molecule inside the bacterial cell wall (Sherman et al.,1996). Musser and Ramaswamy (Ramaswamy & Musser, 1998) have observed five different nucleotidic changes in the promoter region of the *ahpC* gene linked to over-expression of *ahpC* that subsequently leads to INH resistance. Initially, researchers proposed that mutations observed in the promoter region of *ahpC* could serve as a surrogate marker for INH resistance (Palomino & Martin, 2014). This, however, was not supported by results of further work which revealed that a spike in the expression of *ahpC* was to be taken as a compensatory mutation to make up for loss in catalase-peroxidase activity rather than assumed to represent INH resistance (Palomino; Sherman et al., 1996).

Effect of mutations of the inhA gene on Isoniazid (INH) resistance

Mutations in the *inhA* gene provide other pathways to understanding INH resistance. Interestingly, mutations in this gene are also linked to resistance to ethionamide (ETH), a second-line drug structurally related to

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INH and also involved in inhibiting mycolic acid biosynthesis. Six point mutations linked to INH resistance have been identified in the *inhA* locus. These are Ile16Thr, Ile16Thr, Ile21Val, Ile47Thr, Val78Ala and Ile95Pro (Basso, Zheng, Musser, Jacobs, & Blanchard, 1998; Ramaswamy & Musser, 1998). The fact that approximately 70-80% of INH resistance in MTB clinical isolates are attributable to mutations in the *katG* and *inhA* genes (Ramaswamy) implies that there is the need to investigate the nature of the *inhA* in isolates containing mutations in the *katG* gene. Generally, mutations in *inhA* and its promoter region with INH MICs in the range of 0.2 – 1 μgmL^{-1} are usually associated with low-level INH resistance and less common than *katG* mutations (Zhang & Talenti, 2000; Hazbón et al., 2006). Higher levels of INH resistance are usually observed in clinical isolates with mutations in the *inhA* gene and simultaneously in the *katG* gene. The most common *inhA* mutation conferring resistance in INH-resistant clinical isolates has been found to occur in its promoter region (-15C→T) and this feature is usually associated with mono-resistance (Leung et al., 2006).

Effect of kasA gene mutations on Isoniazid (INH) resistance

The *kasA* gene encodes the enzyme, β-ketoacyl-ACP synthase (or β-ketoacyl-acyl-carrier-protein synthase), involved in the biosynthesis of mycolic acids and other fatty acids. Functionally, it is involved in lipid metabolism. Free lipids in the mycobacterial cell wall are extended in length by the type II fatty acid synthase complex and then by *kasA* enzyme into mycolic acids (Kremer, Guérardel, Sudagar, Locht, & Besra, 2002). Commonly, substitution mutations occur in codons 66 (GAT-AAT), codon

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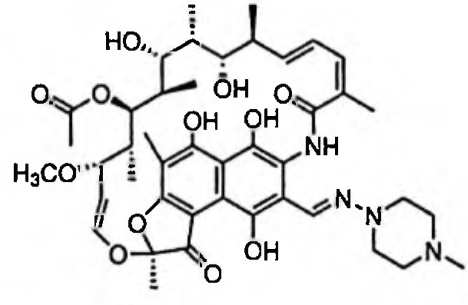
Rifampicin (RIF) and other related Rifamycins

Rifampin (RIF) is a semi-synthetic derivative of rifamycin and is one of the first-line anti-tubercular drugs. Rifampicin, RIF (Figure 2), in concert with INH, acts as the chemotherapeutic linchpin of the DOTS regimen. Rifampicin, RIF, was first introduced as an anti-TB drug in 1972. It is quite potent against TB bacilli, exhibiting an efficient sterilising effect.

Effect of rpoB gene on Rifampicin (RIF) resistance

Extensive studies, using the *E. coli* model, have shed some light on the mode of the bactericidal action of RIF. The DNA-dependent enzyme, RNA polymerase, is made up of subunits α, β, β' and σ' and is involved in transcription essential to cellular survival.



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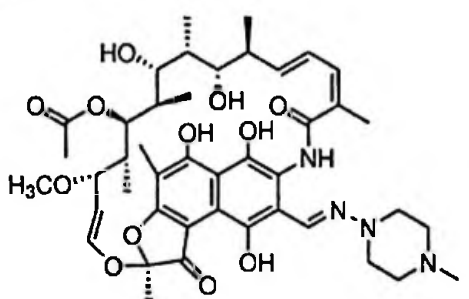

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These subunits making up the structure of the enzyme are encoded, respectively, by the genes *rpoA*, *rpoB*, *rpoC* and *rpoD*. RIF binds to the β-subunit resulting in inhibition of transcription initiation (Ramaswamy & Musser, 1998). Mis-sense mutations and short deletions in the core-central region of RNA polymerase beta subunit gene *rpoB* result in resistance to RIF. Several mutations in this region confer RIF resistance. There are 38 distinct point mutations or short insertions or deletions in the core 81-bp region or the so-called RIF resistance-determining region (RRDR) of *rpoB* codons 507-533 encoding 27 amino acids (Ramaswamy). The common mis-sense mutations occur in codons 531(Ser) and 526(His). Common substitutions are Ser531Leu, His526Tyr and Asp516Val. Most common mutations occur in codons 513 and 526. Mis-sense mutations in codons 526 and 531 tend to characterise high-level resistant isolates. Generally, mutations in codons 531, 526 and 516 (Ser531Leu, His526Tyr, and Asp516Val) are common (Somoskovi, Parsons, & Salfinger, 2001). Some studies have also reported mutations outside of the hot-spot region of *rpoB* in rifampicin-resistant *M. tuberculosis* isolates (Heep et al., 2001).

Mono-resistance to INH is common but mono-resistance to RIF is usually infrequent. It has found that about 90% of all INH-resistant isolates of MTB are also RIF-resistant (Somoskovi et al., 2001). Some workers have proposed, as a result of these findings (Somoskovi et al. 2001), that RIF resistance can serve as a surrogate marker for MDR-TB. One key advantage of RIF therapy is that it is both active against growing TB bacilli and sluggish bacilli (Mitchison, 1979). When administered, RIF produces fewer side effects

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It must be noted that a great number of clinical isolates of TB resistant to RIF exhibit mutations in the *rpoB* gene resulting in conformational changes not conducive to a high affinity for the drug leading to the development of resistance (Telenti et al., 1993; Campbell et al., 2001; Feklistov et al., 2008).

Pyrazinamide (PZA)

Pyrazinamide (PZA) or pyrazine carboxamide (Figure 3) is a pro-drug that is only converted to its active form Pyrazinoic acid (POA) by the enzyme, pyrazinamidase/nicotinamidase (PZase) (Konno, Feldmann, & McDermott, 1997). PZA is a structural analogue of nicotinamide and it is also an important first-line drug employed in the short-course treatment regimen for TB. It gained importance in the treatment of MDR-TB more than 30 years ago (Matchison, 1985) and is specifically effective against classical MTB but not other mycobacteria such as *M. bovis,* which is resistant to PZA. A key advantage of PZA is its ability to inhibit growth of semi-dormant bacilli residing at low pH (Mitchison), since it is efficacious at low pH (pH 5.5), where the intra-cytoplasmic concentration of POA rises intensely. Unfortunately, at pH 5.5, PZA activity is less than satisfactory leading to MICs in the range of 6.25 – 50 μgmL^{-1}. The acidic intracellular environment created as a result is non-conducive to the activity of a vital fatty acid synthase

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Pyrazinamide (PZA)

Pyrazinamide (PZA) or pyrazine carboxamide (Figure 3) is a pro-drug that is only converted to its active form Pyrazinoic acid (POA) by the enzyme, pyrazinamidase/nicotinamidase (PZase) (Konno, Feldmann, & McDermott, 1997). PZA is a structural analogue of nicotinamide and it is also an important first-line drug employed in the short-course treatment regimen for TB. It gained importance in the treatment of MDR-TB more than 30 years ago (Matchison, 1985) and is specifically effective against classical MTB but not other mycobacteria such as *M. bovis,* which is resistant to PZA. A key advantage of PZA is its ability to inhibit growth of semi-dormant bacilli residing at low pH (Mitchison), since it is efficacious at low pH (pH 5.5), where the intra-cytoplasmic concentration of POA rises intensely. Unfortunately, at pH 5.5, PZA activity is less than satisfactory leading to MICs in the range of 6.25 – 50 μgmL^{-1}. The acidic intracellular environment created as a result is non-conducive to the activity of a vital fatty acid synthase

resulting, eventually, in cell death (Zimhony, Vilchèze, & Jacobs Jr., 2004). In a study of isolates from Peru, it was found that 59% of MDR isolates were also PZA-resistant (Saravia et al., 2005). Because of the technical demands involved, susceptibility testing for PZA is not a routine practice in the TB management programmes of many countries, and, as a result of this, there is a paucity of information on the extent of PZA resistance. It has also been shown that PZA resistance strongly correlates with MDR-TB isolates from South Africa (Louw et al., 2006).

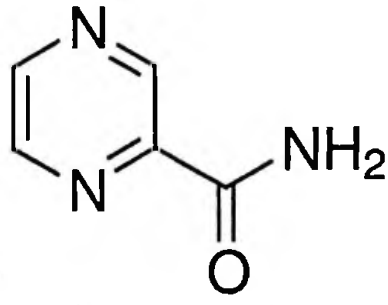

Figure 3: Molecular structure of Pyrazinamide (PZA)

Effect of pncA gene mutations on Pyrazinamide resistance

The enzyme, pyrazinadaze, PZase, is encoded by the *pncA* gene (Scorpio & Zhang, 1996). It has been observed that mutations in the *pncA* gene can cause a reduction in the activity of PZase leading to resistance to PZA by MTB clinical isolates (Lemaître, Sougakoff, Truffot-Pernot, & Jarlier, 1999; Cheng, Thibert, Sanchez, Heifets, & Zhang, 2000). Though extensive efforts have gone into characterising mutations in the *pncA* gene in PZA-resistant isolates, comparisons only vary on the basis of geographical location and in terms of mis-sense mutations, base insertions or deletions, and complete deletions (Lemaitre et al., 1999; Cheng et al., 2000; Suzuki, Suzuki, Tamaru,

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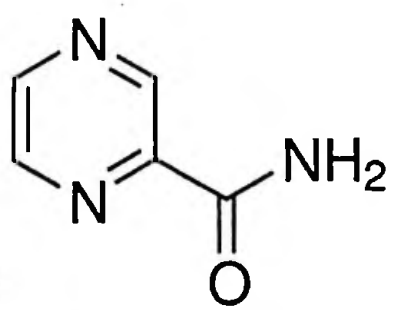

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Katsukawa, & Oda, 2002; Jureen, Werngren, Toro, & Hoffner, 2008; Ando et al., 2010). Though *pncA* gene mutations may be diverse and varied, there exists evidence for clustering of mutations within different positions of the gene, such as at amino acid residues 3 – 17, 61 – 85, and 132 – 142 (Scorpio, 1997; Park et al., 2001). It is thought that the diversity of mutations within the *pncA* gene could be due to adaptation in terms of mutagenesis and/or flaws in DNA mis-match repair mechanisms (Mitchison, 1985). However, it should be observed that though approximately 72-97% of PZA-resistant clinical isolates have mutations in the *pncA* gene (Scorpio et al., 1997; Lamaitre et al., 1999; Cheng et al., 2000; Louw et al., 2006), there are still some PZA-resistant strains without mutations in the *pncA* gene.

Ethambutol (EMB)

Ethambutol (EMB) or (+)-2, 2'-(ethylenediimino)-di-1-butanol (Figure 4), is another potent tuberculostatic and essential first-line drug. It plays a crucial role in the treatment of drug-resistant TB. Ethambutol, EMB, may also be considered an important anti-mycobacterial drug because, when administered in treatment regimens, it enhances the efficacy of other companion drugs, such as the aminoglycosides, rifamycins and quinolones.

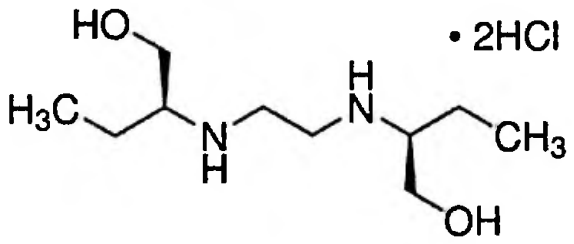

Figure 4: Molecular structure of Ethambutol (EMB) as the hydrogen chloride

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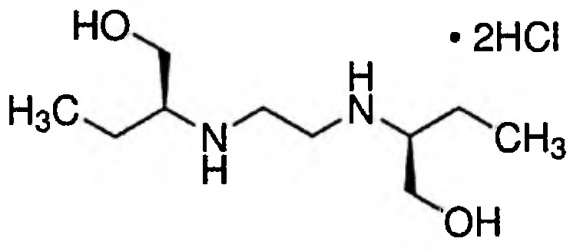

Figure 4: Molecular structure of Ethambutol (EMB) as the hydrogen chloride

The MICs of EMB for MTB fall in the range 0.5 – 2μg mL^{-1}. EMB is active against replicating bacilli but ineffective against non-replicating bacilli. It disrupts an arabinosyl transferase (*embB*) enzyme essential for cell wall biosynthesis (Takayama & Kilburn, 1989). EMB inhibits the polymerisation of cell wall arabinan of arabinogalactan and of lipo-arabino-mannan and initiates the accumulation of D-arabino-furanosoyl-P-decaprenol, an intermediate in arabinon biosynthesis (Wolucka, McNeil, Hoffmann, Chojnacki, & Brennan, 1994; Zhang & Yew, 2009).

Effect of embB gene mutations on Ethambutol resistance

In the chemotherapy of EMB, arabinosyl transferase, encoded by the gene *embB* has been proposed as the target (Telenti et al., 1997). Three genes implicated in EMB resistance have been identified (Telenti et al., 1997). The *embB* gene is organised on the basis of an operon with *embA, embB* and *embC.* The operon is ordered as *embCAB* and the three genes share approximately 65% homology. This operon encodes for trans-membrane proteins (Telenti et al., 1997). Mutations in the *embCAB* operon system are implicated in EMB resistance (Telenti et al.). Common mutations found in EMB-resistant isolates occur in codon 306 giving rise to 3 different amino acid substitutions; that is, Met→Val, Leu and Ile (Ramaswamy et al., 2000; Mokrousov, Otten, Vyshnevskiy, & Narvskaya, 2002). It has been observed that point mutations in the *embB306* codon locus occur in 50 to 60% of all EMB-resistant clinical isolates (Ramaswamy et al., 2000) whiles *embB306* mutations can also occur in EMB-sensitive clinical isolates (Mokrousov et al., 2002).

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Despite several studies on EMB resistance in MTB, the real role played by mutations in codon *embB306* in the development of EMB resistance and multi-drug resistance has not been fully understood. Significantly, about 35% of EMB-resistant strains (MIC<10μg mL^{-1}) do not harbour *embB* mutations (Zhang & Yew, 2009), suggesting that other mechanisms may be at play in EMB resistance.

However, phenotypic or culture-based testing of EMB resistance is extremely difficult since results obtained are not often reproducible. Thus, the onus rests on the appropriate application of molecular-based methodologies in the effective management of EMB-resistant TB. Therefore, further work is required to fully elucidate the mechanism of EMB-resistance.

Streptomycin (STR)

Streptomycin (STR), as shown in Figure 5, is an aminocyclitol aminoglycoside antibiotic and it is the oldest drug employed in the treatment of TB. SM was first employed to treat TB in 1948 (British Medical Research Council, 1948). Streptomycin (STR), exerts its bactericidal action on actively growing bacilli at MICs of 2 – 4 μgmL^{-1}but not intra-cellular or non-growing bacilli (Mitchison, 1985). The drug acts on ribosomes disrupting translation proof-reading by binding to the 16S rRNA (Moazed & Noller, 1987). This action leads to inhibition of protein synthesis (Gale, Cundliffe, Reynolds, Richmond & Waring, 1981). Proof-reading involves a decoding of aminoacyl-tRNA, which is a key step in the translation of mRNA. Thus, a disruption of the decoding process by STR results in inhibition of mRNA

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translation (Benveinste & Davies, 1973). Generally, for bacteria, STR resistance is induced by acetylation of the STR molecule by amino-glycoside modifying enzymes (Davies & Wright, 1997). For MTB, however, resistance to STR seems to be due to mutations in the S12 ribosomal protein, encoded by the *rpsL* gene (Douglas & Steyn, 1993) and mutations in the *rrs* gene encoding the 16S rRNA protein (Finken, Kirschner, Meier, Wrede, & Böttger, 1993). These mutations are point mutations and they result in single amino acid substitutions.

The linchpin for real pharmacological activity of STR is the ribosome moiety, which is also a highly conserved structure involved in the translational step during the action of STR. This ribosome also comprises an rRNA domain and several polypeptides including the ribosomal protein S12 (Carter et al., 2000).

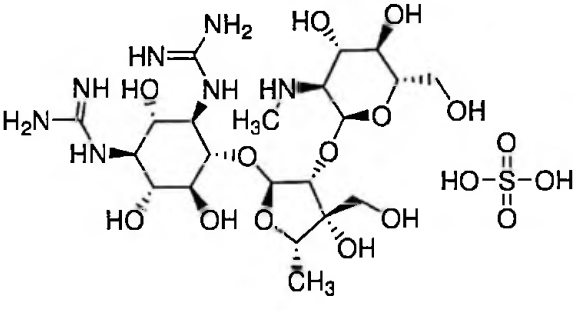

Figure 5: Molecular structure of Streptomycin (STR) as the dihydrogen sulphate

Indeed, this ribosome is generally conserved in other mycobacteria as well such as naturally STR-resistant species as *Mycobacterium avium*, *Mycobacterium gordonae* and *Mycobacterium schulgai*. This fore-going feature could indicate also a possible involvement of bacterial cell

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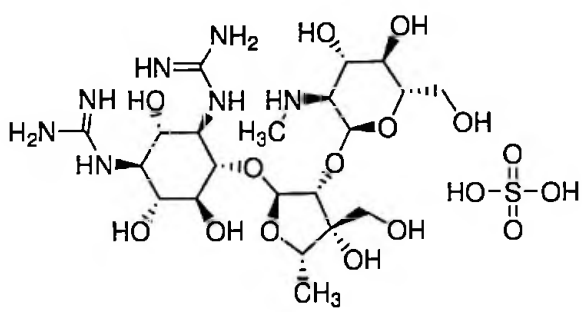

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permeability effects in the development of STR resistance (Honoré et al., 1994).

The effect of rpsL and rrs gene mutations on Streptomycin (STR) resistance

Both the *rpsL* gene (also known as the 30S ribosomal subunit protein S12), and the *rrs* gene (also known as the 16S rRNA) play an important role in the pharmacology of SM as an anti-microbial agent. Mutations linked to SM-resistance have been identified in genes encoding the ribosomal protein S12, *rpsL*, (Honore et al., 1994) and genes encoding 16S rRNA, or *rrs* (Douglas & Steyn, 1993). Mechanistically, there seems to be synergy between *rpsL* and *rrs* as the former stabilises the highly conserved pseudo-knot structure of the latter (Jnawali & Ryoo, 2010). Thus, amino acid point substitutions in *rpsL* gene lead to alterations in the core structure of 16Sr RNA triggering resistance to SM (Jnawali). Structural alterations in 16S rRNA disrupt interactions between STR and the *rrs* gene leading to resistance (de Stasio, Moazed, Noller, & Dahlberg, 1989).

Overall, mutations in both the *rpsL* and *rrs* genes are the major driving forces of SM resistance (Honore et al., 1994). These genetic mutations in *rpsL* and *rrs* account for approximately 50% and 20% of all SM-resistant strains, respectively. The most common mutations in the *rpsL* gene is the Lys→Arg substitution at codon 43 (Jnawali & Ryoo, 2010). Also significant, but less common, is the mutation at codon 88 (Jnawali).

For the *rrs* gene, mutations occur in the loops of its coiled structure clustering around two regions between nucleotides 530 and 915 (Jnawali & Ryoo, 2010). Interestingly, however, between 20 and 30% of SM-resistant

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The Second-line Anti-tuberculosis Drugs

Though less efficacious, these second-line drugs are employed to treat MDR-TB where treatment periods of up to 9 months may be required. An MDR-TB isolate has been defined by WHO as any MTB isolate with concomitant resistance to isoniazid and rifampicin. An XDR-TB isolate has also been defined by WHO as any MDR-TB isolate which is also resistant to at least two of the oral second-line drugs and one injectable second-line drug.

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Second-line anti-tuberculosis drugs, such as amikacin and kanamycin (aminoglycosides), capreomycin, viomycin and enviomycin (polypeptides), ofloxacin, ciprofloxacin and gatifloxacin, levofloxacin (fluoroquinolones), ethionamide (a nicotinic acid derivative), D-cycloserine (a D-alanine analogue), prothionamide (a thionamide) and clofazimine, have been employed in TB treatment for some time. Currently, the latest generation of anti-TB agents include drugs such as the di-aryl-quinoline derivative, Bedaquiline (Protopopova et al., 2007; de Jonge, Koymans, Guillemont, Koul, & Andries, 2007; Worley & Estrada, 2014) or Sirturo™ (Johnson & Johnson, New Brunswick, New Jersey, US.), and the nitromidazo-oxazole derivatives Delamanid (Matsumoto et al., 2006: Szumowski & Lynch, 2015), Pretomanid (Stover et al., 2000; Momin, Thien, Krittaphol & Das, 2016) and TBA-354 (Kmentova et al., 2010; Denny, 2015). The latter, TBA-354 is being touted as a representative of the 'next generation' class of nitro-imidazo-oxazines because of successes in the treatment of MDR-TB (Tasneen et al., 2015; Upton et al., 2015)

Molecules Re-modelled as anti-Tuberculosis drugs

Recently, a few known molecules have been re-modelled for their anti-mycobacterial activity. These molecules include the oxazolidinone derivatives, Linezolid (Cox & Ford, 2012, as cited in D'Ambrosio et al., 2015) and Tedizolid phosphate (Vera-Cabrera et al., 2006).

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Tuberculosis Diagnosis

The linchpin of procedures involved in the control and management of TB disease is the identification of the aetiolgic agent, MTB. Successful identification of the bacillus ultimately leads to a better diagnosis. In the infected patient, identification of MTB in the laboratory from matrices, such as sputum, pleural aspirates, broncho-alveolar lavage (BAL), would always be done in tandem with the presence of an unusual chest x-radiograph and the existence of the classical symptons of TB. In rare cases, a positive identification is difficult to achieve because certain cellular characteristics of the TB bacillus (such as its lipoid wall) render it almost impermeable to staining agents and dyes.

Laboratory diagnosis of tuberculosis

Laboratory diagnosis can be done by phenotypic identification of the MTBC species, microscopy, culture test, biochemical tests as well as molecular genetic or DNA tests.

Phenotypic Identification of the MTBC

Decontamination of Mycobacterial sample matrices

In the phenotypic identification of mycobacterial species including members of the MTBC, decontamination is first done by isolating the slow-growing and docile mycobacterial species to produce a sample matrix suitable mainly for microscopy and inoculation onto media for culture. The decontamination process also renders sample handling less dangerous and,

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depending on the process being applied, up to 60% of the mycobacterial load could also be killed (WHO, 1998).

Traditional decontamination methods include the NaOH-NALC method (Kubica, Dye, Cohn, & Middlebrook, 1963), the Petroff (or NaOH) method (Petroff, 1915; Sharma, Misra, Gandham, Jadhav, Angadi, & Wilson, 2012), the Oxalic acid method (Kent & Kubica, 1985) and the Sodium Lauryl Sulphate method (Narasiman, Mathur, & Pamra, 1972). Recently, a decontamination technique involving Chlorhexidine and Squalamine (Asmar & Drancourt, 2015) has also been introduced.

The Universal Sample Processing solution (USP) methodology (Chakravorty, Sen, & Tyagi, 2005; Chakravorty & Tyagi, 2005) is another method of decontaminaion. Basically, the USP solution consists of 4 to 6 M Guanidinium hydrochloride (GuHCl), 50 mM Tris-Cl, pH 7.5, 25 mM EDTA, 0.5% Sarkosyl, and 0.1 to 0.2 M β-mercaptoethanol. The USP method is particularly useful in cases of extra-pulmonary TB. This method involves treating sputum samples with 1.5 to 2X the volume of the USP solution followed by vortexing or shaking, incubation and centrifugation (5,000 to 6,000 x g). The formed pellet is then used for downstream analysis.

The primary aim of all decontamination processes is to enhance the viability of TB bacilli for growth by killing 'competing' or contaminating and fast-growing and usually less fastidious non-mycobacterial micro-organisms. Generally, these traditional methods of decontamination can be modified in one way or the other to yield excellent results.

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Two other convenient methods of decontamination, where the decontaminant also serves as transport medium for sputum samples are the Tri-sodium phosphate (TSP) method (Jena & Panda, 2004) and the Cetyl Pyridinium Chloride (CPC) (usually also containing 1% Sodium chloride) method (Smithwick, Stratigos, & David, 1975; Pardini et al, 2005). The TSP and the CPC methods are particularly useful if logistical constraints (i.e. long distances to be traversed between sampling sites, field storage sites and the laboratory) regarding sample acquisition exist. A major drawback to the CPC method is that residual CPC partially inhibit growth on M7H10 agar medium in downstream manipulations of CPC-isolated mycobacteria (Smithwick et al., David, 1975; Kant & Kubica, 1985).

Diagnosis by microscopy

Microscopy continues to play an indispensable role in TB case detection, at least in developing countries, where the burden of the disease is highest. The operational advantages inherent in microscopy, as a diagnostic tool, are the simple procedures involved, low cost, speed and high specificity. White light microscopy and fluorescence microscopy are usually employed.

White light microscopy

This is a first-line screening method and it is, particularly, suited to resource-stretched settings. By far, it is the most widely employed diagnostic technique, being usually carried out following WHO standard operating procedures (SOP's) and methodologies.

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Gerald, 2013) in order to render them safe for culturing and smear microscopy.

In Ghana, as is the case in many developing countries, direct white light microscopy is the main tool of diagnosis or detection of pulmonary TB in the healthcare system. This test is also referred to as Sputum Smear Microscopy (SSM) since the usual test sample or matrix is sputum expelled or coughed up from the presenting patient. Sputum smear microscopy is simple, robust, rapid and technically less demanding. If performed optimally, the limit for the microscopic detection of acid-fast bacilli in sputum samples is within the range 10^4-10^5 bacilli mL^{-1} (Someshwaran, Shreeram, Deshpande & Gnanaprakash, 2016). A major drawback of the SSM method is its inability to distinguish between MTB and other mycobacteria. The staining technique of choice for SSM detection of TB is the Ziehl-Neelsen (ZN) staining technique, where MTB and other mycobacteria appear as red-to-pink rods on a blue background (Kent & Kubica, 1985; Walker, 2001). A slight modification of the ZN technique, called the Kinyoun technique, has also been applied with success in TB microscopy (Ellis & Zabrowarny, 1993; Hussey & Zayaitz, 2008). The Kinyoun staining method is basically a 'cold' ZN technique since no heat is applied in the procedure, but the concentration of the methylene blue is increased.

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microscopy yields yellow fluorescing rods in a dark cosmos-like background (Bloom, 1994). The NTP of Ghana has recently rolled out a programme to introduce fluorescence microscopy in selected district hospitals in a bid to improve the TB case detection rate (Ghana NTP, 2012). Work continues to be carried out through the modification of these traditional staining methodologies (Shrestha, Bhattacharya, Lekhak, & Rajendra, 2005; Ghana NTP; WHO, 2012).

Detection of MTBC by culture method

Culture is extremely important in the study of members of the MTBC as it provides the starting material for phenotypic and genotypic analysis. The main purpose of mycobacterial culture is to isolate members of the MTBC and NTM, quite devoid of contaminating organisms, for identification and for downstream analyses.

Members of the MTBC can be cultured using solid and liquid media. The MTBC are characterised by a remarkably slow-growing rate, approximately doubling in population every 24 hours. The culture technique is, generally, considered the 'Gold Standard' for determination of specificity and sensitivity in TB case detection. Traditionally, solid media employed for the culture of members of the MTBC are egg-based media, like Loewenstein-Jensen medium, Ogawa medium and Coletsos® medium (Coletsos, 1971; Idigoras, Perez-Trallero, Alcorta, Gutierrez, & Muñoz-Baroja, 1995) and agar-based media, such as Middlebrook 7H10 or Middlebrook 7H11. Liquid media (broth media) employed for culture of the members of the MTBC include Middlebrook 7H9 and Middlebrook 7H12. Both Ogawa and Coletsos® media

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are modifications of Loewenstein-Jensen medium, the former devoid of asparagine and the latter incorporating a higher proportion of pyruvic acid (as sodium pyruvate) to enhance the growth of *M. bovis.* Recently, the semi-automated or fully-automated Mycobacterial Growth Indicator Tube or MGIT® Systen (Becton & Dickenson, USA) has been introduced for the cultivation and recovery of mycobacteria. In the MGIT® system (Tortoli et al., 1999; Tortoli, Benedetti, Fontanelli, & Simonetti, 2002; Kontosa et al., 2004; Siddiqi & Rüsch-Gerdes, 2006), the tube containing a modified Middlebrook 7H11 broth is bolstered up with oleic-acid-albumin-dextrose-catalase (OADC) enrichment supplements and appropriate bacteriostatic agents (as the PANTA antibiotic mixture) to mitigate contamination by accompanying microbes. The MGIT® system incorporates a fluorescence-based detector which detects fluorescence after the consumption of oxygen by the growing mycobacteria. Other automated systems are the Bract/ALERT 3D System (bioMerieux Dignostics, France) and the VersaTREK System (ThermoScientific, USA), both of which employ a modified Middlebrook 7H11 broth.

Biochemical characteristics of the MTBC observed from growth features on culture

Biochemical characteristics of the mycobacteria are exploited for the phenotypic identification of members of the MTBC and other mycobacteria. For a developing country like Ghana, an accurate determination of the causative agents of mycobacterial disease in samples is extremely important for disease control and management. In the past, traditional methods (based

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on biochemical characteristics) were employed to identify common mycobacteria implicated in infection with varying degrees of success. These traditional methods employed in the phenotypic identification have since increased in number. They include, among other techniques, colony morphology, niacin production and accumulation, nitrate reductase activity, catalase activity, growth kinetics, urea hydrolysis, production of pyrazinamidase and resistance or sensitivity to thiophene-2-carboxylic acid hydrazide (TCH) and other agents. Application of these methods is hindered by the slow growth rate of, particularly, members of the MTBC. Also, encumbering these methods is the possibility of the subjective interpretation of the features encountered in colony morphology and cross-resistance to drugs. The trait of subjective bias in the worker does not always allow for an unequivocal identification of the specie involved (Djelouadji, Raoult, Daffé & Drancourt, 2008; Hall & Roberts, 2006).

More novel methods such as High-performance Liquid Chromatography (HPLC) identification based on mycolic acids present in the cell wall of mycobacteria (Butler & Guthertz, 2001) as well as other chromatographic methods based on Volatile Organic Compounds (VOCs) have also been introduced. The latter method has generally been termed as 'breath-testing' and in one study could discriminate between sputum samples containing only MTB and those containing mixed infections (Pavlou & Turner, 2004; Buszewski, Kesy, Ligor, & Amann, 2007; Phillips et al., 2007).

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Some observed growth features

Growth Velocity

Growth characteristics of mycobacteria can be grouped into two classes: Fast or Rapid Growers and Slow Growers. The former are usually those growing within the first 7 days upon inoculation while the latter are those observed to grow beyond the first seven days.

The ribosomal RNA (rRNA) make up of about 80% of the RNA of all mycobacteria and just about 1 unit of mycobacterial DNA. Genetic studies in the growth characteristics of mycobacteria have revealed that the quantum of RNA depends on the number of ribosomal RNA operons, *rrn*, and also on the efficiency of how they transcribe themselves into RNA. Mycobacteria usually harbour one or two *rrn* operons regulated by the promoters P1 and P2. Whereas slow growers like the MTBC and *M. leprae* harbour the general *rrnA* operon (also found in all mycobacteria), the rapid growers harbour, additionally, the *rrnB* operon. The *rrnB* operon is located on the *tyrS* gene (a gene functionally involved in the translation mechanism probably by catalysing the attachment of L-tyrosine onto tRNA) (Verma, Avinash, & Wami, 1999; Arnvig, Gopal, Papavinasasundaram, Cox, & Colston, 2005).

Colony Morphology

Observations of the growth of mycobacteria colonies on solid media reveal two characteristic features: smoothness or roughness. These morphological features may additionally be either opaque or shiny (Tasso, Martins, Mizuka, Saraiva, & Silva, 2003).

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Pigmentation and growth of Mycobacteria

Pigmentation remains one of the broad categorising features of mycobacterial growth. Whereas some mycobacterial colony species develop a yellow colouration independent of white light stimulus, other colony species develop carotenoid colouration (pink to reddish yellow) in the presence of light. The latter are termed photochromogens while the former are termed scotochromogens. A third class of colony species, termed non-chromogens, is charcterised by a dull-yellow to cream colouration either in the presence of light or darkness (Bernardelli, 2007).

Effect of p-Nitro-benzoic acid (PNB) on growth of Mycobacteria

The presence of PNB offers a means of differentiating between sub-groups within the MTBC. Usually, PNB included in media inhibits the growth of classical MTB, *M. africanum*, *M. bovis* and *M. microti* (Palomino, Leão, & Ritacco, 2007). Non-tuberculous mycobacteria are resistant to PNB (Giampaglia, Martins, Inumaru, Butuem, & Telles, 2005; Nepali, Ghimire, Khadka, & Acharya, 2008).

Effect of Thiophene-2-carboxylic acid hydrazide (TCH) on growth of Mycobacteria

Members of the MTBC (including only *M. afriacnum subtype I*) are resistant (uninhibited by) to TCH except *M. bovis* and *M. africanum* subtype II (i.e. *M. bovis* and *M. afriacnum* are sensitive to TCH). Thus, the inclusion of TCH in LJ medium and other media cannot prevent the growth of classical MTB and *M. africanum* subtype I (Koneman, 1988). This test, therefore, provides a means of differentiating *M. bovis* and *M. africanum* subtype I from

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other members of the MTBC. This test should be applied with caution since some NTM species are also positive for the test.

Effect of Violet Crystal on the growth of Mycobacteria on McConkey Agar

Non-tuberculous mycobacteria (NTM) such as *M. chelonae, M. abscessus* and *M. fortuitum*, are able to grow on McConkey agar without violet crystal, but other mycobacteria such as classical MTB and *M. bovis* cannot grow in this medium except in the presence of violet crystal. This effect provides a means of differentiating MTB and *M. bovis* from NTM.

Effect of Sodium chloride on growth of Mycobacteria

The presence of Sodium chloride can also be employed to differentiate between some mycobacterial species. For example, *M. fortuitum*, an NTM, has the ability to grow in LJ medium impregnated with 5% Sodium chloride.

Cord-formation in alcohol-acid resistant Mycobacterial Cells

Cord formation is a microscopic feature of mycobacterial cell isolates in which the longitudinal axis of the bacilli are parallel to that of the cords (Tasso, Martins, Mizuka, Saraiva, & Silva, 2003). Cord-formation ability is attributable to the presence of the so-called 'cord factor', a glycol-lipid composed of mycolic acid molecules found in the mycobacterial cell walls (Palomino, Leão, & Ritacco, 2007). Cord-formation is a defining feature in ZN microscopy where cords are evident from rough non-chromogenic colonies. This microscopic feature indicates the presence of classical MTB. NTM are indicated by the absence of cords (Tasso et al.).

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Biochemical tests used in the identification of MTBC species

The Niacin Test

Niacin is produced by all mycobacteria through a redox mechanism in the energy metabolism process. However, the MTBC accumulates its niacin because of the activity of nicotianimide adenine dinucleotide (NAD) and its inability to process the resulting niacine (Leão et al., 2004; Palomino, Leão, & Ritacco, 2007). As a result of the accumulated niacin in MTBC cultures, cyanogen chloride (which is formed in the presence of citric acid, between chloramine T and Potassium thyocyanate) breaks the pyridine ring of the niacin forming an aldehyde, γ-carboxyglutamate, which binds with the aromatic amine producing a yellow coloration. Thus, a yellow coloration is imparted and this indicates the presence of the MTBC.

Nitrate Reduction Test

Mycobacterial cellular membranes harbour the enzyme, Nitrate Reductase, which is capable of reducing nitrates to nitrites. Mycobacteria can use this enzyme as a means to source nitrogen (Palomino, Leão, & Ritacco, 2007). Thus, the presence of nitrate (usually as sodium nitrate) in liquid cultures of MTBC can be reduced to nitrite by Nitrate Reductase. The formation of the nitrite can be confirmed by the formation of a furchsia colour (Bernardelli, 2007) following the addition of a thio-amide, sulfalinamide, and the amine salt, dihydrochloride-N-naphthyl ethylenediamine.

Catalase Test

Catalase is an anti-oxidant-eliminating enzyme and, therefore, capable of eliminating or mopping up classical anti-oxidant species such as the

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peroxide moiety (as hydrogen peroxide) which is produced usually during cellular respiration. This action by catalase produces water and free oxygen (Palomino, Leão, & Ritacco, 2007). Extensive research work indicates that there are two classes of the catalase enzyme - Thermolabile and Thermostable. Studies have indicated that the classical MTB and *M. bovis* (both very important members of the MTBC) may be controlled mainly by the thermolabile-type catalase, since it has been observed that its catalytic activity is inhibited at 68°C for both species. In contrast, it has been observed that other mycobacteria are able to maintain catalase activity well beyond 68°C. In this test, bubbles of oxygen appear by the action of the enzyme as it eliminates hydrogen peroxide, in a reaction matrix containing 30% perhydrol and 10% Tween 80 and liquid cultures of the MTBC or other mycobacteria. A quantitative estimate can be made of the enzymatic activity by measuring the height of the bubbles (Bernardelli, 2007).

Urease Test

In mycobacterial biosynthesis, the urease enzyme, encoded by the *ureABC* operon, hydrolyses urea and forms two molecules of ammonia in the process (Palomino, Leão, & Ritacco, 2007). The test involves employing a phenol red medium, where, when the production of ammonia occurs, drastically increases the pH with an attendant colour change to fuchsia (purplish red colour).

Bromocresol Purple Medium Test

This test distinguishes classical MTB isolates from *M. bovis* isolates. The medium is basically LJ imperegnated with Bromocresol purple. Classical

peroxide moiety (as hydrogen peroxide) which is produced usually during cellular respiration. This action by catalase produces water and free oxygen (Palomino, Leão, & Ritacco, 2007). Extensive research work indicates that there are two classes of the catalase enzyme - Thermolabile and Thermostable. Studies have indicated that the classical MTB and *M. bovis* (both very important members of the MTBC) may be controlled mainly by the thermolabile-type catalase, since it has been observed that its catalytic activity is inhibited at 68°C for both species. In contrast, it has been observed that other mycobacteria are able to maintain catalase activity well beyond 68°C. In this test, bubbles of oxygen appear by the action of the enzyme as it eliminates hydrogen peroxide, in a reaction matrix containing 30% perhydrol and 10% Tween 80 and liquid cultures of the MTBC or other mycobacteria. A quantitative estimate can be made of the enzymatic activity by measuring the height of the bubbles (Bernardelli, 2007).

Urease Test

In mycobacterial biosynthesis, the urease enzyme, encoded by the *ureABC* operon, hydrolyses urea and forms two molecules of ammonia in the process (Palomino, Leão, & Ritacco, 2007). The test involves employing a phenol red medium, where, when the production of ammonia occurs, drastically increases the pH with an attendant colour change to fuchsia (purplish red colour).

Bromocresol Purple Medium Test

This test distinguishes classical MTB isolates from *M. bovis* isolates. The medium is basically LJ imperegnated with Bromocresol purple. Classical

MTB isolates induce a pH-dependent colour discharge of the medium from purple to yellow (Kent & Kubica, 1985; Kubica et al., 2006). This test therefore distinguishes classical MTB from *M. bovis* isolates amongst the MTBC.

Lebek Medium Test

Lebek medium (Deutsches Institut für Normung, 1993) is a semi-solid medium employed to assess the oxygen preference of mycobacterial species (Niemann, Richter & Rüsch-Gerdes, 2000). Whereas classical MTB isolates exhibit an aerophilic characteristic by growing on the surface of the medium and on the walls of the glass tube/slants just above the surface, *M. bovis* isolates exhibit microaerophilic characteristics by growing below the surface and well within the body of the medium. Thus, culturing MTB isolates in Lebek medium affords a means of distinguishing *M. bovis* from classical MTB.

Pyrazinamidase Test

The *pncA* gene encodes the bacterial intracellular bacterial enzyme, Pyrazinamidase, (PZAse), in mycobacteria. PZAse hydrolyses the drug pyrazinamide, PZA, into the pro-drug pyrazinoic acid in classical MTB, into which PZA is believed to be transported by passive diffusion. Through natural selection, some mycobacteria present a mutation in the *pncA* gene, which renders them resistant to PZA. Naturally, classical MTB and *M. canetti* (out of the MTBC) do not harbour this resistance-conferring mutation in the *pncA* gene, and therefore this biochemical property can be exploited as a test to differentiate classical MTB (positive PZAse test) and *M. canetti* (positive

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PZAse test) from the rest of the MTBC (negative PZAse test) (Zhang & Mitchison, 2003; Palomino, Leão, & Ritacco, 2007).

Testing for Drug Resistance in the MTBC

One of the most important aspects of TB research is in the area of resistance or susceptibility of the TB bacilli to anti-microbial agents employed to eliminate them from the infected individual. Drug susceptibility testing is particularly important for chemotherapeutic reasons and for the tracking of resistant strains in circulation. A variety of methods exist, based on either phenotypic or genetic properties of the TB bacillus. These methods can also be carried out either in liquid or on solid medium matrices. Each methodology has its own advantages and inherent pitfalls.

Phenotypic methods of drug resistance testing of the MTBC

Phenotypic methods of drug resistance testing refer to the conventional or traditional growth-based drug susceptibility testing (DST) methods employed to determine the susceptibility or resistance of TB isolates to the drugs employed to combat them. As discussed earlier, the existence of mutations in certain specific genes are linked to drug resistance. These mutations are usually point mutations characterised by single nucleotidic changes in the DNA sequence. For example, most RIF-resistant isolates are defined by point mutations in an 81-bp segment in the so-called rifampicin-resistance- determining-region, RRDR (Ramaswamy & Muser, 1998).

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various concentrations (i.e., break-point concentrations) using either a proportional technique or an absolute concentration technique for interpretation. These conventional phenotypic DST methods were, usually referred to as the 'Gold standard' for drug resistance studies. Now, they are considered as the imperfect 'Gold Standard' because of the following reasons: (1) phenotypic methods are fairly slow as they depend on mycobacterial growth on or in a suitable medium, (2) for some mutations giving rise to cases of low level but clinically significant RIF resistance, molecular methods may yield a more realistic resistance profile and (3) some drugs, such as EMB (growth of micro-colonies, susceptibility problems in broth) and PYZ (inoculum size sensitivities and the required low pH for PYZ dissolution) (Van Deun, et al., 2009; Van Deun, Wright, Zignol, Weyer, K & Rieder, 2011; Rigouts et al., 2013; Van Deun et al., 2013). Another reason is the fact that phenotypic susceptibility testing protocols for the second line drugs have not been standardised. Yet, despite all these advantages, molecular methods of resistance-testing have not replaced phenotypic methods.

Phenotypic methods, by and large, will persist for sometime because molecular methods require levels of expertise not universally available now. Also, only a few well-equipped laboratories capable of carrying out efficient molecular assays for resistance-determination are available and, more importantly, most molecular assays are at best laboratory developed tests (LDT's). In addition, not all drug resistance-associated mutations are known and also not all mutations can be associated with phenotypic drug resistance because of the problem of 'silent' mutations.

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The minimal inhibitory concentration–drug susceptibility testing (MIC-DST) of anti-tuberculosis drugs

The first drug susceptibility test (DST) of MTB was carried out by Canetti and co-workers (Canetti et al., 1969). These tests involved the inoculation of bacterial culture suspensions on LJ medium containing serially diluted anti-TB drugs. Many DST methods have subsequently been developed, including the use of anti-TB drug impregnated discs (Wayne & Kraus, 1966) or strips referred to as the Etest (Wanger & Mills, 1996; Hazbon et al., 2000).

Other phenotypic methods include the calorimetric test, the automated non-radiometric Mycobacterial Growth Indicator (MGIT) 960 test system (Palomino, Traore, Fissette, & Portaels, 1999; Tortoli, Benedetti, Fontanelli, & Simonetti, 2002), the radiometric BACTEC 460TB method (Siddiqi, 1995; Siddiqi, Libonati, & Middlebrook, 1981) and the Alamar Blue Assay system (Franzblau et al., 1998). Whereas the Etest and Alamar blue assay are based on visual detection of growth of mycobacterial species, the BACTEC and MGIT systems are based on automatic detection of mycobacterial growth in broths containing standard concentrations of anti-TB drugs. A drawback to these broth-based systems is the lack of possibility for the assessment of morphology of the growths. Thus, features of over-growth by atypical mycobacteria and contamination are bound to impact on the reliability of these methods (Tortolli et al.).

The MIC-DST methodology (Klingeren, Dessens-Kroon, van der Laan, Kremer, & van Soolingen, 2007), which has been adopted in this thesis

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work, is based on the principle of resistance of mycobacterial species to tuberculostatics on solid 7H10 agar in a 25-well plate format. Each well contains different concentrations of tuberculostatics. In the test, stock mycobacterial suspensions of turbidities ranging from 2 to 10 $x10^5$cfu mL^{-1} are prepared from bacterial culture harvests and inoculated in 10-μL volumes into each well. The plates are then incubated at 37°C. The plates are then read on the 5^{th}, 12^{th} and 19^{th}day. The Minimum Inhibitory Concentration, MIC, is taken as the lowest concentration of a tuberculostatic inhibiting 99% of the delivered inoculums from growing.

Molecular (Genotypic) methods of drug resistance testing in the MTBC

Molecular or genotypic methods of resistance testing have the inherent advantage of providing information on drug resistance patterns in cases where a phenotype-based DST is unavailable due to unviable or contaminated isolates. They include techniques, such as DNA sequencing (Parish & Stoker, 1999), dideoxy fingerprinting (Felmlee, Lin, & Whelen, 1995; Williams et al., 1994), heteroduplex analysis (Williams et al., 1999), Restriction Fragment Length Polymorphism (RFLP) (Parish & Stoker; Wegenack et al., 1997), single stranded conformational polymorphism (SSCP) (Victor et al., 1997; Telenti et al., 1993) and various other reverse-line probe techniques (De Beenhouwer et al., 1995; Rosau et al., 1997). Each technique is perculiar, with its own advantages and experimental challenges, but the widespread application of DNA Sequencing, which may be considered as the clear 'gold standard' is hindered by cost. Some of these methods are discussed below.

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Phosphorus-32 Dot Blot Hybridisation of the rpoB gene for RIF Resistance-testing

Dot-blot hybridisation is one of the techniques employed to detect resistance-associated mutations in members of the MTBC (Victor et al., 1999; Victor & van Helden, 2001). Generally, this method is used in screening for mutations associated with drug resistance. Possibility also exists for the dot-blot hybridisation method to be adapted to study other loci. This technique is versatile for mutational analysis. It is best applied when the initial focus is on the mutations most prevalent in a population or geographical area of interest. The *rpoB* gene was also selected for this thesis because it has been recommended to be employed as a surrogate marker for the detection of MDR-TB by the WHO primarily because RIF mono-resistance is rare (Ramaswamy & Musser, 1998; Chen et al., 2012). Also, it has been observed that 95-98% of mutations in the so-called 81-bp RRDR of the *rpoB* gene are linked to resistance (Soini & Musser, 2001; Siddiqi et al., 2002). The *rpoB* gene codons 516, 526, and 531 have been used in this thesis because these codons contain most of these mutations.

The technique works best when well-characterised controls are included in order to unequivocally interpret results of dot-blot hybridisation runs. Positive controls are mutants while negative controls are wild-type isolates. The well-characterised controls are probes designed to capture the most prevalent drug resistance-associated mutations in the high burden region of interest. The mutations would have been known well-characterised from prior base-line studies.

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Operationally, the following experimental flow is recommended for the Dot Blot hybridisation technique: PCR amplification → confirmation of amplification by gel electrophoresis → labeling of probe for region of interest → dot-blot hybridization → over-exposure of first time autoradiography to ensure that samples were sufficiently blotted (various modes of washing stringency) → final autoradiography.

The Genotype® MTBDRplus Assay

The GenoType® MTBDR*plus* assay (HAIN Lifescience GmbH, Nehren, Germany) is a reversed line blot assay designed with an enhanced sensitivity to detect a broader spectrum of mutations in the *rpoB* and *inhA* genes, particularly, in the regulatory region of the latter gene (Hillemann, Rüsch-Gerdes, & Richter, 2007).

It employs oligonucleotide probes complimentary to mutations associated with resistance to both INH and RIF. The GenoType® MTBDR*plus* assay test includes nylon membrane strips onto which species-specific oligonucleotide probes containing the most commonly-occurring mutations associated with resistance to INH and RIF (the two first-line drugs whose resistance define MDR-TB), which have been immobilised as parallel lines. In the procedure, DNA is first isolated, usually, from lysed culture harvests followed by multiplex PCR with biotinylated primers to yield biotinylated PCR products. The biotinylated single-stranded PCR products are hybridised to the mutant probes. Streptavidin, labelled with alkaline phosphatase, is then added; and this binds with any biotinylated hybrid formed with any specific probe (i.e. in the PCR product) on the reverse line blot. Incubation is then carried out with

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5-bromo-4-chloro-3-indolyl phosphate and nitro-tetrazolium (BCIP/NBT) chromagen resulting in a purple coloration. The pattern of bands formed is indicative of the species of MTBC.

The GenoType® MTBDR*plus* assay has been designed to detect mutations in *rpoB*, *katG*, and *inhA* genes in clinical strains of *M. tuberculosis*. The GenoType® MTBDR*plus* has been extensively evaluated in a variety of settings (Lacoma et al., 2008; Chen et al., 2012). Typically, a reversed line strip of the GenoType® MTBDR*plus* assay test (Figure 6) incorporates 27 probes as follows: control probes which have been designed to represent a Conjugate Control (CC), a multiplex PCR Amplification Control (AC) and an MTBC-specific Control (TUB).

For the detection of rifampicin resistance, one *rpoB* gene amplification control; eight *rpoB* wild-type controls *rpoB* WT1 to *rpoB* WT8 (505 to 533); four mutant *rpoB* probes represented by MUT1 codon D516V, MUT2A codon D526Y, MUT2B codon H526D and MUT3, codon S531L have been included. To detect isoniazid resistance are one *katG* gene amplification control; one *katG* wild-type probe WT codon 315, and two *katG* mutant probes represented by *katG* MUT1 (AGC→ACC) S315T1 and *katG* MUT2 (AGC→ACA) S315T2. Also, included for the detection of isoniazid resistance but on the *inhA* gene are one *inhA* gene amplification control; *inhA* wild-type probes WT1 and WT2 for positions -15 and -16 of the gene regulatory region; four mutant *inhA* probes represented by MUT1 (mutation C→T in position - 15), MUT2 (mutation A→G in position -16), MUT3A (mutation T→C in position

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-8) and MUT3B (mutation T→A in position 8). Finally, for ease of comparison of band colours, a colour intensity bar, M, is added.

The GenoType® MTBDR*plus* assay has been shown to be very accurate as revealed in a meta-analysis of publications by Ling and co-workers (2008). They found that, when compared with culture and phenotypic DST, the GenoType® MTBDR*plus* assay had an approximate sensitivity and specificity for RIF-resistance of 98.1% and 98.7% and for high-level INH resistance of 84.3% and 99.5%, respectively.

Strain Identification of the MTBC

Some of the methods employed in the strain identification of members of the MTBC in the populations studied were the Immunochromatographic test and Genotypic tests. The former was represented by the Capilia TB-Neo Immunochromatographic test (testing for presence or absence of the protein, MPB64). The Genotypic tests comprised the classical 16S rDNA diagnostic PCR amplification based on the insertion sequence, IS*6110*, and the Reversed Line Probe Assays:

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3. Multiplex Ligation-dependent Probe Amplification (MLPA) Assay (this test has a distinct advantage of both Strain Identification and Genetic Analysis of drug resistance).

The Capilia TB-Neo Immunochromatographic Test

The liquid culture broths of the MTBC have been found out to exude more than 33 different proteins (Cole et al., 1998). The most important of these proteins is MPB64, a 24-kDa protein that had initially been isolated from culture filtrates of the Tokyo strain of *M. bovis* BCG, otherwise referred to as *M. bovis* BCG Tokyo (Harboe et al., 1986; Nagai, Wiker, Harboe, & M. Kinomoto, 1991). The perculiar nature of the MPB64 protein stirred up the possibility of diagnostic applications (Nagai et al.; Abe, Hirano, & Tomiyama, 1999). Thus, while members of the MTBC are able to express this protein, MOTTS and other atypical mycobacteria have mutations on their genomes rendering MPB64 expression impossible. Analysis of the MPB64 base gene in a panel of Capilia-negative isolates (Hirano, Aono, Takahashi, & Abe, 2004) revealed that all the isolates in the panel (12 isolates) had mutations in the MPB64 gene. Five different mutations were found, and with no epidemiological links between the patients providing the isolates, the mutations were assumed as commonly occurring within the MPB64 gene. The genetic function of the MPB64 gene is unknown but it has been observed to strongly induce a delayed-type hypersensitivity reaction similar to purified protein derivatives in guinea pigs exposed to MTBC bacilli. In human TB

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patients, the reaction is lymphocyte proliferation (Harboe et al., 1986; Nakamura et al., 1998). It has also been observed that DNA vaccines (BCG vaccine) expressing MTBC antigen MPB64 immunisation in a murine model led to a more variable protection level against an aerosol exposure to MTBC. This observation clearly indicates the underlying importance of MPB64 to the MTBC.

Using this property of the MTBC secreting MPB64, the Japanese company, TAUNS, developed an İmmuno-chromatographic assay, called the Capilia® TB-Neo Test (TAUNS, Numazu, Japan, and TAUNS Laboratories, Inc., Shizuoka, Japan), which is used to discriminate between members of the MTBC and mycobacteria other than tuberculosis strains (MOTTS) or other atypical mycobacteria (Ramos, Carvalho, Ribeiro, & Guimarães, 2016). The test cartridge (Plate 2,) comprises a sample pad, a reagent pad, a nitrocellulose membrane and an absorbent pad. When applied into the well, the sample (a suspension of harvested MTB isolates in an extraction buffer) travels through the membrane using capillary action. The test itself consists of a cartridge. The test principle is basically a double antigen sandwich type reaction where monoclonal antibodies raised in mice against the MPB64 antigens secreted by the MTBC are conjugated to colloidal gold particles. The antibody-colloidal gold complex, recognises an epitope on the MPB64 antigen (to which it has been raised in the host mice) in the test sample and binds to it. The resulting antibody-colloidal gold-MPB64 complex conjugate, further flows into the test zone where monoclonal antibodies have been immobilised on the membrane as a solid support. The monoclonal anti bodies here also recognise another

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epitope (to which the monoclonal antibodies have been raised) on the MPB64 antigen (already bound in the colloidal gold complex conjugate) and bind to it. A reddish purple colour is then imparted as a strip in approximately 15 minutes in the presence of the test antigens. The Capilia test offers a convenient and cost-effective platform for the accurate screening of cultured TB isolates in TB endemic regions.

The advantages in the application of the Capilia test include its sensitivity and specificity being comparable to nucleic acid probe-based methods, no specialised equipment is required, and it is fast and easy to perform. As such, it increasingly being applied in clinical practice (Hasegawa et al., 2002; Chikamatsu et al., 2014; Ramos et al., 2016).

Genotypic Identification of the MTBC

The complete genome of MTB is characterised by densely-packed coding regions, which together constitute 4.4 Mbp. It has a very high cytosine (C) and guanine (G) content. The coding regions are represented by approximately 4,000 protein-coding sequences (Cole et al., 1998). There are very few point-mutations in the MTB genome, which do not usually lead to amino acid sequence changes in the final protein product. Thus, the MTB genome is characterised by few silent mutations. Furthermore, recombinations in the MTB genome seem to be through transposons. These transposons are important since their simplest forms, called insertion sequences (ISs), form the basis for discrimination between the various strains of the MTBC. Insertion sequences are small mobile genetic sequences that

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encode only their capacity for transposition and regulation (Jagielski et al., 2016). Insertion of IS elements tends to cause gene disruptions and may lead to the alteration of the expression of adjacent genes. In the MTBC genome, a total of 29 different IS elements, belonging to 4 IS families, have been identified (Cole et al.). The strain, *Mycobacterium tuberculosis* H37Rv, harbours eight of these IS elements in more than a single copy, with three occurring in three copies (IS*1557*) or more (IS*1081* and IS*6110*) (Gordon & Supply, 2005).

The most extensively studied IS element is IS*6110* which is a member of the IS*3* family. The IS*6110* element was first described in *Enterobacteriaceae* (McAdam et al., 1990) and it is 1,355 bp long with imperfect 28-bp terminal inverted repeats (TIRs) at its ends and contains two partially overlapping open reading frames, *orfA* and *orfB*. These two open reading frames code for a transposase enzyme (McAdam et al. ; Thierry et al., 1990a; Thierry at al., 1990b). The insertion sequence, IS*6110,* is unique to members of the MTBC occurring in multiple copies in most of its members, with the exception of *M. bovis*, which may contain only one to five IS*6110* sequences. Generally, the copies of IS*6110* vary from 0 to 25; and it is scattered throughout the genome. The IS*6110* prefers genetic spots like the Direct Repeat (DR) locus, the phospholipase C gene (*plc*) region, the Pro-Pro-Glu (PPE) family genes, the *dnaA-dnaN* intergenic region, or other ISs. Due to its widespread distribution, IS*6110* has been exploited as a genetic marker to speciate MTB strains. Its presence has also been exploited in such diverse applications as diagnosing TB infections in mummified remains (Salo,

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There is a plethora of molecular biological techniques designed to discriminate between the various strains of the MTBC. Presently, only segments of the genome are sequenced to provide relevant genetic information about different isolates (Mostrom, Gordon, Sola, Ridell, & Rastogi, 2002). Molecular biology techniques based on the 16S rRNA gene and commercial test kits (all part of NAATs) have, to a very large extent, replaced conventional biochemical methods of identification of mycobacteria.

***16S rDNA PCR Assay for* IS*6110* (*Diagnostic* PCR *for the* MTBC)**

This is a test based on amplification of IS*6110* found in members of the MTBC but absent in other mycobacteria. The IS*6110* sequence is one of the more common targets for molecular-based diagnosis of MTBC. The test involves the amplification of a 208 bp fragment of IS*6110* (Kolk et al, 1992; Kox, van Leeuwen, Knijper, Jansen, & Kolk, 1995; Kolk, Kox, Vanleeuwen, Kuijper, & Jansen, 1998) using the primers pMyc14 - GRGRTACTCGAGTGGCGAAC (forward primer) and pMyc7 - GGCCGGCTACCCGTCGTC (reverse primer). This primer pair offers an accurate diagnostic PCR method for the MTBC because of its high sensitivity. The test is sensitive even on sample containing as little as 100fg MTB DNA (equivalent to 20 bacterial cells). There are other diagnostic MTBC primer sets

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such as the INS1F-INS2R and the Pt18F-INS2R primer sets (Kolk et al.; Kox et al.; Kolk et al.). The primer set, INS1F –CGTGAGGGCATCGAGGTGGC and INS2R –GCGTAGGCGTCGGTGACAAA amplifies a 245bp fragment of the IS*6110* locus and a 301bp fragment of the recombinant *M. smegmatis* strain 1008 DNA (with a modified IS*6110* sequence) usually employed as an internal control. Also, the primer set, Pt-18F – GAA CCG TGAGCGCA TCG AGG and INS2-R GCG TAG GCC TCG GT GACAAA, is also used in diagnostic PCR for the MTBC, which amplifies a 249 bp fragment of the 16S rDNA locus and a 305 bp control fragment from recombinant *M. smegmatis* strain 1008 DNA, also as an internal control (Kolk et al.; Wilson et al., 2003). In both cases the recombinant *M. smegmatis* strain 1008 used has its IS*6110* sequence modified by the insertion of a 56bp fragment to render it suitable to be employed as an internal control (Kox et al.).

Reversed Line Probe Assays

Line probe assays employ nitrocellulose membrane strips with genus-specific and species-specific oligonucleotide probes immobilized on them for the identification of mycobacteria strains from culture isolates. Usually, lysed DNA is of sufficient purity to be used as samples in line probe applications.

The mechanism involves lysed DNA, usually from colonies harvested from cultures, hybridising to the probe, which is complementary to the lysed DNA for a positive outcome. A positive result is indicated by a colour change imparted by the labelled oligonucleotide probe after appropriate treatment in a

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colour reaction. They are additionally termed 'reversed' line because of the oligonucleotide complimentality matching between probe and sample oligonucleotide.

Examples of line probes include the GenoType® DNA strip reversed line blot and GenoType MTBDR*plus*® tests (HAIN Lifescience GmbH, Nehren, Germany), the AccuProbe gene probe methodology (Gen-Probe, San Diego, Ca, USA), the Speed-Oligo Mycobacteria (Vircell, Granada, Spain) and the INNO-LiPA reversed line blot assay (formally Innogenetics BV, Ghent, Belgium, and now Fujeribio, Japan).

These reversed line blot assays target nucleotide differences within the 16S and/or 23S rRNA regions of the mycobacterial genome. The reversed line probe assays can also detect MTBC members as well as several commonly encountered NTM species. For example, the AccuProbe gene probe methodology, which also targets the 16S rRNA gene can be applied conveniently to assign mycobacterial species to the MTBC.

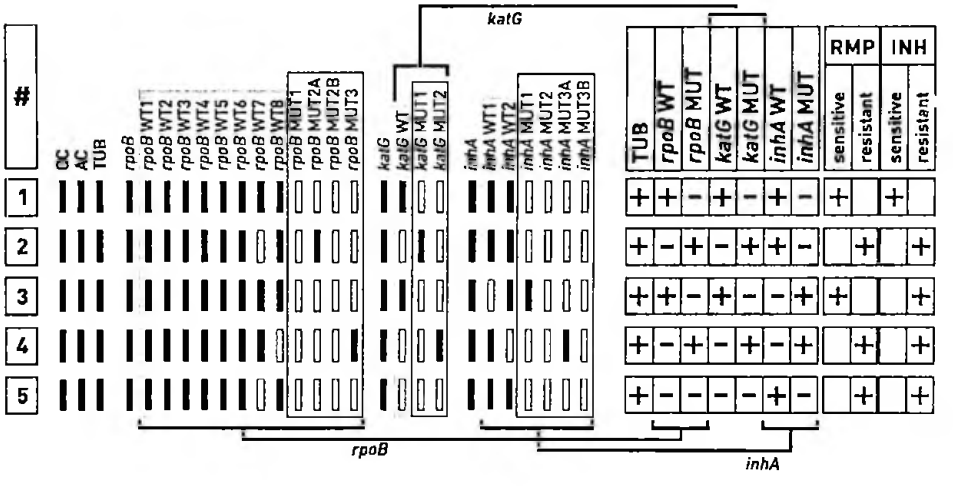

Figure 6: The GenoType® MTBDR*plus* strip test format

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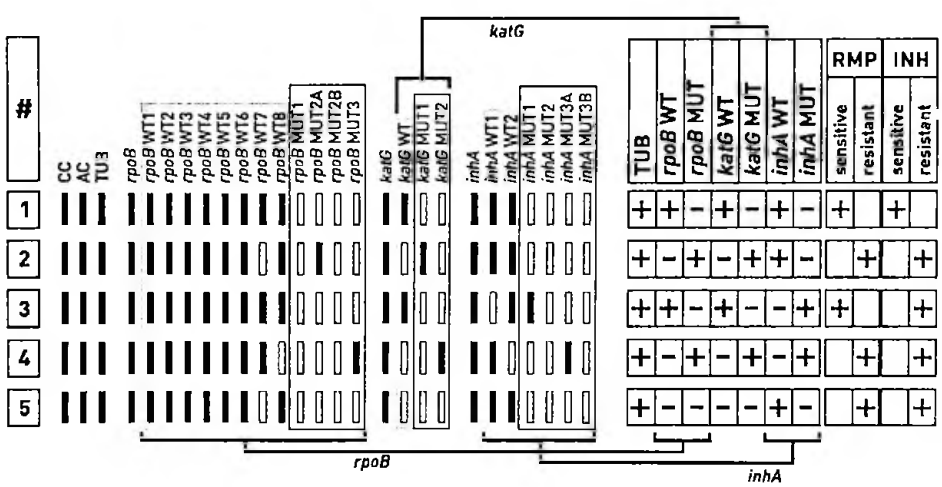

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Probes employed in the AccuProbe method do not, however, allow for the identification to the species level (Evans, Nakasone, Sutherland, de la Maza, & Peterson, 1992; Richter, Weizenegger, Farh, & Rusch-Gerdes, 2004).

The 'regions of difference' (RD's), have also been used to define genetic differences in the MTBC. For example, it has been found that 1-16 regions of difference (RD1-16) are absent in *Mycobacterium bovis* BCG when compared to *Mycobacterium tuberculosis* H37Rv (Behr et al., 1999). With advances in comparative genomics, the typing paradigm of the MTBC has been expanded to include new Large Sequence Polymorphisms (LSPs), and Single Nucleotide Polymorphisms (SNPs), for a more efficient and standard genetic analysis (Huard et al., 2006) of mycobacteria, in general, and the MTBC, in particular.

The GenoType® MTBC Test

The GenoType® MTBC reversed line blot test targets nucleotide differences within the 16S and/or 23S rRNA regions and, thus, is used in differentiating members of the MTBC. This assay also exploits polymorphisms in the *gyrB* gene and the RD1 deletion in *Mycobacterum bovis* BCG (Richter, Weizenegger, Farh, & Rusch-Gerdes, 2004).

The GenoType® MTBC Test is usually used in the identification of the various sub-species of the MTBC. The principle underlying the GenoType® MTBC Test is as follows:

The GenoType® MTBC Assay test kit includes nylon membrane strips onto which species-specific oligonucleotide probes have been immobilised as parallel lines. To carry out the test, DNA is usually isolated from lysed culture

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Streptavidin labelled with alkaline phosphatase is then added and this binds with any biotinylated hybrid formed with any specific probe (i.e. in the PCR product) on the reverse line blot. Incubation with 5-bromo-4-chloro-3-indolyl phosphate and nitro-tetrazolium (BCIP/NBT) chromogen then results in a purple coloration. The pattern of bands created is indicative of the species.

As a line probe assay, the turn-around time between sample and results is about 4 – 6 hours for the GenoType® MTBC Assay test. The test also returns both a high sensitivity and specificity i.e. >90% (Russo, Tortoli, & Menichella, 2006). A drawback is the reported instances of cross-reactivity particularly amongst some rare NTM's (Tortoli, Pecorari, Fabio, Messino, & Fabio, 2010).

Typically, the GenoType® MTBC assay (based on nucleotide differences within the 16S and/or 23S rRNA regions and also polymorphisms in the *gyrB* gene and the RD1 deletion in *M. bovis* BCG) is able to speciate or differentiate between the following MTBC members: *M. tuberculosis*, *M. bovis* (as *M. bovis* ssp. *bovis*), *M. caprae* (as *M. bovis* ssp. *caprae*), *M. bovis* BCG, *M. microti* and *M. africanum* I.

The INNO-LiPA Mycobacteria Assay

The INNO-LiPA® Mycobacteria v2 reversed line blot assay targets the DNA polymorphism in 16S-23S ribosomal RNA spacer region and differentiates other mycobacteria from the MTBC (Miller, Infante, & Cleary,

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The principle of the assay is based on reversed line blot hybridization, as follows: The INNO-LiPA® Mycobacteria v2 reversed line blot assay test kit includes nylon membrane strips onto which species-specific oligonucleotide probes have been immobilised as parallel lines. DNA is usually isolated from lysed culture harvests and used to carry out the test. A Multiplex PCR with biotinylated primers is then carried out to yield Biotinylated PCR products. These biotinylated PCR products are hybridized to specific oligonucleotide probes, which have been immobilised on a nitrocellulose membrane support as parallel lines.

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After hybridisation, streptavidin, labelled with alkaline phosphatase, is added and this binds with any biotinylated hybrid formed on the reversed line blot. Incubation of the strips with 5-bromo-4-chloro-3-indolyl phosphate and nitro-blue tetrazolium (BCIP/NBT) chromogen results in a purple coloration. The pattern of bands is compared to a key or standard strip with patterns indicative of the species.

The INNO-LiPA assay is particularly useful in diagnosing MOTT infections in immune-compromised patients for better management.

Multiplex Ligation-dependent Probe Amplification (MLPA) assay (Genetic Method for Strain Identification and Resistance testing)

Multiplex ligation-dependent probe amplification Assay (MLPA) is a multiplexed PCR-based assay which can be used to identify single-point polymorphisms (SNPs) through the amplification of sequence-specific uniquely designed probes rather than the amplification of target DNA (Erlandson et al, 2003; Hogervorst et al., 2003). The MLPA assay has been applied successfully in the screening of human DNA for certain rare genetic disorders (Erlandson; Hogervorst). Recently, MLPA has been applied successfully to characterise and also to determine resistance-associated genes amongst members of the MTBC (Bergval et. al., 2007; Beyene et al, 2009; Bergval et al., 2012a; Bergval et al., 2012b; Stucki et al., 2012; dos Santos et al., 2017). MLPA is robust, flexible and specific and can be applied to simultaneously genotype isolates and also detect resistance-associated

After hybridisation, streptavidin, labelled with alkaline phosphatase, is added and this binds with any biotinylated hybrid formed on the reversed line blot. Incubation of the strips with 5-bromo-4-chloro-3-indolyl phosphate and nitro-blue tetrazolium (BCIP/NBT) chromogen results in a purple coloration. The pattern of bands is compared to a key or standard strip with patterns indicative of the species.

The INNO-LiPA assay is particularly useful in diagnosing MOTT infections in immune-compromised patients for better management.

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mutations in isolates circulating in TB-endemic regions. In laboratories in resource-stretched countries, where drug-susceptibility testing is not common-place, MLPA can be adopted to test for the most commonly occurring mutations implicated in drug resistance in a particular geographical region.

MLPA Probe Selection

Probes for investigating Drug Resistance

For this study, the drug resistance markers (targeted by probes rpoB-522, rpoB-526G, rpob-526T, rpoB-531, rpoB-176, inhA-15, katG-315, and embB-306) were chosen on the basis that in a typical batch of clinical isolates 70-85% of RIF resistance, 65-80% of INH resistance and 45-65% of EMB resistance would be captured (Musser, 1995; Sreevatsan et al., 1997a; Telentti, 1997; Riska, Jacobs, & Alland, 2000; Ramaswamy et al., 2003). In the current study, all the probes chosen (to prepare the probe mix) targeted resistance-associated mutations except probe embB-306, which, intentionally, was chosen to target the wild-type sequence in isolates because codon 306, where the mutation is found, normally can harbour many different nucleotidic changes (Sreevatsan et al. ; Ramaswammy & Musser, 1998).

Based on a study of 842 MTB-complex isolates obtained from different geographical areas worldwide Sreevatsan and co-workers (Sreevatsan et al., 1997b) have classified members of the complex into three genetic groups according to the two most common polymorphisms occurring. These polymorphisms occur in the genes encoding catalase-peroxidase and the A subunit of the gyrase genes. Assignments to any one of the three groups is

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based on a combination of polymorphisms at *kat*G (codon 463) and *gyr*A (codon 95) as shown in the scheme below (Figure 7).

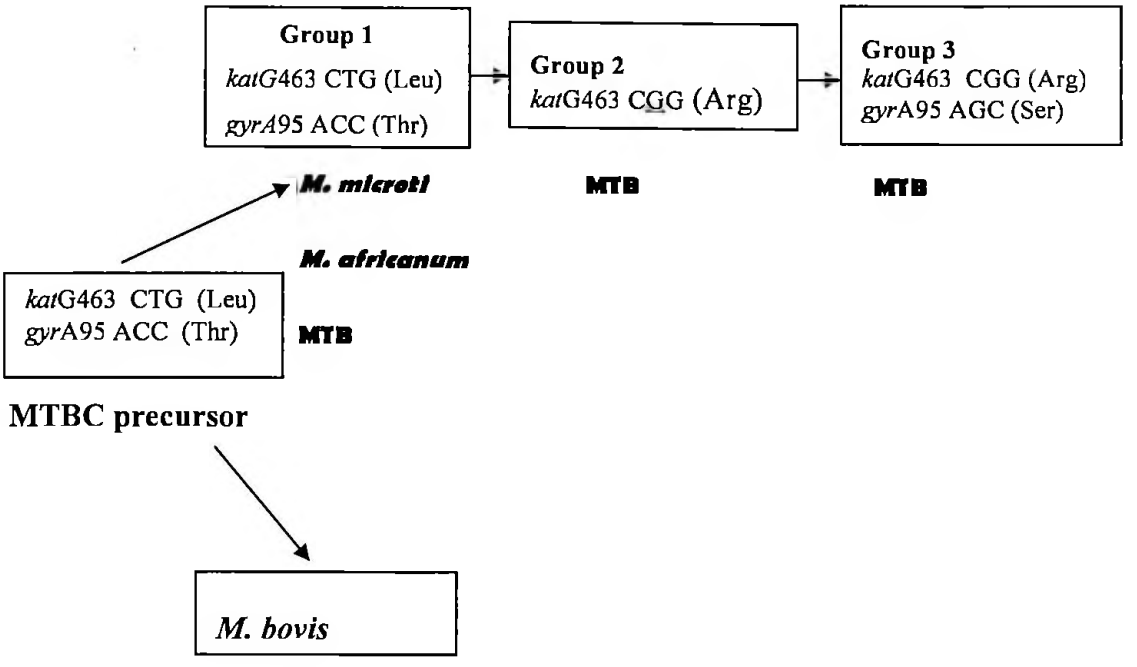

Figure 7: Scheme adopted from Sreevatsan et al., 1997, depicting classification of the MTBC into PGG's. (Legend: Group I – *kat*G codon 463 CTG (Leu) and *gyr*A codon 95 ACC (thr); Group II – *kat*G codon 463 CGG (Arg) and *gyr*A codon 95 ACC (Thr); Group III – *kat*G 463 CGC (Arg) and *gyr*A codon 95 AGC (Ser)

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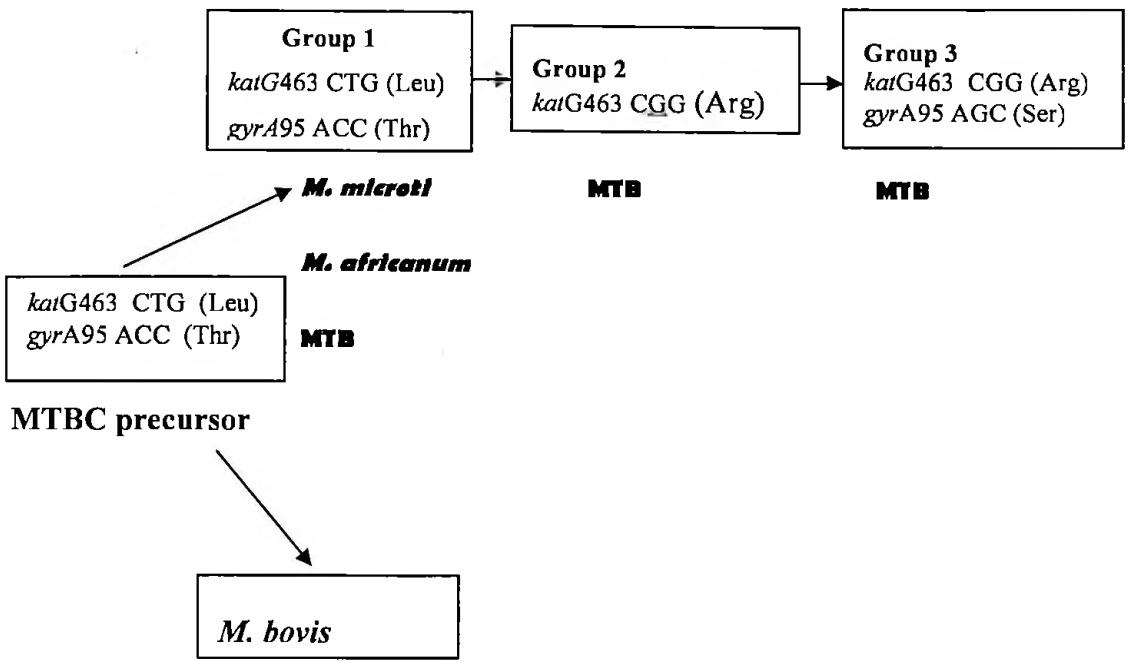

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The protocol of MLPA

The protocol of MLPA involves 3 key steps; (1) an overnight hybridisation step, (2) a ligation step and (3) a multiplex PCR step (Figure 8).

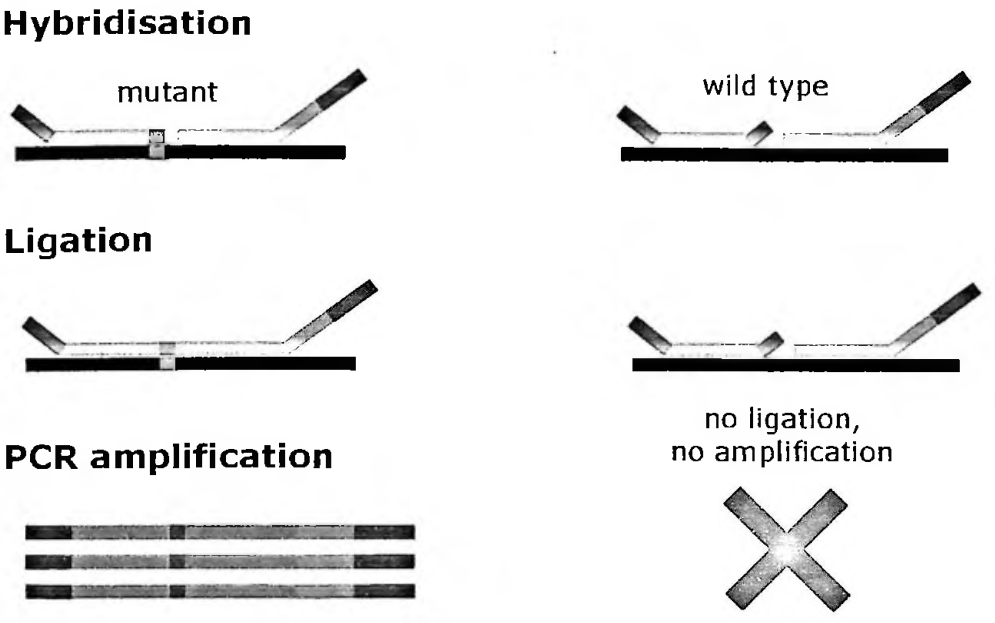

Figure 8: A Procedural Scheme of the MLPA Assay

Legend: The probe PCR mix consists of sequence-specific sequences (green), a primer sequence (blue) for the multiplex PCR, and a stuffer sequence (grey), of variable length designed to give a characteristic base-pair weight for the different SNPs (for analysis by gel electrophoresis or capillary electrophoresis). A SNP or mutation is depicted in red. Naturally-occurring unmutated or wild-type DNA is depicted entirely by black (right hand side) and therefore the SNP (red) is absent. Hence, the probe will not hybridise completely leading to a situation where ligation and amplification cannot take place.

Strain Identification

The existence of polymorphic DNA in the MBTC has spurred the discovery of many methods for the typing of circulating isolates for epidemiological purposes. The most widely applied method is based on the

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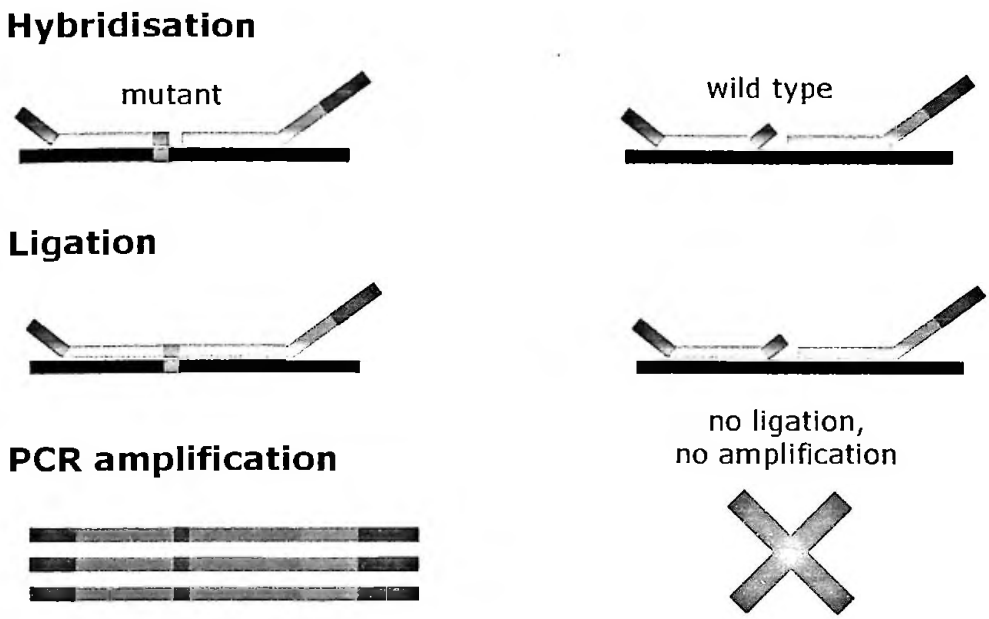

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The existence of polymorphic DNA in the MBTC has spurred the discovery of many methods for the typing of circulating isolates for epidemiological purposes. The most widely applied method is based on the

highly mobile insertion element IS*6110*, which is found, usually in multiple copies in the genome of the MBTC as discussed earlier.

Spoligotyping

Spacer oligonucleotide typing, commonly termed Spoligotyping, is a genotyping technique applied in the study of the epidemiology of the MBTC (Kamerbeek et al., 1997). Spoligotyping yields a fingerprint, usually called a spoligotype, and consists of a binary sequence of a length of 43 characters each of which denotes the presence or absence of spacers.

Spoligotyping exploits the observed polymorphism in the 'direct repeat' (DR) region, a structure that belongs to a family of repeats called clustered repetitive interspersed palindromic repeats (CRISPRs) found in bacterial and archaeal genomes (Pourcel Salvignol, & Vergnaud, 2005). The technique of Spoligotyping has gained widespread application in the epidemiological studies of the MBTC and has enabled the classification of isolates into distinct strains. It is, particularly, useful in transmission studies.

Certain eukaryotic species genomes contain these CRISPRs, which define a family of widely encountered repetitive DNA elements. These elements were first discovered in *Escherichia coli* (Ishino, Shinagawa, Makino, Amemura, & Nakata, 1987) but subsequently have been found in approximately 40% of bacteria and about 90% of archaea (Pourcel, Salvignol, & Vergnaud, 2005; Sorek, Kunin, & Hugenholtz , 2008; Makarova et al., 2011).

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However, the DNA polymorphism in the characteristic 'direct repeat' (DR) chromosomal locus of the MTBC genome can be exploited to offer another typing technique. This DR region has been extensively described after sequencing the DR in the tuberculosis vaccine, *M. bovis* BCG (Hermans et al., 1991). Sequencing results indicated that the DR region of *M. bovis* BCG consists of directly repeated sequences of 36 bp each, which are interrupted or interspersed with non-repetitive spacers each 35 to 41 bp in length. The sequencing data also revealed that *M. bovis* BCG had 49 copies of the DR and that the occurrence of the region is variable among other MTBC strains. Usually the DR region in members of the MTBC contains one or a number of the insertion element, IS*6110*. A comparison of these DRs for different strains revealed that the order of spacers in the DRs seemed to be the same and deletions and/or insertions also occur.

As opposed to DRs, spacers may usually be present once in a DR region, but sometimes may be two either separated by a single DR or several other DRs and their spacers. Any DR with its neighbour being a non-repetitive spacer is termed a 'Direct Variant Repeat' (DVR).

The mechanisms governing the occurrence of DRs and the spacers they contain have not been fully understood. The presence or absence of the 43 spacers in the DR locus can be determined by hydrising the amplified spacer region to a set of complimentary oligonucleotides immobilised onto a nylon support. These immobilised oligonucleotides represent each of the unique spacer DNA sequences, and the method of their determination is basically

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'**spacer oligonucleotide typing**' thus the acronymised as 'SPOLIGOTYPING' for this test (Kamerbeek et al., 1997).

The Principle and Mechanism of Spoligotyping

The spoligotyping technique involves the detection of the presence or absence of these 43 unique spacers in the DR of MTBC by a reverse line blot hybridization methodology (Kamerbeek et al., 1997).

Since the reverse primer is biotinylated, after hybridisation all the reverse strands (immobilized on the nylon support or membrane) also become biotinylated and, therefore, can be subjected to chemiluminiscence detection after appropriate treatment.

Obtained results can be presented in a binary mode, that is, present (1) or absent (0). These results can be easily digitised and interpreted and therefore lead to situations where inter-laboratory comparisons can be made (Dale et al., 2001). There are available major database platforms online for the query and analysis of spoligotyping patterns: the SpolDB4 (http://www.pasteur-guadeloupe.fr:8081/SITVITDemo/), the MIRU-VNTR*plus* (http://www.miru-vntrplus.org/MIRU/), SITVIT WEB – MTB Genotyping Worldwide Database Online Consultation (http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/) (Demay et al., 2012) and the TB-Insight – Tuberculosis Tracking and Control (http://www.tbinsight.cs.rpi.edu/) (Ozcaglar et al., 2011; Shabbeer et al., 2012a; Shabbeer et al., 2012b).

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Spolygotyping has gained wide-scale usage since its discovery because of its inherent advantages. These advanatages include the following:

1. Spoligotyping can be performed directly on clinical samples, and this offers an additional advantage of applicability to non-infectious sample matrices without the need for culturing mycobacteria;
2. Spoligotyping is extremely sensitive, requiring DNA material of the order of as little as 10fg (10^{-15}g) or the equivalence of DNA obtained from about 3 bacterial cells (Kulkarni, Sola, Filliol, Rastogi, & Kadival, 2005) to provide interpretable signals;
3. Spoligotyping can also be applied to non-viable cultures, paraffin-embedded sections and ZN smears;
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archaeological sources, such as skeletal or mummified remains (Baron, Hummel, & Herrmann, 1996; Donoghue, Spigelman, Zias, Gernaey-Child, & Minnikin, 1998; Zink et al., 2003)

5. It has been observed that fragments with a minimum continuous length of about 72bp can be analysed by spoligotyping;
6. Since the DR region of the MTBC, which is the region being analysed with Spoligotyping, is far more conserved than that being analysed by IS*6110*-RFLP typing, Spoligotyping can be applied to the identification of members of the MTBC to the species and sub-species level, since most *M. bovis* strains lack spacers 39-43 while all *M. bovis* BCG lack spacers in positions 3, 9 and 16; and *M. tuberculosis* strains lack spacers in positions 33 to 36 (Plykaytis et al., 1993). Also, for the Beijing family, spacers are lacking in all positions save the last 9 when applying the 43-spacer platform;
7. Spoligotyping is faster than IS*6110*-RFLP, with the time required to analyse a batch being less than 48 hours. It is also robust and reproducible, relying on a simple binary result format; and
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Thus, the full utility of Spoligotyping is best realised when it is used in tandem with MIRU-VNTR. Since its introduction, as an analytical tool, two automated variants of Spoligotyping have been proposed. The first, called Luminex, employs synthetic oligonucleotide probes immobilized on microspheres. Detection is through fluorophores attached to the spheres and hybridised PCR amplicons. The main advantage here is reproducibility due to the elimination of membrane hybridisation and manual reading of spoligotype patterns. Another advantage is the increased throughput as batches of 96 isolates each can be analysed in a single experimental run as opposed to the batches of 45 employed by the traditional system (Cowan, Diem, Brake, & Crawford, 2004; Zhang et al., 2010). The second variant employs a multiplexed-primer extension based on a Matrix-assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS). Here, spacers are subjected to multiplex PCR using primer extensions and the amplicons analysed by a mass spectrophotometer. This variant even provides a greater reproducibility and higher throughput, better ease-of-use and more efficient data analysis (Honish et al., 2010). However, the higher-than-normal price cost hinders its popular use.

Multi-locus Interspersed Repetitive Units-Variable Number Tandem Repeats (MIRU-VNTR) for Strain Identification

The existence of minisatellite sequences

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Frothingham and Meeker-O'Connel (1998) described the first VNTR-type loci detected on the MTBC genome. They comprised 11 elements, made up of 5 major polymorphic tandem repeats (MPTRs), namely, MPTR-A, MPTR-B, MPTR-C, MPTR-D and MPTR-E; and 6 exact tandem repeats (ETRs), namely, ETR-A, ETR-B, ETR-C, ETR-D, ETR-E and ETR-F. The MPTRs contain 10bp repeats separated by unique 5bp spacer sequences and have a widespread presence in mycobacteria ranging from the MTBC and atypical mycobacteria like *M. kansasii, M. szhulgai* and *M. gordonae* (Hermans, van Soolingen, & van Embden, 1992). On the other hand, the ETRs define and represent large tandem repeats, and they can vary in length from 53 to 79bp. So far, all 6 ETRs and only one MPTR locus (specifically MPTR-A) have been found to be polymorphic, through DNA sequencing, and therefore can be employed in a genotyping technique. However, of this lot, MPTR-A has been found to possess a low variability (MPTR-A has only 3 allelles) and ETR-F has two types of tandem repeats with different lengths. Thus, only 5 ETR loci (ETR-A to ETR-E) are used to genotype MTB.

The first MIRUs were described as tandem repeats of 46 to 101bp described as 41 loci in the genomes of the standard strains, CDC155, *M. tuberculosis* H37 Rv, and AF2122/97 (McHugh, Newport, & Gillespie, 1997;

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Mazars et al., 2001). Amongst the 41 loci, two, namely, VNTR0580 or locus 4 and VNTR3192 or locus 31, were later found to be the same as the ETRs designated as ETR-D and ETR-E (Frothingham & Meeker-O'Connel, 1998).

Presently, the MIRU-VNTR typing technique, based on the most polymorphic loci is used to speciate between different strains of the MTBC by measuring the number and length (or weight) of tandem repeats at each locus of each sample or strain (Supply et al., 2006). It has been observed that variability at each MIRU-VNTR typing locus depends on factors, such as geographical origin of sample and the sample strain's natural variability.

Generalised MIRU-VNTR protocol

Basically, the MIRU-VNTR protocol involves the PCR amplification of each locus by using specific primers complementary to the flanking regions (of the locus) and analysing the PCR amplicons through gel electrophoresis. The size of the amplicons indicates the number of tandem repeats which is determined by comparison to the known size of the repeat unit within each locus of interest. Gel electrophoresis analysis of amplicon fragments is generally fully automated by the use of Capillary Electrophoresis/Sequencing and multiplex PCR involving fluorescently-labelled primers.

It is now convenient to perform MIRU-VNTR experiments in which the PCR products are analysed by a DNA sequencer. The inclusion of a DNA sequencer means it is best to perform the initial PCR as multiplexes targeting 3 loci each. Thus, 8 PCR 'mixes' are prepared where each 'mix' targets 3 loci out of the 24 loci. For each multiplex PCR mix, one primer per

Mazars et al., 2001). Amongst the 41 loci, two, namely, VNTR0580 or locus 4 and VNTR3192 or locus 31, were later found to be the same as the ETRs designated as ETR-D and ETR-E (Frothingham & Meeker-O'Connel, 1998).

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oligonucleotide pair is tagged with a specific fluorescent dye. Work in this study involved the analysis of PCR products by a DNA sequencer.

The first runs of the MIRU-VNTR technique was based on a 12-loci format, but this had a low discriminatory power unless used together with IS*6110*-RFLP typing. Applying the 12-loci protocol to strains with a low copy number of the IS*6110* element improved the discriminatory power.

Presently the MIRU-VNTR protocol, based on a 24-loci (including the original 12-loci) format is used in most studies. The 24-loci format is more informative and thus, it finds a wide range of applications in infection and transmission studies and phylogenetics, with a comparable discriminatory power to that of IS*6110*-RFLP profiling (Oelemann et al., 2007). This has led to it being described as the new 'Gold Standard'. Table 2 depicts the various aliases for the 24 loci targeted by MIRU-VNTR. A drawback to the 24-loci MIRU-VNTR analysis is the high cost fueled by the use of several primers to study all the 24 loci. The high cost of the 24-loci MIRU-VNTR analysis may now be minimised by the use of a 15-loci (including 6 previously investigated loci) format, which has been found to cover approximately 96% of the polymorphisms detected in MTB strains (De Beer et al., 2014).

The results of MIRU-VNTR are expressed in a series of simple digits where each digit represents the number of tandem repeats at a particular locus. This simplified expression of result output gives the possibility for the comparison of results on web-based platforms, such as the MIRU-VNTR*plus* platform, http://www.miru-vntrplus.org/, (Allix-Béguec, Harmsen, Weniger, Supply, & Niemann, 2008; Weniger, Krawczyk, Supply, Niemann, &

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Harmsen, 2010) and the SITVITWEB database and the SpolDB4 (http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/), hosted by the Pasteur Institute, Guadaloupe (Demay et al., 2012; De Beer, Ködmön, van Ingen, Supply, & van Soolingen, 2014). The latter comprises of MIRU-VNTR and Spoligotyping data from over 150 countries. Also of significance is the TB-Insight website which provides the possibility to determine the major genetic lineages of MTBC isolates given its spoligotypes and optionally 24-loci MIRU-VNTR obtained through various methods.

MIRU-VNTR typing is fast, sensitive, reproducible and easy to perform and provides a high throughput. It exhibits a higher resolution than spoligotyping. Its utility is best appreciated when it is applied in tandem with spoligotyping. In this thesis, MIRU-VNTR typing method was employed, in addition to MLPA and Spoligotyping, to type all isolates in thematic areas defined.

Online analysis of spoligotype and MIRU-VNTR data for assignment of MTB isolates into lineages

The TB-Insight online platform

The TB-Insight website is an online platform which provides the possibility to determine the major genetic lineages of MTB isolates from available spoligotype (and optionally 24-loci MIRU-VNTR) data following a set of iterative rules.

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Table 2

Aliase Designations of the 24 loci Targeted by MIRU-VNTR

Loci by MIRU-VNTR convention	Alias 1	Alias 2
154	MIRU 02	
424	Mtub04	VNTR 42
577	ETR C	VNTR 43
580	MIRU 04	ETR D
802	MIRU 40	
960	MIRU 10	
1644	MIRU 16	
1955	Mtub21	
2059	MIRU 20	
2163b	QUB11b	
2165	ETR A	
2347	Mtub29	VNTR 46
2401	Mtub30	VNTR 47
2461	ETR B	VNTR 48
2531	MIRU 23	
2687	MIRU 24	
2996	MIRU 26	
3007	MIRU 27	QUB5
3171	Mtub34	VNTR 49
3192	MIRU 31	ETRE
3690	Mtub39	VNTR 52
4052	QUB26	MIRU 02
4156	QUB-4156	QUB4156c, VNTR 53
4348	MIRU 39	

Legend: ETR = Exact Tandem Repeat; QUB = Queen's University Belfast series

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2165	ETR A	
2347	Mtub29	VNTR 46
2401	Mtub30	VNTR 47
2461	ETR B	VNTR 48
2531	MIRU 23	
2687	MIRU 24	
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The platform provides the RULE and CBN methods to predict and categorise isolates into the major MTBC genetic lineages (Shabbeer, Ozcaglar, Yener, & Bennett, 2012).

The RULE method

The RULE method relies on a set of rules derived from experience-based knowledge about genetic lineage characteristics of the MTBC (Shabbeer, Ozcaglar, Yener, & Bennett, 2012). The following genetic lineages are predicted by the RULE method (Figure 9).

The RULE method uses a knowledge-based set of rules to classify isolates into specific strain groups or phylogenetic lineages, based on the presence or absence of spacer sequences in the spoligotype pattern and the values of the MIRU loci (Shabbeer Ozcaglar, Yener, & Bennett, 2012). The TB-Lineage platform basically requires data in the form of spoligotypes and can also use MIRU. It achieves this by exploiting the following features:

1. Deletion of contiguous spacers.
2. The value of, or the number of repeats observed at MIRU locus 24 (if MIRU 24< 2 then the isolate is assumed modern, else ancestral.) In the event that MIRU locus 24 is not available, then the TB-Lineage platform will, using a Bayesian network, estimate if the strain is modern or ancestral.
3. The set rules are evaluated sequentially until a determination on lineage is made by a rule.

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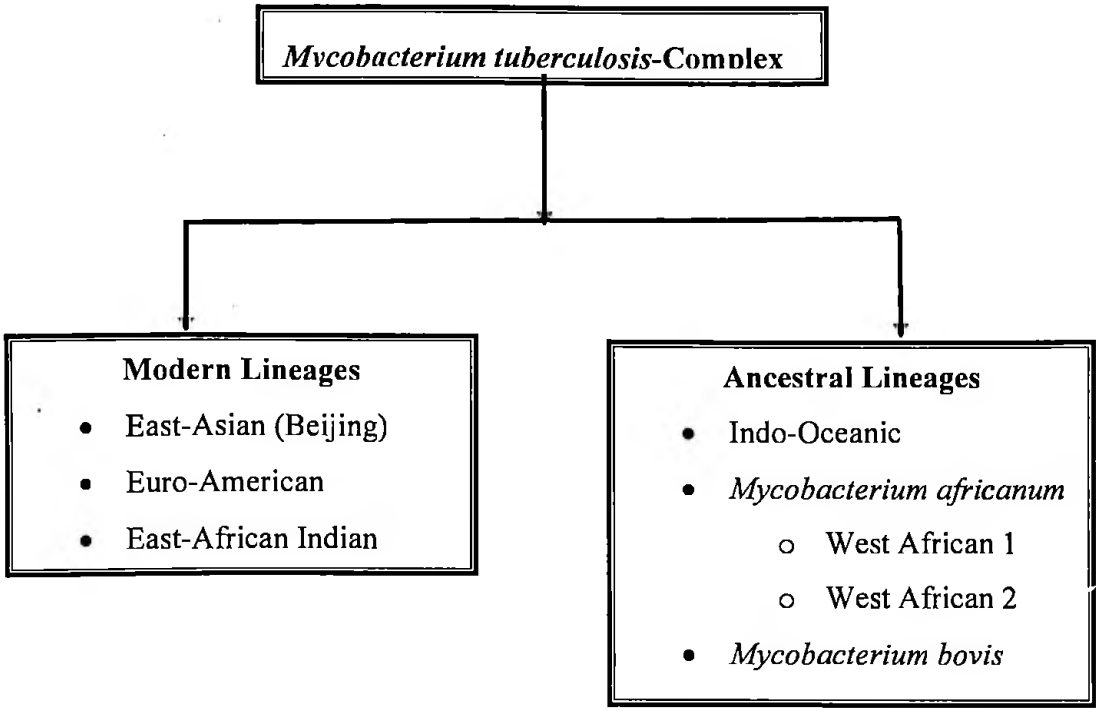

Figure 9: Categorisation by the TB-Insight platform into genetic lineages defined by the US Centre for Disease Control (CDC)

The CBN Method

In the CBN method, major lineages are predicted using a CONFORMAL BAYESIAN NETWORK protocol. CBN can predict major lineages using spoligotypes alone, MIRU alone, or both (Shabbeer, Ozcaglar, Yener, & Bennett, 2012). It predicts by:

1. employing a hierarchical Bayesian network that uses multiple biomarkers obtained from PCR-based typing methods, spoligotyping and MIRU typing.
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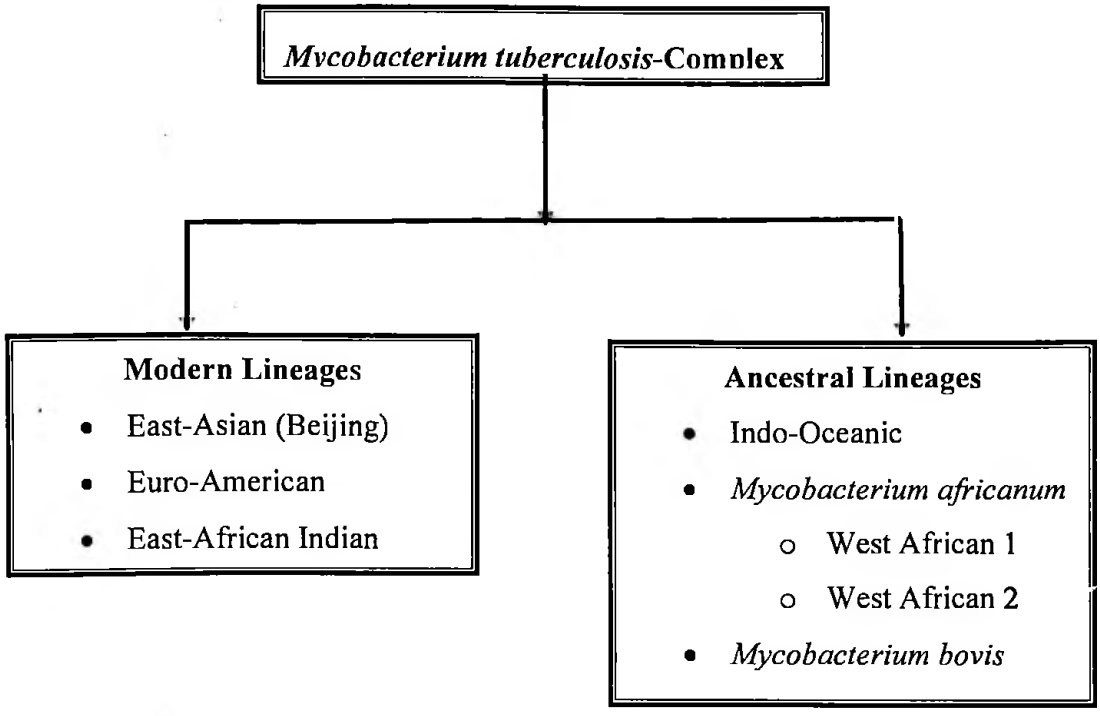

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3. making predictions using one or more types of biomarkers based on availability. A distinct disadvantage here is that the CBN method is unable to discriminate between the West African 1 and West African 2 clades of MAF. The CBN platform assumes *M. africanum* for both.

The KBBN method for SITVIT CLADE assignment

The TB-Insight platform applies a Knowledge Based Bayesian Network (KBBN) model to further delineate the MTBC based on the SITVIT phylogenetic information as defined by the Institut Pasteur, Guadaloupe. The KBBN model uses data to refine rule-based classifiers for incomplete or ambiguous rules (Aminian et al., 2014). The KBBN also serves as a predictive model for the 69 MTBC clades found in the SITVIT international collection. KBBN is a Probability-based model, which incorporates expert rules into Bayesian networks for classification of MTBC clades. The KBBN predictions are done using spoligotypes only and MIRU is not considered. The 69 SITVIT Clades that are predicted have been listed in Table 3.

The SITVIT WEB online platform

The SITVIT WEB online application (Demay et al., 2012) employs three molecular marker types to categorise the MTBC into phylogenetic lineages or groups. These molecular markers are Spoligotypes, VNTRs and MIRUs. The MIRU analysis provided by the SITVIT WEB online application is based on a 12-loci method, while five (5) Exact Tandem Repeats (ETR's) are used in the VNTR analysis of MTBC.

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Table 3
MTBC Clades found in the SITVIT Web portal, Istitut Pasteur, Guadaloupe

Clade	Clade	Clade
AFRI	H	PINI
AFRI_1	H1	PINI1
AFRI_2	H2	PINI2
AFRI_3	H3	S
Beijing	H3-Ural-1	T
BOV	H37Rv	T1-RUS2
BOV_1	H4-Ural-2	T2
BOV_2	LAM	T2-Uganda
BOV_3	LAM1	T3
BOV_4-Caprae	LAM11-ZWE	T3-ETH
Cameroon	LAM12-Madrid1	T3-OSA
Canetti	LAM2	T4
CAS	LAM3	T4-CEU1
CAS1-Delhi	LAM4	T5
CAS1-Kili	LAM5	T5-Madrid2
CAS2	LAM6	T5-RUS1
EAI	LAM8	T-Tuscany
EAI1-SOM	Manu_ancestor	Turkey

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EAI3-IND	Manu3	X3
EAI4-VNM	Microti	ZERO
EAI6-BGD1		
EAI7-BGD2		
EAI8-MGD		
EAI3-IND	Manu3	X3

Legend: These clades represent branches of the phylogeny of the MTBC and are each characterised as originating from a single common ancestor together with all their lineal progeny or descendants. Some of the clades are nested and can provide information on the evolutionary path of the MTBC.

The MIRUVNTRplus Online Platform

The MIRUVNTR*plus* online suite or platform provides another means of genotyping or assigning MTB strains to known lineages and/or clades by comparing strain patterns with those of 186 reference strains representing the major MTBC phylogenetic lineages (Allix-Béguec, Harmsen, Weniger, Supply, & Niemann, 2008;Weniger, Krawczyk, Supply, Niemann, & Harmsen, 2010).

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The service is publicly available at http://www.miru-vntrplus.org/ and its use is free. Comparisons during query can be based on Spoligotyping, MIRU-susceptibility-typing, RD, and SNP data, or by a combination of different data types. Tree dendrogram generation based on Unweighted Pair Group Method with Arithmetic Mean (UPGMA) or Neighbour-Joining (NJ) clustering algorithms is also provided by the service.

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CHAPTER THREE

MATERIALS AND METHODS

Thematic Study Areas

The design for this study covered the molecular characterisation of MTBC strains in circulation in three key thematic population niches in the Ghanaian population. These thematic areas are as follows:

1. the penal system of Ghana, that is, inmates of the penal system;
2. the non-institutionalised population niche of Ghana consisting of mainly patients (outside of prisons) presenting in Health Centres/Hospitals closest to a prison; and
3. the decedent population, defined by diagnosis of TB during routine autopsies.

Selection of population study areas for Themes 1 and 2

Assessment of diagnostic facilities in selected prisons and health centres/hospitals for tuberculosis

Diagnostic facilities in prisons and the hospitals closest in proximity to a prison were assessed in terms of their readiness to effectively control and manage TB. The assessment involved, among other things, cross-referencing TB Registers in Prison Infirmaries with those in hospitals to find out the frequency of a positive diagnosis of TB in prison inmates and the attendant error in positive diagnosis of inmates in the last 6 months prior to the

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commencement of the study. Cross-referencing also gave information about the frequency/prevalence and error in a correct positive diagnosis of TB infections in patients presenting at health centres or hospitals closest to penal facilities. The information garnered was used to determine the sample sizes for the three thematic areas of the study.

The assessment survey explored the feasibility of undertaking the study in the Ghanaian penal system to gain information that could be used to investigate the population structure of the MTBC in Ghana. The survey data were then used to identify sample collection points in selected or candidate prisons through:

1. the inspection and assessment of the suitability of existing infirmary and/or laboratory facilities as collection points for smear-positive sputum samples;
2. assessing internal (individual prisons) and institutional (the Prison Service) policy on TB diagnosis and management;
3. examining the problems associated with TB diagnosis and management in the penal system; and
4. the assessment of the general health facilities and the level of expertise available in each prison infirmary.

The relationship between prisons and/or prison infirmaries and Regional/District Hospitals for TB diagnosis and management was also appraised through inspection and cross-referencing of TB Laboratory Registers for the capture; and the assessment of the policy on newly-diagnosed and follow-up of inmates infected with TB.

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The following prison infirmaries and hospital laboratories were assessed (Table 4):

Table 4

Prison infirmaries and hospital laboratories assessed

Prison Infirmary	Hospital Laboratory
Akuse Prisons	Akuse Government Hospital
Ankaful Contagious Disease Prisons	Ankaful Leprosarium Laboratory
Ankaful Main Prisons	Ankaful Leprosarium
Ankaful Prisons Annex	Ankaful Psychiatric Hospital
Borstal	Koforidua Government Hospital
James Camp	Nsawam Medium Security Prisons Laboratory
James Fort	Nsawam Government Hospital
Koforidua Prisons	Winneba Government Hospital
Nsawam Medium Security Prisons	Ussher Polyclinic
Sunyani Prisons	Sunyani Regional Hospital
Tamale Prisons	Tamale Teaching Hospital
Winneba Prisons	Winneba Hospital
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James Fort	Nsawam Government Hospital
Koforidua Prisons	Winneba Government Hospital
Nsawam Medium Security Prisons	Ussher Polyclinic
Sunyani Prisons	Sunyani Regional Hospital
Tamale Prisons	Tamale Teaching Hospital
Winneba Prisons	Winneba Hospital
Yendi Prisons	Yendi Hospital

Selected Prisons (Theme 1) and Hospitals (Theme 2) after Assessment

A total of 10 prisons were selected as the sampling sites for Theme 1 while 14 main hospitals in close proximity to the prisons were initially selected (Table 5) as sample collection points for Theme 2. The regions from where prisons and hospitals were selected are shown in an Administrative Map of Ghana (Appendix A). However, a few satellite health centres also located within the districts in which selected prisons and hospitals were found were also included to make up the sample size for Theme 2.

Study Populations

The populations were prison inmates, patients presenting at hospitals or health centres, and decedents screened for TB during routine post-mortems at hospitals.

Prison inmates

The prison inmates comprised convicted and remanded inmates. Convicted inmates refer to inmates duly convicted by a competent Ghanaian Court of Law. Remanded inmates refer to inmates whose cases have not been determined to completion by any competent Ghanaian Court of Law. Remanded inmates could also have their cases in the process of being determined. All the prison inmates were male.

Inclusion criteria for prison inmates

Since prisons have been recognised to have a greater prevalence of TB than in the general population (Chaves et al., 1997; MacIntyre, Kendig, Kummer, Birago, & Graham,1997; Long Njoo, & Hershfield, 1999) and with

Selected Prisons (Theme 1) and Hospitals (Theme 2) after Assessment

A total of 10 prisons were selected as the sampling sites for Theme 1 while 14 main hospitals in close proximity to the prisons were initially selected (Table 5) as sample collection points for Theme 2. The regions from where prisons and hospitals were selected are shown in an Administrative Map of Ghana (Appendix A). However, a few satellite health centres also located within the districts in which selected prisons and hospitals were found were also included to make up the sample size for Theme 2.

Study Populations

The populations were prison inmates, patients presenting at hospitals or health centres, and decedents screened for TB during routine post-mortems at hospitals.

Prison inmates

The prison inmates comprised convicted and remanded inmates. Convicted inmates refer to inmates duly convicted by a competent Ghanaian Court of Law. Remanded inmates refer to inmates whose cases have not been determined to completion by any competent Ghanaian Court of Law. Remanded inmates could also have their cases in the process of being determined. All the prison inmates were male.

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the Ghana Prison Service transferring all known TB cases to its contagious diseases facility, CDP, at Ankaful in the Central Region, the defining criterion used was an active cough for at least a week in a prison.

Table 5

Selected Prisons and Hospitals

Region	Prison selected	Hospital
Greater Accra	James Fort	KBTH
	James Camp	Ridge Hospital
		La General Hospital
		Achimota Hospital
Central	Winneba	Winneba Hospital
	Cape Coast	Cape Coast Hospital
	Ankaful Main	Ankaful Leprosarium
		Ankaful Psychiatric
Ashanti	Kumasi Central	KATH
Eastern	Nsawam Medium Security	Nsawam Government Hospital
		Koforidua Regional
Brong Ahafo	Sunyani	Sunyani Regional
Northern	Tamale	TTH
	Yendi	Yendi Hospital

Inmates with an active cough lasting for at least a week and, particularly, in prison cells where TB had recently been diagnosed in an inmate (determined by cross-referencing TB Registers in the nearest District

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Inmates with an active cough lasting for at least a week and, particularly, in prison cells where TB had recently been diagnosed in an inmate (determined by cross-referencing TB Registers in the nearest District

Hospital with Prison TB Registers) were eligible and included. The contents of the 'Information Sheet' (Appendix B) and 'Certification of Verbal Informed Consent' (Appendix C) were explained and administered to all inmates who consented to be part of the study. Two sputum samples each, comprising a mid-night sample (sample 'A') and an 'on-the-spot' sample (sample 'B'), given in the presence of a health official, were obtained from the inmates using the 'Inmate Sputum Sample Collection and Biodata Form' (Appendix D).

Patients presenting to hospitals/health centres

Both male and female patients routinely presenting to the selected hospitals were recruited into the study.

Inclusion Criteria for Patients Presenting

The inclusion criteria for patients presenting were the standard Ghana NTP criteria for suspected TB patients visiting the OPD or Chest Clinic of the selected hospital. These standard criteria were persistent cough for at least 2 weeks, chest pain, fatigue, night sweats, fever, weight loss and loss of appetite. Two sputum samples each, comprising a mid-night sample (sample 'A') and an 'on-the-spot' sample (sample 'B'), given in the presence of a health official, were obtained from the patients using the 'Patient Sputum Collection and Clinical Data Form' (Appendix E). The contents of the 'Information Sheet' (Appendix B) and 'Certification of Verbal Informed Consent' (Appendix C) were explained and administered to all patients who consented to be part of the study. Ethical Clearance was sought and obtained from the

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Ethical Review Committee on Research Involving Human Subjects (ERCRIHS) of The Ghana Health Service, Ministry of Health (Appendix F).

Decedents Screened for TB during Routine Post-mortems

Samples were obtained from decedents (both male and female) during routine post-mortems at the Department of Pathology, Korle-Bu Teaching Hospital, Accra. Data was recorded on the 'PM Specimen Collection Form' (Appendix G).

Inclusion Criterion for Decedents

The main inclusion criterion was the selection of decedents who had not been diagnosed with TB prior to death. This was intentional from the stand-point of experimental design in order to diagnose TB at post-mortem.

Determination of Sample Sizes

The sample size was computed using Fisher's Exact Test. This statistical test method was chosen because the study, under all the outlined thematic areas, yields one of two possible outcomes, that is, TB present or TB absent and, can thus be assigned two possible categorical values. The possibility therefore exists for the definition of the relationship between these categorical values by the use of 2X2 Contingency Tables.

The Fisher Exact Test is represented by the following equation:

$$d = z\sqrt{p}\frac{(1 \quad p)}{n}$$

Where d = margin of error

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p = incidence or expected frequency of infection

n = number of samples or sample size

Sample size determination for prison inmates

For prison inmates, the incidence or expected frequency (p) was determined during the assessment survey of prison facilities and cross-referencing of prison TB registers and the hospital (closest proximity) TB data as a frequency of infection of 83.3% (19 positives out of 23 suspected cases – using coughing as the sole criterion). The margin of error (d) was determined as 5% (1 out of 19 positives failing to grow on culture). These gave a required sample size (n) of 214 coughing prison inmates as shown in Table 6.

Sample size determination for Patients presenting to Hospitals

For patients presenting to the selected hospitals, the incidence or expected frequency was also determined during the assessment and survey of each hospital (closest proximity to a selected prison) TB registers and data and cross-referencing them with TB registers, as a frequency of infection of 30.0% (that is, an average 61 positives out 326 presenting patients), and the margin of error was set at 5% (i.e. about 3 out of 61 positives failing to grow on culture). These gave a sample size of 323 presenting patients who were enrolled for the study (Table 6).

Sample size determination for Decedents for Routine Post-mortem diagnosis of TB

In obtaining the sample size for the number of decedents to be screened for TB in routine post-mortems, the 'Cause of Death' Records was

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cross-referenced with records at the Department of Pathology at the KBTH and the Pathologist co-investigator in this aspect of the study was also interviewed. The incidence or expected frequency of a positive diagnosis of TB in a cadaver (positive tuberculous lesion found in an infected organ) in routine post-mortems was determined as 143/3167 (i.e. 4.6%, from cross-referencing the records and data). The margin of error was set at 1.5% because, from experience, the pathologist had approximately 3 misclassifications for TB in 200 autopsies. This data yielded a sample size of 733 decedents to be screened for TB at post-mortem (Table 6).

Table 6

Determination of sample sizes for the population types

Population Type	Statistical Parameter			
	Z(at 95% CI)	Expected frequency (%) p	Margin of Error(%) d	Sample Size (n)
Prison Inmates	1.96	83.3	5	214
Patients Presenting	1.96	30	5	323
Decedents	1.96	4.6	1.5	733

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Age charateristics of population groups

Table 7

Age characteristics of 214 coughing prison inmates sampled

Inmate Category	Number (%)	Age Range/yrs	Median Age/yrs	Mean Age/yrs (s.d.)
Convicts	108 (50.5)	18 - 78	30.0	34.0 (8.8)
Remands	106 (49.5)	16 - 83	31.0	32.3 (7.6)
Total	214 (100)	16 - 83	30.0	30.6 (9.2)

Legend: Values in brackets are percentages in Column 2 and Standard Deviation values in Column 5; s.d. = standard deviation

Table 8

Demographic Characteristics of 323 sampled patients presenting at selected hospitals

Inmate Category	Number	Age Range/yrs	Median Age/yrs	Mean Age/yrs (s.d.)
Female	114	17 - 88	35.0	35.0 (6.8)
Male	209	15 - 87	33.0	36.3 (8.6)
Total	323	15 - 88	34.0	35.6 (9.2)

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Table 9

Age Characteristics of 733 cadavers sampled. Values in brackets are percentages

Sex of Cadaver	Number (%)	Age Range (Years)	Median Age (Years)	Mean Age±s.d.(Years)
Male	449 (61.1)	8 - 98	32.7	30.8 ±12.8
Females	284 (38.9)	7 - 97	34.2	33.6 ±9.6
Total	733 (100)	7 - 98	33.6	32.6 ±9.2

Sample Processing

Preparation of Loewenstein-Jensen medium

All Loewenstein-Jensen medium slants were prepared according to standard IUATLD/WHO protocol (Kent & Kubica, 1985). Eggs were procured from a familiar source where poultry are only fed with feedstuffs devoid of antibiotics. The working bench in a Bio-safety level P3 environment was cleaned and disinfected. A total of 23 eggs (to yield approximately 1L of egg-mass) were cleaned carefully with plain soap and water, and then soaked in 70% ethanol for 15 minutes and allowed to dry. Each egg was first cracked into a small sterile vessel in order to check its freshness and then transferred into the sterile blender bowl. Sufficient eggs were added to obtain the volume needed. The eggs were homogenised in a sterile blender (Kenwood, UK) into a mass. Salt solution was then added. The salt solution was made up of 2.4g

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anhydrous mono-potassium dihydrophosphate (KH_2PO_4), 0.24g Magnesium sulphate ($MgSO_4 \cdot 7H_2O$), 0.6g Magnesium citrate, and 3.6g L-Asparagine dissolved in double distilled pyrogen-free water and the volume topped up to 600mL with the same distilled water. A volume of glycerol (12mL) was then added to the mixture for the preparation of glycerol-impregnated slants to support the growth of classical MTB. A volume of 20mL 2% Malachite green solution was then added.

All components were gently mixed (to avoid bubbles) to homogeneity. The whole mass was then filtered through a sterile cotton gauze/dressing. The resulting mixture was then dispensed in 6 to 8-ml volumes into sterile 16 x 125 mm screw-capped tubes. The tubes were now placed on the racks so as to achieve an appropriate slope (about 20° angle) and then placed in an inspissator pre-set at 80°C for 45 minutes. The tubes were then removed from the inspissator, cooled, and labelled appropriately (batch, date and type). The screw-caps were then tightened, the tubes placed in plastic bags and stored in an upright position at 4°C (refrigerator) until retrieved for use. For a batch impregnated with pyruvate, 7.2g of Sodium pyruvate was used in place of the 12mL glycerol. Pyruvate-impregnated LJ medium encourages the growth of *M. bovis*.

Treatment of sputum samples

All sputum samples were obtained from coughing prison inmates and patients presenting to selected hospitals. Cetyl-pyridinium chloride, CPC, (containing 0.5% sodium chloride) was employed as both a transport medium

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and a decontaminant for all sputum samples because of the long periods of sample storage as a result of the long distances covered during sample collection (Smithwick, Stratigos & David, 1975; Philips & Kaplan., 1976; Selvakumar, Narayana, Suryanarayanan, & Umapathy, 1993; Pardini et al., 2005). Standard protocol was employed in the decontamination and culture of sputum samples. Sterile double-distilled pyrogen-free water was added to the 50-ml mark in each tube containing sputum in CPC and then centrifuged at 3000g for 15 minutes. Tubes were then left for 15 minutes to reduce aerosols, before decanting the supernatant. Sample sediments were then re-suspended in 1–2ml sterile distilled water and then inoculated (100µl) onto Löwenstein-Jensen (LJ) media slants in replicates – one glycerol-impregnated slant and the other pyruvate-impregnated slant. Tubes were then loosely screwed and then incubated at 37°C by laying them onto a slanting bed for 4 days before standing them upright on test-tube racks. Tubes were monitored daily for the first 7 days (for fast or rapid growers) and then, thereafter, weekly for eight weeks. Whenever growths occurred, they were carefully observed and the nature of colour and general morphology of the growths were recorded. All growths were harvested into approximately 0.5ml sterile double distilled water and also preserved in Middlebrook 7H9 broth (enriched with Oleic acid-albumin-dextrose-catalase, OADC) and then stored at -20°C until retrieved for analysis.

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Samples with suspicious tuberculous lesions diagnosed at post-mortem

Sample Acquisition and Initial Treatment

Samples were obtained from organs displaying lesions indicative of tuberculosis in the opinion of the Pathologist during routine autopsies at the Korle-Bu Teaching Hospital. Approximately 2 – 3g of any suspicious tuberculous specimen was excised into a sterile screw capped polypropylene container which was then stored under ice in an ice chest. The organ site was noted and recorded by the attending Technician. The ice chest was then sent to the laboratory and the samples stored at -20°C until retrieved for analysis.

In a laminar flow hood in a P2 facility, about 1– 2g of the lesioned sample was cut into a fresh sterile polypropylene vessel and 1mL of sterile Phosphate buffered saline, PBS, (pH 7.4) added. The sample was then carefully macerated and homogenised with a sterile scapula and plastic pestle, after which the supernatant was then transferred into a fresh 15mL Eppendorf tube and 10mL sterile PBS added, and the contents vortexed briefly. Using a disposable dropping pipette, 3mL of the suspension was aliquoted into a fresh 15mL Eppendorf tube to serve as starting material to be decontaminated using the NaOH-NALC method.

Decontamination, Microscopy, Primary Culture and Isolation of Samples obtained from Post-Mortem

All samples (i.e. each with 3mL starting sample) to be decontaminated were arranged on a rack with each tube 1 space apart to avoid contamination. An equal volume, 3mL, of decontaminating solution (3% Sodium hydroxide/0.5% N-Acetyl-L-Cysteine/1.45% Sodium citrate) was added to the

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sample, screw-capped tightly and vortexed for 15 - 30 seconds. It was then allowed to stand in the hood for 15 minutes.

At the end of the 15 minutes, 15mL of PBS was added to neutralise the suspension after which it was centrifuged at 3000xg for 15 minutes. Care was taken to ensure that samples were exposed to the NaOH during the decontamination process for not more than 20 minutes so that there was no 'over kill' of mycobacterial species in the suspension. The supernatants were then discarded and the formed pellet re-suspended in 2mL sterile phosphate buffer, pH 6.8, (by sucking and expelling buffer); after which, approximately 200-250µL of re-suspended pellet was each inoculated on 2 LJ slants (one incorporating glycerol and the other pyruvate). Finally, about 100µL of re-suspended pellet was smeared on a pre-cleaned labelled slide and the slide dried to be subjected to Ziehl-Neelsen staining. The slants were then incubated at 37°C and observed weekly for growth.

Microscopy, Primary Culture and Isolation of Sputum Samples

Ziehl-Neelsen (ZN) microscopy was performed on all sample sediments (products after decontamination) prior to inoculation onto LJ medium for acid-fastness. Ziehl-Neelsen microscopy was performed according to standard protocol (Lannette, Balows, Hauser Jr., & Shadomy, 1985) in a P2 laminar flow hood. Firstly, all labelled slides were cleaned with 70% ethanol, using sterile non-buff gauze dressing. Approximately 100 to 200µL of the harvested suspension was applied onto each slide to yield a circular zone (approximately 1 cm in diameter) and then heat-fixed using a hot

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plate. The slides were then flooded with carbol-fuchsin solution on a staining rack, to completely cover smeared areas while heating the slides from the under-side with a Bunsen flame (until vapour was visible but not to the point of boiling) and then left to stand for about 5 minutes. The slides were then rinsed with running sterile distilled water to rid them completely of the carbol-fuchsin solution. Thereafter, using a dropping pipette, the slides were decolourised completely with 20% sulphuric acid for 5 minutes, drained and then rinsed again until no traces of carbol-fuchsin was visible in the running water. The slides were then finally counter-stained with 0.3% Methylene blue, and then left to air-dry in a slanting position on a slide holder, before microscopic examination.

Confirmatory microscopy and sub-culture on Coletsos Medium

For purer colonies, all stored harvests were sub-cultured on Coletsos medium (bioMerieux Clinical Diagnostics, Marcy l'Etoile, France) as follows: a sterile plastic loop (0.5 cm diameter) was dipped into each stored harvest in Middlebrook 7H9 medium and used to streak the surface of the Coletsos slant. Slants were then incubated at 37°C in an atmosphere containing 5% carbon dioxide. Cultures were checked for their morphology and each harvested into 2mL of Middlebrook 7H9 medium. Sub-cultured harvests, realised from all positive growths and preserved in sterile distilled water, were again tested for acid-fastness using ZN-microscopy.

plate. The slides were then flooded with carbol-fuchsin solution on a staining rack, to completely cover smeared areas while heating the slides from the under-side with a Bunsen flame (until vapour was visible but not to the point of boiling) and then left to stand for about 5 minutes. The slides were then rinsed with running sterile distilled water to rid them completely of the carbol-fuchsin solution. Thereafter, using a dropping pipette, the slides were decolourised completely with 20% sulphuric acid for 5 minutes, drained and then rinsed again until no traces of carbol-fuchsin was visible in the running water. The slides were then finally counter-stained with 0.3% Methylene blue, and then left to air-dry in a slanting position on a slide holder, before microscopic examination.

Confirmatory microscopy and sub-culture on Coletsos Medium

For purer colonies, all stored harvests were sub-cultured on Coletsos medium (bioMerieux Clinical Diagnostics, Marcy l'Etoile, France) as follows: a sterile plastic loop (0.5 cm diameter) was dipped into each stored harvest in Middlebrook 7H9 medium and used to streak the surface of the Coletsos slant. Slants were then incubated at 37°C in an atmosphere containing 5% carbon dioxide. Cultures were checked for their morphology and each harvested into 2mL of Middlebrook 7H9 medium. Sub-cultured harvests, realised from all positive growths and preserved in sterile distilled water, were again tested for acid-fastness using ZN-microscopy.

Screening for the MTBC

Capilia TB-Neo Immunochromatographic Assay (ICA) for presence or absence of the protein, MPB64

All isolates obtained from primary culture of samples which were elicited from prison inmates, patients presenting to hospitals, and decedents diagnosed with TB at post mortem were subjected to confirmatory ZN microscopy and then further sub-cultured on Coletsos medium. The sub-cultures on Coletsos medium were subjected to the Capilia TB-Neo ICA test by following the manufacturer's instructions, with slight modifications, as follows:

A volume of 200µL each of the supplied extraction buffer was dispensed into appropriately labelled 0.5mL screw-capped tubes using an Eppendorf repeater pipette. A loop full (1mm diameter platinum micro-loop) each of grown sub-cultured colony was placed into a tube containing extraction buffer. The screw cap of each tube was then fixed firmly, and vortexed vigorously but briefly to obtain a uniform suspension. Each uniform suspension was then used as test material. An aliquot of 100µL of each suspension was pipetted into the sample hole in each cartridge. The reading area was then observed and recorded. A purple-red line in both the test area (T) and the control area (C) was recorded as a positive result while a purple-red line in the control area only was recorded a negative result.

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The 16S rDNA PCR assay (Diagnostic PCR for the MTBC)

All harvested isolates were screened for their MTBC status by subjecting them to the 16S rDNA PCR, which is diagnostic for members of the MTBC. The 16S rDNA PCR (using primers pMYCF-unlabelled and pMYCR) is designed based on the amplification sequences of in the presence of the insertion element IS*6110,* which is characteristic of ~~the~~ members of the MTBC. The presence of a 208 bp fragment indicates the presence of a member of the MTBC (Kox, van Leeuwen, Knijper, Jansen, & Kolk, 1995; Kolk, Kox, Vanleeuwen, Kuijper, & Jansen, 1998).

Isolated bacterial cells were lysed to release DNA by placing a loop full (5mm diameter) of grown colonies from the Coletsos slants into 200µL (in screw-capped 0.5 mL Eppendorf tubes) of sterile double distilled pyrogen-free water and heating on a heating block at 98°C for 30 minutes. The cellular debris was then spun down (3000rpm) in a microfuge (Eppendorf, Germany) and 180µL of the supernatant pipetted into fresh tubes. This was then used as DNA template. A PCR mixture was prepared as follows: The mixture comprised 4µL 25mM $MgCl_2$, 0.4µL dNTP mixture (containing 25mM each), 0.5µL of 10µM pMYCF forward primer 5'-GRGRTACTCGAGTGGCGAAC-3', 0.5µL of 10µM pMYCR reverse primer – 5'-GGCCGGCTACCCGTCGTC-3' (Kox et al., 1995), 5µL of 10X Gold Star Buffer; 0.3µL Gold Star Taq Polymerase, 38.3µL water (Baker) and 1µL DNA template. The total reaction volume was 50µL. PCR was programmed in 'hot start' mode as follows: 10 minutes at 95°C; then 34 cycles of (30 seconds at 95°C; 30 seconds at 55°C; 30 seconds at 72°C) and then finally held at

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72°C for 5 minutes. The success of the PCR was verified by the presence of a 208bp fragment after 1.5% agarose gel (Seakem, Lonza) electrophoresis in 1X TBE (pH 7.4) run at 80V for 30 minutes (EC135-90 Power Pack, E-C Apparatus Corporation, USA).

Phenotypic Resistance-testing of Isolates

DST using the Minimum Inhibitory Concentration Method (DST-MIC).

The MIC-DST methodology (Klingeren, Dessens-Kroon, van der Laan, Kremer, & van Soolingen, 2007), which is based on the principle of resistance of mycobacterial species to tuberculostatics on solid 7H10 agar in a 25-well plate format was carried out on all isolates.

Preparation of Mycobacterial Suspensions for inoculation

All primary MTB isolates (harvested from LJ slants) which were sub-cultured on Coletsos medium for purer isolates, and harvested into Middlebrook 7H9 broth (using $2mm^2$ sterile loops) were then confirmed as MTB or NTM by the Capilia TB-Neo ICA test.

In a Level II laboratory, and in a Laminar Flow safety cabinet, a suspension of $2\text{-}10x10^5$ cfu/mL was prepared in a stoppered bottle containing water and glass beads (2mm diameter). The suspensions were adjusted to have a density of approximately 1 McFarland unit (equivalent to 3 X 10^8CFU/mL) using an Oxoid™ Turbidometer (ThermoFisher Scientific, USA). Suspensions of atypical mycobacteria to be employed for quality control purposes were prepared to a density of approximately 0.2 McFarland

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units (approximately 6 X 10^7 CFU/mL). The stoppered bottles and their contents were then shaken on an orbital shaker for 20 minutes at 350 rpm.

Preparation of Middlebrook 7H10 medium

The Middlebrook 7H10 medium used in the 25-well plate format was prepared according to standard protocol (Klingeren, Dessens-Kroon, van der Laan, Kremer, & van Soolingen, 2007). Middlebrook 7H9 (Sigma-Aldrich, USA) was weighed (9.5g) into a 500-mL bottle and 450mL double distilled pyrogen-free water and 2.5mL glycerol (BDH, Poole, UK) added. The bottles were then placed in a water bath at 100°C until all the Middlebrook 7H9 media dissolved. The agar was then autoclaved (10 minutes at 121°C), cooled to 50°C in a water-bath and 50mL of pre-warned Oleic acid-Dextrose-Catalase (OADC) enrichment broth added to the contents of each autoclaved bottle. A pH of 6.6 ±0.2 was maintained at the point of solidification of the agar.

Preparation of Tuberculostatic Stock solutions

The Tuberculostatic stock solutions were prepared to standard concentrations (Table 10). All stock solutions were dissolved in double-distilled pyrogen-free water, unless otherwise indicated.

Preparation of Middlebrook 7H10 containing Tuberculostatics at standard concentrations.

All bottles were appropriately labelled and the correct volume of stock tuberculostatic added (Table 11). Middlebrook 7H10 containing OADC was then dispensed in 110mL volumes into each labelled bottle containing the tuberculostatic and the bottle placed on a heating block.

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Table 10

Stock solutions of Tuberculostatics

Tuberculostatic	Stock Concentration	Comment
Isoniazid (INH)	1 000 or 10 000mg/L	5 or 20mg/L final
Rifampicin (RIF)	1 000mg/L	Dissolved in DMSO
Ethambutol (EMB)	10,000mg/L	
Streptomycin (STR)	10,000mg/L	
Pyrazinamide (PYZ)	10,000mg/L	Warmed until 50°C
Amikacin (AMK)	1,000 or 10,000mg/L	5 or 20mg/L final
p-Amino salicylic acid,	1,000mg/L	

Preparation of the 25-well plates

The 25-well plates were prepared according to standard procedure (Klingeren, Dessens-Kroon, van der Laan, Kremer, & van Soolingen, 2007). All plates for a batch of isolates worked on were appropriately labelled (including an orientation mark to indicate the first well and the direction of reading for growths). All 25 bottles containing the prepared tuberculostatics in Middlebrook 7H10 medium were then loaded onto the WFA (Bosch, Germany) pumping machine (Klingeren et al.) following the SOP designed for the WFA pumping machine.

The WFA pumping machine was then calibrated to allow a pumping volume for one 25-well plate at a time, and then all plates in a batch filled with the Middlebrook 7H10 containing the first-line tuberculostatics at the calculated concentrations as shown in Column 2 of Table 11.

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Table 11

Serial concentrations of tuberculostatics as delivered in respective wells of each 25-well plate

Bottle code	Tuberculostatic concentration, µg/mL	µL/120mL	Working Solution (µg/mL)
Control series	Control		
AMK series	AMK, 5.0	600	1000
INH series	INH, 0.1	120	100
RIF series	RIF, 0.1	120	100
PAS	PAS, 1.0	120	1000
EMB series	EMB, 1.0	120	1000
STR series	STR, 1.0	120	1000

A layout of a typical 25-well plate containing Middlebrook 7H10 medium is depicted in Figure 10.

Inoculation of isolate suspensions onto wells of 25-well MIC-plate

This was carried out in Biosafety Level III environment and in a LamiBiohazard Safety Cabinet (CleanAir® Techniek b.v., The Netherlands). Each isolate, as a suspension, was inoculated in volumes of 10µL into each well except 'Contr 1/100'well, using a repeater pipette. The absolute turbidity of mycobacterial isolate suspension per well being approximately 2-10x10^3 CFU. For the suspension required for the 'Contr 1/100' well for each isolate, 10 µL was pipetted into a tube containing 990µL double-distilled pyrogen-free water. This was then swirled gently before being inoculated onto the 'Contr 1/100' well (Figure 10).

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INH **0.2**	INH 0.1	**AMK** **5.0**	Control 1/100	Control 1
RIF 0.2	RIF 0.1	INH 2.0	INH 1.0	INH 0.5
PAS **1.0**	RIF 5.0	RIF 2.0	**RIF** **1.0**	RIF 0.5
EMB 20.0	EMB 10.0	**EMB** **5.0**	EMB 2.0	EMB 1.0
STR 20.0	STR 10.0	**STR** **5.0**	STR 2.0	STR 1.0

***Figure 10*: Lay-out of a 25-well plate for the DST-MIC test**

Legend: Contr 1= Inoculum at density of McFarland 1; Contr 1/100 = 1/100 of Contr 1; AMK= Amikacin (to speciate atypical mycobacteria); INH=Isoniazid; RIF = Rifampicin; PAS = p-Amino salicylic acid (to speciate atypical mycobacteria); EMB = Ethambutol; STR = Streptomycin (concentrations are in µg/mL). All bold face concentraions are the break-point MIC values and gold-shaded cells indicate positions of break-point concentrations of the first-line drugs in the plate.

After inoculation, the lids were rotated 90° to allow for space between the lid and the plate for the evaporation of excess water vapour from the wells. They were then incubated at 35°C. After 48 hours, the lids were rotated back to normal position and then secured in place with adhesive tape and incubated further in the presence of sufficient humidity and approximately 5% carbon

INH **0.2**	INH 0.1	**AMK** **5.0**	Control 1/100	Control 1
RIF 0.2	RIF 0.1	INH 2.0	INH 1.0	INH 0.5
PAS **1.0**	RIF 5.0	RIF 2.0	**RIF** **1.0**	RIF 0.5
EMB 20.0	EMB 10.0	**EMB** **5.0**	EMB 2.0	EMB 1.0
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dioxide (made possible by a Carbon dioxide generator, Midi CO_2 Incubator, ThermoFisher Scientific®, USA). All plates were incubated and observed for growth for a maximum period of at least 28 days before being discarded.

Reading and Interpretation of growths.

The plates were checked within the first 3 days for contamination by other bacteria. The main reading was then carried out on the 5^{th}, 12^{th} and 19^{th} days. Most definitive readings were made on the 12^{th} day and beyond. The following points were considered in interpreting growths in the wells:

1. The 'Contr 1' well was always observed first. The rest of the wells in a plate were read only if there was growth in this well. Reading was postponed for a few more days if the initial growth was minimal.
2. Growth on the 'Contr 1/100' well was taken to represent 1% of mycobacteria observed to grow on the other wells. The test was repeated if no growth occurred even after waiting.
3. If no growth was observed except in well containing a break-point concentration of tuberculostatic, a comparison was made with the growth observed in the 'Contr 1/100' well where the break-point concentration (or MIC) was taken as that of the next well if the growths were approximately equal.

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4. In the case that growth in the break-point well was less than that in the 'Contr 1/100' well, the strain was considered sensitive at the assigned break-point concentration.
5. In the case that growth in the break-point well was more than that in the 'Contr 1/100' well, the concentration of the next well was considered as the MIC and the strain was considered as resistant.
6. In the case that growths were observed on the well labelled AMK (amikacin-impregnated well), their morphology was carefully assessed and the PAS (p-Amino salicylic acid-impregnated) well also observed for growths. Atypical mycobacteria are usually resistant to both AMK and PAS. Morphologically, MTB is usually rough whilst atypical mycobacteria are smooth.

The INNO-LiPA Mycobacteria Assay (Genotypic Speciation)

All samples categorised as atypical or NTM by the Capilia TB-Neo test or 16S rDNA PCR or the Hain MTBC GenoType test were screened using the INNO LiPA mycobacteria assay to further confirm them as truly atypical. The atypical mycobacteria identified by the Capilia TB-Neo ICA test lacked the protein, MBP64.

A total of 43 isolates out of the 54 cultures obtained from samples of sputum collected from coughing prison inmates were initially found to be acid-fast by ZN microscopy. Thirteen out of these 43 were confirmed as NTM

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DNA Isolation for the INNO-LiPA Mycobacteria assay

Milli Q water (100 µL) was pipetted into appropriately labelled 1.5-mL 'tight seal' micro centrifuge tubes. In a bio-safety hood, one small loop full of cells each was transferred to the respective tubes containing the 100µL MilliQ water. The bacterial suspensions were heat-killed at 98°C for 30 min on a heating block to lyse the bacterial cells. The lysates obtained were then centrifuged (3,000 x g) for 30 seconds.

Preparation of PCR Mix for and PCR of the INNO-LiPA Reversed Line Blot assay

The PCR reaction mix was prepared for all samples, including one blank and two extra samples as shown in Table 12. A final volume of 45 µL of PCR reaction mix was added to the PCR tubes. DNA lysate (5 µL) was added to the PCR reaction mix using a filter-plugged tip.

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Table 12

PCR mix for INNO LiPA PCR Assay

Constituent	Volume
mQ water	24.7 µL
Amplification buffer	10 µL
MYC primer solution	10 µL
Taq polymerase	0.3 µL
Total PCR mix volume	45.0µL

Milli Q water (5 µL) used in preparing the lysates was pipetted into the PCR mix in the blank tube as the negative control of the PCR. The tubes were vortexed gently to mix and then set onto a Hybaid thermal cycler for amplification. The PCR programme consisted of an initial 4 minutes at 95°C; followed by 35 cycles of (30 seconds at 95°C; 30 seconds at 62°C; 30 seconds at 72°C) and finally held at 4°C until removed and stored.

Visualization of the PCR products

A 50 ml 3% Nusieve 3:1 Agarose gel was prepared and Ethidium bromide added to the dissolved agarose to a final concentration of 0.5 µg/ml, swirled, poured and allowed to solidify. PCR product, 4 µL, and 1µL 5x DNA sample loading buffer were added and mixed by pipetting and then loaded into the wells. DNA marker VIII (Sigma-Aldrich, USA), 5 µL was applied in the first slot and the gel slab electrophoresed for 35 minutes at 100 V; after which a photograph of the gel on a UV transilluminator was taken and recorded. A positive result for atypical mycobacteria was indicated by a single fragment at

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Visualization of the PCR products

A 50 ml 3% Nusieve 3:1 Agarose gel was prepared and Ethidium bromide added to the dissolved agarose to a final concentration of 0.5 µg/ml, swirled, poured and allowed to solidify. PCR product, 4 µL, and 1µL 5x DNA sample loading buffer were added and mixed by pipetting and then loaded into the wells. DNA marker VIII (Sigma-Aldrich, USA), 5 µL was applied in the first slot and the gel slab electrophoresed for 35 minutes at 100 V; after which a photograph of the gel on a UV transilluminator was taken and recorded. A positive result for atypical mycobacteria was indicated by a single fragment at

approximately 480bp (García-Agudo, Jesús, Rodríguez-Iglesias, & García-Martos, 2011; Frothingham & Wilson, 1993).

INNO-LiPA Reversed Line Blot Analysis using the ProfiBLOT II Machine

The amplicons were subjected to the INNO-LiPA Reverse Line Blot Assay using the ProfiBLOT II machine (Tecan, Maennedorf, Switzerland) following the manufacturer's SOP and operating instructions. Briefly explained: The machine was set up together with supplied buffers pre-warmed to given temperatures per the manufacturer's instructions. For each sample, exactly 20 µL denaturation solution was pipetted into a separate groove of the 30-groove plastic tray. PCR product, 20 µl, was mixed with the denaturation solution for each sample by pipetting up and down. A reversed line blot strip was added at the top corner of the sample tray containing denaturation solution and PCR product with a pair of tweesers. The programme prompts displayed on the instrument's read-out were then followed and the tray inserted. After running to conclusion (after approximately 2 hours), the tray was removed from the machine and the strips left to dry in the tray to reveal the purple-brown coloration strips whenever there was hybridisation.

The dried reversed line blot strips were then pasted in a result log book and compared with the provided interpretation chart. Care was taken to make sure that bands were as strong as or stronger than the *Mycobacterium* genus band on the interpretation chart.

Interpretation of results of the INNO-LiPA Reversed Line Blots

For a correct interpretation of the results obtained, only those bands which were as strong as or stronger than that of the Universal Control (UC)

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Interpretation of results of the INNO-LiPA Reversed Line Blots

For a correct interpretation of the results obtained, only those bands which were as strong as or stronger than that of the Universal Control (UC)

were considered as successfully analysed samples. Samples were considered as NTM or atypical mycobacteria if only the bands for the Universal Control (UC) and the Conjugate Control (CC) were visible and the PCR product was also clearly visible on the gel. Samples were considered not to contain *Mycobacteria* when only the Conjugate Control (CC) band was visible and the PCR product was also clear on the gel. In situations where no PCR product was visible on the gel and no or only a weak signal of the *Mycobacterium* genus line was visible, the test was repeated with a new lysate.

Phosphorus-32 Dot Blot Hybridisation of the *rpoB* Gene for RIF Resistance-Testing (Genotypic Resistance-testing of Isolates)

Polymerase Chain Reaction (PCR) of the *rpoB* gene

Amplifications were carried out in a Bio-Rad Dyad® DNA Peltier Thermal Cycler (Bio-Rad Laboratories Inc., Ca, USA). Samples were subjected to PCR amplifications with the primers TR8 and TR9 for the *rpoB* gene codons 516, 526 and 531. Due to logistical contraints with the procurement of ^{32}P-δATP (Amersham Life Science, UK), only the isolates obtained from prison inmates and patients presenting at the selected hospitals were subjected to this test.

Preparation of samples for PCR of the rpoB gene

In a laminar flow microbiological safety hood, mycobacterial cultures on Coletsos slants were carefully harvested into 2ml polypropylene screw-capped tubes containing 1ml of sterile double distilled water. These were

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Preparation of samples for PCR of the rpoB gene

In a laminar flow microbiological safety hood, mycobacterial cultures on Coletsos slants were carefully harvested into 2ml polypropylene screw-capped tubes containing 1ml of sterile double distilled water. These were

heated at 98°C for 20 minutes to lyse bacterial cell walls and release DNA and also to destroy PCR-inhibiting proteases. Upon cooling at room temperature, a 100µl aliquot each was added to 400µl of sterile double-distilled water to provide the crude DNA template stock for PCR.

The PCR master 'mixes' were prepared per 100µl volume reaction mixture consisting of 10X PCR buffer (10µl), $MgCl_2$ (6µl to give a concentration of 2.25mM), dNTP's (8µl to give a final concentration of 0.20mM), *rpoB* forward primer (or TR8) 5'-TGCACGTCGCGGACCTCCA-3' and *rpoB* reverse primer (or TR9) 5'-TCGCCGCGATCAAGGAGT-3'(0.5µl each to give a final concentration of 0.20mM), *Taq* polymerase enzyme (0.3µl or 1.5 Units per reaction), water (69.7µl) and sample DNA (5µl) was added to the mix (95µl). Controls were *rpoB*516, *rpoB*526, *rpoB*531, H37Rv and water blank. Amplifications were carried out using the following parameters: 95°C for 15 minutes; 2 cycles comprising of 94°C for 1 minute and 72°C for 2 minutes; 94°C for 1 minute, then 2 cycles comprising of 71°C for 1 minute and 72°C for 1 minute; 94°C for 1 minute, then 2 cycles comprising of 70°C for 1 minute and 72°C for 1 minute; 94°C for 1 minute, then 40 cycles comprising of 69°C for 1 minute and 72°C for 1 minute; and then finally held at 72°C for 10 minutes.

The presence of PCR amplification product of 157bp was a positive indication (Victor et al., 1999) and all amplifications were confirmed on 1.5% agarose mini-gels incorporating 3µl Ethidium bromide per 60 ml of the molten gel in 1X TBE buffer (pH 8.3). Electrophoresis was run at 90V and 70mA for

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40 minutes. Gel slabs after electrophoresis were first observed on a Vilber-Lourmat UV-transilluminator before being photographed with an Alpha Digidoc® RT2 Gel analysis system.

Labelling and Dot Blot Hybridisation

PCR product (10µl) was heat-denatured at 95°C in 190µl Dot Blot buffer (0.4µl of 0.4M sodium hydroxide containing 25mmol EDTA), rapidly cooled on ice and loaded under suction onto a Hybond-N+ membrane pre-wet with 150µl of dot blot buffer per spot in a dot blot minifold apparatus. Fixation of the denatured DNA onto the Hybond-N+ membrane was achieved by baking at 80°C for 2 hours. Membrane was then pre-hybridised in a buffer (7.2mL 5X SSPE, 0.4mL Denhardt's solution and 0.4mL 10% SDS) at a temperature 10 °C below the annealing temperature of the oligonucleotide probe for at least 30 min.

Radioactive labelling of oligonucleotide probe

The oligonucleotide probe, *rpoB* 531-mu, (10µl or 50pmol) was mixed with T4-kinase (2µL or 20 Units), kinase 10X buffer (5µL), water (27µL) and 1µL (5µCi) ^{32}P-δATP (Amersham Life Science, UK), incubated (37°C for 30 minutes), and inactivated (72°C for 10 minutes) before placing into pre-hybridisation buffer containing membrane. This was hybridised for 60 minutes at 10°C below the annealing temperature of the oligonucleotide probe. The membrane was then first washed in 30ml 2XSSPE at room temperature for 30 min. A final washing was done in 30ml 0.5XSSPE at the annealing temperature for 10 minutes after which the membrane was then sealed in

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plastic, placed in a cassette with a film and stored at -71°C for subsequent autoradiography.

The GenoType® MTBDR*plus* Assay (Genotypic Resistance-testing)

All isolates, from prison inmates, patients presenting at hospitals and decedents at post-mortem, obtained from lysed sub-culture harvests, were also subjected to the GenoType MTBDR*plus*® assay (Hain Lifescience, Nehren, Germany) to test for resistance to Isoniazid and Rifampicin. The GenoType® MTBDR*plus* assay employed in this study is an improvement on the older version and has an enhanced sensitivity to detect a broader spectrum of mutations in the *rpoB* and *inhA* genes, particularly, in the regulatory region of the latter gene (Hillemann et al., 2007). The GenoType® MTBDR*plus* assay was performed according to the manufacturer's instructions.

DNA isolation for GenoType® MTBDR*plus* assay

Milli Q water, 100 µL, was pipetted into appropriately labelled 1.5 mL 'tight seal' microcentrifuge tubes. In a bio-safety hood, one small loop-full of bacterial cells each was transferred to the respective tubes containing 100 µL mQ water. The bacterial suspensions were heat-killed for 30 minutes at 98°C on a heating block. The lysates obtained were then centrifuged (3,000 x g) for 30 seconds.

Preparation of PCR Mix for and PCR of the GenoType® MTBDR*plus* Assay

The PCR reaction mix was prepared for all samples, including one blank and two extra samples as shown in Table 13.

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Table 13

PCR mix for the GenoType® MTBDRplus Assay

Constituent	Volume
PN Mix	38.0 µL
$MgCl_2$ (Hotstar Qiagen kit)	2 µL
Hotstar Taq buffer	5 µL
Hotstar Taq polymerase	0.1 µL
Total PCR mix volume	45.1 µL

Key: PN mix = Primer deoxy-nucleotide mix.

Reaction mix, 45 µL, was added to the PCR tubes. In the biosafety hood, 5 µL lysate in was added to the PCR reaction mix, by using a filter-plugged tip. Milli Q water, 5 µL, used in preparing the lysates were pipetted into the PCR mix in the blank tube as the negative control of the PCR. Tubes were then vortexed gently and then set onto a Hybaid thermal cycler, and PCR performed under the following parameters:

15 minutes at 95°C; then 10 cycles of (30 seconds at 95°C; 2minutes at 58°C) and then 20 cycles of (25 seconds at 95°C; 40 seconds at 53°C; 40 seconds at 70°C) finally held at 4°C until removed and stored for further analysis.

Visualization of the PCR products

A 50 ml 3% Nusieve 3:1 agarose gel was prepared and Ethidium bromide added to the dissolved agarose to a final concentration of 0.5 µg/mL, swirled, poured and allowed to set. PCR product, 4 µL, and 1µL 5x DNA sample loading buffer were added and mixed by pipetting and then loaded into the wells. DNA marker VIII (Boerhinger, Mannheim, Germany), 5 µL was applied in the first slot and the gel slab electrophoresed for 35 minutes at 100

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V. Finally, a photograph of the gel on a UV transilluminator was taken and recorded. A positive result was indicated by 2 or 3 bands on AGE. Thus PCR-positive samples were then subjected to the reversed line blot GenoType® MTBDR*plus* Assay.

GenoType® MTBDRplus assay using the ProfiBLOT II Machine.

The amplicons were subjected to the GenoType® MTBDR*plus* Assay (also a Reversed Line Blot Assay) using the ProfiBLOT II machine (Tecan, Maennedorf, Switzerland) following the manufacturer's SOP and operating instructions. This was carried out as described previously (page 137). The only difference here was that, a reversed line blot strip consisted of oligonucleotide probes defining the most frequent mutations associated with resistance to INH and RIF, instead of a strip probing for NTM.

The GenoType® MTBC Assay

All isolates, obtained from lysed sub-cultures, were subjected to the HAIN GenoType® MTBC assay (Hain Lifescience GmbH, Nehren, Germany). The GenoType® MTBC assay is also a reverse line blot hybridisation assay used in the identification of the various sub-species of the MTBC.

DNA Isolation for the GenoType® MTBC Assay

Isolate lysates used were obtained by harvesting a loop-full of MTBC sub-cultures in 1.5ml tight-seal microfuge tubes containing 100µl Milli Q water. The bacterial suspension was then heat-killed on a water-bath at 98°C for 30 minutes and the contents were then centrifuged (12,000rpm for 1 minute) to

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precipitate cellular debris. The supernatant was then used for the Multiplex Hain Genotype MTBC PCR.

Preparation of PCR Mix for the GenoType® MTBCAssay

The PCR mix was prepared according to the manufacturer's instructions as shown in Table 14.

Table 14

PCR mix for GenoType® MTBCAssay

Constituent	Volume
PN Mix (primer-dNTP mix + buffer)	38.0 µL
$MgCl_2$ (Hotstar Qiagen kit)	2 µL
Hotstar Taq buffer	5 µL
Hotstar Taq polymerase	0.2 µL
Total PCR mix volume	45.2µL

Key: PN mix = Primer deoxy-nucleotide mix.

Reaction mix, 45.2 µL each, was added to the PCR tubes. In the biosafety hood, 5 µL sample DNA lysate was added to the PCR reaction mix, by using a filter-plugged tip. Milli Q water, 5 µL, used in preparing the lysates was pipetted into the PCR mix in the blank tube as the negative control of the PCR. Tubes were then vortexed gently and then set onto a Hybaid thermal cycler, and PCR run under the following parameters:
15 minutes at 95°C; then 10 cycles of (30 seconds at 95°C; 2 minutes at 58°C) and then 20 cycles of (25 seconds at 95°C; 40 seconds at 53°C; 40 seconds at 70°C) finally held at 4°C until removed and stored for further analysis.

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Gel Electrophoresis was run using a 3% Nusieve gel (4.5g per 150ml 1XTBE.). A volume of 5µl of each PCR product was loaded directly in the respective wells. Molecular weight marker XIII, 5µl, (Promega Corporation, IL, USA.) was also loaded and the gel made to run at 120V, 120mA for 40 minutes. Two close bands indicated the presence of members of the MTBC, a single bands indicated non-tuberculous mycobacteria (NTM) or atypical mycobacteria while three bands indicated the presence of *Mycobacterium bovis* BCG.

Further manufacturer's instructions were followed in order to hybridise and detect bound probes using the ProfiBLOTT II machine (Tecan, Maennedorf, Switzerland). The ProfiBLOTT II machine also offers automated incubation, washing and shaking steps. This step was carried out as described previously (page 137). The only differences here were that a reversed line blot strip consisted of 13 oligonucleotide probes with the different genetic mutations defining most members of the MTBC and other mycobacteria instead of a strip probing for NTM and also 40µL of denaturation solution were used instead of 20µL.

Multiplex Ligation-dependent Probe Amplification (MLPA) assay of Mycobacterial Isolates

Multiplex ligation-dependent probe amplification assay (MLPA) is a multiplexed PCR-based assay, which can be used to identify single nucleotide polymorphisms (SNPs) through the amplification of sequence-specific

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Multiplex ligation-dependent probe amplification assay (MLPA) is a multiplexed PCR-based assay, which can be used to identify single nucleotide polymorphisms (SNPs) through the amplification of sequence-specific

uniquely designed probes rather than the amplification of target DNA (Schouten et al., 2002; Bergval et al., 2007).

DNA Isolation from Culture slants for MLPA

Pure mycobacterial colonies originally harvested from LJ media and stored in Middlebrook 7H9 (Difco, Becton Dickinson, Sparks, MD, USA) were sub-cultured on Coletsos medium (bioMérieux, Marcy L'Etoile, France). A loop-full each of sub-cultured mycobacteria from the Coletsos slants was harvested into 150µl lysis buffer of 10mM TRIS-HCl pH8 (Sigma-Aldrich, USA) with 1mM EDTA (Sigma, USA) and 1% Triton X-100 (BDH Laboratory Supplies, Poole, England) and then vortexed briefly for 10 seconds. Cells were lysed (95°C for 30 min), vortexed again (10 s) and then spun down at 5,000 x g for 3 minutes. Subsequently, 135µl of the supernatant was removed and incubated on a heating block at 37°C for 30 minutes after the addition of 2µl of 5mg/ml DNase-free RNase (Roche Diagnostics, Mannheim, Germany). The samples obtained were then stored at 4°C until required for the MLPA PCR assay and Capillary electrophoresis/sequencing.

Oligonucleotide Probes used in the MLPA

The probes were designed to capture 23 discriminatory genetic loci or markers on the MTB genome (Table 15). The genetic markers targeted by the probes give information about resistance to anti-TB drugs, principal genotypic groups (PGGs) and mycobacterial species-type (Schouten et al., 2002) (These probes were manufactured by MRC-Holland (Amsterdam, The Netherlands).

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Table 15

All the Oligonucleotide Probes constituting the MLPA 'Probe Mix'

Probe	Length of probe (bp)	Target/Information
embB-306	142	EMB resistance marker, Wild-type
katG-315	160	INH resistance marker
inhA-15	178	INH resistance marker
16S rRNA	202	16S ribosomal RNA gene, MTB-complex specific
rpoB-176	229	RIF resistance marker
rpoB-531	256	RIF resistance marker
rpoB-526G	265	RIF resistance marker
rpoB-526T	274	RIF resistance marker
rpoB-522	283	RIF resistance marker
IS6110	301	Insertion Element IS*6110*, MTB-complex specific
gyrA-668	310	Putative genotype marker, specific for PGG 1 and 2
katG-463	319	Genotype marker, specific for PGG 2 and 3
gyrA-95	328	Genotype marker, specific for PGG 1 and 2
gyrA-90	337	FQ resistance marker
gyrA-94	346	FQ resistance marker
mutT2-58	355	Genotype marker, specific for Beijing 2
ahpC-46	364	Genotype marker, specific for CAS1 spoligotype
ogt-12	373	Genotype marker, specific for Beijing 2
ogt-15	382	Genotype marker, specific for Haarlem
ogt-37	391	Genotype marker, specific for Beijing 3 and 4
Ag-85C	409	Genotype marker, specific for LAM RD RIO spoligotype
TbD1	418	Absent in modern MTB strains
RD9	430	Genotype marker, only present in modern *M. tuberculosis*

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A different probe targets a mutation in the sample DNA with a variable 'stuffer' to give it a different length, hence, a different weight so that PCR products can be analysed by gel electrophoresis (Schouten et al.). In order that the products could be analysed by capillary electrophoresis the primer set was labelled with 6-carboxyfluorescein.

Probes for MLPA Genotyping

The case for the Principal Genotypic Groups (PGGs)

For efficient discrimination of genotypes to be encountered in sample populations in the various themes of this study, genotypic markers *gyrA* (codon 95) and *katG* (codon 463) were included because of their ability to categorise isolates broadly on the basis of the three Principal Genotypic Groups, PGGs, *viz.* PGG 1, PGG 2 and PGG 3 (Sreevatsan et al., 1997; Donoghue et al., 2004; Kong et al., 2007). The categorisation into PGG's is possible because of the nature of the SNPs at *katG* (codon 463) and *gyrA* (codon 95).

Probes for Putative Virulent Strains

To help identify strains reputed to be virulent, such as the Haarlem and the Beijing lineages, genes like the *ogt* codon 15 (for speciating the Haarlem lineage) and *mutT2* codon 58, *mutT4* codon 48, *ogt* codon 12, and *ogt* codons 37 (for speciating the Beijing lineage) were included in the probe 'mix' (Rad et al., 2003). For an exhaustive definition of the MTBC as a distinct group, probes targeting the 16S rRNA gene and IS*6110*, a sequence exploited for the typing of MTB through restriction fragment length polymorphism (RFLP)

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analysis (van Embden et al., 1993), were included to serve as internal controls and to speciate the MTBC.

Probes for 'Ancient' and 'Modern' Lineages

In order to categorise isolates on the basis of 'modern' and 'ancient' TB, a probe targeting the TbD1 region on the genome of 'ancient' TB was included. Usually, the TbD1 region is absent on the genome of 'modern' TB or classical TB. Here, all the other members of the MTBC are considered 'ancient' TB (Brosch et al., 2002; Baker, Brown, Maiden, & Drobniewski, 2004).

MLPA Analysis

MLPA was performed on all samples following the standard protocol, albeit with slight modifications, developed by MRC-Holland, Amsterdam, The Netherlands (Schouten et al., 2002; Bergval et al., 2007). The MLPA protocol involved three key steps; *viz.* an overnight hybridisation step, a ligation step and a multiplex PCR step.

Step 1: Hybridisation

RNase-treated (Promega, USA) DNA lysate template (2µl) was added to 3µl double-distilled water, melted at 98°C for 15 min, instead of 10 min (Bergval et al., 2007) or 5 min (Schouten et al., 2002) applied in the past. Melting was carried out in a PTC-100 thermal cycler (Bio-Rad Laboratories Inc., Ca., USA). Mycobacterial DNA has a high GC content and it has been observed that increasing the melting time leads to an increased signal strength and hence the sensitivity of the assay (Bergval et al.). The advantages of extension of 'melting' have been observed in methylation MLPA (Nygren et

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al., 2005). A volume of 3µl of the MLPA HYBRIDISATION mix, prepared by adding 1.5µL probe mix to 1.5µL MLPA buffer supplied, was added to each melted sample mixture. The mixture was hybridised overnight for at least 12 hours at 60°C in a PTC-100 Peltier Thermal cycler (MJ Research, USA).

Step 2: Ligation

Ligation was effected by adding 32µL of'Ligation mix', (a mixture of 3µL each of the supplied Ligase-65 buffer A and Ligase-65 buffer B, water (25µL) and the ligation enzyme Ligase-65, (1µL) to the hybridisation product (8µL) after over-night hybridisation. Total volume per sample now was 40µL. Ligation was then made to proceed for 15 minutes at 54°C. The ligase was then inactivated by heating at 98°C for 5 minutes.

The **'LIGATION Mix'** was prepared as follows for N samples:

	1 sample	(N+8*) samples
Ligase-65 buffer A	3µl	(N+8) x 3µl
Ligase-65 buffer B	3µl	(N+8) x 3µl
Water	25 µl	(N+8) x 25µl
Then Vortexed/mix well:		
Ligase-65 enzyme	1µl	(N+8)x1 µl
	32µl	e.g. vol. V

(* = Correction for 8-Channel Multi-pipette used)

Step 3: Multiplex PCR

Thereafter, the ligase-65 enzyme was inactivated for 5 minutes at 95°C. The ligated products (10µl each) were added to each 40µl PCR reaction mix comprising 30 µl of MLPA PCR Mix (4 µl 10X SALSA PCR buffer, 26

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µl water), 10 µl of POLYMERASE Mix (MLPA PCR primers (2µL), MLPA PCR dilution buffer (2 µL), water (5.5µL), and Taq polymerase enzyme (0.5µL). One of the primers was labelled with 6-carboxyfluorescein so that Fragment Analysis or Capillary Electrophoresis could be performed to determine sizes picked by the probes. This was then amplified (35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute, and followed, finally, by an extension at 72°C for 20 minutes) for which only 1 primer pair was required. The characteristic feature in MLPA analysis, depending on the nature of probes and their targets, is that only successfully hybridised and ligated probes are amplified (but not target DNA) during the PCR step and this allows mutants or wild-types to be identified (Figure 8, Chapter 2). Each mutation or SNP is targeted by a probe characterised by a different 'stuffer' length (Figure 8). Thus, the PCR fragment products can be separated and identified by ordinary gel electrophoresis or Capillary Electrophoresis (Schouten et al., 2002; Anthony et al., 2005). The PCR fragment products were confirmed and analysed by 2% agarose gel electrophoresis before being subjected to Cycle Sequencing and Capillary Electrophoresis.

Cycle Sequencing of Targeted Loci

All the genes targeted by probes in the probe 'mix' after the multiplex PCR were sequenced to confirm the sequence specificity of the MLPA assay. The primers used to amplify these genetic loci prior to sequencing are depicted in Table 16.

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Primer	Sequence	Product size (bp)	Locus
katG315 FW	5'-CATGAACGACGTCGAAACAG-3'	233	*katG* codon 315
katG315 RV	5'-CGAGGAAACTGTTGTCCCAT-3'		
katG463 FW	5'-TCCCGTTGCGAGATACCTT-3'	300	*katG* codon 463
katG463 RV	5'-AGGGTGCGAATGACCTTG-3'		
embB306 FW	5'-CTCCTCCTCAGGCCGTTC-3'	293	*embB* codon 306
embB306 RV	5'-AGACTGGCGTCGCTGACAT-3'		
gyrA95 FW	5'-GGTGCTCTATGCAATGTTC-3'	236	*gyrA* codon 95
gyrA95 RV	5'- GGGCTTCGGTGTACCTCAT-3'		
inhA15 FW	5-CGAAGTGTGCTGAGTCACACCG-3'	203	*inhA* regulatory region _15
inhA15 RV	5'-TCCGGTAACCAGGACTGAAC-3'		
ogt FW	5'-GAAGATCGCATGATTCACTAC-3'	234	*ogt* codons 12, 15, and 37
ogt RV	5'-GTCGGTTCCCCGGAGGTCAAG-3'		
mutT2 FW	5'-GAACTTCCCGGCGGTAAGGTC-3'	149	*mutT2* codon 58
mutT2 RV	5'-AGCGTCGTCGTGCCGTTCAAC-3'		
mutT4 FW	5'-GAATCACATGGACGCCCAACC-3'	132	*mutT4* codon 48
mutT4 RV	5'-AACCCTCCAGCCGATGTTTCG-3'		
rpoB 2F	5'-CCCAGGACGTSGAGGCSATCAC-3'	537	*rpoB* codons 522, 526, and 531
rpoB 2R	5'-GGCGSGGYGASACGTCCATGTA-3'		
rpoB 7F	5'-CTTCTCCGGGTCGATGTCGTTG-3'	365	*rpoB* codon 176
rpoB 7R	5'-CGCGCTTGTCGACGTCAAACTC-3'		

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embB306 FW	5'-CTCCTCCTCAGGCCGTTC-3'	293	*embB* codon 306
embB306 RV	5'-AGACTGGCGTCGCTGACAT-3'		
gyrA95 FW	5'-GGTGCTCTATGCAATGTTC-3'	236	*gyrA* codon 95
gyrA95 RV	5'- GGGCTTCGGTGTACCTCAT-3'		
inhA15 FW	5-CGAAGTGTGCTGAGTCACACCG-3'	203	*inhA* regulatory region _15
inhA15 RV	5'-TCCGGTAACCAGGACTGAAC-3'		
ogt FW	5'-GAAGATCGCATGATTCACTAC-3'	234	*ogt* codons 12, 15, and 37
ogt RV	5'-GTCGGTTCCCCGGAGGTCAAG-3'		
mutT2 FW	5'-GAACTTCCCGGCGGTAAGGTC-3'	149	*mutT2* codon 58
mutT2 RV	5'-AGCGTCGTCGTGCCGTTCAAC-3'		
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Cycle sequencing of the PCR products was done in both directions according to the method outlined previously by Anthony et al. (2005). The cycle PCR was carried out in 25µL volumes containing 2.5 µL 10X Hot Goldstar PCR buffer (Eurogentec); 2 µl 25 mM $MgCl_2$; 0.2 µL 25 mM dNTP mixture (Amersham Bioscience); 0.15 µl (5 units/µL) Hot Goldstar DNA polymerase (Eurogentec); 0.25 µL 10 µM of each primer, 18.6 µL water (Baker brand) and 1 µL of crude DNA template. Thermal cycling was performed using a PTC-100 Peltier Thermal Cycler (MJ Research, USA).The PCR consisted of 10 min at 95°C, then 35 X (96°C /30 sec, 60°C / 30 sec, 72°C / 60 sec). PCR success was verified by 1.5% agarose gel electrophoresis. The PCR products obtained therefrom were diluted 10-fold (i.e. 1/10) with purified Baker water and then subjected to sequencing using the Dideoxy Chain Termination method with the Big Dye Terminator cycle sequencing Kit (Applied Biosystems, Ca, USA), following the manufacturer's instructions. Cycle PCR, prior to actual sequencing, was carried out, using ¼ volume reactions with either the forward or reverse primer(Table 16). Sequence analysis/Capillary Electrophoresis was carried out using a 310 Genetic Analyzer (Applied Biosystems, Ca, USA) and following standard procedure (Anthony et al., 2005).

Sequencing data acquired by the 310 Genetic Analyzer was compared with agarose gel electrophoresis analysis of the results of the MLPA PCR runs. Results were tabulated with positively identified fragments indicated by a solid square in the Microsoft Wingdings font format.

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Spoligotyping (Spacer Oligonucleotide Typing)

DNA isolation for PCR and Spoligotyping

DNA was obtained from the sub-cultured isolates harvested from Coletsos slants. The colonies were scraped and a loop-full (0.5cm diameter loop) was re-suspended in 500µl of sterile autoclaved double-distilled water in sterile 2-ml screw-capped polypropylene tubes followed by heating at 95°C for 20 min on a pre-heated block to lyse bacterial cell walls and release DNA.

Spoligotyping was performed according to standard procedure (Kamerbeek et al., 1997; van der Zanden et al., 1998). The procedure employed, relies on the PCR amplification of the highly polymorphic Direct-Repeat (DR) locus in the MTB genome.

The DNA obtained in the supernatant from the isolated strains was used for the amplification. Two primers, DRa-bio (5'-GGT TTT GGG TCT GAC CGAC-3') DRb (5'-CCA AGA GGG GAC GGA AAC-3'), were used. Primer DRa-bio is biotinylated. Together, they amplify the Direct-Repeat (DR) locus.

Sample DNA lysate (3µl) of each isolate was added to a final volume of 25µl Readymix PCR reaction mix (containing 10X PCR Buffer, 25mM $MgCl_2$, a primer set of DRa-bio and DRb, RNAse-free water and Taq polymerase) procured from Ocimum Biosolutions (Ocimum Biosolutions Hyderabad, India). PCR was performed using an Applied Biosystems 2720 PCR Machine. The cycling parameters were 3 min at 95°C and then 1 min at

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94°C, followed by 30 cycles of 1 min at 55°C, and 30 s at 72°C, and finally products were held at 72°C for 10 min.

Spoligotyping Analysis

The PCR products obtained after amplification (above) were then hybridised to a Biodyne C membrane containing 43 immobilised synthetic oligomeric spacer sequences derived from the Direct-Repeat region of *M. tuberculosis* $H_{37}Rv$ and *M. bovis* BCG. These represent each of the unique spacer DNA sequences described for the MTB genome (Kamerbeek et al., 1997). Hybridisation with the oligonucleotides immobilised onto the nylon membrane was performed using a 45-lane blotter (Miniblotter 45, Immunetis, Cambridge, Ma., USA). A volume of 20µl of each PCR product was pipetted into capped Eppendorf tubes containing 150µl of 2X SSPE (pH 7.4) with 0.1% Sodium dodecyl sulphate (SDS) at 55°C for 1 hr.

This 2X SSPE-diluted PCR product was heat-denatured at 99°C for 10 minutes and snap-cooled on ice immediately. The supplied membrane (containing the immobilised oligonucleotides complementary to those of the 43 spacers) was washed for 5 minutes in a rotary incubator at 60°C in 250mL2X SSPE containing 0.5% Sodium dodecyl sulphate (SDS).The membrane and a cushion support were then fixed onto the miniblotter following the manufacturer's instructions (Miniblotter 45, Immunetis, Cambridge, Ma, USA). The top right corner of the membrane was cut off as a mark of orientation and the membrane positioned in such a manner that slots

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Spoligotyping Analysis

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This 2X SSPE-diluted PCR product was heat-denatured at 99°C for 10 minutes and snap-cooled on ice immediately. The supplied membrane (containing the immobilised oligonucleotides complementary to those of the 43 spacers) was washed for 5 minutes in a rotary incubator at 60°C in 250mL2X SSPE containing 0.5% Sodium dodecyl sulphate (SDS).The membrane and a cushion support were then fixed onto the miniblotter following the manufacturer's instructions (Miniblotter 45, Immunetis, Cambridge, Ma, USA). The top right corner of the membrane was cut off as a mark of orientation and the membrane positioned in such a manner that slots

in the miniblotter were perpendicular to the line pattern of the immobilised oligonucleotides. Excess fluid was removed from the slots by aspiration. The miniblotter screws were tightened in order to prevent sample mixtures from moving into neighbouring slots. The slots were then filled with the diluted PCR product (snap-cooled and now approximately 170µl) and the membrane hybridised at 60°C for 60 minutes on a horizontal surface witout shaking. After hybridisation samples were removed from the slots by aspiration and the membrane removed from the miniblotter using a pair of forceps.

The membrane was then washed twice in 250mL 2X SSPE/0.5% SDS for 10 minutes at 60°C, after which the membrane was then placed in a rolling bottle (Schott, Duran, SigmaAldrich Co., USA) and allowed to cool in order not to inactivate the peroxidase in the anticipated next step. Streptavidin-peroxidase conjugate, 5.0µL, (Boehringer Mannheim, Germany, strength of 500U/mL) was added to 14mL of 2X SSPE/0.5% SDS and the membrane incubated in this solution for 45 to 60 minutes at 42°C in a rolling bottle. A precaution of shaking/swirling the bottle gently was done before fixing the bottle in the hybridisation oven. The membrane was then washed twice in 250ml of 2X SSPE/0.5% SDS at 42°C for 10 minutes.

The membrane was rinsed twice in 250mL of 2X SSPE for 5 minutes at room temperature, and then incubated for 1 minute in 20mL ECL detection liquid obtained by mixing 10mL of Amersham ECL Detection Reagent 1 and 10mL of Reagent 2 (GE Healthcare, UK). Finally, the membrane was then heat-sealed into a transparent plastic sheet (Nylon PPE, Nalgene, USA) with an impulse sealer and secured in a film cassette (Eastman Kodak, USA).

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The membrane was then developed in a dark room by exposure (5 minutes) to a light-sensitive X-ray film (Eastman Kodak, USA) by washing for 2 minutes in a Kodak GBX 'fixer and replenisher' (Kodak, USA) and rinsing for 1.5 minutes in a Kodak GBX 'developer and replenisher' (Kodak, USA). Bound fragments (presence of spacer) were revealed as black 'square dots' in the image of the membrane after development. White 'square dots' indicated the absence of spacers. The images were then recorded using the Spoligo Excel spread sheet in both octal and binary mode. These modes were then used to query the SITVITWEB data base (Demay et al., 2012).

Regeneration of Membranes for Re-use

Where necessary, the nylon membrane was regenerated, following the manufacturer's instructions, for re-use until all isolates had been spoligotyped. The regeneration procedure involved the dissociation or de-sorption of the hybridised PCR products from the membrane so that it could be used for the next batch of isolates. The membrane was washed twice by incubation in 150 mL 1% SDS at 80°C for 30 minutes in a rotatory shaker. The membrane was thereafter washed in 200mL 20 mM EDTA (pH 8) and finally stored moist by sealing (using an impulse sealer) in a plastic bag containing 10 mL 20mM EDTA at 4°C. This ensured that the membrane was not dehydrated before the next use.

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Multilocus Interspersed Repetitive Units-Variable Number of Tandem Repeats (MIRU-VNTR) Typing

DNA Isolation for MIRU-VNTR

Standard protocol (Supply, 2005) was applied. A loop full of sub-cultured colonies (from Coletsos slants) was re-suspended in 200µL 10 mM Tris-HCl, 1 mM EDTA (pH 7.0), in a safe lock tube. The colonies were lysed to release DNA by heating at 95°C for 30 minutes on heating block in a microbiological security facility. The tubes were then spun at 15,000xg for 1 minute to pellet the cell debris and the supernatant containing the DNA (approximately 170 – 180 µL) transferred into a new tube.

PCR amplification of the MIRU-VNTR

PCR amplification of the different VNTR regions was achieved by employing primers specific for the flanking regions of each VNTR region. Since the analysis of the PCR products was to be done using a DNA sequencer, 8 different multiplex PCRs were performed in order to analyse eight groups of three loci each simultaneously. For each multiplex PCR, one primer per oligonucleotide pair was procured synthesised and tagged with a specific fluorescent dye. The advantage of this system was that in case of amplification failure of some loci in some multiplex reactions, these loci could then be amplified separately in a simplex PCR, as a repeat.

Preparation of Multiplex PCR Mixes

PCR premixes for amplification of the 24 MIRU-VNTR loci were first prepared before addition of DNA template using the commercial QIAGEN

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PCR Mix kit (QIAGEN Benelux B.V., The Netherlands) or the commercial Genoscreen typing kit (Genoscreen, Lille, France). From experience, the final concentration of $MgCl_2$ was made to vary from 1.5 mM (default concentration using the 10X Qiagen buffer) to 3 mM (by adding more $MgCl_2$), according to the specific MIRU locus. The final volume per reaction before addition of template DNA was set at 18 µL. The volume of template DNA added was 2 µL each. In a DNA-free area, the PCR premixes were prepared as shown in Table 17 and Table 18.

A 96-well Semi-skirted PCR microplate (Sorensen Bioscience Inc, Salt Lake City, UT, USA) was each labelled for an isolate. Separations between zones with different multiplexes were also indicated by marker. PCR premix, 18µL, was dispensed into each well of the labelled and marked microplates.

PCR Amplification

In PCR sample addition room (devoid of PCR amplicons), 2 µL of extracted DNA (stored diluted 1:50) was dispensed into each well. Controls included were H37Rv DNA (positive control) and sterile water (negative controls). The PCR microplates were then tightly sealed using an adhesive PCR film. This was done in order to avoid evaporation during amplification. The target loci were amplified in a Perkin Elmer PCR machine using the following cycling parameters: 95°C for 15 minutes, then 40 cycles comprising 94°C for 1 minute, 59 °C for 1 minute and 72°C for 1.5 minutes; then 72°C for 10 minutes and then finally held at 4°C, until retrieved for verification by agarose gel electrophoresis.

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Table 17

Volumes in μL for the discriminatory multiplex PCR mixes for the first 15 loci

Mix	1	2	3	4	5
Loci	4, 26, 40	10,16, 31	0424, 0577, 2165	2401, 3690, 4156	2163b, 1955, 4052
$MgCl_2$ final concentration	3nM	2nM	1.5nM	3nM	1.5nM
H_2O	7.5	8.3	8.7	7.5	8.7
10 X Buffer	2	2	2	2	2
5x 'Q' Solution	4	4	4	4	4
$MgCl_2$ (25mM)	1.2	0.4	0	1.2	0
dNTP (5mM)	0.8	0.8	0.8	0.8	0.8
Primers each[a]	0.4	0.4	0.4	0.4	0.4
HotStart DNA Taq	0.08	0.08	0.08	0.08	0.08
Total premix volume	18	18	18	18	18
Template DNA	2	2	2	2	2
Total Reaction Volume	20	20	20	20	20

Legend: [a] There are 6 primers (3 pairs of 1 forward and 1 reverse each) for each set of 3 loci to be analysed in each multiplex mix. Initial concentration for all unlabeled primers: 20pmol/μL. Initial concentration for the labelled oligonucleotides: 2 pmol/μl for locus 0577, 3690 and 1955, 8 pmol/μl for locus 4052, 20 pmol/μl for locus 4156 and 4 pmol/μl for the other loci.

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Table 18

Volumes in µL for the discriminatory multiplex PCR mixes for the last 9 loci

Mix	6	7	8
Loci	2, 23, 39	20, 24, 27	2347, 2461, 3171
$MgCl_2$ final concentration	2.5nM	1.5nM	2.0nM
H_2O	7.9	8.7	8.3
10 X Buffer	2	2	2
5x 'Q' Solution	4	4	4
$MgCl_2$ (25mM)	0.8	0	0.4
dNTP (5mM) (Roche)	0.8	0.8	0.8
Primers each[a]	0.4	0.4	0.4
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of this analysis was to determine the sizes and eventual separation of all PCR fragments at each locus.

Capillary Electrophoresis and Sequence Analysis

Fragment size of the amplicons was analyzed on an ABI 3730 DNA sequence analyzer (Applied Biosystems, California, USA) and number of copies or repeats at each locus was determined by automated assignment using the Genemapper 4.0 software (Applied Biosystems, California, USA) and the GeneScan software (Thermo Fisher Scientific, USA). In the case of aberrant or discrepant results, the size of the repeats was double-checked by size estimation by comparison to a 50 bp and 100 bp DNA ladder and the positive control (H37Rv) on agarose gels and by cross-comparison to a reference table. Capillary Electrophoresis or Sequence Analysis was done by performing a 'dye set and spectral calibration' on the ABI 3730 DNA sequence analyzer (Applied Biosystems, USA). This was necessary in order for the dye set or combination (on labelled primers) used in the MIRU-VNTR analysis to be successfully detected by the sequencer. The dye combination was 6FAM™, VIC®, NED™, for the amplicons, and ROX™ for the internal size standard. Following the manufacturer's instructions, Spectral Calibration was performed using the ABI DS-31 Matrix Standard Kit (Applied Biosystems, USA), containing 4 oligonucleotides labelled, respectively, with 6FAM™, VIC®, NED™, and ROX™ pre-pooled in a single tube.

Sample Preparation for Injection by Sequencer

A volume of 1μL of PCR product was pipetted into the bottom of the wells of the 96-well semi-skirted PCR plate manufactured for the ABI

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sequencer (Sorensen Bioscience Inc, USA). Care was ensured to get the aliquoted sample to the bottom of the wells without any air bubbles.

Exactly 10 µL of a 'mix' prepared combining 10µL formamide (Applied Biosystems, USA) and 0.2µL of MapMaker 1000 ROX™ internal size standard (BioVentures Inc., Murfreesboro, Tennessee, USA) was aliquoted onto each PCR product in the wells. This was then stored at 4°C until required for attachment onto the plate assembly for auto-sampling in the ABI sequencer.

Following the ABI standard operating procedures (SOP) and the manufacturer's instructions, all necessary files were created in the equipment prior to placing the 96-well plate assembly into the sequencer. The 96-well plate was then placed into the ABI sequencer and the programme executed following the SOP (ABI PRISM® 3730 Genetic Analyzer User Guide). Apparent fragment sizes were then computed by the sequencer using the GeneScan Analysis software (Thermo Fisher Scientific, USA) by comparing and correlating their migration data to those of internal size standard bands. In order to filter the results, software algoritms were followed and the peaks queried in order to delete stutter peaks, spurious peaks, and unequivocally assign all true peaks based on colours imparted by the fluorescent labels. Results files were then re-named and saved to be retrieved for statistical analysis.

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Editing and Sizing of Data and Assigning Alleles using the Genotyper Software

Size data obtained using the GeneScan Software were edited and converted into MIRU-VNTR alleles using the Genotyper software (Thermo Fisher Scientific, USA), while customized files, referred to as templates, were used for each multiplex (or for any of the 3 loci in a multiplex) in calling the alleles. These templates contained allele-calling information for the corresponding loci, as well as macros that were used to automate the genotyping process.

Creation of Final Results Tables and Verification of Results

Creation of final results tables was done by employing a macro called "final table" in the Genotyper software templates. These tables were used to check, and subsequently export the results. The obtained results in excel format were retrieved and analysed by checking all dye labels. Also, spoligotyping data was included in the exported results file and the 'relatedness' or otherwise of batches of isolates in the various panels were analysed on-line using tools available in the Molecular TB and SITVITWEB websites.

The Hunter–Gaston Discriminatory Index (HGDI) Analysis for Discriminatory Power of the Typing Methods Employed

The Hunter–Gaston Discriminatory Index, HGDI, (Hunter & Gaston, 1988), was applied to yield a numerical index of discriminatory power for the

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To also shed light on the efficiency of MIRU-VNTR in typing MTB isolates in the three populations, the discriminatory performance of individual loci and combined loci sets (12-Loci, 15-Loci and 24-Loci) were assessed using the online resources: MIRU-VNTR*plus* website database (MIRU-VNTR*plus*, n.d., Navigate) and the Health Protection Agency VNTR **DI**versity and **C**onfidence **E**xtractor, V-DICE website (V-DICE, n.d., hpa-bioinformatics). These combined loci sets (viz. 12-Loci MIRU-VNTR, 15-Loci MIRU-VNTR and 24-Loci MIRU-VNTR) were derived as groups from loci described variously by researchers (Frothingham & Meeker-O'Connel, 1998; Supply et al., 2000; Supply et al., 2001; Supply et al., 2005). It was envisaged that these analyses would provide information on the most efficient loci and, ultimately, the most appropriate typing method to employ in the typing of MTB isolates in a similar Ghanaian setting.

Where appropriate, the HGDI was calculated using the following formula:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} x_j(x_j - 1)$$

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Where, **N** stands for the total number of strains in the sample population to be typed, **S** stands for the total number of different MIRU-VNTR patterns, and x_j stands for the number of strains belonging to the j^{th} pattern.

As with all diversity indices, the HGDI provides a quantitative means of estimating how many different lineages (or types of species) there are existing in a population data set and at the same time taking into consideration how evenly distributed the basic members of the population are among these lineages (or types of species). There are several methods of estimating or measuring diversity such as the Shannon Index (Shannon, 1948), the Simpson Index (Simpson, 1949) and the Berger-Parker Index (Berger & Parker, 1970), among others. However, each method has its own inherent advantages and disadvantages in terms of applicability to different situations and data sets.

The Hunter-Gaston discriminatory index (HGDI) is the average probability that the molecular typing system employed will assign a different lineage type to two strains that are not related randomly sampled from the data set population of a given taxon. The index is usually expressed as a fraction of 1.0 and, thus, is defined by the range 0.0 – 1.0. Normally, an index of 1.0 obtained through a typing method indicates that, that method was able to distinguish every individual member of a strain population from all the other members of that population. On the other hand, an index of 0.0 would show that all individual members of a strain population were identical, while an index of 0.5 would mean that if one strain was randomly picked from a strain population then there exists a 50% chance that the next strain randomly picked

Where, **N** stands for the total number of strains in the sample population to be typed, **S** stands for the total number of different MIRU-VNTR patterns, and x_j stands for the number of strains belonging to the j^{th} pattern.

As with all diversity indices, the HGDI provides a quantitative means of estimating how many different lineages (or types of species) there are existing in a population data set and at the same time taking into consideration how evenly distributed the basic members of the population are among these lineages (or types of species). There are several methods of estimating or measuring diversity such as the Shannon Index (Shannon, 1948), the Simpson Index (Simpson, 1949) and the Berger-Parker Index (Berger & Parker, 1970), among others. However, each method has its own inherent advantages and disadvantages in terms of applicability to different situations and data sets.

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would be indistinguishable from the first. The HGDI (also sometimes referred to as the Discriminatory Power, D) is basically similar to the original Simpson's Index of Diversity, except that, for the former, the sample size is usually small. Another assumption for HGDI is that, in determining the diversity of strains, consecutive strains are drawn or sampled (from a sample cluster in the population) without replacement. The HGDI is usually the index of choice in Microbiology, and thus it has been employed in the current study.

Where appropriate, confidence intervals (CI) for HGDI (or D) were computed according standard methodology (Simpson, 1949; Grundmann, Hori & Tanner, 2001) as follows:

$$\mathbf{CI} = \left[\mathbf{D} - 2\sqrt{\sigma^2}, \quad \mathbf{D} + 2\sqrt{\sigma^2}\right]$$

Where, D = Hunter-Gaston Discriminatory Index, HGDI; σ^2 = variance. Also,

$$\sigma^2 - \frac{4}{n}\left[\sum \pi_j^3 - \left(\sum \pi_j^2\right)^2\right]$$

Where $\mathbf{n_j}$= number of isolates in the $\mathbf{j^{th}}$lineage; $\mathbf{n}$ = total number of strains in the population of isolates; $\boldsymbol{\pi_j}$= frequency $\mathbf{n_j/n}$ (also the probability of picking a $\mathbf{j^{th}}$-type strain from the population).

Individual loci were categorised as highly (HGDI > 0.6), moderately (0.3–0.6) and poorly (<0.3) discriminatory following criteria set out by Sola and co-workers (Sola et al., 2003).

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Sensitivity Performance Analysis of the various Techniques

Results obtained from the application of the various techniques were analysed in order to provide information on diagnostic parameters, such as sensitivity (Sn), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV) and efficiency (E). These comparisons should assist in the selection of the best technique in efficiently analysing MTB isolates for effective control and management of TB disease in Ghana. For each pair of comparison, the older and more established technique was assumed as 'the Gold Standard'.

In the analysis, the test performance parameters were defined according to standard formulae (McNeil, Keeler, & Alderstein 1975). Sensitivity represents the ability of the technique to identify correctly those isolates with a feature of interest (i.e. having a genetic feature defining lineage type or resistance to a drug) as truly having that feature. Sensitivity is sometimes called the proportion of positive tests or true positive ratio. Specificity is defined as the technique's ability to identify correctly an isolate without feature of interest as truly not having that feature. Specificity is also sometimes referred to as proportion of negative tests or true negative ratio. Positive predictive value for a positive test result from a technique is the proportion of isolates with specific feature of interest, which has correctly been found out or analysed when using only that technique.

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CHAPTER FOUR

RESULTS

The number of prison inmates and the number of coughing inmates enrolled from each prison are presented in Table 19, while their age characteristics are shown in Table 20. Table 21 indicates the age characteristics of prison inmates yielding a positive MTBC isolate.

Table 19

Number of Coughing Inmates Sampled in selected Prisons

Prison	Region	Total Population of Prison	Number of Coughing Inmates Sampled
James Fort	Greater Accra	992	52
James Camp	Greater Accra	452	4
Nsawam	Eastern	2736	38
Winneba	Central	214	15
Ankaful Main	Central	391	19
Kumasi Regional	Ashanti	199	23
Sunyani	Brong-Ahafo	755	20
Tamale Regional	Northern	291	24
Yendi Prison	Northern	102	19
Total number of inmates enrolled			214

A total of 323 were enrolled after satisfying the inclusion criteria and consenting to their participation. The number of presenting patients presenting at the selected hospitals are shown in Table 22, while their demographic characteristics are shown in Table 23.

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Table 20

Age characteristics of coughing inmates sampled (N=214)

Category	Number of inmates sampled	Age range (years)	Median age (years)	Mean age (years)
Convicts	108	18 - 78	30.0	34.0
Remands	106	16 – 83	31.0	32.3
Total	214	16 – 83	30.0	30.6

Table 21

Age characteristics of coughing inmates yielding a positive MTBC isolate

Inmate Category	Number	Age Range/yrs	Median Age/yrs	Mean Age/yrs (s.d.)
Convicts	6	22 - 55	35.5	37.0 (12.6)
Remands	24	19 - 53	28.0	30.9 (8.2)
Total	30	19 - 55	28.5	32.1 (9.6)

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Table 22

Number of patients presenting at Selected Hospitals in Close Proximity to Prisons

Hospital	**Region**	**Number of Presenting Patients Sampled (N=323)**
Korle-Bu Teaching Hospital	Greater Accra	73
Ridge Hospital	Greater Accra	55
La General Hospital	Greater Accra	34
Nsawam Government	Eastern	50
Winneba District Hospital	Central	12
Ankaful Leprosorium	Central	8
Cape Coast Hospital	Central	15
Okomfo Anokye Teaching	Ashanti	34
Sunyani Hospital	Brong-Ahafo	13
Tamale Teaching Hospital	Northern	22
Yendi Government Hospital	Northern	7

Table 23

Age Characteristics of Patients Presenting at selected hospitals (N=323)

Category	Number of patients sampled	Age range/yrs	Median age/yrs	Mean age/yrs
Males	114	17 - 88	35.0	35.0 (6.8)
Females	209	15 - 87	33.0	36.3 (8.6)
Total	323	15 - 88	34.0	35.6 (9.2)

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Table 24 depicts the demographic characteristics of all presenting patients yielding a positive MTBC isolate.

Table 24

Age Characteristics of Patients Presenting at selected hospitals and yielding a positive MTBC isolate (N=170)

Inmate Category	Number	Age Range/yrs	Median Age/yrs	Mean Age/yrs (s.d.)
Female	41	13 - 76	32.0	35.5 (13.9)
Male	129	17 - 71	35.0	38.0 (13.9)
Total	170	N/A	N/A	N/A
Overall	N/A	13 - 76	35.0	37.4 (13.9)

Legend: N/A = not applicable

Table 25 depicts the demographic charcteristics of cadavers sampled during routine post-mortem examinations.

Table 25

Age Characteristics of cadavers examined during routine post-mortems (N=733)

Sex	Number (%)	Age Range/yrs	Median Age/yrs	Mean Age/yrs (s.d.)
Male	449 (61.1)	8 - 98	32.7	30.8 (12.8)
Females	284 (38.9)	7 - 97	34.2	33.6 (9.6)

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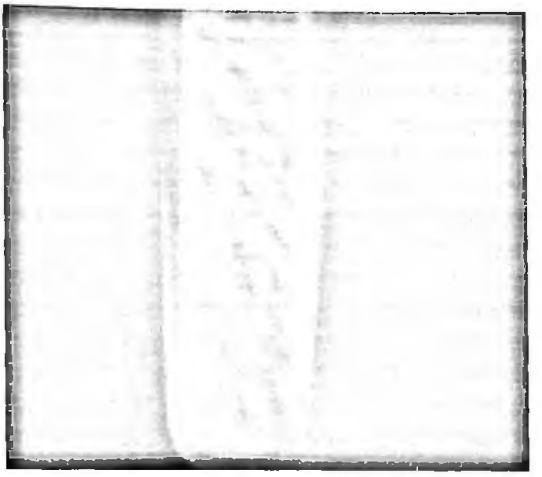

Plate 1: Loewenstein-Jensen slant showing colonies of MTB

A total of 13 out of the 43 sample isolates of prison panel were categorised as NTM or atypical mycobacteria by the Capilia TB-Neo Immunochromatographic test. Thus, 30 prison isolates were classified as having the MPB 64 protein and therefore classified as being members of the MTBC.

All 170 isolates from patients presenting at selected hospitals, 30 isolates from coughing prison inmates and 43 isolates from tissues with tuberculous lesions at PM had the immunogenic protein MPB64. Therefore, they were classified as members of the MTBC. Table 27 depicts all the isolates identified as MTBC by the Capilia TB-Neo Test.

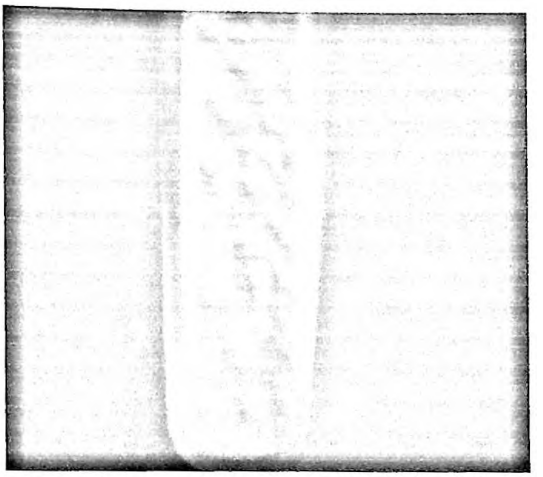

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Table 27

Number of isolates identified by Capilia TB-Neo test

Source of Isolates	MTBC (MPB64 protein present)	NTM (MPB64 protein absent)
Coughing Prison Inmates	30	13
Patients Presenting	170	0
Decedents at Post-mortem	43	0
Total	243	13

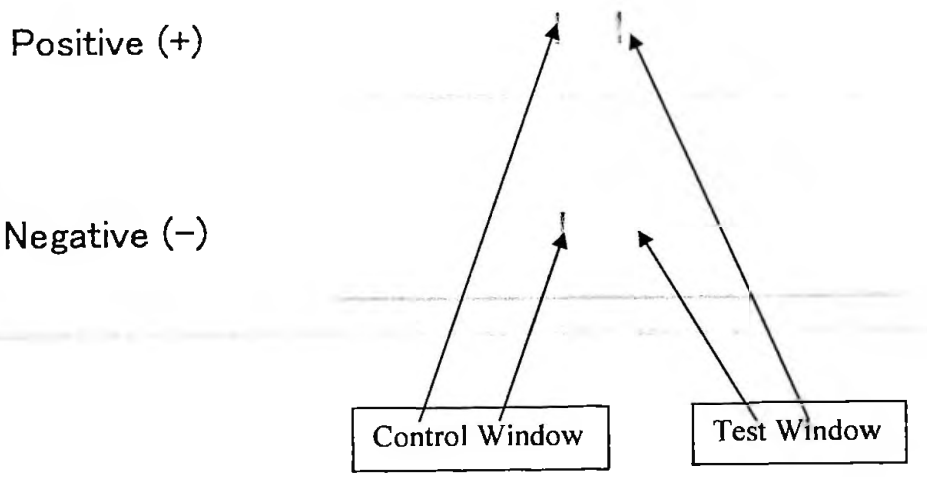

Plate 2: A Cartridge of the Capilia® TB-Neo Test. Legend: In the Negative test result, only one reddish-purple band appears in the control (C) window. In the Positive test result, in addition to the control band, a clear distinguishable reddish-purple band also appears in the test (T) window.

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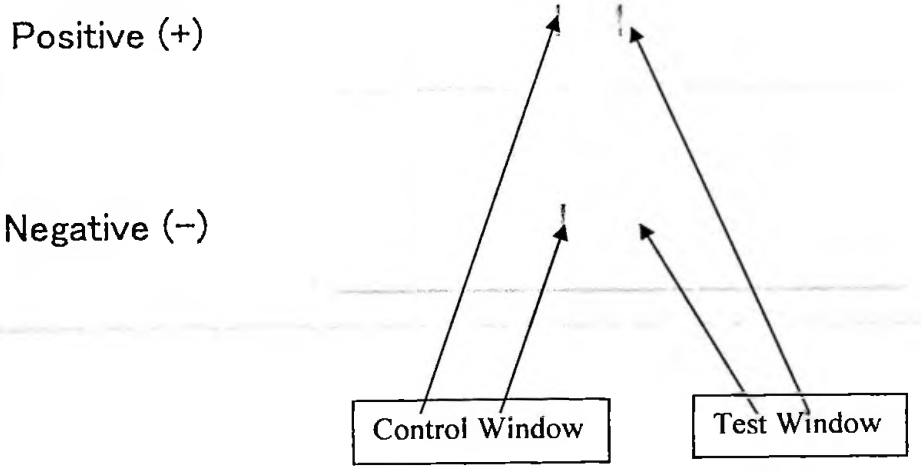

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Isolates identified by the16S rDNA PCR

All 243 isolates, comprising 30, 170 and 43 isolates obtained respectively from coughing prison inmates, patients presenting at the selected hospitals and tissue samples from post-mortem, had the 208bp fragment after being subjected to the 16S rDNA PCR assay indicating that they are members of the MTBC. The 13 isolates from the coughing inmates classified as NTM by the Capilia TB-Neo Immunochromatographic test were also confirmed as such by the 16S rDNA PCR test.

Isolates of NTM identified using BLAST sequencing

Only six out of the 13 isolates had DNA of sufficient purity for successful sequencing analysis by the Basic Local Alignment Search Tool (BLAST) method (Altschul, Gish, Miller, Myers, & Lipman, 1990). After sequence query using the BLAST suite, the 6 NTM isolates were identified as *Mycobacterium peregrenum* (1 isolate), *Nocardia nova* (Nocardiaceae) (1 isolate), *Mycobacterium chelonae* (2 isolates) and *Mycobacterium fortuitum* (2 isolates).

Results of Distribution of isolates among organs with Tuberculous Lesions at Post-mortem.

For the 43 isolates obtained from tuberculous lesions on various organs, lung tissue yielded the greatest number of isolates for both sexes (Figure 11). Overall, 27 isolates were obtained from the 22 male cadavers while 16 were obtained from the 12 females (43 isolates were obtained from 34 cadavers because some cadavers produced more than 1 organ site depicting

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tuberculous lesions in the opinion of the pathologist). Infected organs found exhibited classical tuberculous lesions. Plates 4 and 5 depict liver and lung tissues with tuberculous lesions.

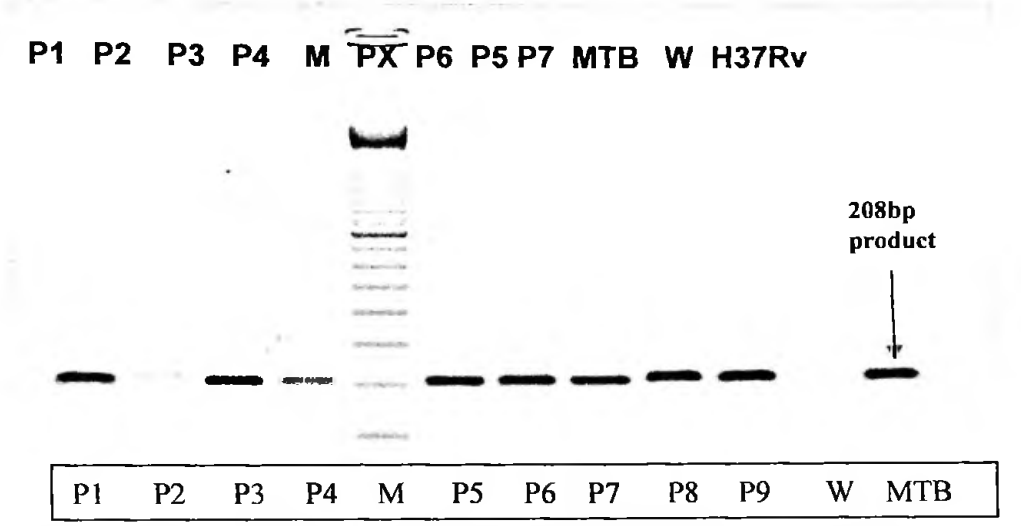

Plate 3: A representative electrophoregram of 16S rDNA PCR products. (Legend: P1 – P9 = samples; M= 100bp Molecular Weight ladder; MTB=TB strain +ve control; W=water HPLC-grade as –ve control)

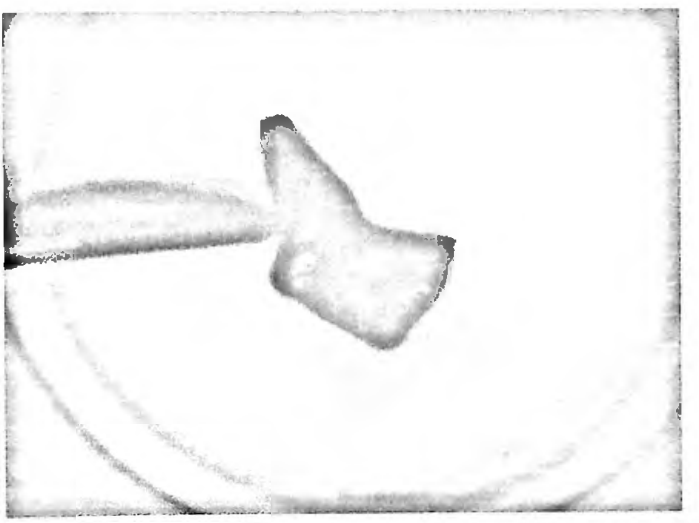

Plate 4: Liver tissue showing two tuberculous nodules.

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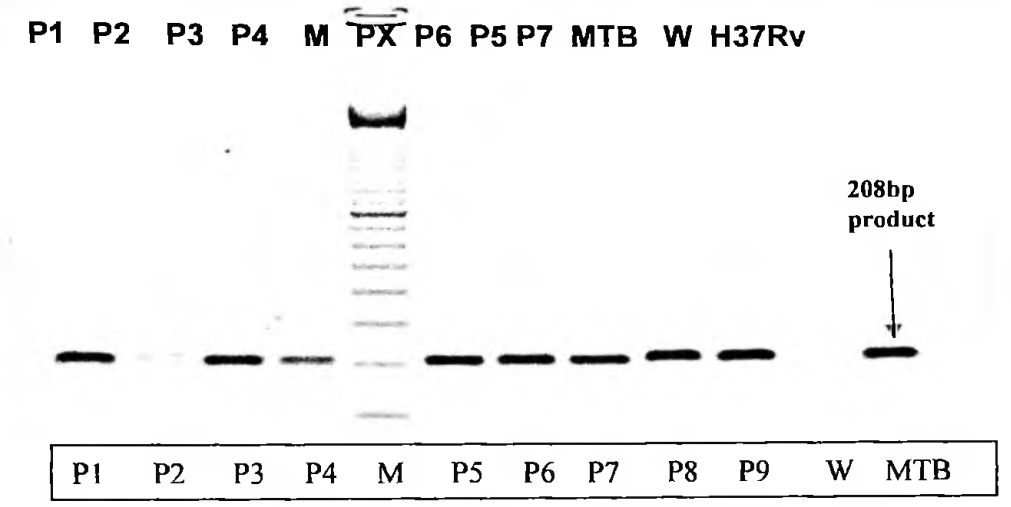

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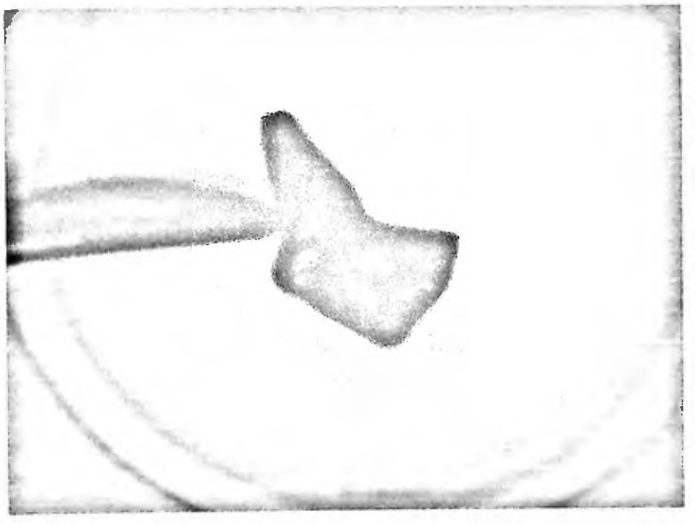

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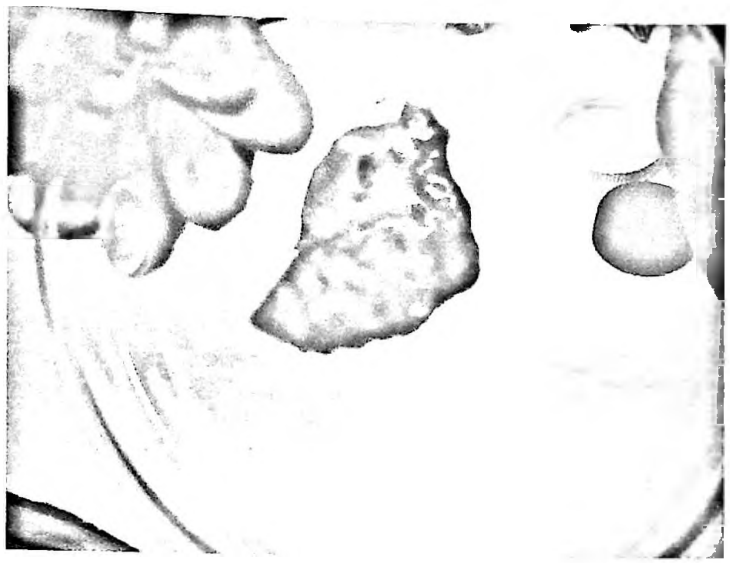


Plate 5: Lung tissue depicting grossly calcified tuberculous lesions.

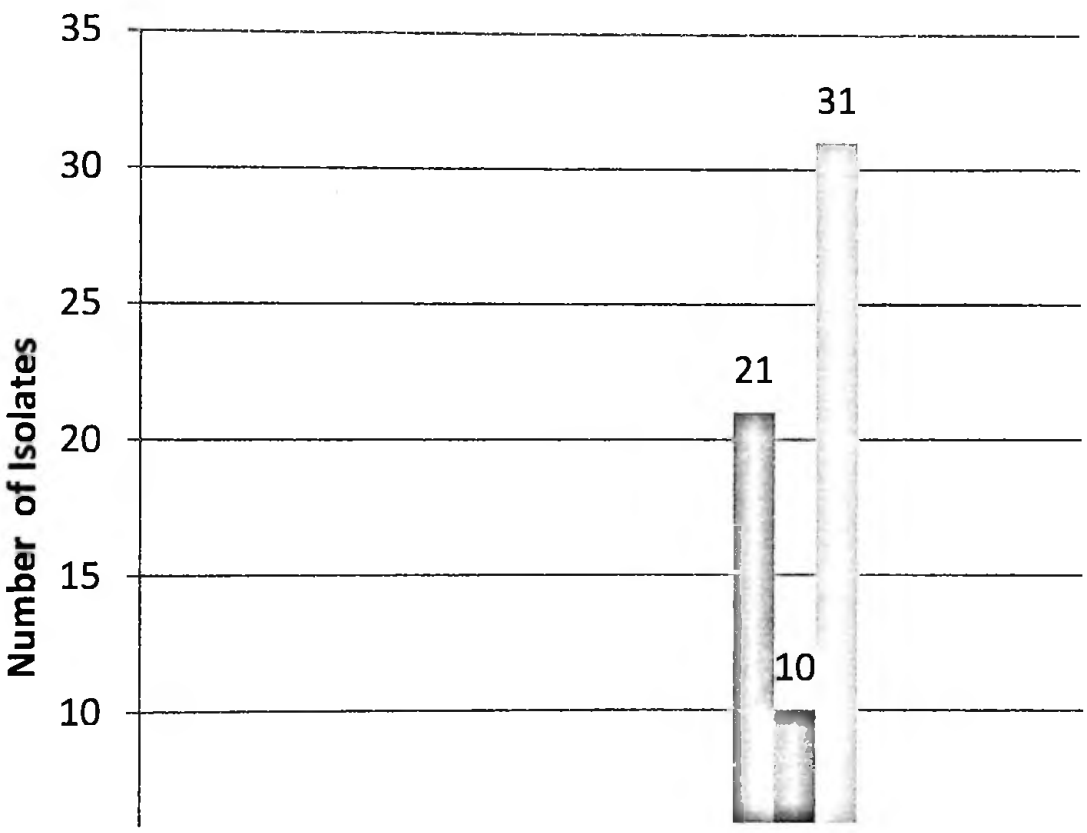
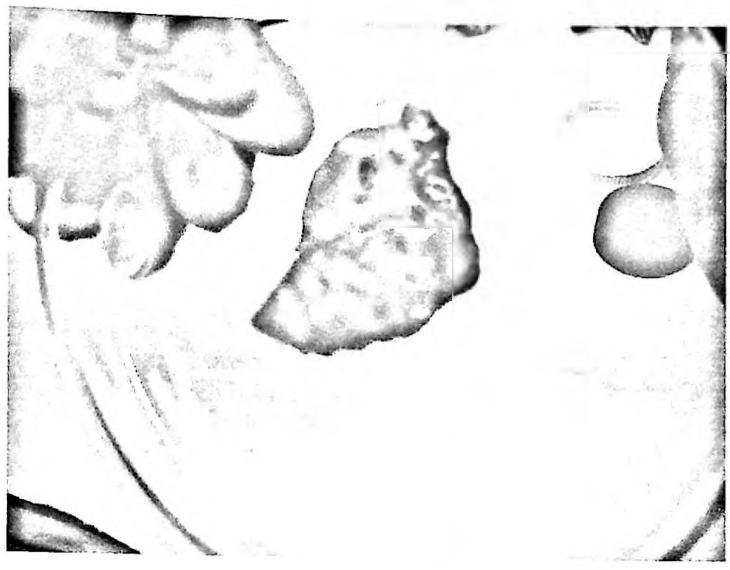


Figure 11: Distribution of isolates among organs with tuberculous lesions at post-mortem in 34 cadavers



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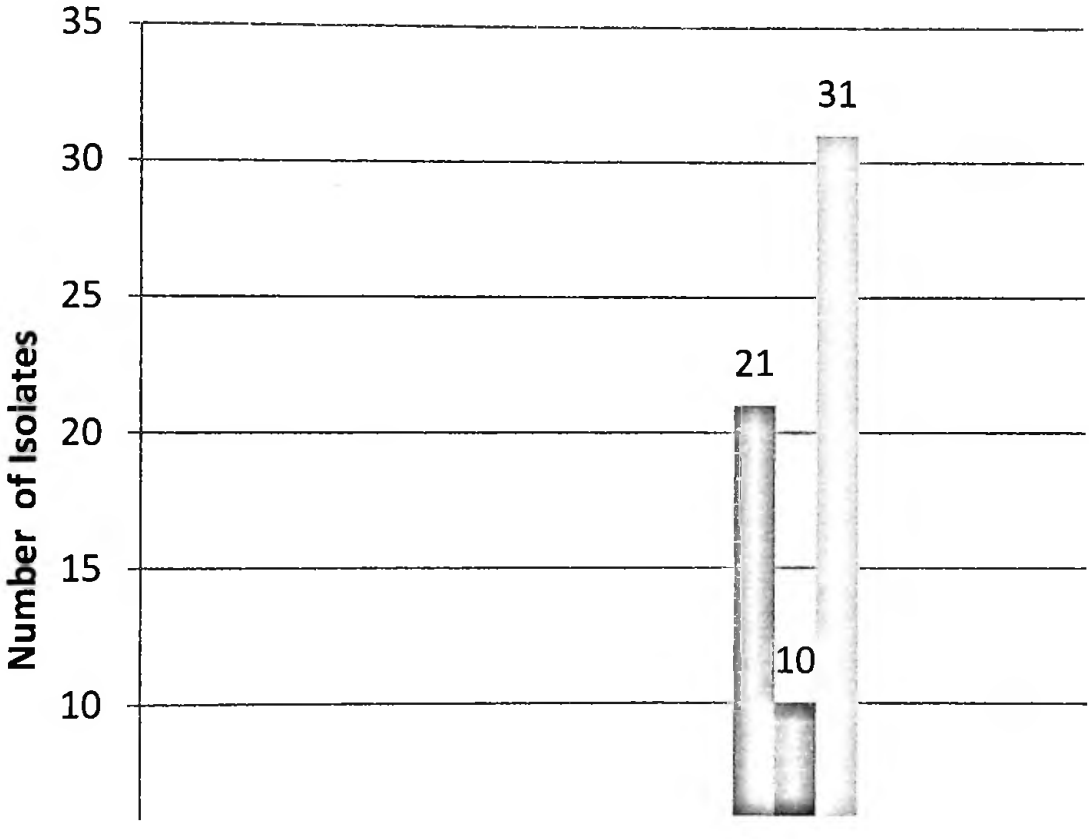

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Results of Phenotypic Resistance of Isolates

Drug-susceptibility of the isolates to the four first line drugs by the Minimum Inhibitory Concentration (MIC) Technique

All the successfully harvested isolates (N=243) from the prison inmates, patients presenting at hospitals and cadavers at post-mortem were subjected to MIC test to assess their sensitivity or resistance to the four first-line anti-tuberculosis drugs – Streptomycin (STR), Isoniazid (INH), Rifampicin (RIF) and Ethambutol (EMB). These drugs form part of the drug regimen of the Ghana National Tuberculosis Programme (NTP). The MIC-DST method has been employed as the 'Gold Standard' comparator for the other resistance-testing methods employed in this study. Observations indicated that resistance to drugs occurred from the second week of incubation and the resistance was unequivocal from growths in the wells containing the break-point concentration of the drugs (Figure 33).

The resistance profiles of isolates and frequencies of resistance to individual drugs among the isolates are shown in Table 28. The mono-resistance to STR was the most prevalent, at 11.9%. This is not far-fetched as abuse (including overuse and misuse) of STR since the 1950's is common place (Ventola, 2015). Prevalence's of mono-resistance to INH and RIF were 2.5% and 1.2%, respectively. There were no EMB mono-resistant isolates. However, some isolates were resistant to combinations of EMB with other drugs. There was no MDR in either the isolates from the prisons or in isolates from cadavers at PM. However, there were MDR-TB isolates (4/170 or 2.4%)

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Drug-susceptibility of the isolates to the four first line drugs by the Minimum Inhibitory Concentration (MIC) Technique

All the successfully harvested isolates (N=243) from the prison inmates, patients presenting at hospitals and cadavers at post-mortem were subjected to MIC test to assess their sensitivity or resistance to the four first-line anti-tuberculosis drugs – Streptomycin (STR), Isoniazid (INH), Rifampicin (RIF) and Ethambutol (EMB). These drugs form part of the drug regimen of the Ghana National Tuberculosis Programme (NTP). The MIC-DST method has been employed as the 'Gold Standard' comparator for the other resistance-testing methods employed in this study. Observations indicated that resistance to drugs occurred from the second week of incubation and the resistance was unequivocal from growths in the wells containing the break-point concentration of the drugs (Figure 33).

The resistance profiles of isolates and frequencies of resistance to individual drugs among the isolates are shown in Table 28. The mono-resistance to STR was the most prevalent, at 11.9%. This is not far-fetched as abuse (including overuse and misuse) of STR since the 1950's is common place (Ventola, 2015). Prevalence's of mono-resistance to INH and RIF were 2.5% and 1.2%, respectively. There were no EMB mono-resistant isolates. However, some isolates were resistant to combinations of EMB with other drugs. There was no MDR in either the isolates from the prisons or in isolates from cadavers at PM. However, there were MDR-TB isolates (4/170 or 2.4%)

in samples obtained from patients presenting at the selected hospitals. These MDR strains depicted patterns with resistance to at least an additional drug each. Two strains were resistant to all the first-line drugs. One strain was poly-resistant to 3 drugs, that is, STR, INH and EMB but not MDR since it did not show a concomitant resistance to RIF.

Table 28

Drug Resistance Profiles (DST-MIC) of TB isolates from various population groups (N=243)

Resistance Profile	Source of Isolate: Patients Presenting at Selected hospitals	Coughing Prison Inmates (n=30)	Post-mortem (n=43)	Total	Prevalence (%)
STR^R only	16	7	6	29	11.9
INH^R only	6	0	0	6	2.5
RIF^R only	2	1	0	3	1.2
STR^R+INH^R	7	1	0	8	3.3
STR^R+EMB^R	1	0	0	1	0.4
STR^R+INH^R+EMB^R	1	0	0	1	0.4
MDR Patterns					
STR^R+INH^R+RIF^R	1	0	0	1	1.6
INH^R+RIF^R+EMB^R	1	0	0	1	
STR^R+INH^R+RIF^R	2	0	0	2	
Total (%)	37/170 (or 21.8%)	9/30 (or 30%)	6/43 (or 14.0%)	52/243 (or 21.4%)	20.3

in samples obtained from patients presenting at the selected hospitals. These MDR strains depicted patterns with resistance to at least an additional drug each. Two strains were resistant to all the first-line drugs. One strain was poly-resistant to 3 drugs, that is, STR, INH and EMB but not MDR since it did not show a concomitant resistance to RIF.

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RIF^R only	2	1	0	3	1.2
STR^R+INH^R	7	1	0	8	3.3
STR^R+EMB^R	1	0	0	1	0.4
STR^R+INH^R+EMB^R	1	0	0	1	0.4
MDR Patterns					
STR^R+INH^R+RIF^R	1	0	0	1	
INH^R+RIF^R+EMB^R	1	0	0	1	1.6
STR^R+INH^R+RIF^R	2	0	0	2	
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STR^R+EMB^R	1	0	0	1	0.4
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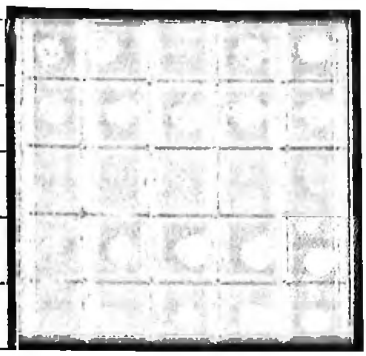
Total (%)	37/170 (or 21.8%)	9/30 (or 30%)	6/43 (or 14.0%)	52/243 (or 21.4%)	20.3

INH 0,2	INH 0,1	**AMK 5**	Contr 1/100	Contr
RIF 0,2	RIF 0,1	INH 2	INH 1	INH 0,5
PAS 1	RIF 5	RIF 2	**RIF 1**	RIF 0,5
ETH 20	ETH 10	**ETH 5**	ETH 2	ETH 1
STR 20	STR 10	**STR 5**	STR 2	STR 1

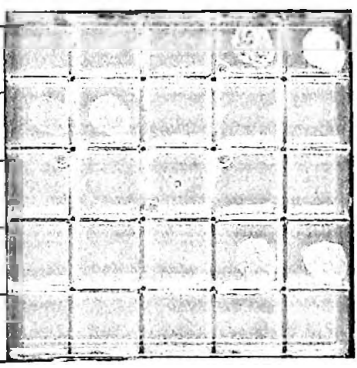


An INH^R-EMB^R-STR^R strain

INH 0,2	INH 0,1	**AMK 5**	Contr 1/100	Contr
RIF 0,2	RIF 0,1	INH 2	INH 1	INH 0,5
PAS 1	RIF 5	RIF 2	**RIF 1**	RIF 0,5
ETH 20	ETH 10	**ETH 5**	ETH 2	ETH 1
STR 20	STR 10	**STR 5**	STR 2	STR 1

A Multi-Drug Resistant (MDR) strain (i.e. Isoniazid-resistant, INH^R, and Rifampicin resistant RIF^R which is also Streptomycin-resistant, STR.

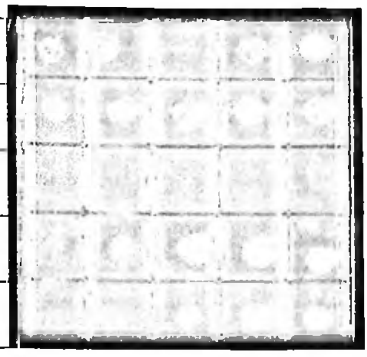
INH 0,2	INH 0,1	**AMK 5**	Contr 1/100	Contr
RIF 0,2	RIF 0,1	INH 2	INH 1	INH 0,5
PAS 1	RIF 5	RIF 2	**RIF 1**	RIF 0,5
ETH 20	ETH 10	**ETH 5**	ETH 2	ETH 1
STR 20	STR 10	**STR 5**	STR 2	STR 1



A strain sensitive to all the drugs

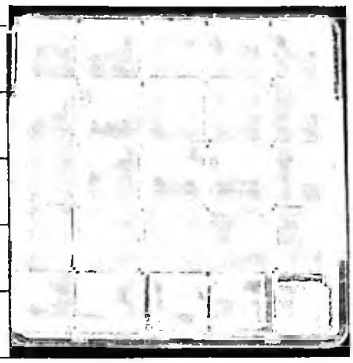
Figure 12: Resistance of TB isolates in 25-well MIC-DST plates containing break-point concentrations of drugs in Middlebrook 7H10 medium are indicated by shaded wells in the scheme on the left. The Break-point concentrations in mg/L are: AMK, 5.0; INH, 0.2; RIF, 0.2; PAS, 1.0; EMB, 5.0 and STR, 1.0)

INH 0,2	INH 0,1	**AMK 5**	Contr 1/100	Contr
RIF 0,2	RIF 0,1	INH 2	INH 1	INH 0,5
PAS 1	RIF 5	RIF 2	**RIF 1**	RIF 0,5
ETH 20	ETH 10	**ETH 5**	ETH 2	ETH 1
STR 20	STR 10	**STR 5**	STR 2	STR 1



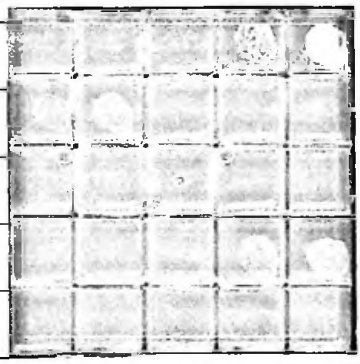
An INH^R-EMB^R-STR^R strain

INH 0,2	INH 0,1	**AMK 5**	Contr 1/100	Contr
RIF 0,2	RIF 0,1	INH 2	INH 1	INH 0,5
PAS 1	RIF 5	RIF 2	**RIF 1**	RIF 0,5
ETH 20	ETH 10	**ETH 5**	ETH 2	ETH 1
STR 20	STR 10	**STR 5**	STR 2	STR 1



A Multi-Drug Resistant (MDR) strain (i.e. Isoniazid-resistant, INH^R, and Rifampicin resistant RIF^R which is also Streptomycin-resistant, STR.

INH 0,2	INH 0,1	**AMK 5**	Contr 1/100	Contr
RIF 0,2	RIF 0,1	INH 2	INH 1	INH 0,5
PAS 1	RIF 5	RIF 2	**RIF 1**	RIF 0,5
ETH 20	ETH 10	**ETH 5**	ETH 2	ETH 1
STR 20	STR 10	**STR 5**	STR 2	STR 1



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Results of Genotypic Resistance among the Isolates

Genetic resistance based on Phosphorus-32 Dot Blot Hybridisation

Genetic resistance based on the *rpoB* gene codons 526 and 531 are presented for only strains isolated from coughing inmates and those from patients presenting at the selected hospitals. Isolates obtained from TB diagnosed at PM were not tested by this method due to logistical contraints with the procurement of ^{32}P-δATP (Amersham Life Science, UK).

Results of the ^{32}P-Dot Blot Hybridisation revealed that seven (7) isolates obtained from the presenting patients had mutations in the *rpoB* gene (codon 531) and therefore resistant to RIF. This technique also revealed that only one (1) isolate from the coughing prison inmates also had a mutation in the *rpoB* gene (codon 531). Blinded sequencing, performed by an independent laboratory, not privy to the Dot Blot data, indicated that the mutation was the *rpoB* codon 531-TTG mutation. Six (6) out of the seven (7) isolates from patients presenting at the selected hospitals were also found to harbour mutations in codon 531 on the *rpoB* gene. The mutation was a codon 531-TTG mutation. The other isolate had a mutation at codon 526 (*rpoB* codon 526-ACC).

Results of Genetic resistance based on GenoType® MTBDR*plus* Assay

A total of 16 isolates obtained from samples collected from patients presenting at the selected hospitals were found to be resistant to INH by the GenoType® MTBDR*plus* assay (Table 29). Nine (9) out of these 16 isolates harboured the

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katG MUT1 mutation (AGC→ACC) whiles the remaining 7 isolates contained the *inhA* MUT1 (C→T in position 15) mutation. Fourteen (14) of the 16 INH-resistant isolates were also found to be resistant to INH by the MIC-DST method, which is the 'Gold Standard'. Five (5) isolates obtained from patients presenting at the selected hospitals were found to be resistant to RIF. Four (4) of these RIF-resistant isolates contained the *rpoB* MUT3 S531L mutation whiles one (1) isolate carried the *rpoB* MUT2B H526D mutation. All these 5 isolates were also found to be RIF-resistant by the 'Gold Standard', the MIC-DST method.

The GenoType® MTBDR*plus* assay found 1 isolate each from the prisons inmates to be resistant to INH and RIF (Table 29). The INH-resistant isolate carried the *inhA* MUT1 (C→T in position 15) while the RIF-resisatnt isolate carried *rpoB* MUT3 S531L mutation. These two were also confirmed as such by the MIC-DST method. The ability of the MIC-DST method to identify most of the isolates as resistant to the first line drugs, indicates that it can be used for early detection in the absence of equipment for the sophisticated GenoType® MTBDR*plus* assay. No isolate amongst those obtained from post-mortem samples was found to be resistant to either INH or RIF (Table 29).

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Table 29

Results of Genotypic Resistance based on the GenoType® MTBCDRplus Assay

Population	Isoniazid-resistant isolates, INH^R			Rifampicin-resistant isolates, RIF^R		
	inhA MUT1 (C→T in position 5) mutation	*katG* MUT (AGC→ACC) mutation	Total	*rpoB* MUT3 S531L mutation	*rpoB* MUT2B H526D mutation	Total
Prison Inmates	1	0	1	1	0	1
Presenting Patients	7	9	16	4	1	5
Decedents	0	0	0	0	0	0
Total	8	9	17	5	1	6

Results of Genetic characterisation

Genetic characterisation based on Multiplex Ligation-dependent Probe Assay (MLPA)

The 136 isolates from samples obtained from patients presenting at the selected hospitals classified as MTB PGG2 (Table 30) included also members of the sub-groups MTB Haarlem (14), MTB (121) and MTB LAM RD-Rio (1) based on MLPA analysis (Table 31).

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Table 30

Principal genotypic groups (PGGs) of MTBC isolates detected by MLPA using various probes

	Principal Genotypic Group (PGG)			
Source of Isolate	PGG 1	PGG 2	PGG 3	Total
Coughing prison inmates	1	27	2	30
Patients Presenting at selected hospitals	15	136	19	170
Tissues at post-mortem	11	32	0	43
Total	28	195	20	243

Also, 10 out of the 15 isolates of this panel (presenting patients) classified broadly as PGG1 (Table 30) were further classified as *M. africanum/bovis* while 2 each were classified as Ancestral TB/*M. canetti* and MTB Beijing respectively (Table 31). Of the 30 isolates obtained from coughing prison inmates, 2, 27 and 1 were classified as PGG1, PGG2 and PGG3, respectively (Table30). The 27 PGG2 isolates included 1 isolate each further sub-classified as MTB LAM RD-Rio and MTB Haarlem (Table 31). Of the 11 PGG1 isolates obtained from post-mortem (Table 30), 10 were further sub-classified as *M. africanum/bovis* and 1 as Ancestral TB/*M. canetti* (Table 31).

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Table 32

Number of isolates from the various Population groups identified as resistant to INH, RIF and EMB by MLPA analysis

Population Type	INH^R	RIF^R	EMB^R
Coughing Prison Inmates	1	1	1
Patients Presenting	16	5	6
Tissues from Post-mortem	0	0	0

Legend: INH^R=Isoniazid-resistant; RIF^R=Rifampicin-resistant; EMB^R=Ethambutol-resistant

Similarly, five of the six isolates detected by MLPA as being resistant to EMB were also found to be resistant to EMB by the MIC-DST method. Resistance patterns were first visualised by 2% agarose gel electrophoresis and then confirmed by Fragment Analysis using Capillary Electrophoresis (Anthony et al., 2005). Agarose gel electrophoresis preliminarily revealed fragments indicative of resistance-associated mutations and species or lineage.

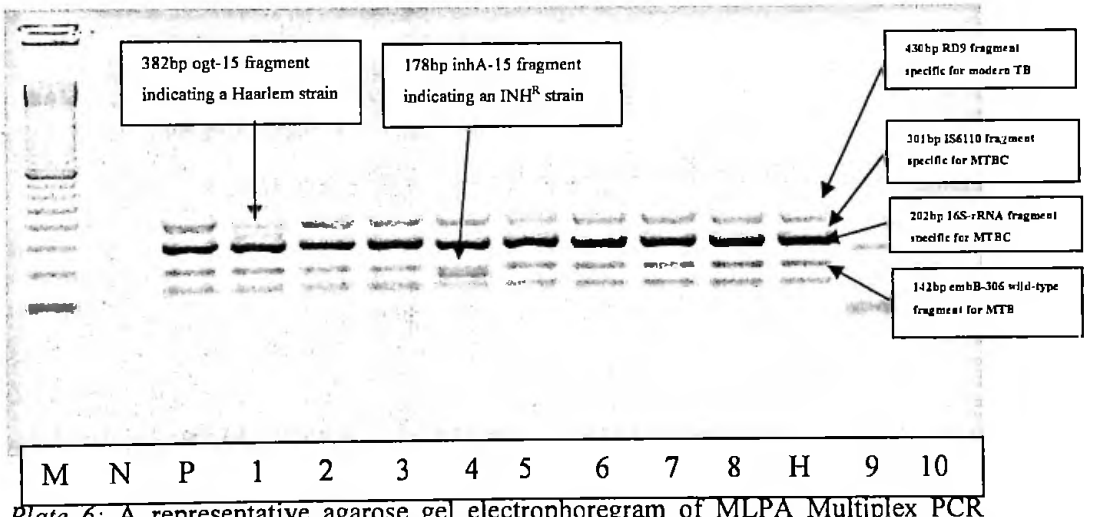

Plate 6: A representative agarose gel electrophoregram of MLPA Multiplex PCR products (Legend: M = Molecular weight Marker; N = Milli Q water as Negative control; P = MTB Strain 72 as positive control; H = MTB H37Rv; Sample 1 = Haarlem strain (note presence of fragment 382bp from *ogt*-15 probe); Sample 4 = INH resistant isolate (note fragment at 178bp from *inhA*-15 probe); 2, 3, 5, 6, 7, 8, 9, 10 = Susceptible and classical MTB Samples).

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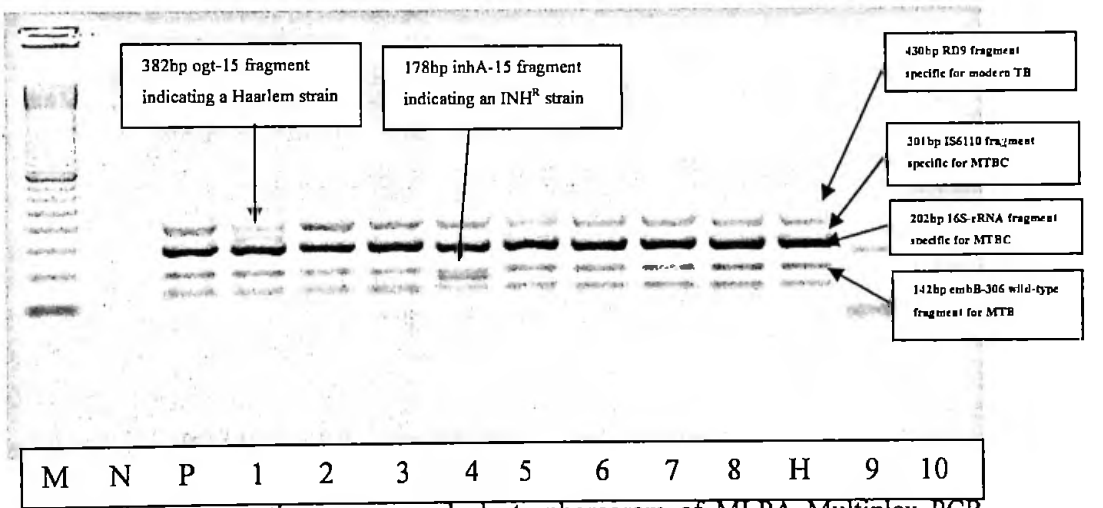

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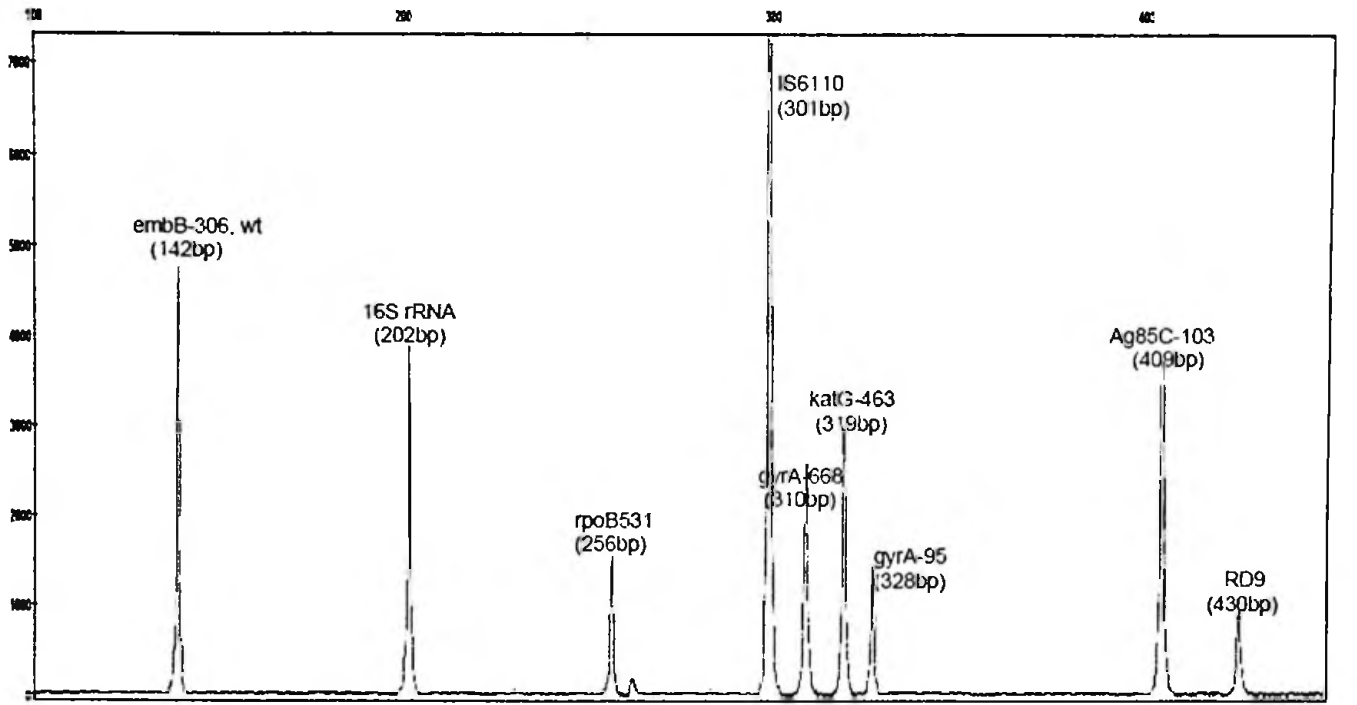
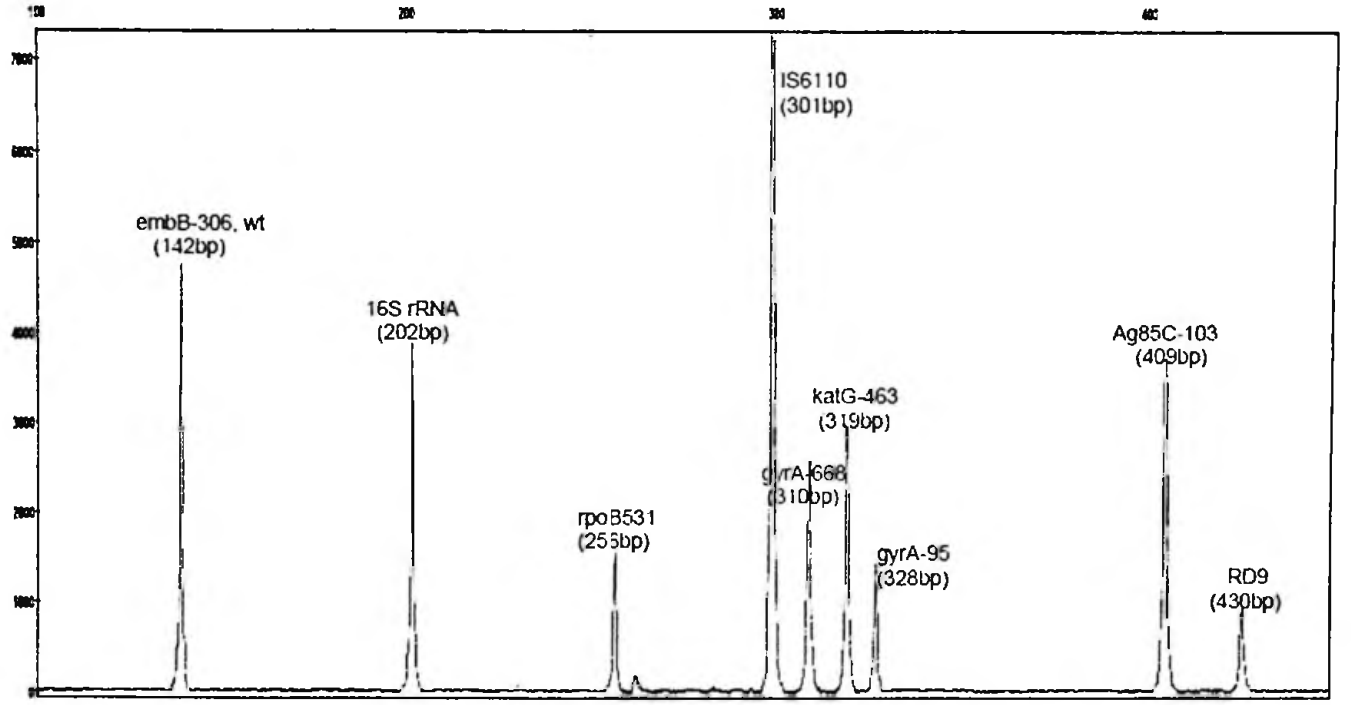


Figure 13: Capillary electrophoresis spectrum for a Rifampicin-resistant and LAM RD-Rio strain (Legend: embB-306 (142bp fragment)=Wild-type EMB resistance marker; 16S rRNA (202bp fragment)=16S ribosomal RNA gene, MTB-complex specific; rpoB-531 (256bp fragment)=RIF resistance marker; IS6110 (301bp fragment)=Insertion Element IS6110, MTB-complex specific; gyrA-668 (310bp fragment) =Putative genotype marker, specific for PGG 1 and 2; katG-463 (319bp fragment)=Genotype marker, specific for PGG 2 and 3; gyrA-95 (328bp fragment)=Genotype marker, specific for PGG 1 and 2; Ag-85C (409bp fragment)=Genotype marker, specific for LAM RD-RIO lineage (LAM9 Spoligotype); RD9 (430bp fragment)=Genotype marker, only present in modern M. tuberculosis).



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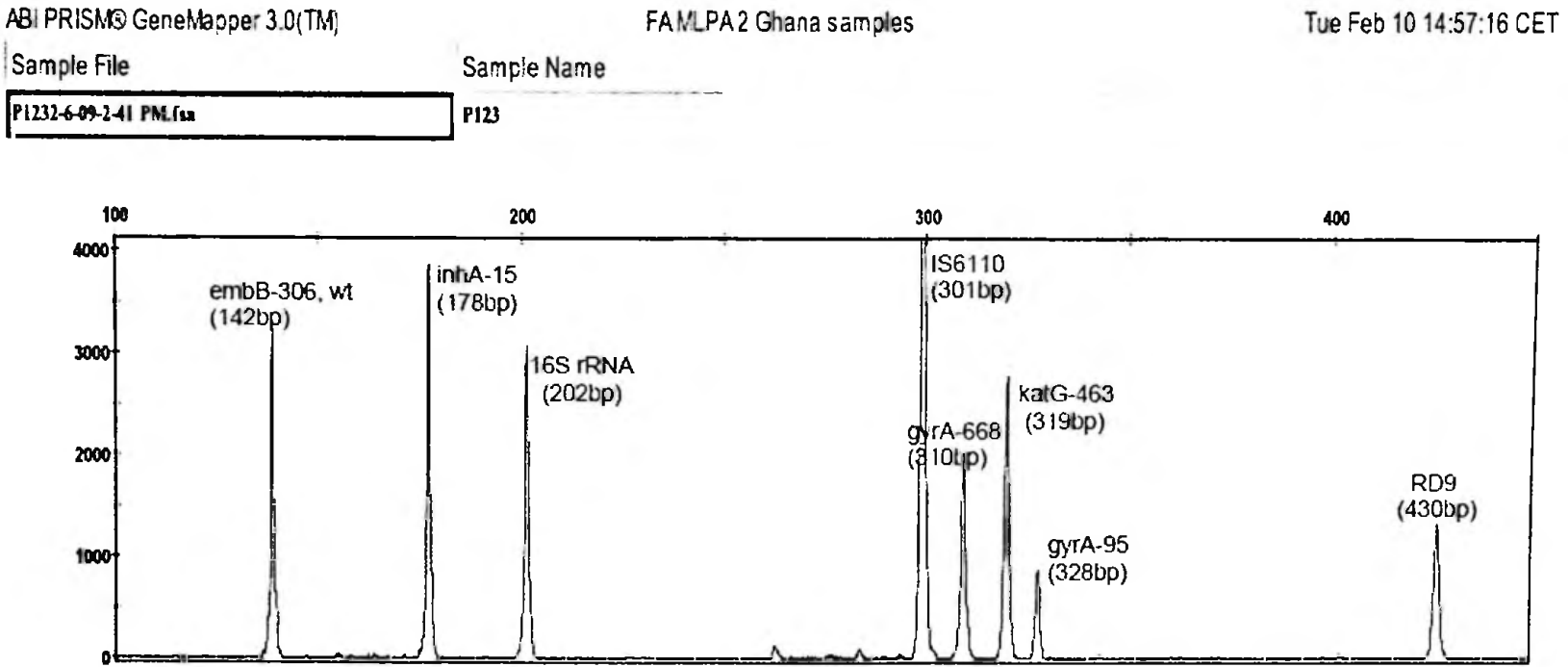

Figure 14: Capillary electrophoresis spectrum for an Isoniazid-resistant strain (Legend: embB-306 (142bp fragment)=Wild-type EMB resistance marker; inhA-15 (178bp fragment)=INH resistance marker;16S rRNA (202bp fragment)=16S ribosomal RNA gene, MTB-complex specific; IS*6110* (301bp fragment)=Insertion Element IS*6110*, MTB-complex specific; gyrA-668 (310bp fragment)=Putative genotype marker, specific for PGG 1 and 2; katG-463 (319bp fragment)=Genotype marker, specific for PGG 2 and 3; gyrA-95 (328bp fragment)=Genotype marker, specific for PGG 1 and 2RD9 (430bp fragment)=Genotype marker, only present in modern *M. tuberculosis*).

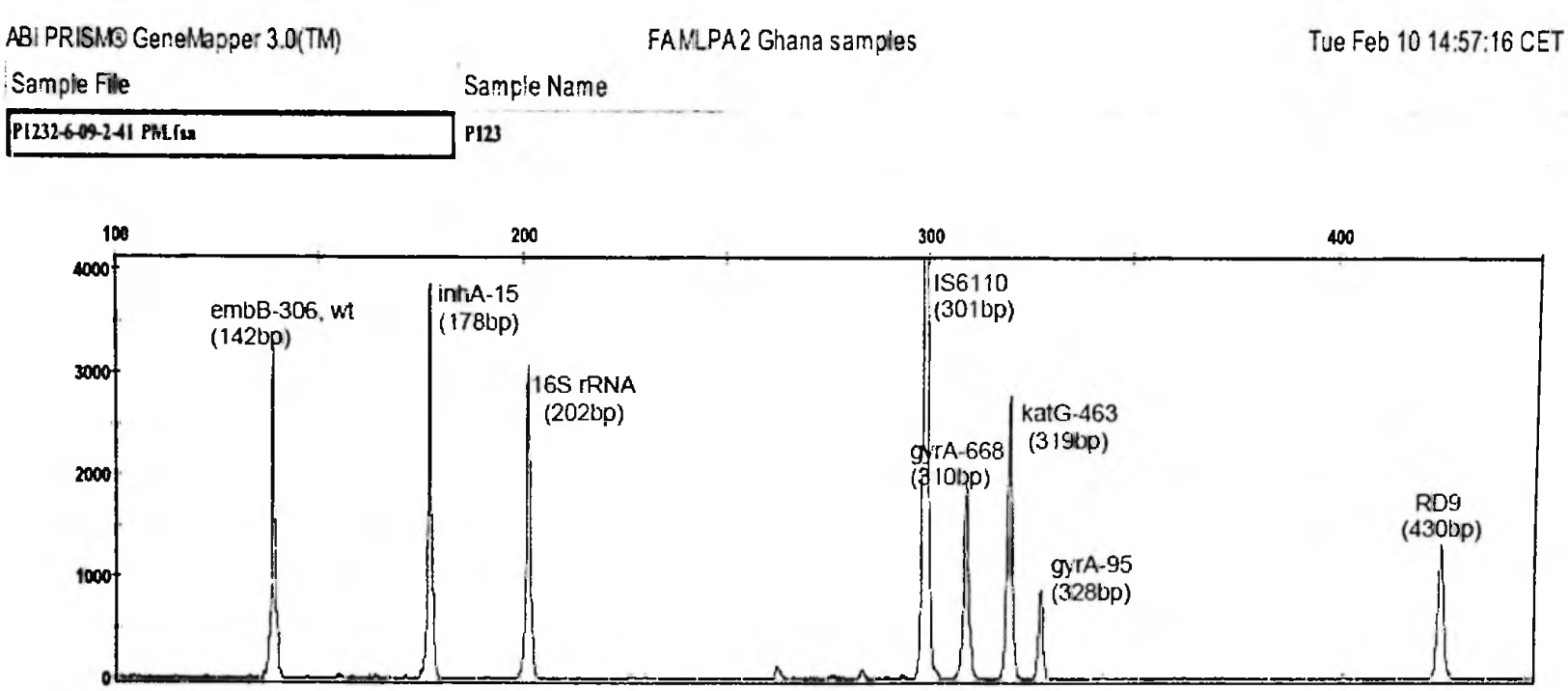

Figure 14: Capillary electrophoresis spectrum for an Isoniazid-resistant strain (Legend: embB-306 (142bp fragment)=Wild-type EMB resistance marker; inhA-15 (178bp fragment)=INH resistance marker;16S rRNA (202bp fragment)=16S ribosomal RNA gene, MTB-complex specific; IS*6110* (301bp fragment)=Insertion Element IS*6110*, MTB-complex specific; gyrA-668 (310bp fragment)=Putative genotype marker, specific for PGG 1 and 2; katG-463 (319bp fragment)=Genotype marker, specific for PGG 2 and 3; gyrA-95 (328bp fragment)=Genotype marker, specific for PGG 1 and 2RD9 (430bp fragment)=Genotype marker, only present in modern *M. tuberculosis*).

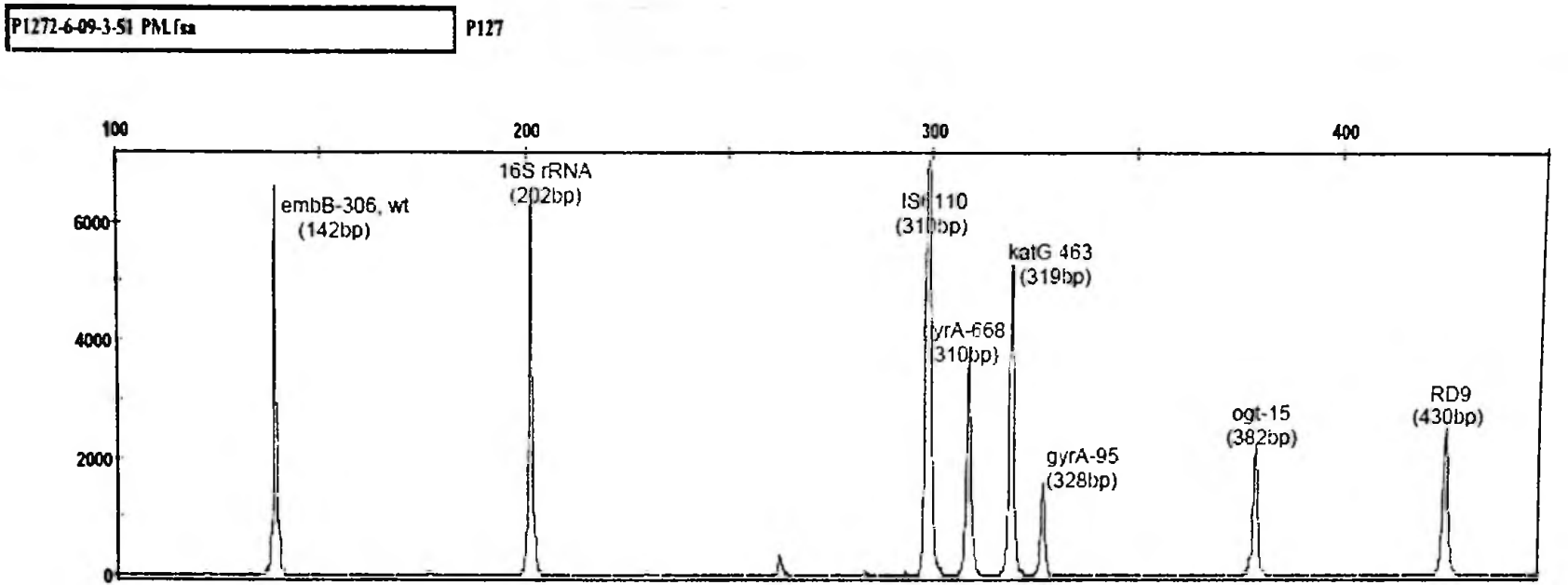

Figure 15: Capillary electrophoresis spectrum for a Haarlem strain (Legend: embB-306 (142bp fragment)=Wild-type EMB resistance marker; 16S rRNA (202bp fragment)=16S ribosomal RNA gene, MTB-complex specific; IS6110 (301bp fragment)=Insertion Element IS*6110*, MTB-complex specific; gyrA-668 (310bp fragment) =Putative genotype marker, specific for PGG 1 and 2; katG-463 (319bp fragment)=Genotype marker, specific for PGG 2 and 3; gyrA-95 (328bp fragment)=Genotype marker, specific for PGG 1 and 2; ogt-15 (382bp fragment)= Genotype marker for Haarlem lineage; RD9 (430bp fragment)=Genotype marker, only present in modern *M. tuberculosis*).

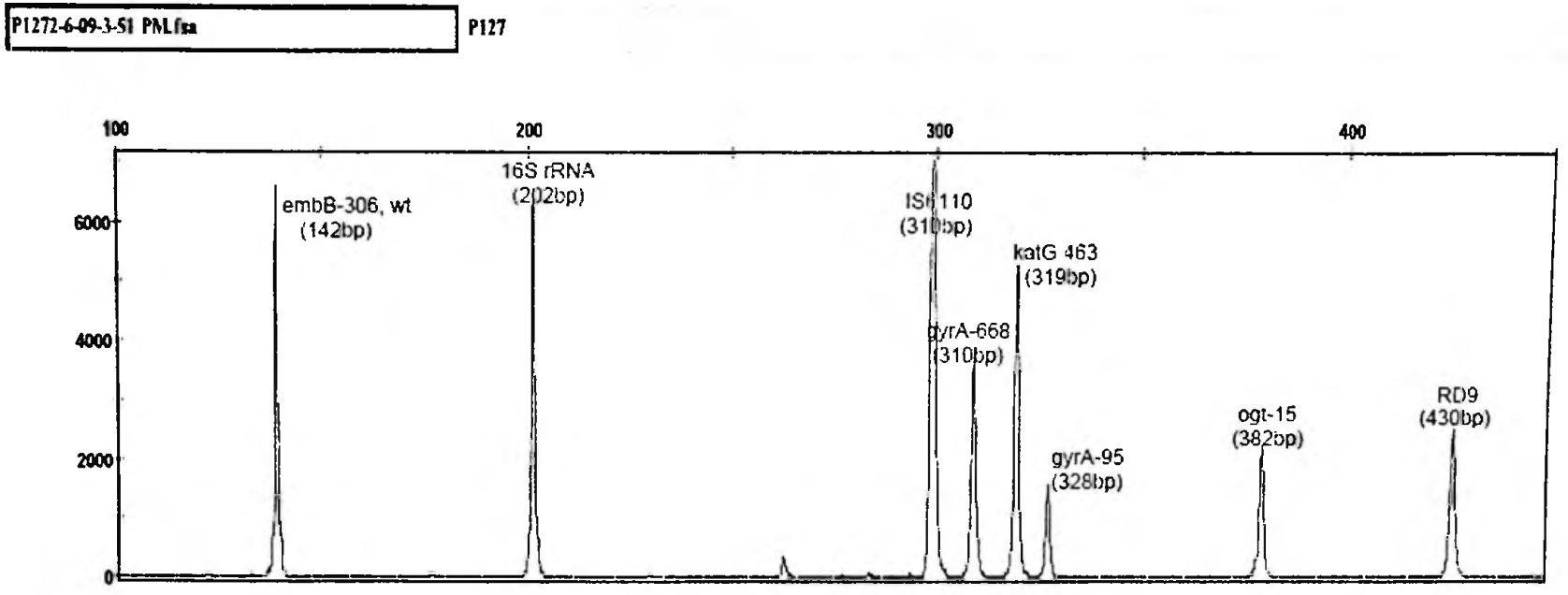

Figure 15: Capillary electrophoresis spectrum for a Haarlem strain (Legend: embB-306 (142bp fragment)=Wild-type EMB resistance marker; 16S rRNA (202bp fragment)=16S ribosomal RNA gene, MTB-complex specific; IS6110 (301bp fragment)=Insertion Element IS*6110*, MTB-complex specific; gyrA-668 (310bp fragment) =Putative genotype marker, specific for PGG 1 and 2; katG-463 (319bp fragment)=Genotype marker, specific for PGG 2 and 3; gyrA-95 (328bp fragment)=Genotype marker, specific for PGG 1 and 2; ogt-15 (382bp fragment)= Genotype marker for Haarlem lineage; RD9 (430bp fragment)=Genotype marker, only present in modern *M. tuberculosis*).

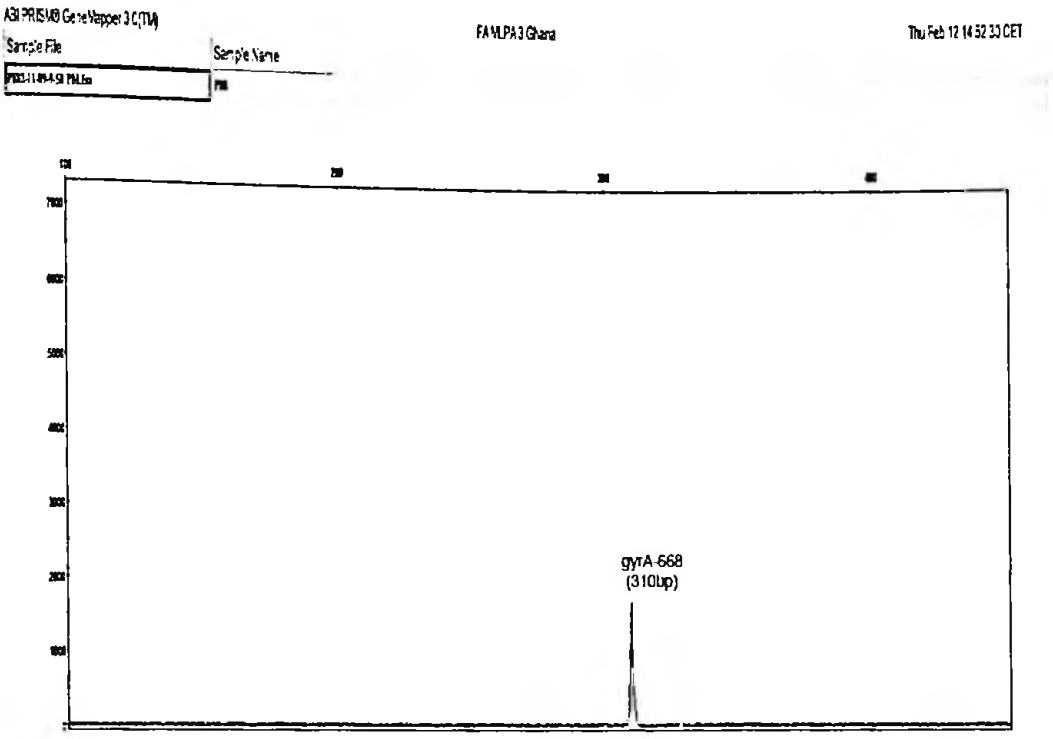

Figure 16: Capillary electrophoresis spectrum for an NTM strain (Legend: gyrA-668 (310bp fragment) =Putative genotype marker, specific for PGG 1 and 2. Note that there are no other diagnostic fragment peaks).

Results of Genetic characterisation of NTM isolates by BLAST sequencing and INNO-LiPA Mycobacteria Assay

Nine isolates out of the 13 isolates (classified as NTM by the Capilia Neo-TB ICA and 16S rDNA PCR Assay) and identified by BLAST-sequencing were also subjected to the reversed line blot INNO-LiPA assay as shown in Table 33.

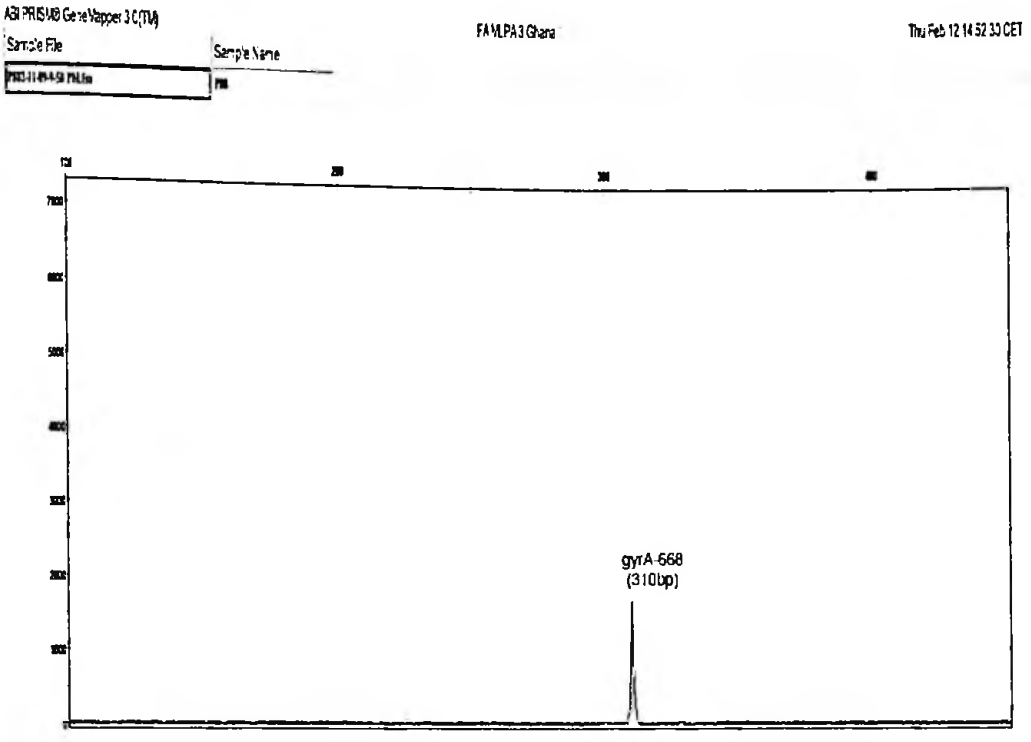

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Table 33

Classification of NTM by BLAST Sequencing and the INNO-LiPA Assay

Sample ID	BLAST Sequencing	INNO-LiPA Mycobacteria Assay
P09	*Nocardia nova*	NR
P13	*M. immunogenum* (related to *M. abscessus*)	*M. chelonae complex* (Group III, *M. abscessus*)
P14	*M. mucogenum*	NR
P17	*M. porcinum*	NR
P18	*M. porcinum*	NR
P71	*M. peregrenum*	*M. fortuitum -M. peregrenum*
P88	*M. abscessus*	*M. chelonae* complex (Group III, *M. abscessus*)
P91	*M. chelonae*	*M. chelonae* (Group I)
P102	*M. fortuitum*	NR

Legend: NR = non reactive

Characteristics of isolates based on the GenoType® MTBC Assay

All the isolates from the three sources were subjected to the GenoType® Assay (version 2.0) in order to broadly characterise isolates as to whether they were members of the MTBC or the several commonly encountered NTM species. The test characterises MTBC isolates as - *Mycobacterium tuberculosis*, *Mycobacterium bovis* (as *Mycobacterium bovis* ssp. *bovis*), *Mycobacterium caprae* (as *Mycobacterium bovis* ssp. *caprae*), *Mycobacterium bovis* BCG, *Mycobacterium microti* or as *Mycobacterium africanum* I (MAF I). Results are displayed in Table 34.

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P18	*M. porcinum*	NR
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P102	*M. fortuitum*	NR

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Table 34

Isolates identified by the GenoType® MTBC Assay

Source of Isolate	Type of Isolate				
	MTB	MAF I	*M. bovis*	*M. bovis* BCG	Total
Coughing Prison Inmates	29	1	0	0	30
Patients Presenting	162	8	0	0	170
Tissues at Post-mortem	32	11	0	0	43

Key: MAF I = *M. africanum* I

The results were determined after PCR and agarose gel electrophoresis. The electrophoregrams were interpreted as follows: two close bands indicated members of the MTBC; single bands indicated non-tuberculous mycobacteria (NTM) or atypical mycobacteria while 3 bands indicated the presence of *M. bovis* BCG. Plate 7 shows an electrophoregram of mycobacterial reference strains used for the GenoType® MTBC Assay. Plates 7 and 8 depict results of agarose gel electrophoresis after PCR for reference strains and samples respectively.

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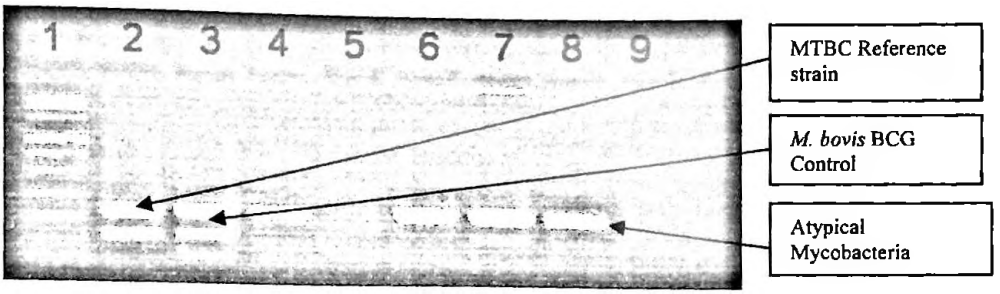


Plate 7: Agarose Gel Electrophoregram of PCR products of reference mycobacterial strains using the GenoType® MTBC Assay (Legend: 1 = Molecular Marker VIII; 2 =MTBC; 3 =*M. bovis* BCG; 4, 6 = atypical mycobacteria; 5 =Negative control; 7 =*M. gordonae*; 8 =*M. malmoense*; 9 =Blank).

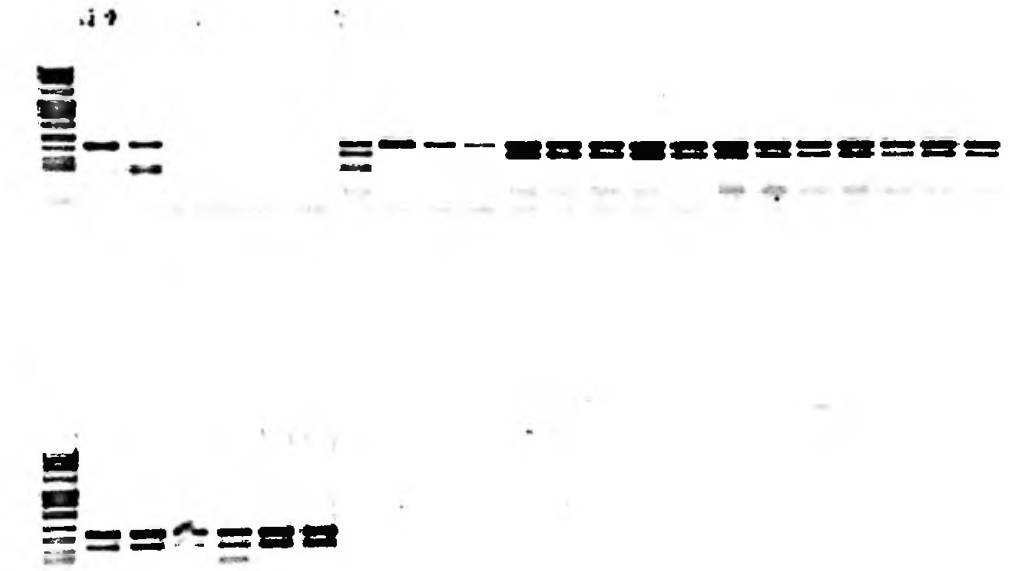
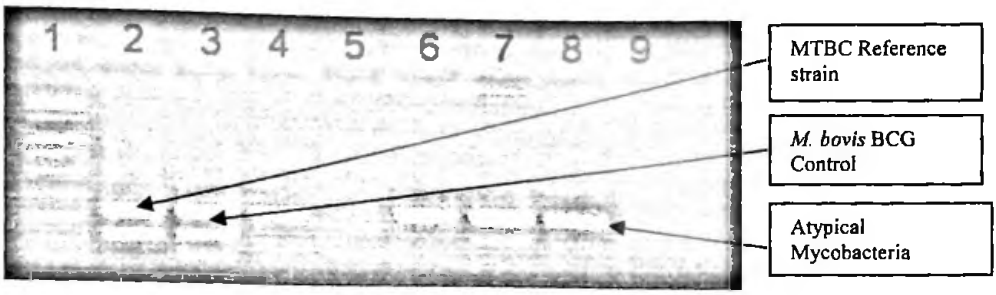


Plate 8: A Representative agarose gel electrophoregram of GenoType® MTBC PCR of isolates. (Legend: Single bands indicate atypical or non-tuberculous mycobacteria, double bands indicate MTBC, and triple bands indicate *M. bovis* BCG control).



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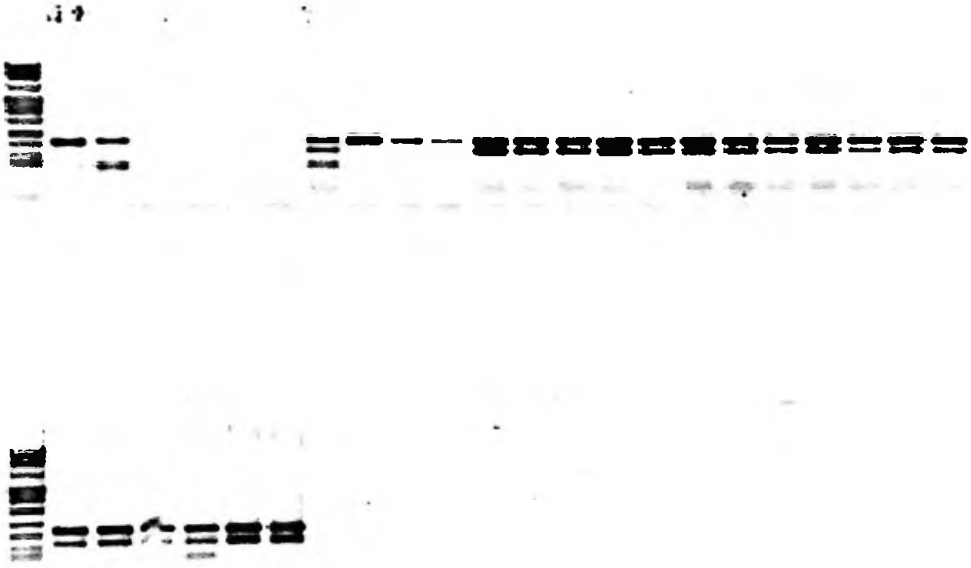

Plate 8: A Representative agarose gel electrophoregram of GenoType® MTBC PCR of isolates. (Legend: Single bands indicate atypical or non-tuberculous mycobacteria, double bands indicate MTBC, and triple bands indicate *M. bovis* BCG control).

Results of Spoligotyping (Diversity of MTB Spoligotypes)

All isolates in the 3 panels were successfully subjected to Spoligotyping in order to garner information on the MTB spoligotypes in circulation and to find out if the spoligotype diversity was the same for all these thematic areas *viz.* the prisons, patients presenting at hospitals and TB diagnosed at PM. Spoligotyping was carried out after PCR to amplify the DR region of isolates with the primer set DRa-bio and DRb. Plate 9 shows a gel electrophoregram being used to verify PCR of the DR region of the MTB.

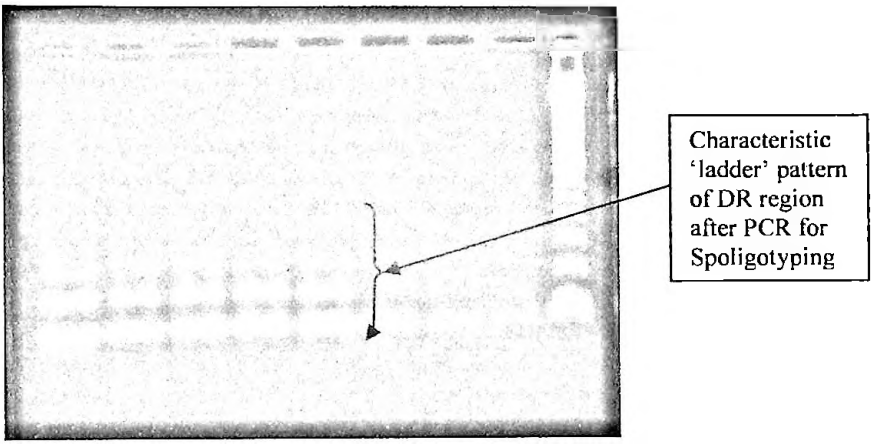

Plate 9: A 1.5% Agarose gel electrophoregram verifying PCR amplification of the DR region of MTB for Spoligotyping

Plate 10 depicts the Spoligotype patterns obtained for a panel of isolates. Solid dots indicate the existence of spacers while empty spaces indicate a lack of spacers.

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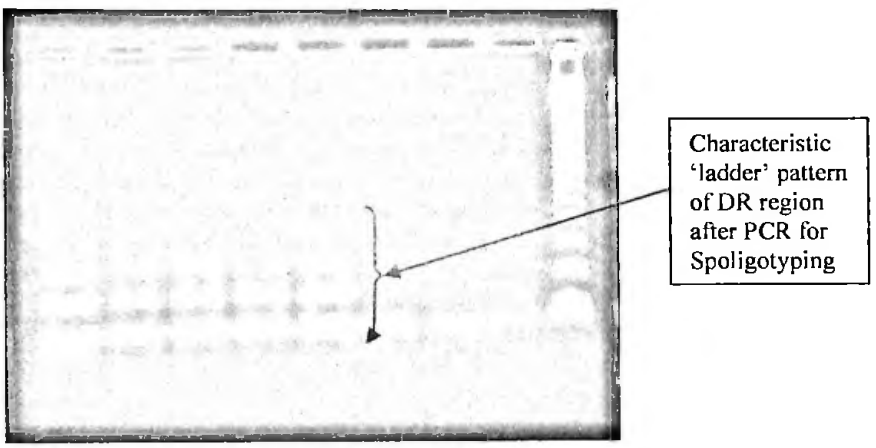

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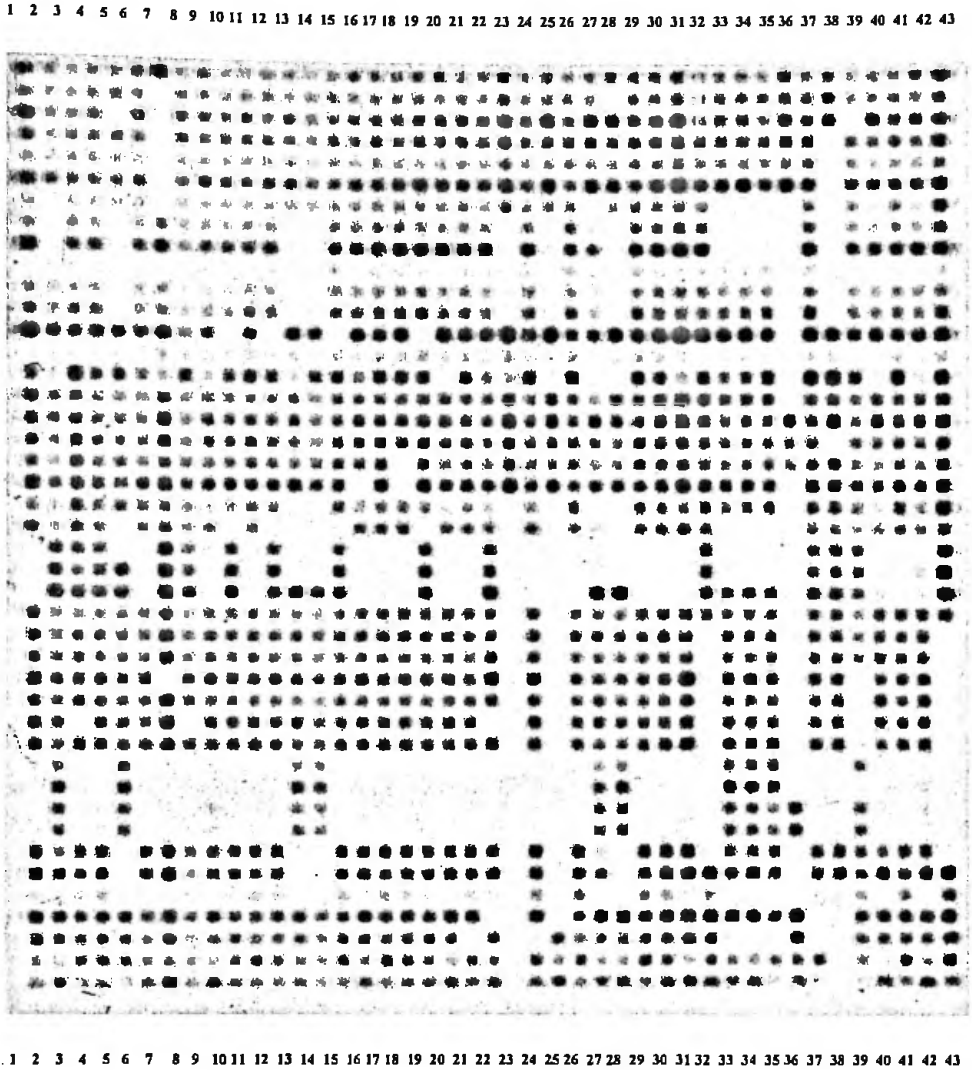

Plate 10: Spoligotype patterns of a panel of MTB isolates. (Legend: Dark squares indicate hybridisation (existance of spacers) while blank squares indicate a lack of hybridisation (absence of spacersd) in the DR region of the MTB genome).

Diversity of MTBC Spoligotypes from the Coughing Prison Inmates

All 30 isolates were spoligotyped and the patterns used to query the SITVIT WEB Internet suite, the MIRU-VNTR*plus* website and TB-Insight web tool. Table 34 depicts the different spoligotypes of isolates obtained from the coughing prison inmates.

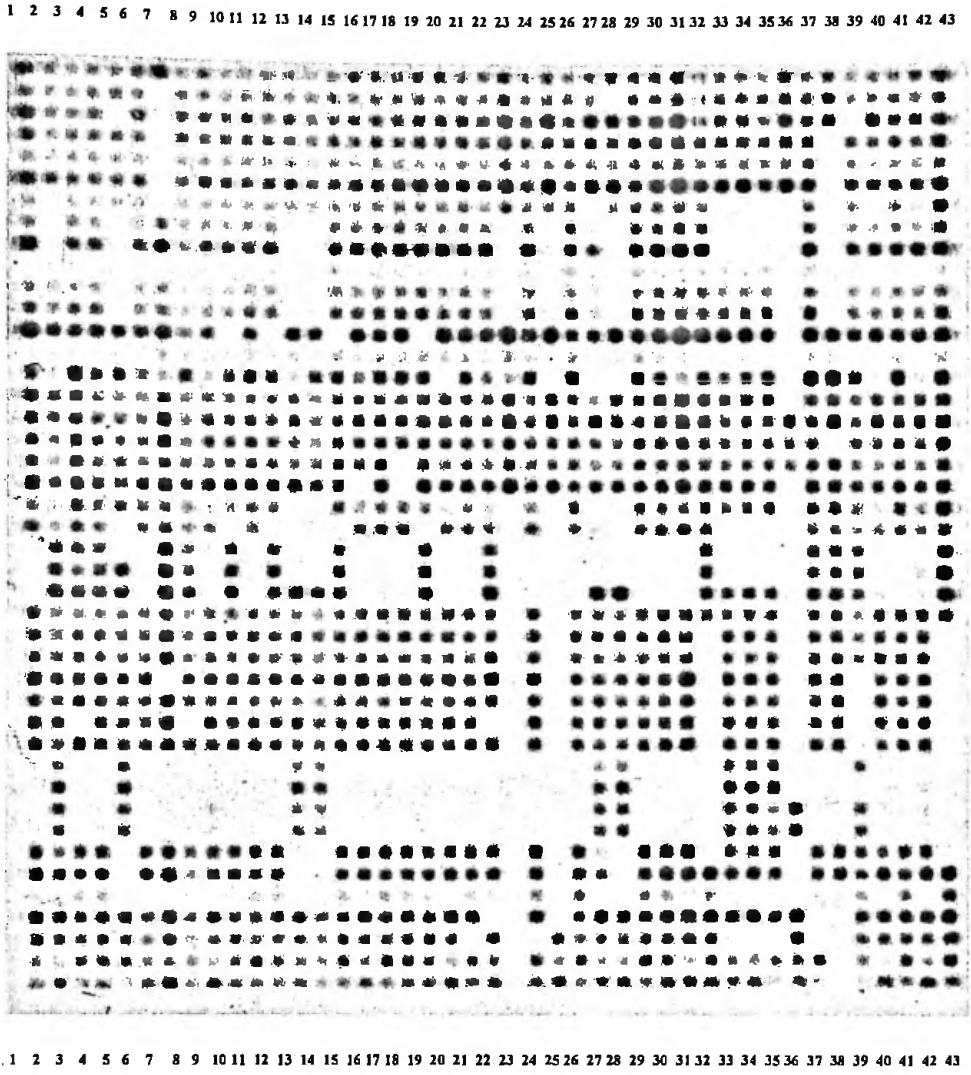

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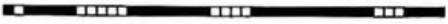
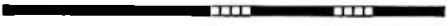
Table 35

Spoligotypes of isolates obtained from coughing prison inmates

SPOLIGOTYPE PATTERN[d]	SITVIT WEB Clade by KBBN (TB-Insight)	Major Lineage by Rules (TB-Insight)	Major Lineage by CBN (TB-Insight)	Frequency
	T	Euro-American	Euro-American	12
	Cameroun	Euro-American	Euro-American	1
	Cameroun	Euro-American	Euro-American	7
	T3	Euro-American	Euro-American	6
	AFRI_2	West African 1	M. africanum	1
	LAM	Euro-American	Euro-American	1
	T	Euro-American	Euro-American	1
	Cameroun	Euro-American	Euro-American	1
Total				30

These spoligotypes displayed in Table 35, upon querying the SpolDB4 database hosted by the Pasteur Institute, Guadaloupe (The SITVIT Database, n.d., Query Tools), yielded unambiguously finer classifications in terms of the Signature Type (ST) or Signature International Type (SIT) and the SpolDB4

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Spoligotypes of isolates obtained from coughing prison inmates

SPOLIGOTYPE PATTERN[d]	SITVIT WEB Clade by KBBN (TB-Insight)	Major Lineage by Rules (TB-Insight)	Major Lineage by CBN (TB-Insight)	Frequency
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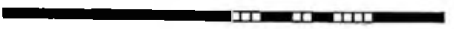
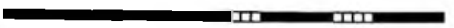
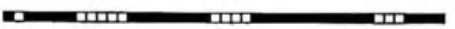
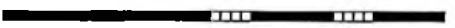
	Cameroun	Euro-American	Euro-American	1
	Cameroun	Euro-American	Euro-American	7
	T3	Euro-American	Euro-American	6
	AFRI_2	West African 1	M. africanum	1
	LAM	Euro-American	Euro-American	1
	T	Euro-American	Euro-American	1
	Cameroun	Euro-American	Euro-American	1
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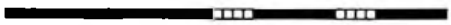
lineage assigned each spoligotype. Table 36 displays the SpolDB4 SITs and lineages of the MTB isolates from the prisons inmates.

Table 36

SpolDB4 SITs and lineages of the MTB isolates from the prisons inmates

SPOLIGOTYPE PATTERN[d]	SpolDB4 ST or SIT	SpolDB4 Lineage	Frequency
	53	T1	12
	167	T1	1
	61	LAM10_CAM	7
	772	LAM10_CAM	1
	42	LAM9	1
	Orphan		6
	319	AFRI_2	1
	Orphan		1
Total			30

Unweighted Pair Group Method with Arithmetic Mean (UPGMA) trees showing the spoligotype lineage diversities of MTBC isolates of the various population groups are as follows: Figure 17 depicts the spoligotype lineage diversity of the 30 MTBC isolates realised from the coughing prison inmates; Figure 18 depicts the spoligotype lineage diversity of 24 MTBC isolates from the sub-population of remanded inmates.

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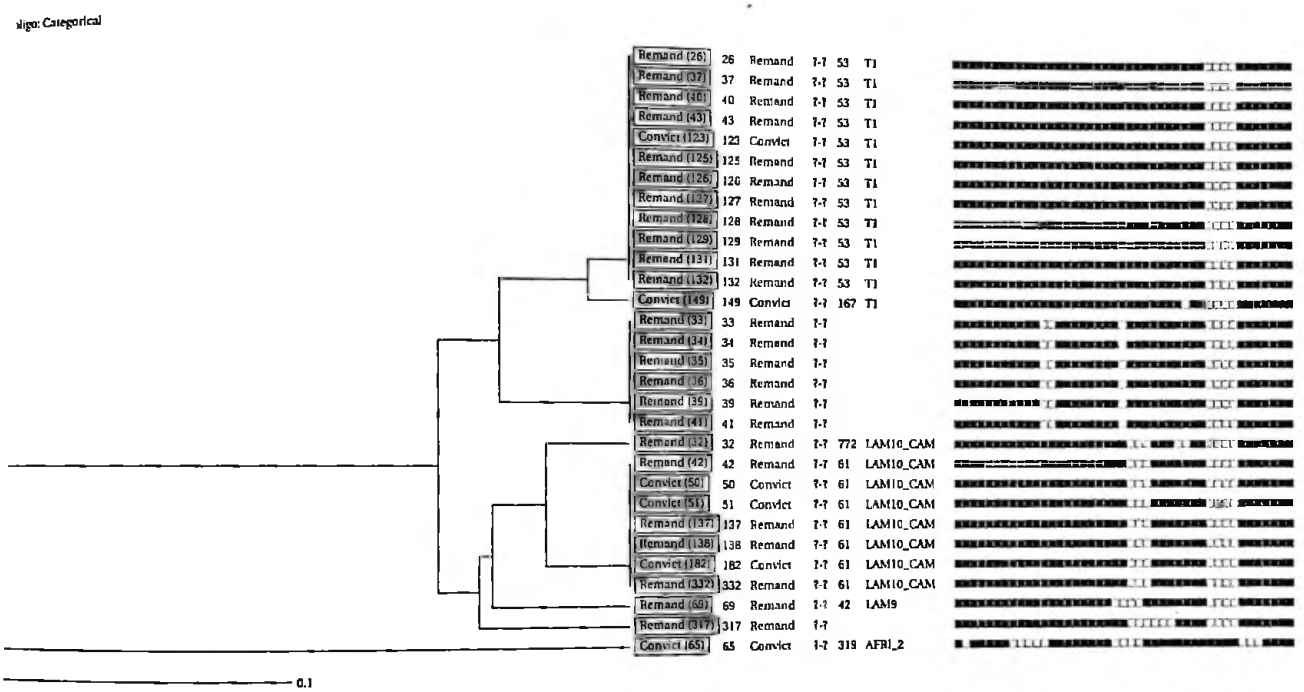
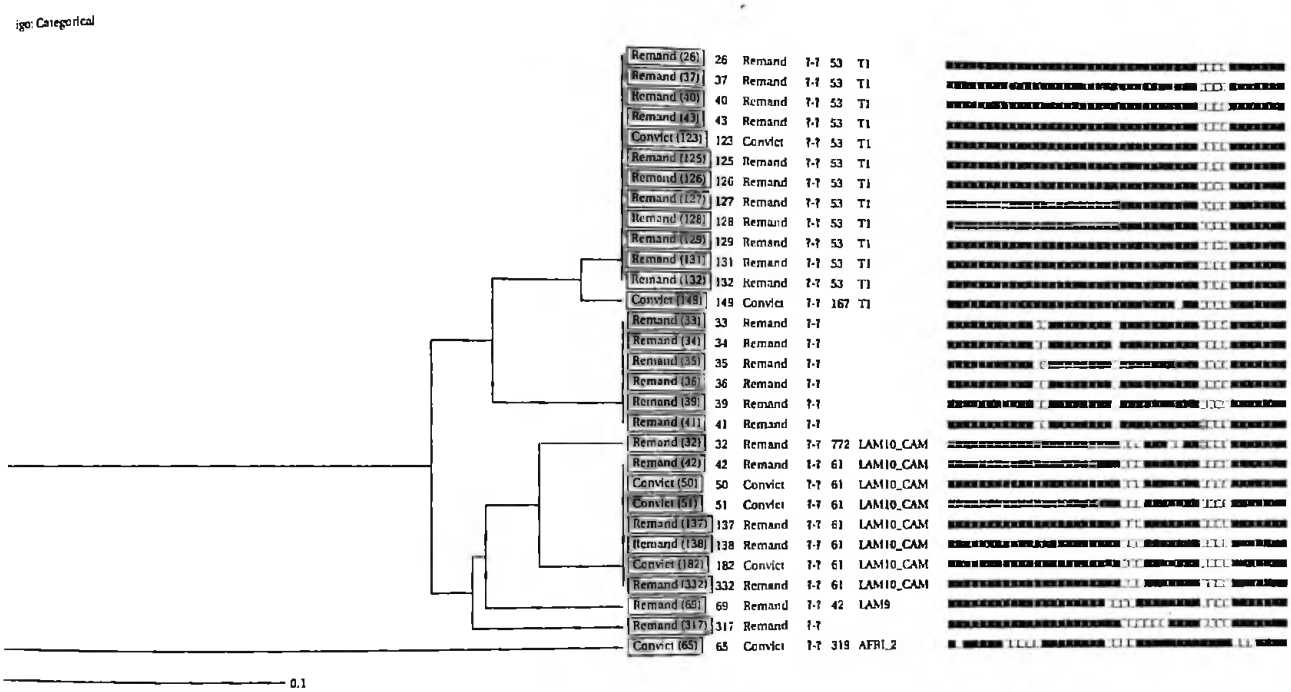


Figure 17: A UPGMA tree showing the Spoligotype lineage diversity of the 30 MTBC isolates from the coughing inmates from the selected prisons. Weighting distance measure set at Categorical=1. (Legend: UPGMA= Unweighted Pair Group Method with Arithgmetic Mean)



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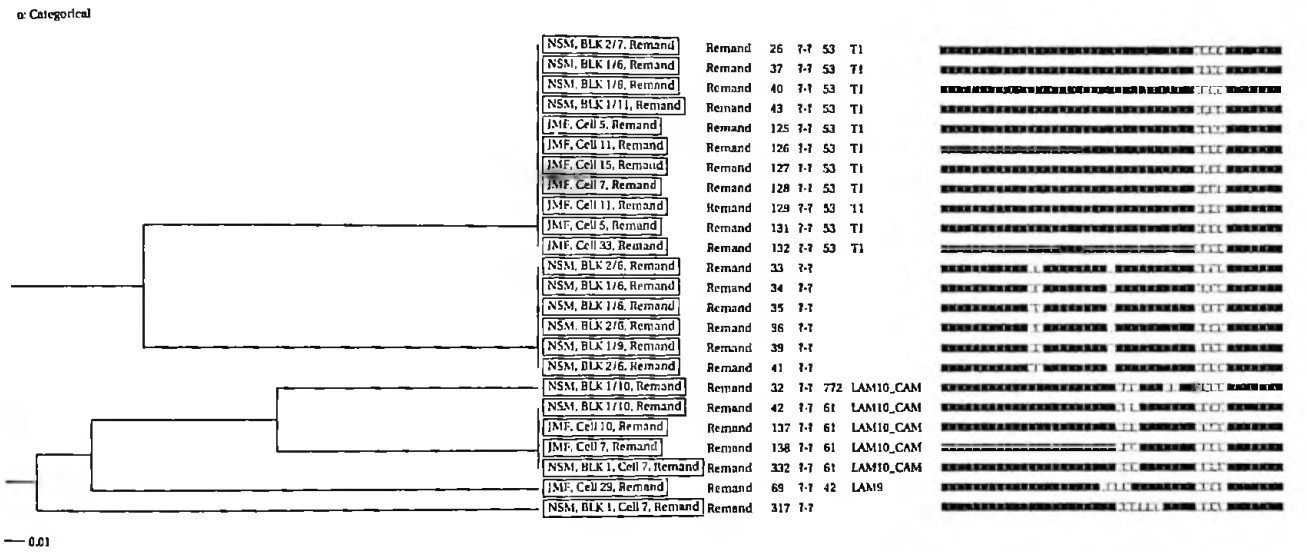


Figure 18: A UPGMA tree showing the Spoligotype lineage diversity of 24 MTBC isolates from the sub-population of remanded coughing inmates from the selected prisons. Weighting distance measure set as Categorical=1. (Legend: UPGMA= Unweighted Pair Group Method with Arithmetic Mean).

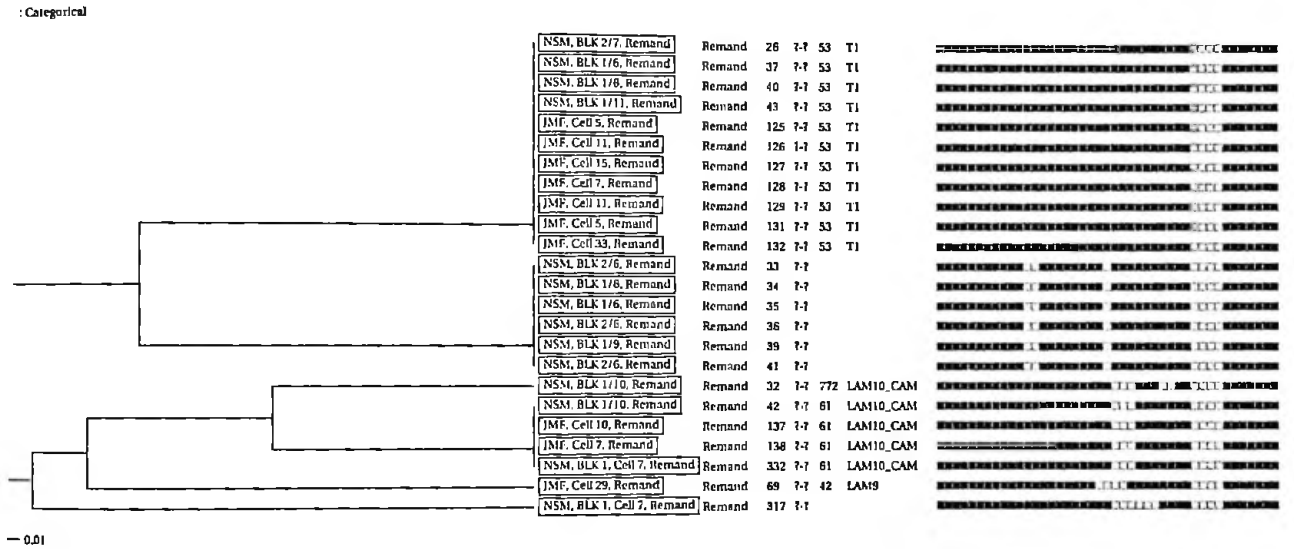


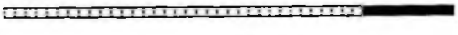
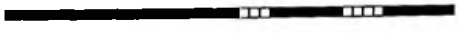
Figure 18: A UPGMA tree showing the Spoligotype lineage diversity of 24 MTBC isolates from the sub-population of remanded coughing inmates from the selected prisons. Weighting distance measure set as Categorical=1. Legend: UPGMA= Unweighted Pair Group Method with Arithmetic Mean).

Diversity of MTBC Spoligotypes from patients presenting at the selected hospitals

Analysis by MIRU-VNTR was successful for all 170 isolates obtained from patients presenting at the selected hospitals in close proximity to the prisons. Preliminary analysis with the SITVIT WEB Internet suite as well the MIRU-VNTR*plus*® website, SpolDB4 and TB-Insight web tool revealed the results in Table 37 and Table 38.

Table 37

Isolates from Presenting Patients: Spoligotypes from preliminary analysis with the SITVIT WEB Internet suite, MIRU-VNTRplus website tool and TB-Insight web tool

SPOLIGOTYPE PATTERN[d]	SITVIT WEB Clade by KBBN (TB-Insight)	Major Lineage by Rules (TB-Insight)	Major Lineage by CBN (TB-Insight)	Frequency
	Beijing	East Asian	East Asian	2
	Cameroun	Euro-American	Euro-American	12
	Cameroun	Euro-American	Euro-American	46
	Cameroun	Euro-American	Euro-American	3
	Cameroun	Euro-American	Euro-American	2
	Cameroun	Euro-American	Euro-American	1
	Cameroun	Euro-American	Euro-American	1
	T	Euro-American	Euro-American	36
	T	Euro-American	Euro-American	1

Diversity of MTBC Spoligotypes from patients presenting at the selected hospitals

Analysis by MIRU-VNTR was successful for all 170 isolates obtained from patients presenting at the selected hospitals in close proximity to the prisons. Preliminary analysis with the SITVIT WEB Internet suite as well the MIRU-VNTR*plus*® website, SpolDB4 and TB-Insight web tool revealed the results in Table 37 and Table 38.

Table 37

Isolates from Presenting Patients: Spoligotypes from preliminary analysis with the SITVIT WEB Internet suite, MIRU-VNTRplus website tool and TB-Insight web tool

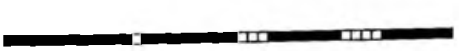
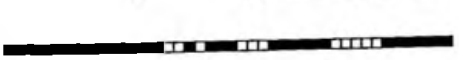
SPOLIGOTYPE PATTERN[d]	SITVIT WEB Clade by KBBN (TB-Insight)	Major Lineage by Rules (TB-Insight)	Major Lineage by CBN (TB-Insight)	Frequency
	Beijing	East Asian	East Asian	2
	Cameroun	Euro-American	Euro-American	12
	Cameroun	Euro-American	Euro-American	46
	Cameroun	Euro-American	Euro-American	3
	Cameroun	Euro-American	Euro-American	2
	Cameroun	Euro-American	Euro-American	1
	Cameroun	Euro-American	Euro-American	1
	T	Euro-American	Euro-American	36
	T	Euro-American	Euro-American	1

Table 37 (cont.)

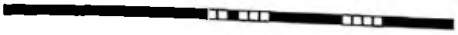
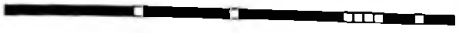
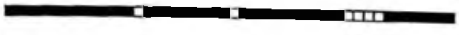
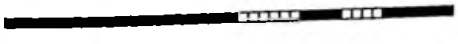
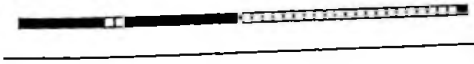
SPOLIGOTYPE PATTERN[d]	SITVIT WEB Clade by KBBN (TB-Insight)	Major Lineage by Rules (TB-Insight)	Major Lineage by CBN (TB-Insight)	Frequency
	T	Euro-American	Euro-American	1
	T3	Euro-American	Euro-American	6
	T2	Euro-American	Euro-American	2
	T2	Euro-American	Euro-American	1
	T2	Euro-American	Euro-American	3
	T3	Euro-American	Euro-American	10
	T3	Euro-American	Euro-American	1
	AFRI_2	West African 1	*M. africanum*	5
	AFRI_2	West African 1	*M. africanum*	2
	AFRI_2	West African 1	*M. africanum*	4
	AFRI_2	West African 1	*M. africanum*	1
	LAM	Euro-American	Euro-American	4
	T	Euro-American	Euro-American	1
	H1	Euro-American	Euro-American	1
	H3	Euro-American	Euro-American	8
	H3	Euro-American	Euro-American	4
	H3	Euro-American	Euro-American	2
	H3-Ural-1	Indo-Oceanic	Euro-American	3
	H3-Ural-1	Indo-Oceanic	Euro-American	1
	EAI	Indo-Oceanic	Indo-Oceanic	1
	Cameroun	Euro-American	Euro-American	4
	Zero	Indo-Oceanic	Euro-American	1
Total				170

Table 37 (cont.)

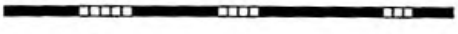
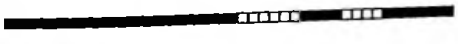
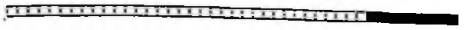
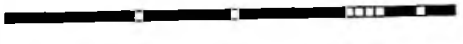
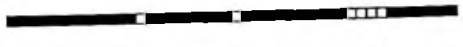
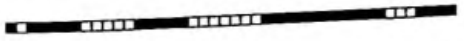
SPOLIGOTYPE PATTERN[d]	SITVIT WEB Clade by KBBN (TB-Insight)	Major Lineage by Rules (TB-Insight)	Major Lineage by CBN (TB-Insight)	Frequency
	T	Euro-American	Euro-American	1
	T3	Euro-American	Euro-American	6
	T2	Euro-American	Euro-American	2
	T2	Euro-American	Euro-American	1
	T2	Euro-American	Euro-American	3
	T3	Euro-American	Euro-American	10
	T3	Euro-American	Euro-American	1
	AFRI_2	West African 1	*M. africanum*	5
	AFRI_2	West African 1	*M. africanum*	2
	AFRI_2	West African 1	*M. africanum*	4
	AFRI_2	West African 1	*M. africanum*	1
	LAM	Euro-American	Euro-American	4
	T	Euro-American	Euro-American	1
	H1	Euro-American	Euro-American	1
	H3	Euro-American	Euro-American	8
	H3	Euro-American	Euro-American	4
	H3	Euro-American	Euro-American	2
	H3-Ural-1	Indo-Oceanic	Euro-American	3
	H3-Ural-1	Indo-Oceanic	Euro-American	1
	EAI	Indo-Oceanic	Indo-Oceanic	1
	Cameroun	Euro-American	Euro-American	4
	Zero	Indo-Oceanic	Euro-American	1
Total				170

Table 38

Isolates from Presenting Patients: SpolDB4 SITs and lineages of spoligotypes of the MTB isolates from the patients presenting at selected hospitals

SPOLIGOTYPE PATTERN[d]	SpolDB4 ST or SIT	SpolDB4 Lineage	Frequency
	1	Beijing	2
	772	LAM10_CAM	12
	61	LAM10_CAM	46
	Orphan	Orphan	3
	2550	-	2
	Orphan	-	1
	Orphan	-	1
	53	T1	36
	167	T1	1
	Orphan	-	1
	Orphan	-	6
	888	-	2
	Orphan	-	1
	73	-	3
	504	-	10
	112	-	1
	319	AFRI_2	5
	329	AFRI_2	2
	331	AFRI_2	4
	Orphan	-	1
	42	LAM9	4
	167	T1	1
	1652	-	1
	50	Haarlem	8

204

Table 38

Isolates from Presenting Patients: SpolDB4 SITs and lineages of spoligotypes of the MTB isolates from the patients presenting at selected hospitals

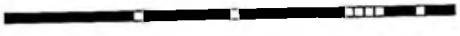
SPOLIGOTYPE PATTERN[d]	SpolDB4 ST or SIT	SpolDB4 Lineage	Frequency
	1	Beijing	2
	772	LAM10_CAM	12
	61	LAM10_CAM	46
	Orphan	Orphan	3
	2550	-	2
	Orphan	-	1
	Orphan	-	1
	53	T1	36
	167	T1	1
	Orphan	-	1
	Orphan	-	6
	888	-	2
	Orphan	-	1
	73	-	3
	504	-	10
	112	-	1
	319	AFRI_2	5
	329	AFRI_2	2
	331	AFRI_2	4
	Orphan	-	1
	42	LAM9	4
	167	T1	1
	1652	-	1
	50	Haarlem	8
			204

Table 38 (cont.)

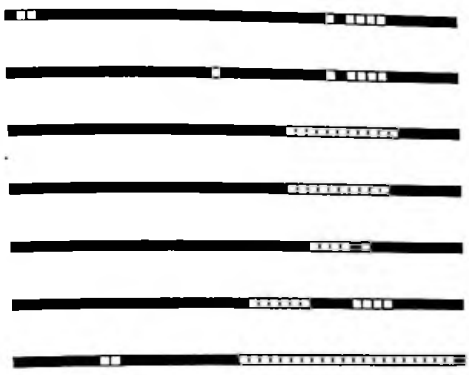
SPOLIGOTYPE PATTERN[d]	SpolDB4 ST or SIT	SpolDB4 Lineage	Frequency
	655	Haarlem	4
	1804	Haarlem	2
	1498	-	3
	374	-	1
	236	-	1
	Orphan	-	4
	Orphan	-	1
Total			170

Figure 19 is an Unweighted Pair Group Method with Arithmetic Mean (UPGMA) tree showing the spoligotype lineage diversity of the 170 MTBC isolates realised from the patients presenting at the selected hospitals.

Table 38 (cont.)

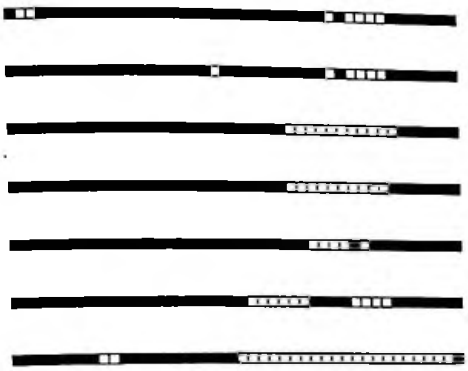
SPOLIGOTYPE PATTERN[d]	SpolDB4 ST or SIT	SpolDB4 Lineage	Frequency
	655	Haarlem	4
	1804	Haarlem	2
	1498	-	3
	374	-	1
	236	-	1
	Orphan	-	4
	Orphan	-	1
Total			170

Figure 19 is an Unweighted Pair Group Method with Arithmetic Mean (UPGMA) tree showing the spoligotype lineage diversity of the 170 MTBC isolates realised from the patients presenting at the selected hospitals.

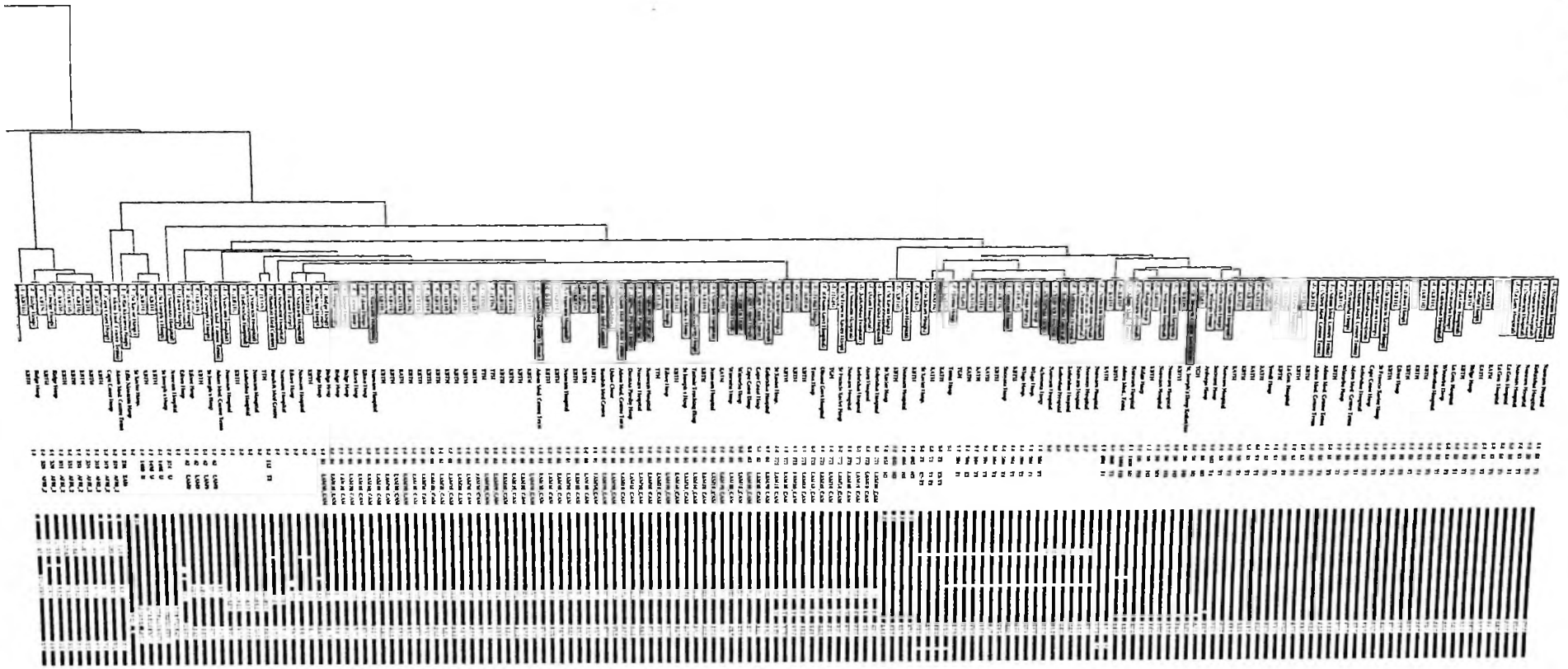


Figure 19: A UPGMA tree showing the Spoligotype lineage diversity of the 170 MTBC isolates from the patients presenting at the selected hospitals. Weighting distance measure set as Categorical=1. (Legend: UPGMA=Unweighted Pair Group Method with Arithmetic Mean).

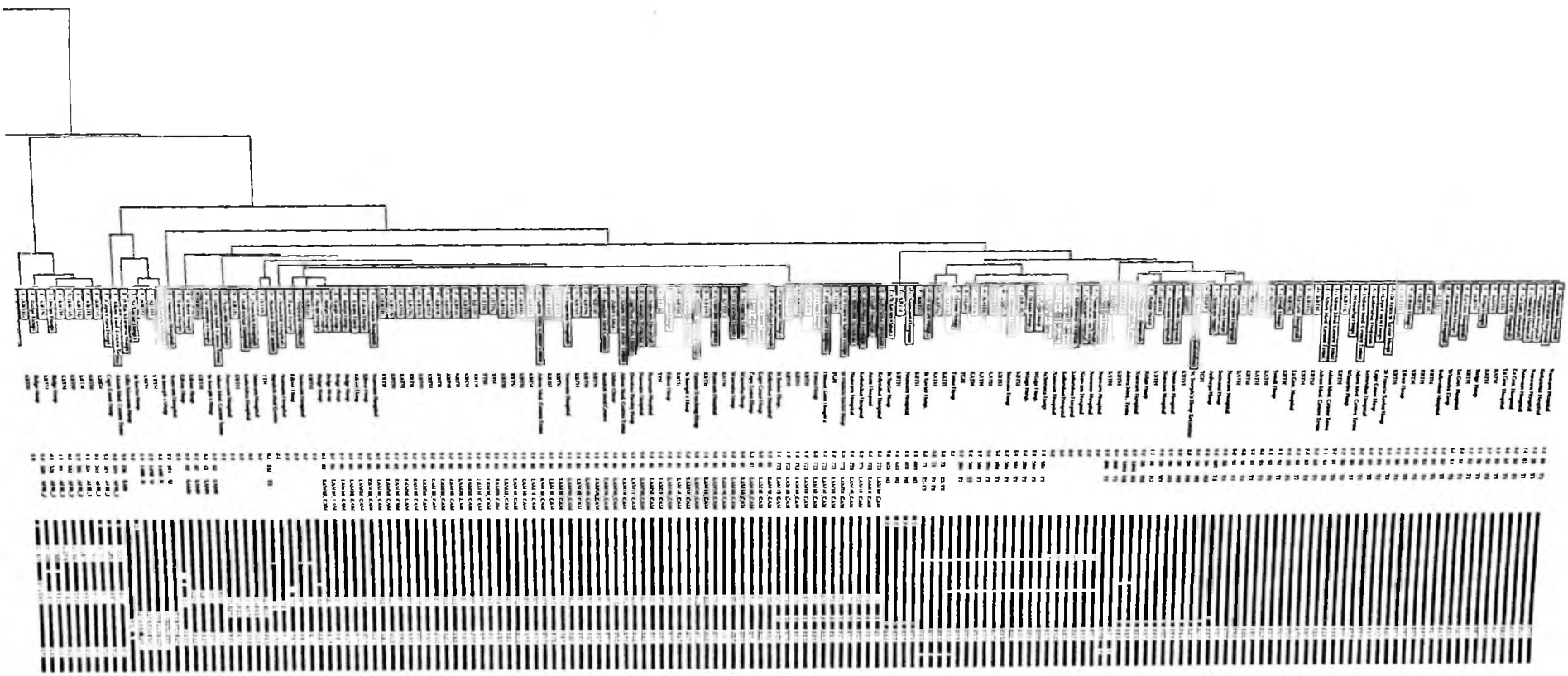


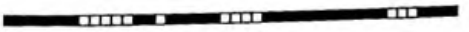
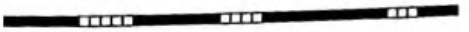
Figure 19: A UPGMA tree showing the Spoligotype lineage diversity of the 170 MTBC isolates from the patients presenting at the selected hospitals. Weighting distance measure set as Categorical=1. (Legend: UPGMA=Unweighted Pair Group Method with Arithmetic Mean).

Diversity of MTB Spoligotypes from TB diagnosed at post-mortem

The 43 isolates obtained from the diagnosis of TB at post-mortem were successfully analysed by spoligotyping. Preliminary query of the SITVIT WEB Internet suite and the MIRU-VNTR*plus* website were successful. The analysis with the KBBN platform on the TB-Insight web tool could not be carried out because it was not functional. However, results obtained using the two other tools could sufficiently shed light on the spoligotype diversity of these isolates as shown in Table 39.

Table 39

SpolDB4 SITs, lineages and frequencies of spoligotypes of the MTB isolates obtained at post-mortem

SPOLIGOTYPE PATTERN[d]	SpolDB4 ST or SIT	SpolDB4 Lineage	Frequency
	504	T3	2
	61	LAM10_CAM	13
	1498	U	1
	50	H3	1
	78	T2-T3	1
	86	T1	1
	115	LAM10_CAM	1
	329	AFRI_2	1
	331	AFRI_2	3
	1867	AFRI_1	3

Diversity of MTB Spoligotypes from TB diagnosed at post-mortem

The 43 isolates obtained from the diagnosis of TB at post-mortem were successfully analysed by spoligotyping. Preliminary query of the SITVIT WEB Internet suite and the MIRU-VNTR*plus* website were successful. The analysis with the KBBN platform on the TB-Insight web tool could not be carried out because it was not functional. However, results obtained using the two other tools could sufficiently shed light on the spoligotype diversity of these isolates as shown in Table 39.

Table 39

SpolDB4 SITs, lineages and frequencies of spoligotypes of the MTB isolates obtained at post-mortem

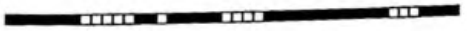
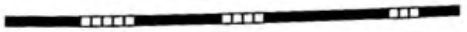
SPOLIGOTYPE PATTERN[d]	SpolDB4 ST or SIT	SpolDB4 Lineage	Frequency
	504	T3	2
	61	LAM10_CAM	13
	1498	U	1
	50	H3	1
	78	T2-T3	1
	86	T1	1
	115	LAM10_CAM	1
	329	AFRI_2	1
	331	AFRI_2	3
	1867	AFRI_1	3

Table 39 (cont.)

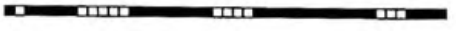
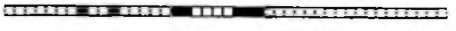
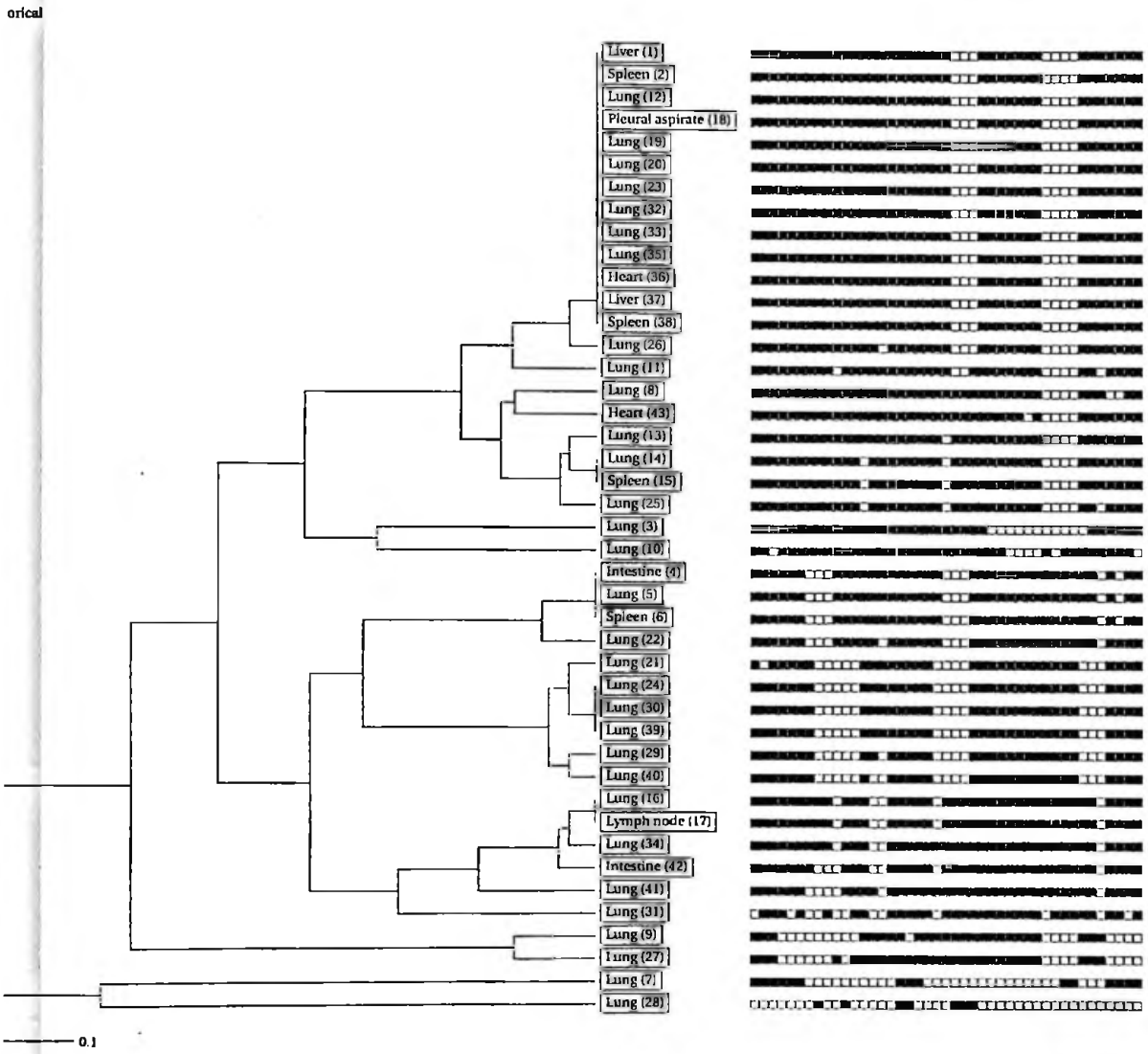
SPOLIGOTYPE PATTERN[d]	SpolDB4 ST or SIT	SpolDB4 Lineage	Frequency
	Orphan	-	2
	Orphan	-	1
	Orphan	-	1
	2407	AFRI_2	1
	200	X3	1
	319	AFRI_2	1
	Orphan	-	1
	Orphan	-	1
	Orphan	-	1
	Orphan	-	1
	Orphan	-	1
	Orphan	-	1
	Orphan	-	1
	Orphan	-	1
	Orphan	-	1
	Orphan	-	1
Total			43

Figure 20 is an Unweighted Pair Group Method with Arithmetic Mean (UPGMA) tree showing the spoligotype lineage diversity of the 43 MTBC isolates realised from cadavers during routine post-mortems.

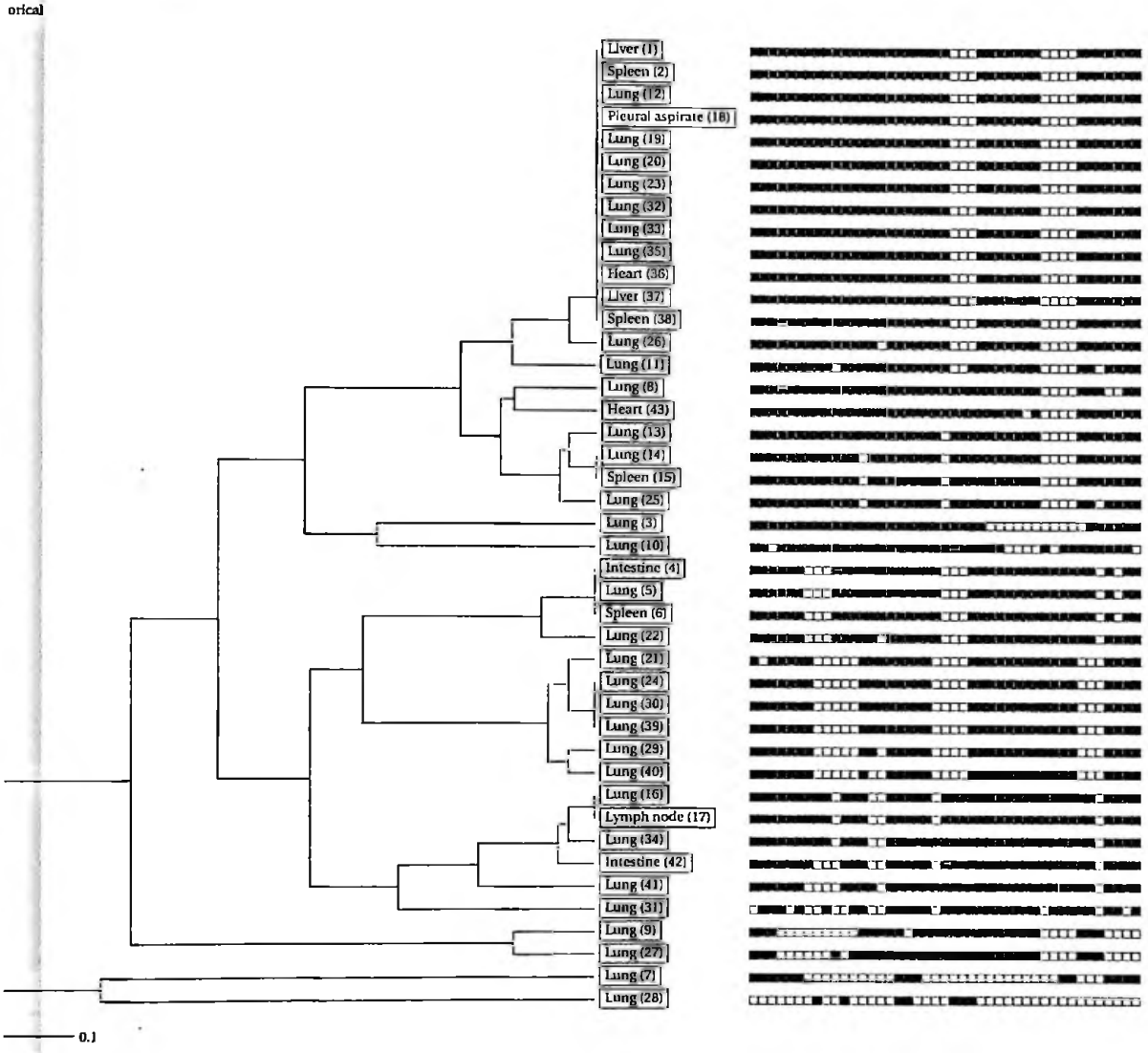
Table 39 (cont.)

SPOLIGOTYPE PATTERN[d]	SpolDB4 ST or SIT	SpolDB4 Lineage	Frequency
	Orphan	-	2
	Orphan	-	1
	Orphan	-	1
	2407	AFRI_2	1
	200	X3	1
	319	AFRI_2	1
	Orphan	-	1
	Orphan	-	1
	Orphan	-	1
	Orphan	-	1
	Orphan	-	1
	Orphan	-	1
	Orphan	-	1
	Orphan	-	1
	Orphan	-	1
	Orphan	-	1
Total			43

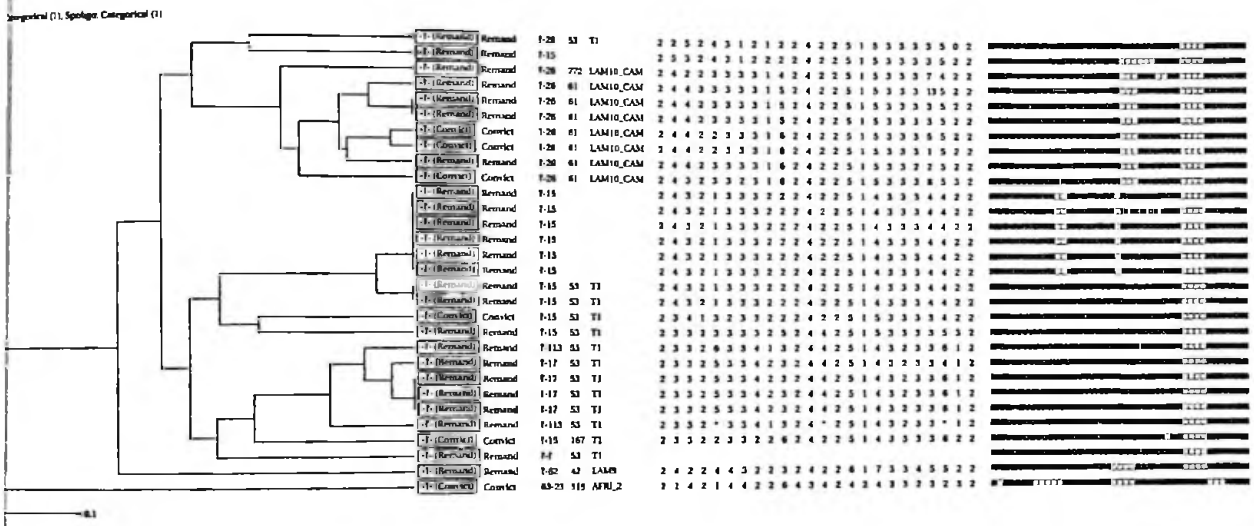
Figure 20 is an Unweighted Pair Group Method with Arithmetic Mean (UPGMA) tree showing the spoligotype lineage diversity of the 43 MTBC isolates realised from cadavers during routine post-mortems.



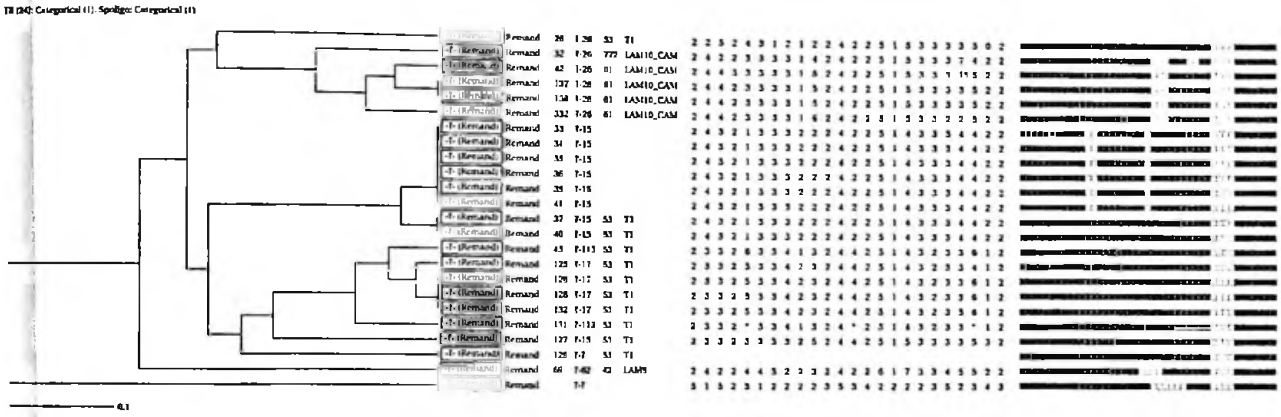
): A UPGMA tree showing the Spoligotype lineage diversity of 43 MTBC isolates from ecimens with suspicious lesions indicative of tuberculosis at post-mortem. Weighting measure set as categorical=1. (Legend: UPGMA= Unweighted Pair Group Method with c Mean).



): A UPGMA tree showing the Spoligotype lineage diversity of 43 MTBC isolates from ecimens with suspicious lesions indicative of tuberculosis at post-mortem. Weighting measure set as categorical=1. (Legend: UPGMA= Unweighted Pair Group Method with c Mean).



1: A UPGMA tree showing the genetic diversity of the 30 MTBC isolates from coughing inmates from the selected based on 24-Loci MIRU-VNTR and Spoligotyping data. Weighting distance measure set as Categorical=1 for both NTR and Spoligotyping.



ı 22: A UPGMA tree showing the genetic diversity of the 24 MTBC isolates obtained from the remanded coughing es from the selected prisons. Weighting distance measure set as Categorical=1 for both the 24-Loci MIRU-VNTR poligotyping.

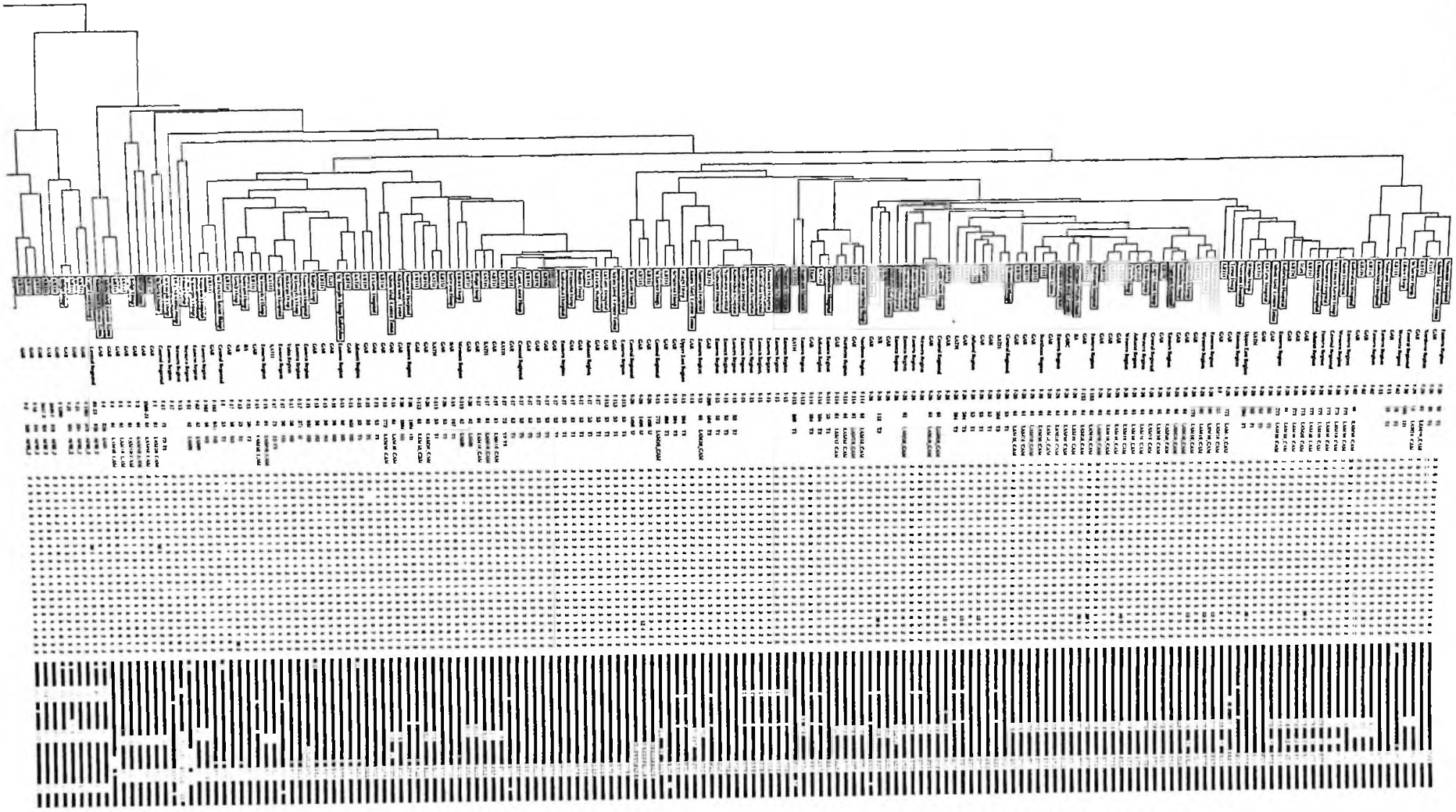
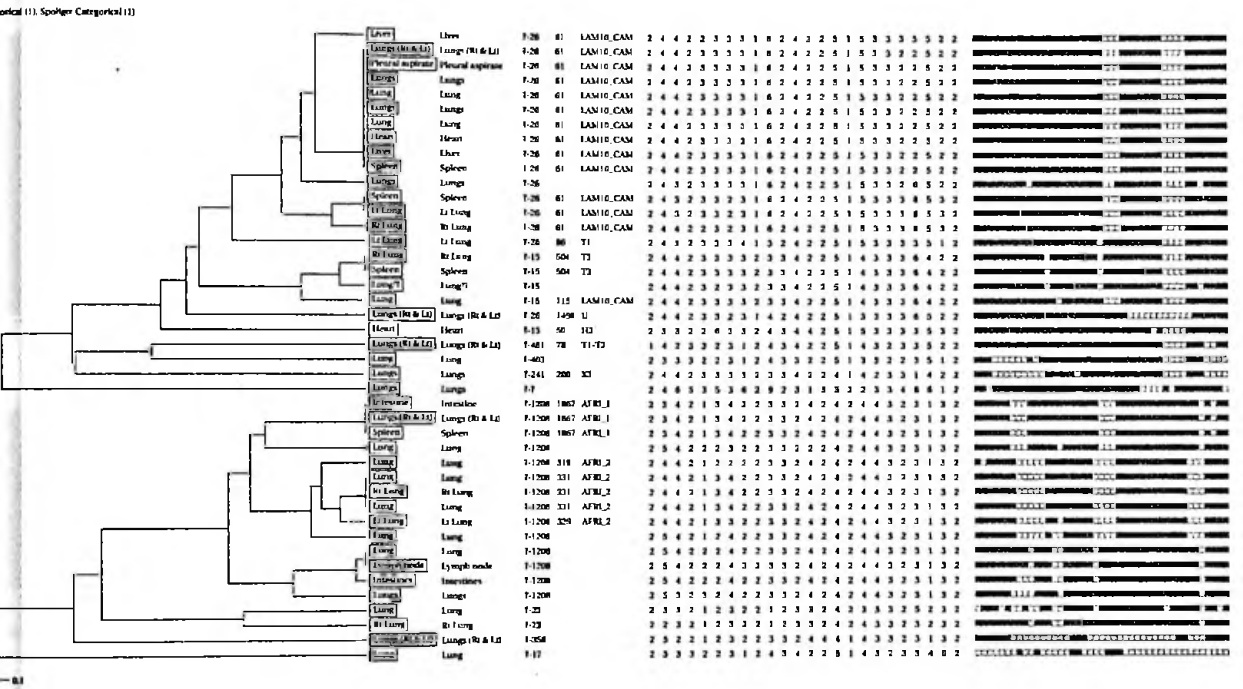


Figure 23: A UPGMA tree showing the genetic diversity of the 170 MTBC isolates obtained from patients presenting at the selected hospitals. Weighting distance measure set as Categorical=1 for both 24-Loci MIRU-VNTR and Spoligotyping.



UPGMA tree showing the genetic diversity of 43 MTBC isolates obtained from the decedents at post-mortem.
stance measure set as Categorical=1 for both the 24-Loci MIRU-VNTR and Spoligotyping.

Observed Alleles

Alleles were observed after MIRU-VNTR analysis on several independent loci for 4 isolates. These alleles are displayed in Table 41.

Table 41

Alleles and their repeats at specific MIRU-VNTR loci

Sample ID	Locus 2996	Locus 802	Locus 424	Locus 577	Locus 2165	Locus 2401	Locus 2163b	Locus 1955	Locus 4052	Locus 2531	Locus 3171
G129	4,5	3,5	3,5	3,4			3,5	3,4	5,6		2,3
G131		3,5				2,4			5,6		
G170			3,4	2,3	2,6					5,6	
G021		3,5				2,4			5,6		

Legend: P = prison isolate; N, WR = patient isolate

Figure 25 below indicates a probable explanation of the summation of spoligotypes in the case of a mixed infection (T. Zozio, personal communication, 9th November, 2016).

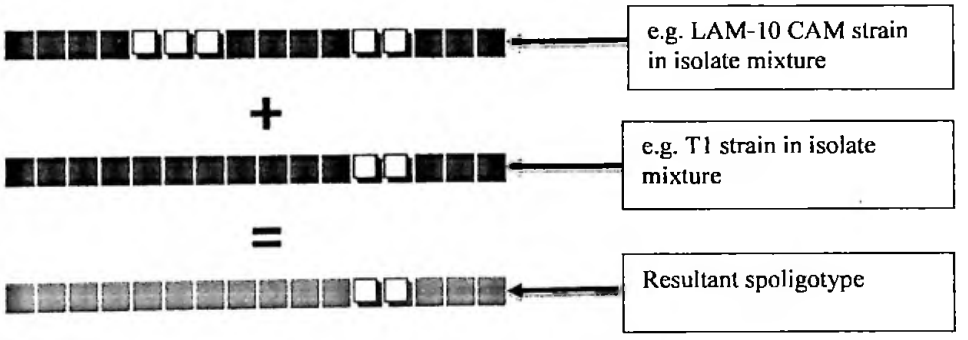


Figure 25: Spacer summation in spoligotyping. The T1 strain completely 'masks' the LAM-10 CAM strain by erroneously providing hybridisation sites for the region of the latter where there are 3 spacers

Results of the Hunter–Gaston Discriminatory Index (HGDI) Analysis for Discriminatory Power of the Typing Methods Employed

Table 42

The Hunter-Gaston Diversity Indices (HGDI) for Typing Methods computed from data using the online resource MIRU-VNTRplus

		Hunter-Gaston Discriminatory Index (HGDI) for Typing Method			
Population Category	Typing Method	43-Spacer Spoligotyping	12-Loci MIRU-VNTR	15-Loci MIRU-VNTR	24-Loci MIRU-VNTR
Coughing Prison Inmates (n=30)	Spoligotyping Only	0.77 (8)	N/A	N/A	N/A
	MIRU-VNTR Only		0.86 (11)	0.93 (20)	0.93 (20)
	Spoligotyping + MIRU-VNTR		0.95 (19)	0.95 (21)	0.95 (21)
Remanded Inmates (n=24)	Spoligotyping Only		N/A	N/A	N/A
	MIRU-VNTR Only		0.87 (12)	0.88 (14)	0.88 (14)
	Spoligotyping + MIRU-VNTR		0.90 (11)	0.93 (15)	0.93 (15)
Presenting Patients (n=170)	Spoligotyping Only	0.8572 (30)	N/A	N/A	N/A
	MIRU-VNTR Only		0.94 (67)	0.99 (107)	0.99 (111)
	Spoligotyping + MIRU-VNTR		0.99 (105)	0.99 (132)	1.00 (140)
Decedents (n=43)	Spoligotyping Only	0.9048 (25)	N/A	N/A	N/A
	MIRU-VNTR Only		0.93 (24)	0.95 (26)	0.95 (26)
	Spoligotyping + MIRU-VNTR		0.95 (28)	0.95 (28)	0.95 (28)

Legend: Values in brackets indicate the number of mycobacterial lineage types revealed by a typing method or a combination of typing methods thereof. Discriminatory Power, D, for each typing method or combination of typing method was computed as the Hunter–Gaston Discriminatory Index HGDI.

Tables 43 to Table 54 refer to results of HGDI analysis of 12-, 15- and 24-Loci MIRU-VNTR typing of all the population groups including the remanded inmate sub-population. The tables include information such as: the Confidence Interval which is also the HGDI value expressed as 95% upper and lower boundaries; K = the number of different repeats occurring at a particular locus in the sample set; max (π) = the fraction of samples with the most frequent repeat number in this locus (range 0.0 to 1.0). Table 54 shows a summary of the most discriminatory loci for distinguishing strains on the selected loci-sets and across population groups.

Table 43

All Prison Inmates 12-Loci MIRU-VNTR Typing - HGDI of various loci obtained in the 12-Loci MIRU-VNTR typing of isolates

VNTR	Locus	Diversity Index	Confidence Interval (95%)	K	Max (π)
802	MIRU 40	0.834	0.768-0.901	8	0.290
2996	MIRU 26	0.600	0.501-0.699	5	0.516
2059	MIRU 20	0.529	0.441-0.617	3	0.581
1644	MIRU 16	0.299	0.093-0.504	5	0.839
960	MIRU 10	0.243	0.049-0.437	4	0.871
3192	MIRU 31	0.243	0.049-0.437	4	0.871
2531	MIRU 23	0.187	0.006-0.368	4	0.903
580	MIRU 4	0.187	0.006-0.368	4	0.903
2687	MIRU 24	0.127	0.000-0.283	3	0.935
4348	MIRU 39	0.065	0.000-0.181	2	0.968
3007	MIRU 27	0.065	0.000-0.181	2	0.968
154	MIRU 2	0.065	0.000-0.181	2	0.968

Legend (Applicable to Tables 42 to 53): Diversity Index (for VNTR data) = also the HGDI and it is the variation of the number of repeats at each locus. It has a range of 0.0 (no diversity) to 1.0 (complete diversity); Confidence Interval = the Precision of the Diversity Index (HGDI), expressed as 95% upper & lower boundaries; K = the number of different repeats occurring at this particular locus in the sample set; max (π) = the fraction of samples with the most frequent repeat number in this locus (range 0.0 to 1.0).

Table 44

Remanded Inmates – HGDI of various loci obtained in the 12-Loci MIRU-VNTR typing of isolates

VNTR	Locus	Diversity Index	Confidence Interval (95%)	K	max(π)
802	MIRU 40	0.823	0.748-0.898	7	0.320
2996	MIRU 26	0.603	0.464-0.742	5	0.560
2059	MIRU 20	0.530	0.415-0.645	3	0.600
3192	MIRU 31	0.230	0.016-0.444	4	0.880
1644	MIRU 16	0.227	0.019-0.434	3	0.880
2531	MIRU 23	0.157	0.000-0.344	3	0.920
960	MIRU 10	0.157	0.000-0.344	3	0.920
580	MIRU 4	0.157	0.000-0.344	3	0.920
4348	MIRU 39	0.080	0.000-0.221	2	0.960
2687	MIRU 24	0.080	0.000-0.221	2	0.960
3007	MIRU 27	0.080	0.000-0.221	2	0.960
154	MIRU 2	0.080	0.000-0.221	2	0.960

Table 45

Presenting Patients - HGDI of various loci obtained in the 12-Loci MIRU-VNTR Typing of Isolates

VNTR	Locus	Diversity Index	Confidence Interval (95%)	K	max(π)
802	MIRU 40	0.696	0.635-0.758	8	0.509
2996	MIRU 26	0.584	0.524-0.644	9	0.579
2059	MIRU 20	0.509	0.497-0.520	3	0.503
960	MIRU 10	0.453	0.367-0.538	6	0.725
1644	MIRU 16	0.371	0.284-0.459	5	0.784
3192	MIRU 31	0.319	0.232-0.405	5	0.819
2531	MIRU 23	0.312	0.224-0.400	6	0.825
580	MIRU 4	0.206	0.126-0.285	6	0.889
2687	MIRU 24	0.192	0.117-0.266	4	0.895
3007	MIRU 27	0.068	0.017-0.120	3	0.965
4348	MIRU 39	0.035	0.000-0.073	3	0.982
154	MIRU 2	0.023	0.000-0.055	3	0.988

Table 46

Decedents (PM samples) - HGDI of various loci obtained in the 12-Loci MIRU-VNTR Typing of Isolates

VNTR	Locus	Diversity Index	Confidence Interval (95%) (95%)	K	max(pi)
802	MIRU 40	0.648	0.566-0.730	4	0.500
2996	MIRU 26	0.603	0.514-0.691	5	0.523
1644	MIRU 16	0.560	0.437-0.683	4	0.614
960	MIRU 10	0.533	0.425-0.641	5	0.614
2531	MIRU 23	0.521	0.459-0.583	3	0.568
2687	MIRU 24	0.513	0.440-0.585	3	0.591
3007	MIRU 27	0.504	0.397-0.611	4	0.636
3192	MIRU 31	0.504	0.397-0.611	4	0.636
2059	MIRU 20	0.502	0.419-0.585	3	0.614
580	MIRU 4	0.214	0.057-0.370	4	0.886
154	MIRU 2	0.090	0.000-0.205	3	0.955
4348	MIRU 39	0.045	0.000-0.130	2	0.977

Table 47

All Prison Inmates - HGDI of various loci obtained in the 15-Loci MIRU-VNTR Typing of Isolates

VNTR	Locus	Diversity Index	Confidence Interval (95%)	K	max(π)
802	MIRU 40	0.834	0.768-0.901	8	0.290
577	ETR-C	0.609	0.460-0.757	6	0.581
2996	MIRU 26	0.600	0.501-0.699	5	0.516
2059	MIRU 20	0.529	0.441-0.617	3	0.581
1644	MIRU 16	0.299	0.093-0.504	5	0.839
960	MIRU 10	0.243	0.049-0.437	4	0.871
3192	MIRU 31	0.243	0.049-0.437	4	0.871
2531	MIRU 23	0.187	0.006-0.368	4	0.903
580	MIRU 4	0.187	0.006-0.368	4	0.903
2687	MIRU 24	0.127	0.000-0.283	3	0.935
2165	ETR-A	0.127	0.000-0.283	3	0.935
4348	MIRU 39	0.065	0.000-0.181	2	0.968
3007	MIRU 27	0.065	0.000-0.181	2	0.968
2461	ETR-B	0.065	0.000-0.181	2	0.968
154	MIRU 2	0.065	0.000-0.181	2	0.968

Table 48

Remanded Inmates - HGDI of various loci obtained in the 15-Loci MIRU-VNTR typing of Isolates

VNTR	Locus	Diversity Index	Confidence Interval (95%)	K	max(π)
802	MIRU 40	0.823	0.748-0.898	7	0.320
2996	MIRU 26	0.603	0.464-0.742	5	0.560
577	ETR-C	0.577	0.373-0.781	6	0.640
2059	MIRU 20	0.530	0.415-0.645	3	0.600
3192	MIRU 31	0.230	0.016-0.444	4	0.880
1644	MIRU 16	0.227	0.019-0.434	3	0.880
2531	MIRU 23	0.157	0.000-0.344	3	0.920
960	MIRU 10	0.157	0.000-0.344	3	0.920
580	MIRU 4	0.157	0.000-0.344	3	0.920
4348	MIRU 39	0.080	0.000-0.221	2	0.960
2687	MIRU 24	0.080	0.000-0.221	2	0.960
3007	MIRU 27	0.080	0.000-0.221	2	0.960
2461	ETR-B	0.080	0.000-0.221	2	0.960
154	MIRU 2	0.080	0.000-0.221	2	0.960
2165	ETR-A	0.080	0.000-0.221	2	0.960

Table 49

Presenting Patients - HGDI of various loci obtained in the 15-Loci MIRU-VNTR Typing of Isolates

VNTR	Locus	Diversity Index	Confidence Interval (95%)	K	max(π)
802	MIRU 40	0.834	0.768-0.901	8	0.290
577	ETR-C	0.609	0.460-0.757	6	0.581
2996	MIRU 26	0.600	0.501-0.699	5	0.516
2059	MIRU 20	0.529	0.441-0.617	3	0.581
1644	MIRU 16	0.299	0.093-0.504	5	0.839
960	MIRU 10	0.243	0.049-0.437	4	0.871
3192	MIRU 31	0.243	0.049-0.437	4	0.871
2531	MIRU 23	0.187	0.006-0.368	4	0.903
580	MIRU 4	0.187	0.006-0.368	4	0.903
2687	MIRU 24	0.127	0.000-0.283	3	0.935
2165	ETR-A	0.127	0.000-0.283	3	0.935
4348	MIRU 39	0.065	0.000-0.181	2	0.968
3007	MIRU 27	0.065	0.000-0.181	2	0.968
2461	ETR-B	0.065	0.000-0.181	2	0.968
154	MIRU 2	0.065	0.000-0.181	2	0.968

Table 50

Decedents (PM samples) - HGDI of various loci obtained in the 15-Loci MIRU-VNTR typing of Isolates

VNTR	Locus	Diversity Index	Confidence Interval (95%)	K	Max (π)
802	MIRU 40	0.648	0.566-0.730	4	0.500
2996	MIRU 26	0.603	0.514-0.691	5	0.523
1644	MIRU 16	0.560	0.437-0.683	4	0.614
960	MIRU 10	0.533	0.425-0.641	5	0.614
2165	ETR-A	0.527	0.474-0.581	3	0.545
2531	MIRU 23	0.521	0.459-0.583	3	0.568
2687	MIRU 24	0.513	0.440-0.585	3	0.591
3007	MIRU 27	0.504	0.397-0.611	4	0.636
3192	MIRU 31	0.504	0.397-0.611	4	0.636
2059	MIRU 20	0.502	0.419-0.585	3	0.614
577	ETR-C	0.492	0.348-0.635	5	0.682
580	MIRU 4	0.214	0.057-0.370	4	0.886
2461	ETR-B	0.133	0.000-0.269	4	0.932
154	MIRU 2	0.090	0.000-0.205	3	0.955
4348	MIRU 39	0.045	0.000-0.130	2	0.977

Table 51

All Prison Inmates – HGDI of various loci obtained in the 24-Loci MIRU-VNTR typing of isolates

VNTR	Locus	Diversity Index	Confidence Interval (95%)	K	max(π)
802	MIRU 40	0.834	0.768-0.901	8	0.290
2163b	VNTR 2163b	0.791	0.718-0.865	7	0.355
4052	VNTR 4052	0.740	0.659-0.821	6	0.355
3690	VNTR 3690	0.712	0.582-0.842	9	0.484
0424	VNTR 0424	0.630	0.494-0.766	7	0.548
1955	VNTR 1955	0.617	0.468-0.767	5	0.581
577	ETR-C	0.609	0.460-0.757	6	0.581
2996	MIRU 26	0.600	0.501- 0.699	5	0.516
4156	VNTR 4156	0.581	0.422-0.739	5	0.613
2059	MIRU 20	0.529	0.441-0.617	3	0.581
2401	VNTR 2401	0.458	0.287-0.629	4	0.710
3171	VNTR 3171	0.424	0.241-0.607	4	0.742
1644	MIRU 16	0.299	0.093-0.504	5	0.839
3192	MIRU 31	0.243	0.049-0.437	4	0.871
960	MIRU 10	0.243	0.049-0.437	4	0.871
2531	MIRU 23	0.187	0.006-0.368	4	0.903
580	MIRU 4	0.187	0.006-0.368	4	0.903
2347	VNTR 2347	0.127	0.000-0.283	3	0.935
2165	ETR-A	0.127	0.000-0.283	3	0.935
2687	MIRU 24	0.127	0.000-0.283	3	0.935
3007	MIRU 27	0.065	0.000-0.181	2	0.968
4348	MIRU 39	0.065	0.000-0.181	2	0.968
154	MIRU 2	0.065	0.000-0.181	2	0.968
2461	ETR-B	0.065	0.000-0.181	2	0.968

Table 52

Remanded Inmates – HGDI of various loci obtained in the 24-Loci MIRU-VNTR typing of Isolates

VNTR	Locus	Diversity Index	Confidence Interval (95%)	K	max(π)
802	MIRU 40	0.823	0.748-0.898	7	0.320
2163b	VNTR 2163b	0.760	0.657-0.863	7	0.400
4052	VNTR 4052	0.733	0.641-0.826	5	0.400
3690	VNTR 3690	0.687	0.557-0.817	7	0.480
1955	VNTR 1955	0.637	0.480-0.794	5	0.560
0424	VNTR 0424	0.627	0.472-0.782	6	0.560
2996	MIRU 26	0.603	0.464-0.742	5	0.560
4156	VNTR 4156	0.580	0.420-0.740	5	0.600
577	ETR-C	0.577	0.373-0.781	6	0.640
2059	MIRU 20	0.530	0.415-0.645	3	0.600
2401	VNTR 2401	0.497	0.313-0.680	4	0.680
3171	VNTR 3171	0.497	0.313-0.680	4	0.680
3192	MIRU 31	0.230	0.016-0.444	4	0.880
1644	MIRU 16	0.227	0.019-0.434	3	0.880
580	MIRU 4	0.157	0.000-0.344	3	0.920
2531	MIRU 23	0.157	0.000-0.344	3	0.920
960	MIRU 10	0.157	0.000-0.344	3	0.920
4348	MIRU 39	0.080	0.000-0.221	2	0.960
2687	MIRU 24	0.080	0.000-0.221	2	0.960
154	MIRU 2	0.080	0.000-0.221	2	0.960
2165	ETR-A	0.080	0.000-0.221	2	0.960
2347	VNTR 2347	0.080	0.000-0.221	2	0.960
3007	MIRU 27	0.080	0.000-0.221	2	0.960
2461	ETR-B	0.080	0.000-0.221	2	0.960

Table 53

Presenting Patients - HGDI of various loci obtained in the 24-Loci MIRU-VNTR typing of Isolates

VNTR	Locus	Diversity Index	Confidence Interval (95%)	K	max(π)
2163b	VNTR 2163b	0.819	0.800-0.838	9	0.246
3690	VNTR 3690	0.756	0.701-0.812	13	0.450
4052	VNTR 4052	0.706	0.667-0.746	9	0.421
802	MIRU 40	0.689	0.627-0.751	8	0.515
577	ETR-C	0.650	0.605-0.696	8	0.485
4156	VNTR 4156	0.604	0.543-0.665	7	0.573
2996	MIRU 26	0.577	0.517-0.638	8	0.585
1955	VNTR 1955	0.568	0.500-0.636	7	0.614
0424	VNTR 0424	0.555	0.496-0.613	7	0.596
2059	MIRU 20	0.509	0.497-0.520	3	0.503
2401	VNTR 2401	0.480	0.423-0.537	5	0.655
960	MIRU 10	0.453	0.367-0.538	6	0.725
1644	MIRU 16	0.371	0.284-0.459	5	0.784
3171	VNTR 3171	0.346	0.268-0.423	5	0.789
2165	ETR-A	0.324	0.234-0.414	8	0.819
3192	MIRU 31	0.319	0.232-0.405	5	0.819
2531	MIRU 23	0.312	0.224-0.400	6	0.825
580	MIRU 4	0.206	0.126-0.285	6	0.889
2347	VNTR 2347	0.192	0.117-0.266	4	0.895
2687	MIRU 24	0.192	0.117-0.266	4	0.895
2461	ETR-B	0.134	0.065-0.203	5	0.930
3007	MIRU 27	0.068	0.017-0.120	3	0.965
4348	MIRU 39	0.035	0.000-0.073	3	0.982
154	MIRU 2	0.023	0.000-0.055	3	0.988

Table 54

Decedents (PM samples) - HGDI of various loci obtained in the 24-Loci MIRU-VNTR typing of Isolates

VNTR	Locus	Diversity Index	Confidence Interval (95%)	K	max(π)
3690	VNTR 3690	0.722	0.615-0.829	8	0.477
4052	VNTR 4052	0.691	0.613-0.770	6	0.432
2163b	VNTR 2163b	0.670	0.580-0.761	6	0.477
802	MIRU 40	0.648	0.566-0.730	4	0.500
1955	VNTR 1955	0.631	0.548-0.714	6	0.477
4156	VNTR 4156	0.613	0.536-0.690	5	0.477
2996	MIRU 26	0.603	0.514-0.691	5	0.523
2347	VNTR 2347	0.591	0.499-0.683	4	0.545
2401	VNTR 2401	0.571	0.473-0.669	5	0.568
1644	MIRU 16	0.560	0.437-0.683	4	0.614
0424	VNTR 0424	0.556	0.415-0.697	5	0.636
960	MIRU 10	0.533	0.425-0.641	5	0.614
2165	ETR-A	0.527	0.474-0.581	3	0.545
2531	MIRU 23	0.521	0.459-0.583	3	0.568
2687	MIRU 24	0.513	0.440-0.585	3	0.591
3007	MIRU 27	0.504	0.397-0.611	4	0.636
3192	MIRU 31	0.504	0.397-0.611	4	0.636
2059	MIRU 20	0.502	0.419-0.585	3	0.614
577	ETR-C	0.492	0.348-0.635	5	0.682
580	MIRU 4	0.214	0.057-0.370	4	0.886
3171	VNTR 3171	0.172	0.027-0.317	3	0.909
2461	ETR-B	0.133	0.000-0.269	4	0.932
154	MIRU 2	0.090	0.000-0.205	3	0.955
4348	MIRU 39	0.045	0.000-0.130	2	0.977

Results of Analysis of Discriminatory Power of individual loci in all loci-sets used

Table 55 shows the Discriminatory Power (measured as HGDI) of individual loci employed in the loci-sets.

Table 55

Summary of the Discriminatory Power of individual loci (measured as HGDI) for the various Loci-sets analysed

Population Category	12-Loci MIRU-VNTR Typing	15-Loci MIRU-VNTR Typing	24-Loci MIRU-VNTR Typing
	Most Discriminatory Locus (HGDI)	Most Discriminatory Locus (HGDI)	Most Discriminatory Locus (HGDI)
Isolates from Inmates	MIRU-40 (0.83)	MIRU-40 (0.83)	MIRU-40 (0.83)
Isolates from Remanded Inmates	MIRU-40 (0.82)	MIRU-40 (0.82)	MIRU-40 (0.82)
Isolates from Presenting Patients	MIRU-40 (0.70)	MIRU-40 (0.83)	VNTR 2163b (0.82)
Isolates from Decedents	MIRU-40 (0.65)	MIRU-40 (0.65)	VNTR 3690 (0.72)

Legend: The HGDI values are shown in brackets.

Results of Sensitivity Performance Analysis of the various Techniques for Drug Resistance-testing and Genotyping of MTBC Isolates

Results of the comparisons among the various methods are indicated in Table 56. The test performance analysis was carried out by considering the

older and more established phenotypic drug susceptibility test (DST) as the 'Gold standard' and comparing the performance of the other tests with it.

Table 56

Sensitivity (Sn), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV) and efficiency (E) of drug resistance tests of the various methods

Test Method	Gold Std	Application	Sn (%)	Sp (%)	PPV (%)	NPV (%)	E (%)
MLPA Assay	MIC-DST (n=243)	INH Resistance Testing	77.8	98.2	82.4	98.2	96.7
MLPA Assay	MIC-DST (n=243)	RIF Resistance Testing	85.7	100	100	99.6	99.6
MLPA Assay	MIC-DST (n=243)	EMB Resistance Testing	100	99.6	83.3	100	99.6
HAIN MTBC-DR+ Assay	MIC-DST (n=243)	INH Resistance Testing	83.3	99.1	88.2	98.7	97.9
HAIN MTBC-DR+ Assay	MIC-DST (n=243)	RIF Resistance Testing	85.7	100	100	99.6	99.6
*P-32 Dot Blot Hybridisation	MIC-DST (n=200)	RIF Testing	85.7	99.0	75.0	99.5	98.5

*Experiment not done for post-mortem isolates hence n=200

The comparison of MLPA and the HAIN MTBC GenoType Assay in differentiating *M. africanum* from other members of the MTBC (for all the 243 isolates) is presented in Table 57.

Table 57 shows the performance of the genotypic test in differentiating *M. africanum* from other members of the MTBC. The older and more established methods were taken as the 'Gold Standard'. Regarding the application of MLPA, it should be noted that MLPA categorises *M. africanum* and *M. bovis* as the same lineage (Bergval et al., 2007) because of the inclusion of only the RD9 probe.

Table 57

Comparison of MLPA and the MTBC GenoType® Assay in differentiating Mycobacterium africanum from other members of the MTBC (N = 243)

Test Method	Gold Standard	Application	Sn %	Sp %	PPV %	NPV %	E %
MLPA Assay	HAIN MTBC GenoType Assay	Differentiating MAF/*M. bovis* from other members of the MTBC	85.0	97.8	77.3	98.6	96.7
MLPA Assay	Spoligotyping	Differentiating MAF/*M. bovis* from other members of the MTBC	73.3	99.5	95.7	96.3	96.2
HAIN MTBC GenoType Assay	Spoligotyping	Differentiating MAF/*M. bovis* from other members of the MTBC	66.7	100	100	95.5	95.9

CHAPTER FIVE

DISCUSSION

Tuberculosis (TB), as a public health problem in Ghana and elsewhere in the developing world, causes considerable morbidity and mortality. Tuberculosis control and management are solely financed by the state and donor agencies and, therefore, considered as 'free TB services'. The economic toll, in terms of the drain on house-hold incomes, cannot be over-emphasised (Blankson, 2012; Ukwaja, Alobu, Igwenyi, & Hopewell, 2013).

Costs incurred in TB control and management are not only usually seen in direct financial terms but also in social terms. The social costs are best seen in terms of patient and/or house-hold care and the social toll on the care-provider. Costs associated with TB infection include pre-diagnosis cost, diagnosis cost, treatment cost and health care/provider cost (Blankson, 2012). Direct costs associated with TB infection could be as much as 107.06% of monthly rural household income (Blankson), implying that in some months the household can incur debts after spending all their income on TB treatment. As a poverty-related infectious disease, TB not only competes with other diseases in the utilisation of financial resources allocated to the public health sector but also leads to a lowering of productivity because of the attendant morbidity in TB infections. To be able to control and manage TB, characterising the MTBC population structure, identifying the circulating strains and their susceptibility/resistance to current drugs available for treatment and proper diagnosis are paramount. Also, knowledge of the

prevalence and the nature of circulating strains and/or lineages of MTB can contribute to the optimisation of infection control programmes and also lead to a more efficient allocation of scarce resources.

Three population groups were studied with the objective of characterising MTBC strains circulating in Ghana. This characterisation was to help define the population structure of the MTBC in Ghana on the basis of circulating strains in selected institutionalised sections of the Ghanaian populace, particularly inmates in selected prisons in the penal system, in patients presenting at selected health centres/hospitals as well as strains diagnosed at post-mortem. Diagnosis of TB in decedents through routine post-mortems is an attempt to capture strains whose effects may well probably have been sub-clinical. Post-mortem policy at Korle-Bu Teaching Hospital rules out autopsies for known TB patients at death, thus, the inclusion of decedents presented a unique opportunity to include strains, which may have been 'missed'.

Prevalence of the MTBC strains

Approximately 80% (24/30) of all the culture-active TB identified in this study emanated from remanded inmates indicating that the remand population should be given utmost attention in the control and management of TB in the Ghanaian penal environment. That the convicted inmate population accounted for approximately 20% (6/30) of all the culture-active TB identified points to the fact that the existing TB control and management protocol being

undertaken by the Ghana Prison Service is fairly efficient and effective in obviating mini-epidemics of the disease. Additionally, 13 samples elicited from these coughing inmates were found to contain agents other than classical MTB; 12 were NTM and one was *Nocardia nova*. This finding may indicate an underlying problem of the possibility of NTM infections occurring together with TB infections in densely populated and cramped institutionalised sections of the Ghanaian population.

Regarding patients presenting at the selected hospitals 170 out of 323 (50.6%) sputum samples were positive for MTB. This is not surprising as these patients satisfied the usual NTP criteria for TB screening and, therefore, could be considered as suspected TB cases. In a recent study (Boakye-Appiah et al. 2016) using the GeneXpert methodology, 118 out 376 (31.4%) suspected TB cases were positive. This prevalence of 31.4% in suspected TB patients should, however, be viewed from the fact that this was a retrospective study whose setting were just two referral hospitals.

A total of 733 cadavers screened for TB at post-mortem (PM) diagnosed TB in 34 cadavers, giving a prevalence of 4.6%. Since the decedents had apparently been admitted to hospital from causes other than TB, the PM diagnoses could be taken as a dignoses for sub-clinical or latent TB. Thus, the problem of sub-clinical or latent TB may pose a challenge in TB control and management.

Genetic Diversity of MTBC strains

The typing methods employed *viz* MLPA, MIRU-VNTR and Spoligotyping successfully revealed the genetic diversity of all the different population groups studied. The MLPA technique broadly categorises isolates on the basis of principal genotypic or genetic groups (PGG) (Ramaswamy & Musser, 1998). The different species-specific probes employed determine the genotyping capacity or efficiency of the MLPA technique. In this study, the *katG*-463 probe was included to genotype for PGG 2 and PGG3 while the *gyrA*-95 probe was included to genotype for PGG1 and PGG2. Probes *mutT*2 and *ogt*-12 were included to detect the MTB Beijing 2 lineage, probe ogt-37 to detect MTB Beijing 3/4, and probe *ogt*-15 to detect MTB Haarlem. The MTB Beijing strain is very important because of its propensity for virulence and spreading (Glynn, Kremer, Borgdorff, Rodriguez, & van Soolingen, 2006; Aguilar et. al., 2010; Ribeiro et. al., 2014; Liu et. al., 2016). The MLPA technique also detects or lumps *M. africanum* and *M. bovis* together as one specie because of the inclusion of only the RD9 probe. Usually in MLPA, the existence of an RD9 probe fragment at 430bp alone indicates classical or modern TB while the existence of a TbD1 probe fragment at 418bp alone indicates *M. africanum* and/or *M. bovis* (Bergval et al., 2007). However, if both fragments (430bp for RD9 and 418bp for TbD1) occur together then Ancestral TB and/or *M. canetti* is indicated (Bergval, 2007).

A total of 243 isolates (all from the three population groups together) were identified to belong to the various genetic groups. Over 80% (195)

belonged to PGG2 and the rest to PGG1 (11.5%) and PGG3 (8.2%) (Table 25).

Presenting Patients

The 136 isolates from samples obtained from patients presenting at the selected hospitals classified as MTB PGG2 included also members of the sub-groups MTB Haarlem (14) and MTB LAM RD-Rio (1) based on MLPA analysis. Also, 10 out of the 14 isolates of this panel classified broadly as PGG1 were further classified as *M. africanum/bovis* while 2 each were classified as Ancestral TB/*M. canetti* and MTB Beijing respectively using MLPA. This is not far-fetched as PGG 1 contains the ancient lineages like, Beijing, BOV, CAS, EAI, and Manu (Sreevatsan et al., 1997; Mbugi et al., 2016). This candidate is not aware of data from any previous study categorising MTBC isolates from Ghana on the basis of Principal Genetic Groups. However, work on MTBC isolates from other African countries (Mbugi; Mbugi et al., 2015) reveal an association of the PGG 2/3 group with positive HIV serology. The PGG 2/3 group contains evolutionarily-recent developed Euro-American lineages such as the Haarlem, LAM, S, T, and X (Mbugi).

The predominant spoligotype was the LAM_CAM10 series – 58/170, made up of 46 isolates with SIT 61 and 12 isolates with SIT 772 and the T1 (SIT 53) made up of 36 isolates (Figure 19 and Figure 23). These observations are similar to what has been reported in other West African countries (Homolka et al., 2008; Ani et al., 2010; Traore et al., 2010; Cadmus, Hill, van

Solingen, & Rastogi, 2011; Yeboah-Manu et al., 2011; Ouassa et al., 2012; Lawson et al., 2012; Thumamo et al., 2012; Gehre et al., 2013).

Analysis with the MIRUVNTR*plus* (http://www.miru-vntrplus.org/) web tool using spoligotyping data only revealed that the clustering rate was 82.4% (or 0.824) (Figure 19). The most prevalent lineage was the Cameroon lineage, (42.94%) followed by the Ghana lineage (31.18%), the West African 1 lineage (6.47%) and the Uganda I lineage (3.53%). The Latin-American-Mediterranean (LAM) lineage and multiple matches, the Beijing lineage and the East African-Indian (EAI) lineage were low in prevalence.

Coughing Prison Inmates

Of the 30 isolates obtained from coughing prison inmates, only one was classified as PGG1, 2 were PGG3 and as high as 27 were classified as PGG2. The 27 PGG2 isolates included 1 isolate each further sub-classified as MTB LAM RD-Rio and MTB Haarlem (Table 29).

Analysis with the MIRUVNTR*plus* (http://www.miru-vntrplus.org/) web tool and using only spoligotyping data revealed that the Clustering Rate was 73.3% (or 0.733) (Figure 18). The Ghana lineage (63.33%) was the most prevalent followed by the Cameroon lineage (30.0%), the West African 1 lineage (3.33%) and the Latin-American-Mediterranean (or LAM) lineage were the least prevalent (3.33%). A recent study of MTB infections in Ethiopian jails and surrounding communities revealed clustering rates of 28.57% and 31.82% respectively (Mohammed, 2016). The lower clustering rate in this study (Mohammed) was ascribed to the re-activation of a few

dormant index infections within the Ethiopian prisons covered. The higher clustering rate (73.30%) revealed in the current study indicates an active, on-going and recent transmission from multiple index strains.

Three clusters were revealed (Figure 17; Figure 18) with spoligotyping data alone. The first cluster had eight spoligotypes with the same pattern emanating from isolates obtained from five remanded prisoners and three convicts. The three convicts were from the same prison complex (2 of whom were staying in the same cell). The 8 spoligotypes of the first cluster also included 2 spoligotypes from two remanded inmates from the same prison in Accra. The last 3 isolates in this first cluster were from 3 remanded inmates from another prison complex, 2 of whom stayed in adjacent cells in the same block, and 1 stayed in another block. Clearly, this preliminary spoligotyping data set has established that individual inmates co-habitating in close proximity may account for their infection by similar strains (Figure 17; Figure 18). The second cluster had 6 similar spoligotypes all originating from remanded inmates from the same prison. The third cluster had 12 similar spoligotypes originating from 11 remanded inmates and one convicted inmate. Seven (7) of the spoligotypes could be traced to inmates living in the same Main Block of the same prison in Accra. Four (4) of the spoligotypes were from isolates from 4 remanded inmates (Figure 17, page 200; Figure 18, page 201). Prison inmates inter-mingle, particularly at exercise times and during roll calls. Thus, they can be exposed to infection; hence, it is likely that clustering was initiated by infection of different index strains. The penal environment is known to be conducive to the outbreaks of micro-epidemics

(Kamper-Jørgensen et al., 2012; Stucki, 2015; Mohammed, 2016). The risk posed by the high mobility of the remand population into and out of the prison walls due to demands of the investigative and judicial process cannot be ruled out as contributing to the high rate of clustering. The remanded inmates in a prison are usually exposed to a myriad of detention facilities whilst temporarily out of prison to be processed for court or to assist in investigations. This situation leads to contacts with and exposure to inmates of other prisons or detention facilities who may be harbouring TB.

Spoligotyping confirmed a preponderance of the LAM10_CAM (SIT 61) lineage and the T1 (SIT 53) lineage, so ubiquitous in West Africa (Homolka et al., 2008; Ani et al., 2010; Traore et al., 2010; Cadmus, Hill, van Solingen, & Rastogi, 2011; Yeboah-Manu et al., 2011; Ouassa et al., 2012; Lawson et al., 2012; Thumamo et al., 2012; Gehre et al., 2013). In investigating diversity, Spoligotyping data taken together with MIRU-VNTR data provided better discrimination, particularly, in defining infections amongst the coughing inmates to their individual prison cells.

Characteristics and population structure of TB strains and infections diagnosed in decedents

As stated earlier, a total of 51 specimens from organs obtained from 36 cadavers (some cadavers had more than one site infected) with lesions indicative of TB infection (in the opinion of the Pathologist) and treated

(decontamination and culture) had 43 isolates which were proven to be MTBC by the methods employed.

The number of isolates (21/43) obtained from suspicious lesions observed in the lungs was higher in male than in female cadavers (10/43). This could result from the differences in habits between males and females. For example, males indulge in activities such as smoking and have greater geographical mobility, which predisposes them to the risk of TB infection (Neyrolles & Quintana-Murci, 2009; Dogar, Shah, Chughtai, & Qadeer, 2012; Khan, Wako, Ayalew, Tefera, & Tadesse, 2013). Fewer organs of males with tuberculous isolates were the heart (2/43), intestine (1/43) and spleen (3/43). There were no tuberculous lesions or isolates found in liver and lymph nodes, and no acid-fast bacilli were seen in pleural aspirates of males. For female cadavers, isolates were obtained from intestine (1/43) and spleen (1/43) just as for males. However, liver (2/43), lymph node (1/43) and pleural aspirate (1/43) of females had tuberculous isolates with none in heart specimens unlike in the males. These findings clearly indicate that the lung remains the commonest route in TB aetiology. There may be various routes through which the other organs were infected. One intestinal isolate each, from males and females, was confirmed with TB. Intestines are an important site for TB (Ridaura-Sanz, Lopez-Corella, & Lopez-Ridaura, 2012). Infection of organs other than the lungs leads to extra-pulmonary TB (ETB). Extra-pulmonary TB in the intestines caused by *M. bovis* is usually termed as primary intestinal TB (Ridaura-Sanz et al.; Young & O'Connor, 2005) and it has a fairly simple route of infection - through the ingestion of contaminated dairy products from

infected bovines. The secondary form usually develops from an exo-intestinal source, most likely pulmonary, and the infection route is the blood stream or even through the swallowing of lung secretions (Ridaura-Sanz et al.). Involvement of heart tissue is also possible through the blood (haematogeneous route) or direct involvement of the myocardium (Kannangara, Salem, Rao, & Thadepalli, 1984; Rose, 1987; Liu, Nicol, Hu, & Coates, 2013). Two (2) isolates, both from female cadavers, were obtained from liver tissue. Recent studies indicate that hepatic TB is increasingly prevalent because of the HIV/AIDS pandemic (Hickey, Gounder, Moosa, & Drain, 2015). Four (4) cases of splenic TB were found; three emanated from male cadavers while the other was from a female cadaver. The spleen has been reported as the most common abdominal organ infected with TB (Azzam, 2013). This may explain the higher number of splenic cases observed in this study compared to other abdominal organs among the decedent population. Tuberculosis of the spleen (splenic TB) is quite rare and when it occurs, it is usually by dissemination from a primary source, most commonly from the lungs (Azzam). The one (1) isolate obtained from a pleural aspirate most probably may have emanated from a TB infection occurring in the pleural space around the lung (Vorster, Allwood, Diacon, & Koegelenberg, 2015).

Since the cadavers came from patients who died from causes other TB or who were admitted to hospital with diseases other than TB, latent TB could be a significant challenge in the control management of TB. A lack of the

capacity for pre-mortem diagnosis of TB could have serious consequences for the control of TB.

Of the 43 isolates revealed by MLPA technique among the decedent population as high as 74.4% belonged to the Principal Genotypic Group 2 (PGG 2) while the rest belonged to PGG 1. Of the PGG1 isolates, 10 were further sub-classified as *M. africanum/bovis* while the other was Ancestral TB/*M. canetti.*

Spoligotyping revealed that most of the isolates from decedents were mainly of the Cameroon lineage (34.88%) or the West African lineages (34.88%) while 11.63% were orphans. The West African lineages were made up of West African 2 lineage (20.93%), and the West African 1 lineage (13.95%). This is an interesting finding as the prevalence of the West African lineages (1 and 2) identified from prison inmates and presenting patients were far smaller being 5.9% (10/170) among presenting patients and just 3.3% (1/30) among prison inmates. However, it must be noted that these isolates were obtained from sputum samples as opposed to tissue samples from organs with suspicious lesions in the decedents. Furthermore, the common symptomatic feature amongst both the prison inmates and presenting patients was a persistent cough irrespective of duration while for the decedents it was sub-clinical. This finding probably raises questions of infectivity and virulence of the West African clade, since in the past it was the main aetiologic agent of TB in West Africa (Haas et al., 1997; Niobe-Eyangoh et al., 2003; Niemann et al., 2004; de Jong et al., 2005; Brudey et al., 2006; Gagneux et al., 2006; Comas et al., 2009; de Jong, Antonio, & Gagneux, 2010). Other spoligotypes

were the Ghana lineage (9.3%), the Haarlem lineage (4.65%) and the East African-Indian (EAI) and the Uganda I lineages with 2.33%. The total prevalence of approximately 26% for the West African lineages (1 & 2) among decedents is significant since they usually are found in TB infections of presenting patients. This prevalence is similar to the one obtained by the MLPA technique. The clustering rate by Spoligotyping was 41.9% (i.e. < 50%), and this could probably point to a situation of lineage stability. As expected also, strain discrimination was more efficient when Spoligotyping data was applied together with MIRU-VNTR data. Thus a combination of these techniques should enhance identification of MTBC for easy management.

The identification of 36 out of 733 cadavers examined at post-mortem (PM) indicates that better diagnostic tools are required for efficient diagnosis of TB to be able to identify sub-clinical infections. Fortunately, this study also revealed that there was no drug-resistant MTBC diagnosed at PM which indicates that as patients they could not have been on admission harbouring sub-clinical TB, and therefore could not have posed the risk of transmitting drug-resistant TB in the hospitals wards.

In terms of strain diversity, the same strains were identified in the prison population, at PM as well as in patients presenting at the chest units of the selected hospitals routinely for TB screening. Our prisons are crowded; hence, it is not surprising that the strains among them were the same since infected inmates could easily transmit the bacillus to their colleagues.

Presence of Non-tuberculous Mycobacteria (NTM)

Nine isolates identified by BLAST-sequencing were also subjected to analysis using the reversed line blot INNO-LiPA assay. The BLAST sequencing identified two of nine classified as NTM by the Capilia Neo-TB ICA and 16S rDNA as *M. porcinum* and the rest as *M. abscessus, M. chelonae, M. fortuitum, M. immunogenum, M. mucogenum, M. peregrenum* and *Nocardia nova* (Nocardiaceae).

Nocardia nova, which is an unusual isolate in this setting, was obtained from the sputum of a 44 year old convicted artisanal metal worker who complained of intense coughing for a week prior to sampling. He could recall no previous history of hospitalisation for his cough, but explained that the cough varied in intensity from the mild to serious and could be described as 'on and off'. This sample was acid-fast on ZN staining and exhibited a creamy moist, sticky and mildly slimmy morphology on LJ slants. Apart from members of *Mycobacteriaceae*, exhibition of acid-fastness on ZN-staining is a well-known feature of members of *Nocardiaceae, Gordoniaceae, Tackamunellaceae* and *Dietziaceae*. Clearly, this inmate had been infected by *Nocardia nova* and therefore exhibiting a subclinical form of Nocardiosis, save the cough. Nocardiosis is a disease caused by *Nocardia* species found either in the soil or air (Lerner, 1996; Wilson, 2012). It can affect such organs as the lungs, brain and the skin. Immuno-compromised individuals and those on anti-cancer medications are particularly susceptible to *Nocardia* infections. However, whether this inmate was immune-compromised or not was outside

the scope of this study. *Norcardia* also infects birds (Bacciarini, Posthaus, Pagan, & Miserez, 1999; Darzi et al., 2006).

Whiles the INNO-LiPA Mycobacteria assay was unable to detect any specific NTM in five of the samples, BLAST sequencing identified the four as follows: one each belonging to *M. peregrenum*-complex and *M. chelonae* complex (Group I), and two belonging to the *M. chelonae*-complex (Group III). This indicates the superiority in efficacy of the BLAST sequencing over that of the INNO-LiPA Mycobacteria assay. It is likely that if more diverse probes, containing a broader range of single nucleotide polymorphisms (SNPs), are included in the INNO-LiPA Mycobacteria assay, then more NTMs could be identified.

Drug resistance-associated genes and rate of drug resistance among the MTBC isolates from the population groups

About 450,000 people died of TB in Africa, out of 3.2 million prevalent cases in 2014 (WHO, 2015). This could be compounded by the growing challenge of drug-resistant tuberculosis (DR-TB), particularly, multi-drug-resistant tuberculosis (MDR-TB), as well as the extensively drug-resistant tuberculosis (XDR-TB). DR-TB or MDR-TB infection could paint a dismal picture for TB management in Ghana and, indeed, Africa as a whole, particularly with the dual threats of HIV and MDR-TB.

The MIC-DST in this study indicated that resistance to drugs occurred from the second week of incubation and this was clear from growths in the

wells containing the break-point concentration of the drugs (Figure 10). The mono-resistance to STR was the most prevalent, at 11.9%. This is not far-fetched since drug abuse (including overuse and missuse) of STR is common (Ventola, 2015). Prevalences of mono-resistance to INH and RIF were 2.5% and 1.2%, respectively. Some isolates were resistant to combinations of EMB with other drugs. A cross-sectional study in Ghana involving 2,064 consecutive isolates running from 2001 to 2004 (Owusu-Dabo et al., 2006) gave similar results. The study employed the Proportion Method on LJ slants using the same drug panel (as in this atudy) in addition to pyrazinamide, PZA, and thiacetazone, TCZ. Results of this cross sectional study revealed that 76.5% (1,578/2,064) of the isolates were susceptible to all drugs tested whiles 22.9% (472/2,064) of the isolates revealed one form of resistance or the other. This compared favourably with results obtained in this study i.e. 78.6% (191/243) susceptible isolates and 21.4% (52/243) resistant isolates. Also, mono-resistance to STR was the most prevalent, followed by mono-resistances to INH and RIF at 9.0% (185/2,064), 4.3% (89/2,064) and 0.8% (16/2,064) respectively. The good news in this study is that no MDR-TB isolates were found among the prisoners or cadavers at PM. With the overcrowding in our prisons, the presence of MDR-TB could spread easily, since the stifling environmental conditions in most prisons are conducive for the spread of TB (Chaves et al., 1997; MacIntyre, Kendig, Kummer, Birago, & Graham 1997). Sadly, however, there were MDR-TB isolates (4/170 or 2.4%) in samples obtained from patients presenting at the selected hospitals. Unfortunately, two strains were resistant to all the first-line drugs; one strain was poly-resistant to

3 drugs, that is, STR, INH and EMB but not MDR since it did not show a concomitant resistance to RIF. The MDR-TB rate of 2.4% revealed for the presenting patients population compared favourably with the MDR-TB rate of 2.1% (43/2,064) obtained in the earlier work (Owusu-Dabo et al.).

The four MDR isolates were shown to have mutations in the *rpoB* gene by Dot Blot Hybridisation, giving credence to the notion that the *rpoB* gene is a good surrogate marker for MDR-TB. Comparison of the Dot Blot Hybridisation assay results with the MIC-DST results indicated that 5 out of the 7 isolates found to harbour mutations on the *rpoB* genes were also found to be resistant to RIF (mutations in the *rpoB* gene are associated with resistance to RIF). Even though the MIC-DST method is the 'Gold Standard', when combined wth the Dot Blot Hybridisation assay the strains harbouring a mutation in the *rpoB* gene in circulating MTBC can be specifically identified. The disadvantage for a resource-poor country, such as Ghana, is that probes must be designed properly in order to capture the most prevalent mutations and for efficient tracking of resistance-associated mutations using a Dot Blot Hybrisdisation assay. Another disadvantage is the prior knowledge of the sequences of the most prevalent resistance-associated mutations before the designing of the probe. These challenges can hinder the use of the Dot Blot Hybridisation assay since it might be too expensive to employ.

A total of 16 MTBC isolates obtained from samples collected from patients presenting at the selected hospitals were found to be resistant to INH using the HAIN GenoType MTBDR*plus*® Assay. Nine (9) out of these isolates harboured the *katG* MUT1 mutation (AGC→ACC) whiles the remaining 7

isolates contained the *inhA* MUT1 (C→T in position 15) mutation. Fourteen (14) of the 16 INH-resistant isolates were also found to be resistant to INH by the MIC-DST method, indicating that in the absence of equipment and reagents for the more sophisticated HAIN GenoType MTBDR*plus*® Assay, the MIC-DST method could still detect 88% INH-resistant MTBC strains.

Five (5) isolates obtained from patients presenting at the selected hospitals were found to be resistant to RIF by the HAIN GenoType MTBDR*plus*® Assay. Four (4) of these RIF-resistant isolates contained the *rpoB* MUT3 S531L mutation whiles one (1) isolate carried the *rpoB* MUT2B H526D mutation. All these 5 isolates were also found to be RIF-resistant by the 'Gold Standard', the MIC-DST method, indicating the suitability of the HAIN GenoType MTBDR*plus*® Assay for determining resistant-associated mutations in genes. The HAIN GenoType MTBDR*plus*® Assay found 1 isolate each from the coughing prisons inmates to be resistant to both INH and RIF. The INH-resistant isolate carried the *inhA* MUT1 (C→T in position 15) while the RIF-resistant isolate carried *rpoB* MUT3 S531L mutation. These two were also confirmed as such by the MIC-DST method. The ability of the MIC-DST method to identify most of the isolates as resistant to the first line drugs, indicates that it can be used for early detection in the absence of equipment for the sophisticated HAIN GenoType MTBDR*plus*® Assay. No isolate amongst those obtained from post-mortem samples was found to be resistant to either INH or RIF. Previous work (Homolka et al., 2010; Yeboah-Manu et al., 2011; Asante-Poku et al., 2015) on MTBC isolates from Ghana revealed a diverse range of resistance-associated mutations for both INH and

RIF. Evaluating the HAIN GenoType MTBDR*plus*®, by analysing 525 isolates from Ghana revealed that mutations in codon 450 (*rpoB* gene) and codon 315 (*katG* gene) were dominant (Asante-Poku et al.). Their work revealed that 8.2% (14/525) and 1.5% (8/525) were resistant to INH and RIF respectively. The MDR-TB rate was 1.5% (8/525) (Asante-Poku et al.). This work revealed that the resistance rates for INH, RIF and MDR-TB of 9.4% (16/170), 2.9% (5/170) and 2.4% (4/170) respectively compare favourably with the findings of Asante-Poku and co-workers (Asante-Poku et al.). Differences in these resistance rates could be attributed to populations sampled. Whereas the earlier study used a larger population size emanating from a larger geographical area (Asante-Poku et al.), the sampling area in this study only covered regions in which prisons had been selected.

Drug resistance-associated gene mutation patterns revealed by the MLPA technique were quite diverse. The probe sequences employed in the probe 'mix' to detect SNPs associated with drug resistance dictated the drug resistance patterns. Probes employed by MLPA are designed to detect drug resistance-associated mutations for only 3 of the first-line drugs - INH, RIF and EMB (Bergval et al., 2007). That, there was agreement in terms of the MDR-TB rate 2.4% (4/170), indicates that the probes designed to capture resistance-associated mutations had the correct sequences i.e. equivalent to sequences of mutant MTB isolates in circulation, particularly in patients presenting at the selected hospitals.

The 24-loci MIRU-VNTR Typing of Isolates

The 24-loci MIRU-VNTR analysis was successful for all 3 panels of isolates. As described earlier, for a more efficient analysis of genetic diversity, the MIRU-VNTR*plus* web tool was queried simultaneously with both Spoligotyping and MIRU-VNTR data. Clearly, the clustering rates using Spoligotyping data alone was lower than when combined with MIRU-VNTR data, indicating a better discriminatory power when both data sets are combined for diversity analysis (Table 39). This discriminatory power was clearly demonstrated from the UPGMA phylogenetic trees generated for all the population groups using Spoligotyping data alone (Figures 17 to 20) and the combined Spoligotyping and MIRU-VNTR data (Figures 21 to 24).

Indeed, for the prisons data set, whereas Spoligotyping alone revealed three clusters involving 26 isolates, Spoligotyping together with MIRU-VNTR yielded 4 clusters involving 14 isolates. For the 26 isolates, three sets of two spoligotypes each could be traced to three different prison cells, whereas for the 14 isolates the twin analytical tools of Spoligotyping together with MIRU-VNTR two sets of three isolates each could be traced to two different cells and a pair of isolates could also be traced to one prison cell, most likely emanating from the same index strain.

Most discriminatory loci or group of loci for identification of MTBC strains

To further shed light on the population structure of the MTB isolates, the performance of individual MIRU-VNTR loci and combined MIRU-VNTR loci sets was also assessed in order to provide information on the most efficient discriminatory loci to include in typing MTB isolates in the Ghanaian setting. The HGDI values were obtained for all individual loci in all loci-sets (12-loci, 15-loci and 24-loci sets using the online V-DICE proprietary resource) obtained from the website: http://www.hpa-bioinformatics.org.uk/cgi-bin/DICI/DICI.pl. Individual loci were categorised as highly (HGDI>0.6), moderately (0.3–0.6) and poorly (<0.3) discriminatory following criteria set out by Sola et al (Sola et al., 2003).

The locus, MIRU-40 (or VNTR0802), was highly discriminatory (HGDI = 0.65 - 0.83) in all loci-sets and across all population groups. Thus, it was the most highly discriminatory, except for 24-Loci MIRU-VNTR typing of isolates from patients presenting at selected hospitals and the isolates obtained from decedents at post-mortem, where the loci VNTR2163b and VNTR3690 yielded the highest HGDI values of 0.82 and 0.72, respectively. The locus MIRU-40 was also observed to be the most prolific and ubiquitous, appearing in all loci sets categorised as highly discriminatory (HGDI > 0.6) across all population groups. The mean HGDI values for MIRU-40 were 0.78 and 0.75 for 12-Loci and 15-Loci MIRU-VNTR typing, respectively, across all population groups. For 24-Loci MIRU-VNTR typing, where locus MIRU-40 gave the highest HGDI value for isolates obtained from the prison and the

remand sub-population (but not the presenting patients and the decedent samples), the mean value was still high (0.75). These results fully justify the inclusion of MIRU-40 in any loci-set employed to type MTB isolates from similar population groups in Ghana.

Discriminatory power of the various typing methods employed

In this work, the use and inclusion of MIRU-VNTR typing resulted in a better discrimination of the MTB isolates obtained from all the population groups. The HGDI values for Spoligotyping were lower than those for MIRU-VNTR (when each was employed separately as the sole typing method). Also as expected, the combination of Spoligotyping and MIRU-VNTR gave the highest HGDI values across all loci sets for all population groups. The higher HGDI values obtained for MIRU-VNTR (when employed alone) compared to the values for Spoligotyping alone is expected, as MIRU-VNTR typing is more efficient as a typing technique than spoligotyping (Barnes & Cave, 2003; Supply, 2005; Dickman et al., 2010; Mulenga, et al., 2010; Cohen, Wallengren, Wilson, Samuel, & Murray, 2011). Indeed, the discriminating power was even further enhanced when MIRU-VNTR Typing was employed in combination with spoligotyping. An interesting observation was that the HGDI values were the same for 15-loci MIRU-VNTR typing and 24-loci MIRU-VNTR typing (either when used alone or in tandem with Spoligotyping) for all population groups except for patients presenting at the selected hospitals (Table 41). For example, amongst isolates obtained from

the Prison population of coughing inmates (n = 30), the HGDI value obtained for 15-Loci or24-Loci MIRU-VNTR Typing (when either is used alone) was 0.92 while 0.95 was obtained for both (when either is used in tandem with Spoligotyping). In contrast, amongst isolates obtained from the patients presenting at selected hospitals (n = 170), the HGDI value was the same (0.99) for both the 15-loci and 24-loci MIRU-VNTR typing when either is used alone. A near 1.0 value (0.996) was obtained when either typing method was employed in combination with Spoligotyping.

There have been considerable successes in the application of MIRU-VNTR typing in combination with Spoligotyping for the efficient discrimination of lineage strains of MTBC (Supply et al., 2006; Ali et al., 2007; Sharma et al., 2008; Krawczyk et al., 2011; Refaya, Sivakumar, Sundararaman, & Narayanan, 2012; Joseph et al., 2013). The efficiency or discriminatory power of individual loci are varied and may be unique to different populations of circulating MTBC strains in different geographical areas. For example, whilst MIRU4 was least discriminatory for CAS1 strains in Pakistan (Ali), it was the most discriminatory for CAS1 strains in Kerala, India (Joseph). A study in Poland (Krawczyk) revealed MRU40 to be most discriminatory. Work on isolates from a rural area in Kanpur, India (Sharma), revealed MIRU26 to be most discriminatory. A recent study of isolates belonging to the EAI Clade in Tamil Nadu, Sri Lanka, indicated that MIRU39 was most discriminatory (Refaya), whereas work in Kerala, India (Joseph) indicated MIRU39 to be poorly discriminatory for EAI strains.

Thus, for routine practice, the 15-Loci MIRU-VNTR typing method may be employed to give useful epidemiological information about MTB strains circulating in endemic sections of the Ghanaian population. The cost benefits accruing from the use of 15-loci instead of 24-loci are obvious since the high cost associated with the 24-loci MIRU-VNTR analysis would be minimised by the use of a15-loci MIRU-VNTR typing protocol to detect polymorphism in MTB strains (De Beer et al., 2014).

The Problem of Alleles

Querying and filtering revealed double alleles in the MIRU-VNTR data for four isolates (two each from the coughing prison inmates and patients presenting at hospitals). These alleles were observed for each isolate in several independent MIRU-VNTR loci (Table 40). If two alleles are observed in several independent MIRU-VNTR loci, it indicates the presence of a mixed DNA population (Supply, 2005). This mixed population may have emanated from a true mixed infection or DNA contamination in the laboratory. However, if two alleles are observed to occur in a single locus, then the presence of an allelic variant within a clonal isolate is indicated. Thus, these samples represent mixed DNA populations (from two different strains) in each case and clearly not allelic variants. The mixed DNA could have resulted from mixed infections of two different strains or laboratory contamination. However, since religious adherence to good laboratory practice and precautions were observed in sample acquisition, handling and preparation, contamination in the laboratory is improbable.

All the four isolates, namely P129, P131, N170 and WR031, which were indicated as having double alleles, also had the same T1 (SIT 53) spoligotype signature. These results are instructive. Whenever there is a mixed population of T1 (SIT 53) and a LAM-10 CAM strain or a LAM9 strain it is the T1 (SIT 53) spoligotype which is captured and revealed (T. Zozio, personal communication, 2017). As an unwritten rule of thumb in spoligotyping, when there is poly-infection then there is a summation of spacers as indicated in Figure 25 (Zozio). Positionally common (spacers 33 – 36) to both a T1 (SIT 53) strain and either a LAM-10 CAM strain or a LAM9 strain are left unhybridised. The T1 (SIT 53) strain 'erroneously' provides hybridisation sites and completely masks the other spacers in the LAM strains.

Analysis of sensitivities of the various analytical techniques

The application and adoption of any technique in TB control and management depends on its sensitivity (Sn), specificity (Sp), PPV, NPV and efficiency. As seen in terms of both the Sn and Sp, the numbers fell well within the ranges reported (WHO, 2014; Sharma et al., 2015; Havumaki et al., 2017). The WHO quotes ranges of 70-100% and 91-100% for Sn and Sp, respectively, when testing for RIF-resistance (WHO). RIF-resistance testing using the Xpert/RIF platform, also gave values of 96.9%, 99.8%. 99.7% and 98.3% for Sn, Sp, PPV and NPV, respectively (Sharma et al.).

The Sn and Sp ranges for the HAIN GenoType MTB-DR*plus*® in detecting INH-resistance in this thesis are 77 – 90% and 98 – 100%,

respectively. In another work, applying the HAIN GenoType MTB-DR*plus*® to test for INH-resistance gave values of 89.0% and 99.4% for Sn and Sp respectively (Havumaki et al., 2017). Similarly, for RIF-resistance testing, the Sn and Sp values obtained were 90.2% and 98.5%, respectively (Havumaki et al.). However, in all cases, the Phenotypic Indirect Proportion DST method was used as the 'Gold Standard' comparator with critical concentrations of 0.2 µg/mL and 40.0µg/mL for INH and RIF respectively. Also, in a recent work on evaluating the HAIN GenoType MTBCDR*plus*® assay against the Phenotypic Indirect Proportion DST method using MTB isolates fron Ghana, a value of 100% was obtained for both Sn and Sp for RIF resistance, while for INH resistance the respective values were 83.3% and 100% for Sn of Sp (Asante-Poku et al., 2015). In this latter case the 'Gold Standard' DST comparator was the Indirect Proportion Method and employed critical concentrations of 0.2µg/mL and 40.0µg/mL for INH and RIF, respectively. The phenotypic MIC-DST method, which was employed in the current study, is an absolute concentration method and critical concentrations of 1.0µg/mL and 0.2µg/mL for INH and RIF, respectively, were used. The Sn and Sp values obtained in the current study were 85.7% and 100% for INH, and 83.3% and 99.1% for RIF, respectively. These values are in agreement with the findings of Asante-Poku et al (Asante-Poku et al.). The slight differences may emanate from probable difficulties in achieving an optimum and equal inoculum size (for all the 25 wells for each plate) and the viability of isolates in the inocula for the MIC-DST method. These values also fall well within the WHO ranges (WHO, 2014).

The comparison of MLPA and the HAIN GenoType MTBC® Assay in differentiating *M. africanum* from other members of the MTBC for all the 243 isolates was performed against spoligotyping as the 'Gold Standard'. MLPA provides a better sensitivity, however, this is marred by the fact that it lumps *M. africanum* and *M. bovis* as the same lineage (because of the inclusion of only the RD9 probe). Therefore, another technique is required to unequivocally differentiate the two. In terms of RIF resistance testing, all three methods; *viz* MLPA, the HAIN GenoType MTB-DR*plus*® assay and the ^{32}P-Dot Blot Hybridisation, offered the same level of sensitivity. However, the MLPA and the HAIN GenoType MTB-DR*plus*® assay also offered 100% specificity. The HAIN GenoType MTB-DR*plus*® assay was the most sensitive and specific in INH-resistance testing. The MLPA maybe very practical for Ghana as it offers flexibility in terms of the inclusion of new resistance-testing probes with well-characterised mutations of circulating strains as and when they are found. To an extent this also applies to the Phosphorus-32 Dot Blot Hybridisation technique, but a drawback is the use of radio-actively labelled probes, which may pose a challenge because of the risk of contamination and the challenge of disposal of radio-active waste.

CHAPTER SIX

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

Summary

Tuberculosis (TB) disease is one of the oldest and most challenging public health problems confronting the world today. Millions of new TB infections are recorded annually. Sub-Saharan Africa has the highest incidence of pulmonary tuberculosis (PTB) in the world. The emergence of HIV/AIDS pandemic has exacerbated the problem. The emergence of drug-resistant tuberculosis (DR-TB), particularly, multi-drug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) poses a danger for TB control and management.

Genotyping tools are invaluable for genetic characterisation of circulating tuberculosis strains in specified settings. Evaluation of the genetic characteristics will shed light on the population structure of the circulating strains, particularly, in institutionalised sections of the Ghanaian population, such as the prisons, where overcrowding can lead to spread of the infection. Such findings will inform the formulation of better and more efficient control and management strategies for TB.

Tuberculosis is one of the commonest communicable diseases in Ghana. Around 250,000 infections by MTB are recorded each year. Ghana is not one of the WHO-declared 22 high-burden countries. This notwithstanding, TB control and management still remain daunting, mainly due to insufficient financial resources.

Tuberculosis has been detected in prison inmates and patients presenting at various hospitals. Many other also die of causes that may be influenced by TB infection. However, it is believed that there are higher rates of TB infection in prison populations. For efficient control and management of TB in Ghana, it is important to identify and fully elucidate drug resistance patterns and also genotype the strain lineages in new infections and in circulation among the population. Information obtained from such efforts should lead to the design of a more efficient disease management protocol and, ultimately, reduce the problem of sub-clinical TB. Therefore, this study aimed at identifying TB strains, genetic structure circulating among prisoners from selected prisons, patients presenting and decedents at selected hospitals. The efficiencies of various techniques were also assessed.

The panels of MTB isolates obtained, respectively, from coughing prison inmates, newly-diagnosed smear-positive patients presenting at hospitals closest to the prisons and TB diagnosed at post-mortem were subjected to analysis using Drug Susceptibility Testing-Minimal Inhibitory Concentration method (DST-MIC), the Capilia TB-Neo Immunochromatographic Test (ICT), P-32 based Dot Blot Hybridisation on the *rpoB* gene to investigate Rifampicin resistance, Multiplex Ligation-dependent Probe Amplification Assay (MLPA), HAIN GenoType® MTBC Assay, HAIN GenoType MTB-DR*plus*® Test, the INNO-LiPA Mycobacteria Assay (for Non-Tuberculous Mycobacteria found), Spoligotyping and Multi-locus Interspersed Repetitive Units-Variable Number of Tandem Repeats Assay (MIRU-VNTR).

Among the prison panel of isolates, 30 were confirmed as MTBC out of 54 recovered primary cultures of sputum samples elicited from 214 coughing inmates. Forty-three (43) of these 54 primary cultures were acid-fast bacilli (AFB) on Ziehl-Neelsen microscopy while 11 were not. As high as 30 of these 43 were identified as MTBC while 13 were NTM based on the Capilia TB-Neo Test and 16S rDNA PCR. Using BLAST sequencing, six out of the 13 NTM were fully characterised – five as known NTM whilst one (1) was identified as *Nocardia nova*. These NTM were one (1) *Mycobacterium peregrenum*, two (2) *Mycobacterium chelonae* and two (2) *Mycobacterium fortuitum* isolates.

Remanded inmates made up 80% (24/30) of all the confirmed TB cases in the prison population, whiles the rest were made up of convicted inmates. DST-MIC indicated there was no MDR-TB strain in the prison population.

A total of 170 MTBC isolates were found in patients presenting at selected hospitals while 43 MTBC isolates were found in 36 cadavers at post-mortem (some cadavers presented more than one infected organ). DST-MIC indicated there were 4 MDR-TB strains in the 170 MTBC isolates (2.4%) and poly-resistance was also low. There was no resistant isolates amongst the isolates obtained at post-mortem. Streptomycin mono-resistnce was highest at 11.9%. Other significant resistance profiles were INH^R only, 2.5%; RIF^R only, 1.2%; STR^R and INH^R together, 3.3%.

Significant resistance mutations identified were nine (9) out of the 16 INH^R isolates, which harboured the mutations *katG* MUT1 (AGC→ACC), the

inhA MUT1 (C→T in position 15), the *rpoB* MUT3 S531L, the *rpoB* MUT2B H526D and *rpoB* MUT3 S531L.

Spoligotyping indicated a preponderance of the LAM10_CAM (SIT 61) and the T1 (SIT 53) lineages in all the population panels studied. For diversity, spoligotyping data in combination with MIRU-VNTR data provided a better discrimination, particularly, in defining infections amongst the coughing inmates to their individual prison cells. Thus, spoligotyping data together with MIRU-VNTR data can be employed with reasonable success to study transmission dynamics of MTB in the Ghanaian setting.

Analysis of the diversity indices of the various typing methods employing the Hunter-Gaston Diversity Index, HGDI, in this work, indicated that the 24-Loci MIRU-VNTR typing yielded the highest index. The HGDI was even higher when the 24-Loci MIRU-VNTR was used in tandem with the spoligotyping method. However, an interesting observation was that the HGDI values were the same for 24-Loci MIRU-VNTR and the 15-Loci MIRU-VNTR (either when used alone or in combination with Spoligotyping) for the prison and the PM populations. For the presenting patient population an increase was observed from the 15-Loci to the 24-Loci MIRU-VNTR. For individual loci, MIRU 40 (VNTR 0802) was the most versatile, being the most discriminatory for the 12-loci and 15-loci sets MIRU-VNTR analysis across all population groups. This locus (MIRU-40) was also the most discriminatory for the 24-loci MIRU-VNTR analysis, giving the highest HGDI values of 0.83 and 0.82 for the prison population and its sub-population of remanded inmates, respectively. It also provided significantly high values across all

population groups. The locus VNTR2163b provided the highest discrimination index (HGDI=0.82) for presenting patients at selected hospitals, while the locus VNTR 3690 (also referred to as locus Mtub39) gave the highest discriminatory index (HGDI=0.72) for isolates obtained from the decedent population. Thus, the universally recommended ideal set of 24 MIRU-VNTR loci yielded the highest level of discrimination for MTBC strains across all population groups. However, closeness of results obtained for the 15 MIRU-VNTR loci indicates that this could be used to reduce cost.

Sensitivity analysis of the various techniques revealed a highly satisfactory level of sensitivity and specificity (values ranged from 65 – 100%) in defining the population structure of MTBC strains in circulation in the studied populations.

To fully define and contribute to an understanding of the genetic-molecular nature of MTB circulating in the population groups, the typing methods used were quite efficient in providing information that can be used in the management and control of TB in Ghana.

Conclusions

This study attempted at determining the various species, strains and lineages of MTBC in circulation in three very important human population types in Ghana. These population types defined the three thematic areas of this project. The information and data garnered should contribute to the understanding of the population structure of MTBC and thereby help in

combating TB disease in Ghana. Also, the data and information generated by this study can be used in building the requisite database, which is invaluable for the study of transmission dynamics of this dangerous pathogen in the Ghanaian setting. An understanding of the transmission dynamics of MTBC will also serve as a useful tool in designing better control and management protocols of TB in Ghana.

In total, 243 mycobacterial isolates were realised and identified in the three population groups studied. This number was made up of 30, 170 and 43 isolates obtained from coughing prison inmates, patients presenting at selected health centres in close proximity to the prisons and from diagnosis at post mortem respectively. All isolates were identified and confirmed as members of the MTBC. As high as 80% (24/30) of all the TB isolates realised from the prisons came from the remand population. This indicates that the remand prisoners should be given special attention in the control and management of TB. The majority of circulating strains amongst the prison isolates belonged the LAM10_CAM (SIT 61) and the T1 (SIT 53) lineages. However, there was not much drug-resistance among the TB strains isolated from prison inmates.

For isolates obtained from presenting patients, MDR-TB isolates were 2.4% (4/170). Significant resistance mutations identified were nine (9) among16 INH^R isolates, which harboured the *katG* MUT1 mutation (AGC→ACC) whiles the remaining 7 isolates contained the *inhA* MUT1 (C→T in position 15) mutation. Four out of 5 RIF-resistant isolates possessed the *rpoB* MUT3 S531L mutation whiles one isolate carried the *rpoB* MUT2B H526D mutation. One isolate each carried the*inhA*MUT1 (C→T in position

15) and the RIF-resistance *rpoB*MUT3 S531L mutation. The LAM10_CAM (SIT 61) and the T1 (SIT 53) lineages also dominated MTBC strains obtained from the presenting patient population group. While there was no MDR-TB amongst isolates realised from the decedent population, the strains were mainly of the LAM10_CAM (SIT 61) and the T1 (SIT 53) lineages. In terms of circulating lineages, all three population panels mirrored each other and, therefore, TB disease control measures ought to be holistic to be effective.

Analysis of the discriminatory power of the various typing methods by measuring the HGDI indicated that the 24-Loci MIRU-VNTR typing yielded the highest index. The 24-Loci MIRU-VNTR when used in tandem with the spoligotyping method even returned higher HGDI values. Interestingly, the HGDI values obtained were the same for the 24-Loci MIRU-VNTR and 15-Loci MIRU-VNTR (when used alone or in combination with spoligotyping) for the prison and the decedent populations. The MIRU 40 (VNTR 0802) locus was the most adaptable, being effectively the most discriminatory for the 12-loci and 15-loci sets MIRU-VNTR analysis across all population groups. For the prison population and its sub-population of remanded inmates, This VNTR0802 locus was also the most discriminatory in 24-loci MIRU-VNTR analysis, giving the highest HGDI values of 0.83 and 0.82 for prison inmates and remanded prisoners, respectively. The locus VNTR2163b and VNTR 3690 (Mtub39) provided the highest discrimination indices of HGDI of 0.82 and 0.72 for the presenting patients population and the decedent populations, respectively.

The HAIN GenoType® MTBC Assay, HAIN GenoType® MTB-DR+ Test and the MLPA tests were all sensitive and specific in determining resistance associated mutations in the populations studied. However, the MLPA test provides flexibility since probes based on new drug resistance-associated mutations in circulation can always be designed to include the capture of more drug-resistant isolates.

Recommendations

It would be extremely costly in terms of not only finances but logistics for the Ghanaian penal system if every prison inmate is screened and those positive with TB sent to the infectious diseases prison. A mass-screening programme would necessarily involve active case finding initially using microscopy, followed by the Xpert/RIF test and X-radiography. Since there was not much drug resistance, DOTS could still be employed for the management of TB in the penal system in Ghana.

First, based on the current data, a structured TB diagnostic programme should be designed that would both identify a significant number of cases and critically be feasible to implement. This structured programme should involve first isolating (as much as possible) the remand population and then screening them.

Second, since the proportion of drug-resistant isolates found in the current study was low in the general prison population, a co-ordinated DOTS treatment of cohorted prisoners could be undertaken without transfer to the

infectious diseases prison with likely excellent clinical response in the vast majority of cases. Re-screening of patient cohorts after the intensive phase to detect persons who fail to convert to AFB-negative would additionally allow the predicted small number of treatment failures to be identified and transferred to the infectious diseases prison for optimised management.

Third, since pyrazinamide, PZA, is part of the first-line TB drug regimen by the NTP it is being recommended that it should be included in drug-susceptibility testing of MTB isolates.

Fourth, sputum samples of inmates on DOTS should be screened directly for drug-resistant TB. Only those inmates in whom drug-resistant TB is diagnosed should be transferred to the Ankaful Contagious Diseases Prison for optimised treatment and management.

Fith, a policy of screening-at-entry and screening-on-exit of both remanded and convicted persons should be designed and fully implemented by the Ghana Prison Service and the National Tuberculosis Programme. This measure would prevent the acceptance of an infected inmate (remanded or convicted) directly into the penal environment or the release of an infected former inmate directly back into the population outside the penal environment. It may be necessary that the screening regimen adopted should be broadened to cover diseases which may mimick TB e.g. nocardiosis

Sixth, there should be a periodic fumigation of the prison cells in order to improve the general hygiene of the penal system.

Seventh, more resources should be committed for TB screening, diagnosis and case-finding amongst the general Ghanaian population,

Finally, it is being recommended that a comprehensive programme for TB diagnosis at post-mortem be initiated at the Regional Hospitals with the requisite capacity in terms of qualified pathologists, technologists and equipment for effective diagnosis and treatment or management. This should involve sampling and screening of suspicious lesions initially for acid fastness, and then culture to obtain isolates for down-stream analyses.

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APPENDIX A

Administrative Map of Ghana

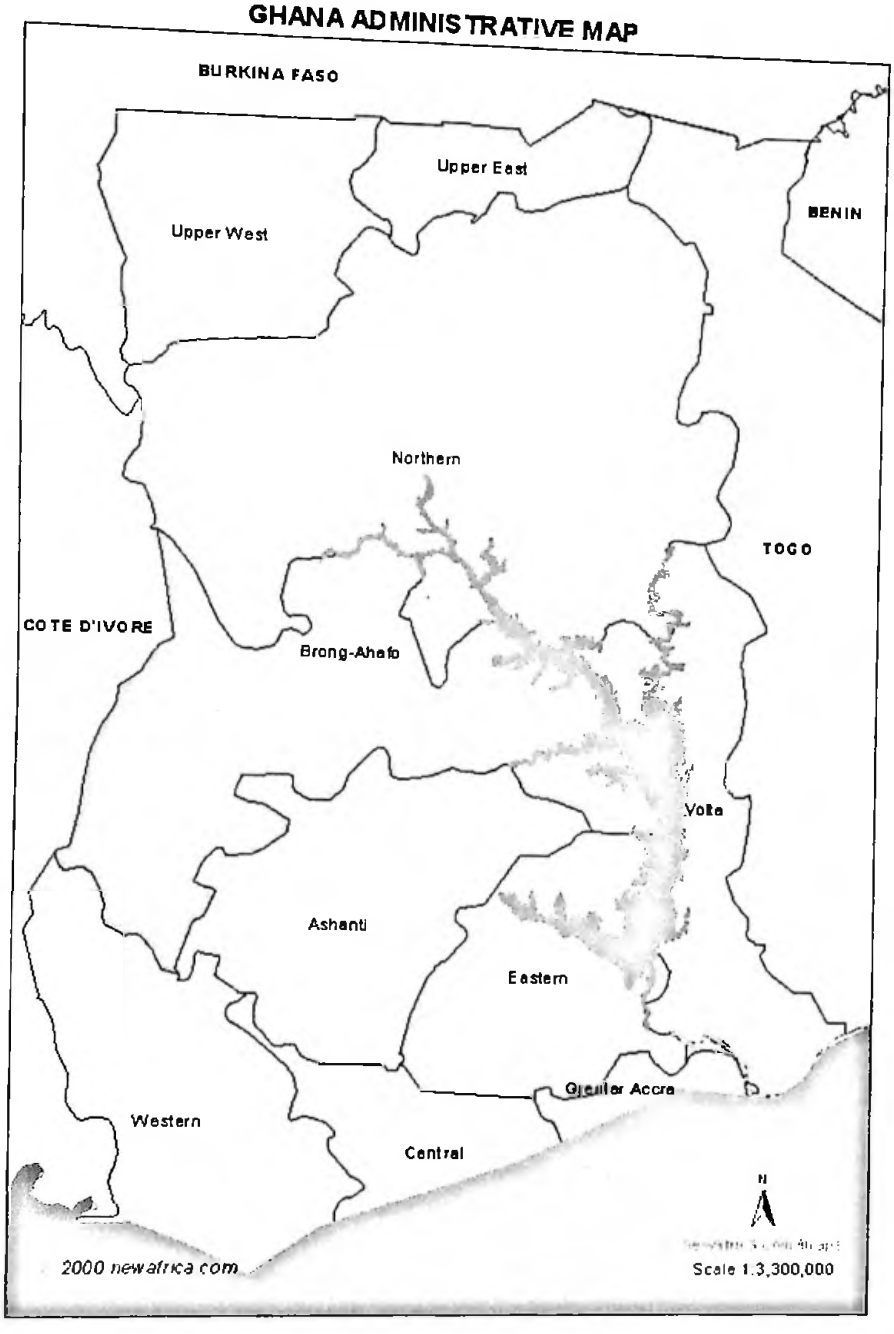

Figure 25: Administrative Map of Ghana showing the Regions from which the Prisons and Hospitals were selected.

APPENDIX B

INFORMATION SHEET
(to be administered together with all consent forms)
MOLECULAR CHARACTERISATION OF *MYCOBACTERIUM TUBERCULOSIS*-COMPLEX IN SELECTED PRISONS AND HOSPITALS IN GHANA.

You are being invited to participate in a study to investigate the genetic nature of Mycobacterium tuberculosis (the bacterium which causes Tuberculosis disease) in selected prisons and hospitals in Ghana. The results of this study will help in the Molecular Characterisation of the different Mycobacterium tuberculosis (MTB) strains in circulation in the selected prisons and in patients presenting at the hospitals.

Information generated on the molecular-genetic nature of the circulating MTB strains can be used by used by stake-holders like the Ghana National Tuberculosis Programme (NTP) establish drug resistance patterns in the country, build a base-line data on the different MTB strains in circulation, provide essential inputs in the formulation of TB drug policy and help achieve the espoused aim to screen all patients, who do not experience a sputum conversion within 2 months of DOTS, for MDR-TB. The information would also help the Ghana Prison Service effectively combat and manage tuberculosis disease among inmates in selected prisons in particular and in the whole penal system in general. The outputs realised at the end of the study will lead to better control and management practices for tuberculosis in prisons and the general Ghanaian population.

The procedures to be used in this study will involve routine tuberculosis management practices and novel procedures such as the proposed molecular techniques. At least two (2) sputum samples will be collected from you as is done routinely if you are suspected of having tuberculosis. These will be transported to the laboratory and subjected to the routine NTP tests and the proposed molecular techniques.

Though you may have routinely provided a sample, your participation in the study is ***completely voluntary.*** *You are free to refuse permission to participate and this will in no way affect how you would be treated at the local hospital or clinic. If, at any point in time during the study, you take the decision not to participate any further, you are at liberty to do so immediately without any further discussion and it will have no consequences for you.*

All information related to your participation would be kept strictly confidential. If you have any problems or questions about the study, feel free to contact any of the following:

1. The Ghana Prisons Officer-in-Charge of your Prison.
2. Mr. Oti Kwasi Gyamfi, Cellular and Clinical Research Centre, RAMSRI/GAEC, Kwabenya. Tel: 0244297230.

APPENDIX C

CERTIFICATION OF VERBAL INFORMED CONSENT

MOLECULAR CHARACTERISATION OF *MYCOBACTERIUM TUBERCULOSIS*-COMPLEX IN SELECTED PRISONS AND HOSPITALS IN GHANA.
SAMPLE ACQUISITION IN PRISONS AND HOSPITALS

I, ---------- of ----------, having understood the contents of the attached Information Sheet, after it has been thoroughly explained together with this consent form to me in the English / Akan / Dagarti / Dagbani / Ga-Dangme / Ewe / Frafra / Hausa / Kasena / Nzema / etc. language, hereby agree to participate in this survey and that my sputum sample be subjected to the molecular analysis as stated in the proposal.

Name of Inmate/Patient: ----------

Sex: ----------

Age: ----------

Signature/Thumbprint of Patient: ----------

Witness: ----------

Date: ----------/----------/----------

APPENDIX D

INMATE SPUTUM SAMPLE COLLECTION AND BIODATA SHEET

Institutional

Prison / Region: Date:

Inmate Identification Number: Sex: M/ F Age/yr:
Weight/kg:

Dimensions of Cell: No. of Inmates in Cell:

Length of Sentence term: Date commenced:

Employment before conviction: Profession:

Previous convictions with dates and prisons (if any):

Health

Initial Health Complaint: *Primary Inclusion Criterion*

Other TB History (if known):

Other Clinical Diagnosis and History (if known):

Sputum Sample Elicited & Date: Mid-night: Yes/No
On-the-spot: Yes/No

Chest Radiography: NORMAL
ABNORMAL

ZN Microscopy for AFB: POSITIVE NEGATIVE

Medications (if any):

Comments by Prisons Medical Officer (if any):

Our Comments (If any):

APPENDIX E

PATIENT SPUTUM COLLECTION AND CLINICAL DATA SHEET

PERSONAL DATA

Name (PatientID).....

Sex: Male ( ) Female ( ): Age:

Address.....

CLINICAL INFORMATION

DiagnosticCentre.....

Patient ID.....

Disease Classification: Pulmonary ( ) Extra-pulmonary ( )

Category of case: (i) New Case ( ), (ii) Relapse ( ), (iii)Treatment Failure ( ), (iv) Follow-up ( )

If yes to (ii), (iii), or (iv)

How many months.....

BACTERIOGICAL EXAMINATION

Smear Result (A)..... (B).....

Diagnosis Date (A)..... (B).....

CPC Added ( )

Officer-in-Charge.....

APPENDIX F

ETHICAL CLEARANCE

GHANA HEALTH SERVICE ETHICAL REVIEW COMMITTEE

In case of reply the number and date of this Letter should be quoted.

My Ref. :GHS-ERC: 3
Your Ref. No.

Health Research Unit
Ghana Health Service
P. O. Box GP-184
Accra

17th August 2006

Tel: +233-21-681109
Fax : 233-21-226739
Email: Hannah.Frimpong@hru-ghs.org

ETHICAL CLEARANCE
ID NO: GHS-ERC: 05/5/06

The Ghana Health Service Ethics Review Committee has given approval for the implementation of your protocol title:

"Application of Molecular Techniques in the Control and Management of Drug-Resistant Tuberculosis(DR-TB) in Selected Ghanaian Prisons"

PRINCIPAL INVESTIGATOR: Oti Kwasi Gyamfi

This approval requires that you submit periodic review of the protocol to the Committee and a final full review to the Ethical Review Committee (ERC) at the completion of the study. The ERC may observe or cause to be observed procedures and records of the study during and after implementation.

Please note that any modification of the project must be submitted to the ERC for review and approval before its implementation.

You are also required to report all serious adverse events related to this study to the ERC within seven days verbally and fourteen days in writing.

You are requested to inform the ERC and your mother organization before any publication of the research findings.

Please always quote the protocol identification number in all future correspondence in relation to this protocol.

SIGNED.....
PROF. ALBERT GEORGE BAIDOE AMOAH
(GHS-ERC CHAIRMAN)

Cc: The Administrative
GHS Ethics Review Committee
Health Research Unit
Accra

APPENDIX G

PM SPECIMEN COLLECTION SHEET **Path. No.:**
[Cellular & Clinical Research Centre (RAMSRI, GAEC) and Department of Pathology, (KBTH)]

Name of Cadaver: Sex: M / F
Age:

Date of Death: On Admission? Yes / No

Date of Admission: Ward/ Block

Presenting Disease / Ante-mortem Diagnosis:

If TB, Date of Diagnosis: On DOTS? Yes / No

Date of Initiation of DOTS: HIV Status: Positive / Negative

HIV Type: HIV I / HIV II Date of HIV Diagnosis:

On ARVs: Yes / No Date of InitiationARV Therapy:

Date of Autopsy:

Autopsy Disease(s):

Cause of Death:

Specimen Type(s):

Pathologist:

APPENDIX G

PM SPECIMEN COLLECTION SHEET **Path. No.:**
[Cellular & Clinical Research Centre (RAMSRI, GAEC) and Department of Pathology, (KBTH)]

Name of Cadaver:
Age: Sex: M / F

Date of Death: On Admission? Yes / No

Date of Admission: Ward/ Block

Presenting Disease / Ante-mortem Diagnosis:

If TB, Date of Diagnosis: On DOTS? Yes / No

Date of Initiation of DOTS: HIV Status: Positive / Negative

HIV Type: HIV I / HIV II Date of HIV Diagnosis:

On ARVs: Yes / No Date of InitiationARV Therapy:

Date of Autopsy:

Autopsy Disease(s):

Cause of Death:

Specimen Type(s):

Pathologist: